

P-1920



No. 06-027688- -00009-0000

Fecha: 2012-02-27 15:17:08 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 203

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. 06.027.688

Solicitud de patente a nombre de **NOVARTIS AG**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 21 de septiembre de 2010, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. **19414** notificado por fijación en lista el 15 de diciembre de 2011, relacionado con el concepto técnico de fondo emitido por ese despacho, me permito manifestar lo siguiente:

1. MODIFICACIONES NO ACEPTADAS

La señora examinadora indica en el numeral 2 del oficio 19414 que *“La reivindicación 1 menciona como característica un ARN de estructura de doble cadena con por lo menos un extremo romo, que comprende una modificación química que es hidroxipropilfosfodiester. Sin embargo, tal modificación no está sustentada en la descripción, de acuerdo con lo siguiente:*

- *A pesar de lo indicado por el solicitante en su respuesta del 29/08/2011 (folio 144-145) este tipo de cap 3' no había sido divulgado como un alternativa de las modificaciones del siRNA que podrían realizarse, ni en el aparte citado por el solicitante ni en el resto de la descripción;*
- *La modificación química hidroxipropilfosfodiester tampoco corresponde a una de las alternativas que tenía la reivindicación 1 anterior, en la fórmula I general que presentaba (folio 43);*
- *No hay sustento dentro del capítulo descriptivo que muestre que la modificación hidroxipropilfosfodiester estabiliza el siRNA contra la degradación por nucleadas;*

Teniendo en cuenta lo anterior, esta reivindicación se considera una ampliación puesto que es una inconsistencia respecto a la información inicialmente revelada en la solicitud. Por tanto, el capítulo reivindicatorio presentado junto al memorial de fecha 29/08/2011 (folios 168-169) no será tenido en cuenta.”

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Antes de contestar a estos argumentos, el solicitante desea hacer énfasis sobre el hecho que las reivindicaciones abarcan un RNA **de doble hebra** (por ejemplo un siRNA) que comprende un extremo 3' que comprende a su vez un hidroxipropilfosfodiester (C3). El extremo 3' lleva a cabo varias funciones, como se describe en la memoria descriptiva. Un extremo 3' adecuado debe: (1) estabilizar el siRNA, por ejemplo, contra las nucleasas, y (2) permitir que el siRNA medie la interferencia del RNA.

En este sentido, el solicitante se permite manifestar que no todos los extremos 3' son capaces de mediar ambas funciones. Los documentos citados por la Oficina Colombiana de Patentes describen varios extremos 3' los cuales **no son capaces de llevar a cabo ambas funciones**. Entonces, es claro que el solicitante ha sido el primer investigador en demostrar que este extremo 3' en particular es capaz de realizar estas funciones.

Ya teniendo lo anterior claro, respetuosamente nos permitimos decir que no nos encontramos de acuerdo con los argumentos expuestos por la señora examinadora en cuanto a las modificaciones presentadas con nuestra respuesta del 29 de agosto de 2011.

La descripción de la presente invención indica que (1) el extremo 3' del RNAs de acuerdo con la presente invención confiere una alta estabilidad *in vivo* en medio de suero y de crecimiento para los cultivos celulares. Por lo tanto, el dsRNA de acuerdo con la presente invención **no requiere estabilización adicional contra la degradación de nucleasa...** (Por favor, véase la página 11 entre las líneas 9 a 25 de la memoria descriptiva).

También es necesario dirigir la atención de la señora examinadora sobre el hecho que en la descripción también se indican varios siRNAs que comprenden el extremo 3'. Estos incluyen: C3-8646, el cual apunta hacia el gen p2x3 (véase por favor la tabla 1 ubicada en la página 25 de la descripción. Adicionalmente, la figura 1 muestra que este siRNA es efectivo contra el gen p2X3 en células CHO. La descripción también muestra el C3-siRNA, el cual apunta a diferentes genes, hGAPDH (tabla 1). Las figuras 2 y 3 demuestran que este siRNA es efectivo contra su objetivo tanto en células CHO-K1 y en células HeLa.

Sobre este particular, el solicitante se permite adjuntar copia informal de la solicitud WO2007/128477 (Anexo 1) que proporciona información adicional a la que presenta la solicitud en estudio, relacionada con la estabilidad que puede ser proporcionada por el extremo 3' reclamado para varios RNA de doble hebra contra nucleasas, en la invención del presente caso.

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En efecto, dicho documento demuestra y corrobora lo indicado en la presente invención en cuanto a que varios siRNAs comprenden el extremo 3' reclamado. Estos incluyen dos siRNAs que comprenden un 3' hidroxipropilfosfodiéster que apunta a VEGFR2, principalmente a VEGFR2-C3-siRNA1 y VEGFR2-C3-siRNA2 (véase por favor la tabla 3); y dos siRNAs que comprenden un 3' hidroxipropilfosfodiéster que apuntan a Tie2, principalmente Tie2-C3-MOE-siRNA1 y Tie2-c3-MOE-siRNA2.

En este sentido, amablemente dirigimos la atención de la señora examinadora sobre el hecho que estas moléculas también comprenden un 2'-MOE sobre al menos dos pares de bases de nucleótidos de cada hebra o cordón. Las figuras 3 y 4 de dicho documento también muestran que el 3' hidroxipropilfosfodiéster fue capaz de estabilizar el siRNA. La descripción indica que, primero: los siRNAs fueron incubados en ácido gástrico de ratón por 2 horas (figura 3) y que no se observó ninguna degradación en los casos de C3 siRNA y C3-MOE siRNA, en tanto que se observó degradación de siRNA tipo nativo luego de 30 minutos.

Sobre este particular, respetuosamente nos permitimos decir que aunque consideramos que la información contenida en la presente solicitud es suficiente para entender la invención, también consideramos importante citar el documento **WO2007/128477** mencionado anteriormente, dado que el mismo proporciona información adicional útil para la señora examinadora en cuanto a que los siRNAs que comprenden el extremo 3' reclamado son capaces de mediar la interferencia de RNA en pruebas con ratones. (Por favor, véase el ejemplo 12 y las figuras 7 y 11).

Aunado a lo anterior, también nos permitimos decir que el documento citado también indica que la estabilidad en el fluido intestinal obtenido de los lavados intestinales de rata, reveló casi la completa degradación del siRNA tipo nativo luego de 15 minutos, en tanto que para el compuesto paterno en el MOE o/h siRNA, C3-siRNA, C3-siRNA y C3-Moe SiRNA se observó para 60 minutos (figura 4). Por favor, véase la página 38, primer y segundo párrafos.

Adicionalmente, nos permitimos presentar junto con esta respuesta datos adicionales (Anexo 2) que demuestran la eficacia del 3' hidroxipropilfosfodiéster.

Estos datos muestran la secuencia de siRNA hacia mFVII que comprende el 3' hidroxipropilfosfodiéster. Este siRNA ha incrementado la estabilidad en suero de rata en 24 horas. El siRNA también fue capaz de mediar la interferencia de RNA.

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Por consiguiente, todos estos datos demuestran que siRNA apunta a diferentes genes (p2x3, hGAPDH, VEGFR2, Tie2 y mFVII) cada uno con el 3' hidroxipropilfosfodiester. En estos varios casos, el cap extremo 3' es capaz de realizar las dos deseadas funciones. El extremo 3' puede: (1) estabilizar el siRNA, por ejemplo, contra nucleasas, tanto como en suero como en fluido intestinal; y (2) permitir que el siRNA medie la interferencia del RNA.

En resumen, nos permitimos manifestar que contrario a los argumentos expuestos por la señora examinadora, la presente invención muestra claro sustento para el extremo 3' reclamado tanto en la memoria descriptiva como en los datos aportados por el cliente y en la solicitud relacionada aportada por el solicitante.

De otra parte, en cuanto a que el extremo 3' reclamado no corresponde a una de las alternativas descritas en la anterior reivindicación 1. Respetuosamente nos permitimos decir que tampoco nos encontramos de acuerdo con dicha apreciación, toda vez que en la antigua reivindicación 1 se establece la fórmula I donde R1 es Y-Z, en donde Y es O (oxígeno), y Z es alquilo, sustituido por un heteroátomo adicional, en donde el heteroátomo es O (oxígeno) (por favor dirigirse a la página 32 reivindicación 1 como fue originalmente presentada). Así las cosas, de manera respetuosa nos permitimos decir que, contrario a lo manifestado por la señora examinadora, la modalidad en cuestión se encontraba abarcada por la reivindicación 1 originalmente presentada.

En gracia de discusión, también se hace importante señalar de manera respetuosa que: aunque no se hubiera cubierto dicha modalidad por el capítulo reivindicatorio original, el solicitante estaría igualmente en todo su derecho y de acuerdo con lo establecido en el artículo 34 de la Decisión 486 para reclamarla luego introduciendo dicha modalidad en las reivindicaciones mediante modificación a la solicitud, dado que, como se demostró anteriormente, dicha modalidad se encuentra **debidamente sustentada en la descripción** y no va más allá del alcance de la divulgación contenida en la solicitud inicial.

Por lo tanto, respetuosamente solicitamos a ese despacho retirar dichas objeciones y proceder a evaluar el capítulo reivindicatorio que fue presentado con nuestra respuesta del 29 de agosto de 2011 dado que con el mismo se superan todas las objeciones planteadas por la señora examinadora en el Oficio No. 08910 del 3 de junio de 2011 y que fueron mantenidas en el Oficio No. 19414 del 15 de diciembre de 2011.

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De otra parte, la señora examinadora indica que la composición farmacéutica de la reivindicación 11 debe ser definida por las secuencias específicas de RNA.

Sobre este particular, nos permitimos decir que no resulta ser necesario definir las secuencias, dado que como se muestra en la descripción y en los datos adicionales citados anteriormente, el extremo 3' reclamado es útil para siRNAs de muchas secuencias distintas que apuntan a muchos genes diferentes. Por consiguiente, resulta a todas luces, innecesario que las reivindicaciones requieran ser limitadas a secuencias particulares.

2. EVALUACIÓN DE LOS REQUISITOS DE PATENTABILIDAD.

La señora examinadora indica en su concepto técnico que los documentos D1 (WO02/096927), D2 (WO03/070918), D3 (Czauderna y colaboradores) y D4 (Hamada y colaboradores) afectan la novedad y el nivel inventivo de la presente solicitud.

En relación con los argumentos indicados por la señora examinadora, nos permitimos respetuosamente reiterar que los extremos 3' reclamados realizan dos funciones sobre los siRNAs:

- 1) Incrementar la estabilidad, por ejemplo contra la nucleasas en el suero y en los fluidos intestinales, y
- 2) Permitir la función del SiRNA.

En este sentido, necesariamente hacemos hincapié sobre el hecho que ninguno de los documentos citados muestra un extremo 3' que realice las dos funciones anteriormente señaladas y que tenga una estructura como el extremo 3' reclamado en la presente solicitud.

En ese orden de ideas, respetuosamente nos permitimos decir que muchos de los extremos 3' descritos en dichos documentos no son capaces de realizar al mismo tiempo las dos funciones anteriormente encontradas para los extremos 3' de la invención en estudio. Es más, dichos extremos 3' reportados en la técnica anterior no pueden ser empleados sobre siRNAs.

Aunado a lo anterior, el solicitante desea manifestar que la selección de un extremo 3' funcional no puede ser realizada de manera teórica. Solamente las pruebas empíricas conducirían al hallazgo de un extremo 3' con función, tal como el extremo hidroxipripilfosfodiéster (C3) reclamado en la presente invención.

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En efecto, el documento D1 (WO02/096927) no muestra la eficacia de un RNAds con un extremo romo y un Cap 3' como el reclamado en la solicitud en estudio.

De hecho, el documento D1 reporta una lista interminable de posibles modificaciones para el extremo 3' en la página 45 de dicha anterioridad. Sobre este particular, también es necesario hacer énfasis sobre el hecho que dicha lista es derivada de una publicación anterior: Beaucage y colaboradores del año 1993 publicada en la revista Tetrahedron 49: 1925 (véase por favor la página 45 entre las líneas 16 y 17 de D1). (Anexo 3).

En este sentido, el documento de Beaucage y colaboradores no resulta pertinente en cuanto a la tecnología de SiRNA, de hecho, dicho documento hace referencia a una tecnología distinta, es decir a **oligonucleótidos anti-sentido (ASO)** o DNA **de una sola hebra**. Es más, Researcher Pal Saetrom (2004 Bioinformatics 20:3055-3063 (Anexo 4)) ha establecido que el “anti-sentido y la RNAi son tecnologías muy diferentes”, conclusiones relacionadas con que los ASO no necesariamente aplican para siRNA y viceversa.

Aunado a lo anterior, es necesario señalar que los ASO difieren en estructura de los siRNAs. En efecto, los oligonucleótidos anti-sentido usados en Beaucage y colaboradores son DNA de hebras sencillas. En claro contraste, los siRNAs reclamados son RNA de doble hebra. Tampoco el mecanismo de acción de los ASO y los SiRNAs es similar. Los oligonucleótidos anti-sentido pueden hibridizar hacia mRNA creando un híbrido RNA-DNA, el cual es escindido (cortado) por la enzima RNaseH. Alternativamente, la hibridación bloquea estéricamente la traslación. Véase por favor Pilowsky et al, 1994 Clin. Exp. Pharm. Physiol. 21:935-44 (Anexo 5). En consecuencia, ninguno de los mecanismos de los ASO son empleados o usados por los SiRNAs.

La interferencia de RNA involucra la escisión (ruptura) de una larga doble hebra de RNA por medio de la enzima ribonucleasa III (llamada Dicer). En este sentido, el solicitante amablemente dirige la atención de la señora examinadora a los artículos de Elbashir et al 2001 (Anexo 6) y de Hammond et al 2001. Science 293:1146 (Anexo 7) donde se muestra que la actividad biológica de los oligonucleótidos anti-sentido y de los siRNAs involucra entonces, **diferentes enzimas y componentes biológicos**. Saetrom (Anexo 4) concluye entonces que “los dos métodos se basan en distintos mecanismos celulares (véase por favor el resumen).

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Los oligonucleótidos anti-sentido y los siRNA también difieren en su tolerancia a las modificaciones químicas. Czaderna y colaboradores hacen referencia a que las modificaciones de desoxi abásicos invertidos y modificaciones de extremo amino son posibles para estabilizar las moléculas anti-sentido, pero que reducen la actividad de siRNAs cuando todos las cuatro terminales son modificadas (véase por favor la página 2710 de **D3**).

Así las cosas, entonces, incluso si Beaucage y colaboradores ó el documento WO 02/096927 mostraran que la modificación del extremo 3' reclamado pudiera funcionar sobre ASO (lo cual de hecho no lo es), no haría obvio el uso de este extremo 3' en siRNAs.

En este sentido, la señora examinadora podrá notar que varios artículos científicos se han presentado haciendo comparaciones específicas de oligonucleótidos anti-sentido y siRNAs dirigidos a los mismos blancos en el mismo gen. Los resultados con ASO incluso difieren dramáticamente de los resultados con siRNAs. En efecto, en el artículo de Kretschmer-Kazemi Far y colaboradores, publicado en el 2003 en *Nucleic Acids Res.* Número 31:4417-4424 (Anexo 8) y en Vickers y colaboradores publicado en el 2003 en el *Journal Biol. Chem.*, 278: 7108 (Anexo 9), proporcionan evidencia adicional en cuanto a que las tecnologías ASO y RNAi son distintas.

En resumen, los ASO y los SiRNAs difieren en su estructura, tolerancia a la modificación química, en el mecanismo de acción y en otros parámetros, lo cual permite concluir que los primeros no podrían ser aplicados a los segundos.

Adicionalmente, los caps 3' específicos enlistados en el documento WO 02/096927 incluyen muchos de los que fueron mostrados para ser ineficaces con siRNAs. Estos incluyen:

- un “residuo abásico invertido (parte)” que fue mostrado al final para ser un in-estabilizador del siRNA y no permitir la actividad del SiRNA cuando está presente sobre los extremos 5' y 3' (Czaderna y colaboradores 2003, véase por favor la figura 4B).
- un “fosfato aminohexilo” como un cap, el cual no fue capaz de estabilizar el siRNA. De hecho, cuando está presente sobre los extremos 5' y 3', dicho fragmento previene la actividad del RNAi (véase por favor Czaderna y colaboradores, 2003 en la figura 4B, específicamente).
- un nucleótido de base modificada”; sin embargo, los nucleótidos de base modificada tal como los 5-propinil-U fueron mostrados por no estabilizar el siRNA ni permitir la actividad del RNAi. Por favor referirse al documento Deleavey y colaboradores 2009 *Curr. Prot. Nucl.*

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Acid Chem. 16.3.1-16.3.22 (Anexo 10) y Terrazas y colaboradores 2009, Nucleic Acids Res. 37:346-353 (Anexo 11).

Por consiguiente, el documento **D1** (WO02/096927) no proporciona ningún dato que muestre que el extremo 3' reivindicado es efectivo en la protección de siRNAs de nucleasas y que permita la actividad de siRNA. De hecho, **D1** proporciona una lista interminable de posibles caps de extremo 3', donde dicha lista se encuentra derivada de un documento dirigido a una tecnología distinta (ASO) que incluye muchos caps de extremo mostrados como no funcionales en siRNAs. Por lo que claramente este documento no puede afectar ni la novedad ni el nivel inventivo de la presente solicitud.

Por su parte, el documento **D2** (WO03/070918) tampoco muestra que la modificación del extremo 3' reclamado pueda proteger el siRNA de la degradación y que permita a la vez la actividad del siRNA. De hecho, **D2** también proporciona una lista de posibles caps de extremo 5' y 3' en las páginas 80 y 81 de dicha anterioridad. No obstante, al igual que en el caso de D1, la lista mostrada en D2 se encuentra derivada de una lista mostrada en la patente estadounidense No. 5,998,203 (véase por favor el documento D2 en la página 80), la cual únicamente proporciona un uso meramente especulativo de caps de extremo sobre ribozimas.

Estas ribozimas son también tecnologías distintas a las del siRNA de WO03/070918 (**D2**) y la tecnología de la presente solicitud. Como se describe más abajo, las ribozimas y los siRNAs difieren en estructura, preferencia de secuencia blanco y en el mecanismo de acción. De hecho, no pueden reconocer las regiones blanco.

En Estructura. Los siRNAs comprenden un RNA de doble hebra y en contraste las ribozimas son una hebra sencilla de RNA con una única estructura secundaria. Distinto al siRNA, la secuencia blanco es dividida en DOS brazos físicamente separados (véase por favor Wang y colaboradores, 2004. Methods Enzymol. 389:244-264 (Anexo 12)). Como se establece en Wang et al y en Miyagishi et al 2003 Antisense Nucl. Acid Drug Dev 13:1-7 (Anexo 13), los siRNAs y las ribozimas también difieren en las secuencias blanco preferidas.

En Mecanismo de acción. Los siRNAs requieren de un complejo silenciador inducido por RNA (RISC) para reconocer y escindir el RNA objetivo, en tanto que las ribozimas no requieren de tal RISC y tienen su propia actividad enzimática. Véase por favor Miyagishi et al. (Anexo 13) y Kawasaki et al. 2004 Differentiation 72:58-64 (Anexo 14).

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En este sentido, la señora examinadora podrá corroborar que varios investigadores han realizado comparaciones específicas de las ribozimas y de los siRNAs que cuyo objetivo son las mismas regiones blanco. Estos investigadores incluyen a Miyagishi y colaboradores y Wang y colaboradores. Estos científicos encontraron que las ribozimas y los siRNAs que hacen blanco en las mismas secuencias no siempre lo hacen de una manera funcional idéntica. Además, muchos de los caps de extremo expuestos en **D2** fueron encontrados luego como caps que no funcionan con siRNA. Estos incluyen:

- un “residuo abásico invertido (parte)” que fue mostrado al final para ser un in-estabilizador del siRNA y no permitir la actividad del SiRNA cuando está presente sobre los extremos 5’ y 3’ (Czauderna y colaboradores 2003, véase por favor la figura 4B).
- un “fosfato aminohexilo” como un cap, el cual no fue capaz de estabilizar el siRNA. De hecho, cuando está presente sobre los extremos 5’ y 3’, dicho fragmento previene la actividad del RNAi (véase por favor Czauderna y colaboradores, 2003 en la figura 4B, específicamente).
- un “nucleótido de base modificada”; sin embargo, los nucleótidos de base modificada tal como los 5-propinil-U fueron mostrados por no estabilizar el siRNA ni permitir la actividad del RNAi. Por favor referirse al documento Deleavey y colaboradores 2009 Curr. Prot. Nucl. Acid Chem. 16.3.1-16.3.22 (Anexo 10) y Terrazas y colaboradores 2009, Nucleic Acids Res. 37:346-353 (Anexo 11).

En este sentido resulta importante señalar que el documento US5,998,203 sobre el cual se basa el documento D2 (WO03/070918) también hace referencia al uso de un 3’ fosfato como un cap de extremo 3’ (véase por favor el párrafo [017]), pero este fue luego mostrado como un cap de extremo que no permite la estabilización del extremo 3’ de un siRNA y no permite la actividad del RNAi. Véase por favor el documento Schwarz y colaboradores del 2002 publicado en Mol. Cell 10:537-548 (Anexo 15) y el documento de Lipardi et al del 2011 publicado en Cell 107:299-307 (Anexo 16). Estas referencias proporcionan evidencia adicional sobre el hecho que el documentos US 5,998,203 en el cual se basa el documento D2, no es una referencia confiable para los caps de extremo de siRNAs.

Así las cosas, respetuosamente nos permitimos manifestar que el documento **D2** (WO03/070918) no proporciona dato alguno relacionado con que el extremo 3’ reclamado en la presente solicitud sea efectivo en la protección de siRNAs de nucleasas y que permita la actividad siRNA. De hecho y en claro contraste, **D2** proporciona una lista enorme de posibles caps de extremo 3’. Esta lista que es derivada de un documento relacionado

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con una tecnología diferente (ribozimas) incluye muchos caps de extremo que luego se ha mostrado que no son funcionales en siRNAs. Incluso si **D2** o el documento US del cual se basa **D2** mostrara que el extremo 3' reclamado fuer funcional sobre ribozimas (lo cual no lo es), no haría obvio el uso del extremo 3' reclamado sobre siRNAs. Motivos por los cuales claramente, este documento tampoco puede afectar ni la novedad ni el nivel inventivo de la presente solicitud.

En relación con el documento **D3** (Czauderna et al) respetuosamente nos permitimos decir que dicha anterioridad no puede afectar ni la novedad ni el nivel inventivo de la solicitud en estudio, toda vez que la misma no reporta ni hace evidente el extremo 3' reivindicado.

En efecto, los extremos 3' descritos por Czauderna (**D3**) son modificación abásicas desoxi invertidas (iB) y modificación de extremo amino (NH₂), ninguna de las cuales, cae dentro del alcance de la presente invención. Aún más, ninguna de las modalidades de Czauderna (**D3**) que comprenden estos extremos 3' tiene un RNA de doble hebra y con extremos romos, como se reclama en la presente invención. Por ejemplo, **D3** describe el uso del iB en la figura 1A, pero en dicha modalidad no se hace la modificación sobre un RNA de doble hebra. El NH₂ y el iB también se describen en la Figura 4A y 4B pero ninguna de las moléculas descritas por **D3** redujeron la interferencia de RNA: "la inducción de RNAi por duplicados de siRNA con una modificación de NH₂ ó iB fue reducida dramáticamente cuando todas las cuatro terminales fueron modificadas" (véase por favor la página 2710, columna 1, primer párrafo). Los extremos por sí mismos no fueron capaces de proteger a la molécula de la degradación. *"Con el fin de probar si las modificaciones terminales eran capaces de incrementar la estabilidad en suero, se incubaron los duplicados de siRNA...en suero...ninguna de las modificaciones de extremo NH₂ o iB estabilizaron los duplicados de siRNA de manera significativa."*

Por consiguiente, la señora examinadora podrá dar cuenta que **D3** no describe el extremo 3' (C3) reclamado y no muestra que pueda (1) proteger una molécula de siRNA contra la degradación y (2) que permita la actividad siRNA. Así las cosas, claramente este documento no puede afectar ni la novedad ni el nivel inventivo de la presente solicitud.

Finalmente, la anterioridad **D4** (Hamada et al), tampoco puede afectar la novedad y el nivel inventivo de la solicitud en estudio, toda vez que la misma no reporta ni hace evidente el extremo 3' reivindicado.

En efecto, **D4** muestra dos tipos de caps de extremo: el 2'-O, 4'-ceten timidina (eT) y el 2-hidroxietil fosfato (hp). Como primera medida se hace

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necesario señalar que ninguno de estos se encuentra abarcado por la presente invención a la luz del capítulo reivindicatorio que no fue aceptado por la señora examinadora. A diferencia del extremo 3' reclamado, los extremos reportados en **D4** no son capaces de proteger el siRNA de la degradación ni permiten la actividad siRNA.

El dsRNA con extremo romo con en el cap de extremo eT fue incapaz de mediar la actividad RNAi, como así se puede corroborar en la página 305 en la oración que une las columnas 1 y 2 de **D4**.

El RNAds con el extremo hp también ha reducido la actividad de interferencia del RNA. Esto también lo pone de manifiesto **D4** en la pagina 305, columna 2. De hecho, la señora examinadora puede corroborar que **D4** indica que “no se detectó un efecto de RNAi mejorado [después] de cuatro días” (páginas 305, columna 2). También podrá notar que **D4** indica que el extremo 3' hp no se muestra sobre el RNAds de extremos romos (véase por favor figura 3 de **D4**).

En resumen, **D4** no reporta ni sugiere extremos 3' que efectivamente medien la actividad de interferencia de RNA. Es más, **D4** no describe el cap de extremo 3' reclamado (C3) y no muestra que pudiera tener la protección de la molécula de siRNA contra la degradación ni que permitiera la actividad siRNA.

En conclusión, nos permitimos manifestar que ninguno de los documentos arriba discutidos y que fueron citados por la señora examinadora muestra que el extremo 3' (C3) reclamado sea capaz de ejercer función sobre una molécula de doble hebra, principalmente ejercer la función de proteger la molécula de la degradación y permitir la actividad de siRNA.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos sea aceptado el pliego reivindicatorio presentado el 29 de agosto de 2011, el cual a todas luces no amplía de ninguna manera el alcance de la solicitud como fue originalmente presentado y conceda el privilegio de patente de invención a la presente invención o si el examinador considera que subsisten razones para objetar la presente invención ruego expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que mi mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: “*habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e*

informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares” y “(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



DILIA MARÍA RODRÍGUEZ D'ALEMAN
C.C. 41.654.882 de Bogotá.
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Clarke, Modet & Cº

COLOMBIA

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ANEXO 1

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CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 November 2007 (15.11.2007)

(10) International Publication Number
WO 2007/128477 A2

- (51) International Patent Classification:
C12N 15111 (2006.01)
- (21) International Application Number:
PCT/EP2007/003867
- (22) International Filing Date:
2 May 2007 (02.05.2007)
- (23) Filing Language:
English
- (24) Publication Language:
English
- (30) Priority Data:
0608838.9 4 May 2006 (04.05.2006) GB
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tellectual Property, CH-4002 Basel (CH).
- (81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH,
- (84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LI, LU, LV, MC, MT, NL, PL,
PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:
— as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))
- Published:
— without international search report and to be republished
upon receipt of that report
- (48) Date of publication of this corrected version:
10 January 2008
- (15) Information about Correction:
see PCT Gazette No. 02/2008 of 10 January 2008

(54) Title: SHORT INTERFERING RIBONUCLEIC ACID (siRNA) FOR ORAL ADMINISTRATION

(57) Abstract: Short interfering ribonucleic acid (siRNA) for oral administration, said siRNA comprising two separate RNA strands that are complementary to each other over at least 15 nucleotides, wherein each strand is 49 nucleotides or less, and wherein at least one of which strands contains at least one chemical modification.

Short interfering ribonucleic acid (siRNA) for oral administration

Background:

RNA interference initially discovered in plants as Post-Transcriptional Gene Silencing (PTGS), is a highly conserved mechanism triggered by double-stranded RNA (dsRNA) and able to down regulate transcript of genes homologous to the dsRNA¹. The dsRNA is first processed by Dicer into short duplexes of 21-23 nt, called short interfering RNAs (siRNAs)². Incorporated in RNA-induced silencing complex (RISC) they are able to mediate gene silencing through cleavage of the target mRNA in the center of the region of homology by Argonaute 2, a component of RISC³. In 2001, Elbashir et al⁴ demonstrated that the direct introduction of synthetic siRNAs would mediate RNA interference gene silencing in *Drosophila* but also in mammalian cells. Since then, siRNA-mediated gene silencing has become a powerful and widely-used molecular biology tool in both target identification target validation studies. Use of siRNAs for gene silencing in animal studies has been described in a limited amount of animal models. Unmodified siRNAs were delivered locally in the eye⁵, intrathecally or intracerebellarly in the central nervous system⁶, and intranasally for the inhibition of respiratory viruses⁷. Intravenous hydrodynamic tail vein injection of unmodified siRNAs has also been studied. This approach allows a rapid delivery, mainly to the liver⁸. A very limited number of studies have been reported on the systemic administration of unmodified siRNAs. Duxbury et al⁹ administered intravenously unmodified siRNAs targeting Focal Adhesion Kinase to an orthotopic tumor xenograft mice model, and observed a tumor growth inhibition as well as a chemosensitization to gemcitabine. Soutschek et al reported the systemic use of highly chemically modified siRNAs for the endogenous silencing Apolipoprotein B. Intraperitoneal administration of most anti-ApoB siRNA at the high dose of 50 mg/kg reduced ApoB protein level and Lipoprotein concentration¹⁰. Despite these examples, *in vivo* use of siRNAs upon systemic delivery requires improvements in order to make this technology widely applicable for target validation or therapeutic applications. Indeed, unmodified siRNAs are subject to enzymatic digestion, mainly by nucleases abundant in the blood stream. In order to improve pharmacological properties of siRNAs several groups investigated chemical modification of these reagents. While the approaches described are very different among themselves and that no systematic study was yet performed, an overview of the results allows to determine the tolerance of siRNAs to chemical modifications. Several

chemistries such as phosphorothioates¹⁷ or boranophosphates¹⁸, 2'-O-Methyl¹⁹, 2'-O-allyl¹⁸, 2'-methoxyethyl (MOE) and 2'-deoxyfluoronucleotides¹⁵ or Locked Nucleic Acids (LNA)¹⁶ have been investigated. These studies highlighted that tolerance for modification is not only chemistry-dependent, but also position-dependent.

The present invention provides a minimally modified siRNA with improved pharmacological properties. The minimally modified siRNAs are 19bp double-stranded RNA modified on the 3'-end of each strand in order to prevent 3'-exonuclease digestion: the 3'-dideoxynucleotide overhang of 21-nt siRNA has been replaced by a universal 3'-hydroxypropyl phosphodisester moiety and the modification of the two first base-pairing nucleotides on 3'-end of each strand further enhances serum stability. Applied intraperitoneally or orally to adult mice, the modified siRNAs displayed higher potency in a growth factor induce angiogenesis model which correlates with their increased serum stability.

Summary:

In one aspect, the present invention provides a short interfering ribonucleic acid (siRNA) for oral administration, said siRNA comprising two separate RNA strands that are complementary to each other over at least 15 nucleotides, wherein each strand is 49 nucleotides or less, and wherein at least one of which strands contains at least one chemical modification.

In one embodiment, the siRNA comprises at least one modified nucleotide.

In another embodiment, the siRNA comprises at least one 3' end cap.

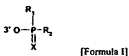
In another embodiment, said modified nucleotide is selected from among 2' alkoxyribonucleotide, 2' alkoxyalkoxy ribonucleotide, a locked nucleic acid ribonucleotide (LNA), 2'-fluoro ribonucleotide, morpholino nucleotide.

In another embodiment, said modified nucleotide is selected from among nucleotides having a modified internucleoside linkage selected from among phosphorothioate, phosphorodithioate, phosphoramidate, boranophosphonate, and amide linkages.

In another embodiment, said two RNA strands are fully complementary to each other.

In another embodiment, said siRNA comprises a 1 to 6 nucleotide overhang on at least one of the 5' end or 3' end.

In another embodiment, the siRNA contains at least one 3' cap, which is chemical moiety conjugated to the 3' end via the 3' carbon and is selected from among compounds of Formula I:



wherein

X is O or S

R₁ and R₂ are independently OH, NH₂, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH₂, SH, carboxylic acid or ester;

or R₁ and R₂ may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S.

In another embodiment, the siRNA contains at least one strand which is complementary over at least 15 nucleotides to the mRNA or pre-mRNA of VEGFR-1, VEGFR-2, VEGFR3, Tie2, bFGFR, IL8RA, IL8RB, Fas, or IGF2R.

In another embodiment, the siRNA contains at least one strand which comprises a sequence selected from SEQ ID NO 1 - 900.

In another embodiment, the siRNA is chosen from the group consisting of SEQ ID NO 901-930.

In another embodiment, the siRNA has a stability in a standard gastric acid assay that is greater than an unmodified siRNA with the same nucleotide sequence.

In another embodiment, the siRNA has a stability in a standard gastric acid assay that is greater than or equal to 50% after 30 minutes exposure.

In another embodiment, the siRNA has a stability in a standard serum assay greater than unmodified siRNA.

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In another embodiment, the siRNA has a stability in a standard serum assay that is greater than or equal to 50% after 30 minutes exposure.

In another embodiment, the siRNA has a stability in a standard intestinal lavage assay that is greater than unmodified siRNA.

In another embodiment, the siRNA has an enhanced oral bioavailability compared to an unmodified siRNA of the same nucleotide sequence.

In one aspect, the invention provides a pharmaceutical composition comprising an siRNA with any one or more of the above properties.

In another aspect, the invention provides an siRNA with any one or more of the above properties for use as a medicament.

In another aspect, the invention provides the use of an siRNA with any one or more of the above properties in the preparation of a medicament for treating an angiogenic disorder.

In another aspect, the invention provides the use of an siRNA with any one or more of the above properties to inhibit an angiogenic process *in vitro*.

Brief Description of the Drawings:

Figure 1a, 1b, 1c, 1d and 1e: Metabolic degradation of unmodified siRNA pGII-siRNA (wild-type siRNA in mouse serum); a-c) Ion Exchange-HPLC analysis of unmodified siRNAs after incubation in mouse serum for 0', 30' and 180'; After 30' of incubation at 37°C, major peak in the Ion Exchange HPLC was isolated and re-injected in LC-MS; d) table of detected molecular weights and their assignments; e) ESI-MS spectrum

Figure 2: Illustration of four double-stranded RNA formats: wild-type (or unmodified) siRNA, MOE o/h siRNA, C3-siRNA and C3-MOE siRNA.

Figure 3: Stability of siRNA in 3 different formats in mouse gastric acid. Samples were incubated at 37°C in mouse gastric acid at a 2 micromolar concentration. Disappearance of parent compound was followed over a 2-6 hours period by quantifying the parent compound band.

Lane 1-7: wild-type siRNA in gastric acid at t=0, 5, 10, 15, 30, 60 and 120 min

Lane 8: ds RNA ladder (30, 21, 19, 16, 13, 10 bp)

Lane 9-13: C3 siRNA in gastric acid at t=0, 5, 10, 15, 30, 60 and 120 min

Lane 16: ds RNA ladder (30, 21, 19, 16, 13, 10 bp)

Lane 17-24: C3-MOE siRNA in gastric acid at t=0, 5, 10, 15, 30, 60 and 120 min

Figure 4: Stability of siRNA in 4 different formats in intestinal lavage. Samples were incubated at 37°C in liver microsomes at a 5 micromolar concentration.

(From left to right)

Lane 1: ds RNA ladder (30, 21, 19, 16, 13, 10 bp)

Lane 2-7: wild-type siRNA in intestinal lavage at t=0, 15, 30, 60, 180 and 360 min

Lane 8-13: moe o/h siRNA in intestinal lavage at t=0, 15, 30, 60, 180 and 360 min

Lane 14-19: C3 siRNA in intestinal lavage at t=0, 15, 30, 60, 180 and 360 min

Lane 20-25: C3-MOE siRNA in intestinal lavage at t=0, 15, 30, 60, 180 and 360 min

Figure 5: Stability of siRNA in 4 different formats in liver microsomes. Samples were incubated at 37°C in intestinal fluid from rat intestinal lavage at a 2 micromolar concentration.

(From left to right)

Lane 1: ds

Figure 6: Stability of siRNA in 4 different formats in mouse serum. Samples were incubated at 37°C in mouse serum at a 2 micromolar concentration. Disappearance of parent compound was followed over a 6 hours period by quantifying the parent compound band.

(From left to right)

Lane 1: ds RNA ladder (30, 21, 19, 16, 13, 10 bp) RNA ladder (30, 21, 19, 16, 13, 10 bp)

Lane 2: wild-type siRNA untreated

Lane 3: moe o/h siRNA untreated

Lane 4: C3 siRNA untreated

Lane 5: C3-MOE siRNA untreated

Lane 6-9: same as 2-5 in liver microsomes t=0

Lane 10-13: same as 2-5 in liver microsomes t=60'

Lane 14-17: same as 2-5 in supernatant S12 t=0

Lane 18-21: same as 2-5 in supernatant S12 t=60'

Lane 2-7: wild-type siRNA in mouse serum at t=0, 15, 30, 60, 180 and 360 min

Lane 8-13: moe o/h siRNA in mouse serum at t=0, 15, 30, 60, 180 and 360 min
Lane 14-19: C3 siRNA in mouse serum at t=0, 15, 30, 60, 180 and 360 min
Lane 20-25: C3-MOE siRNA mouse serum at t=0, 15, 30, 60, 180 and 360 min

Figure 7: Characterization *in cellulo* of 3 formats of anti-VEGFR2 siRNA (2 independent sequences). Wild-type siRNA, C3-siRNA and C3-MOE siRNA were transfected into MSI cells at three concentrations (1, 5, 10 nM). Silencing potency was assessed by measuring VEGFR2 cell surface level by FACS.

Figure 8a and 8b: *In vivo* testing of wild-type siRNA, C3-siRNA and C3-Moe siRNA in a growth factor induced angiogenesis "Agar Chamber" mouse model. Figure 8a shows the results of controls, unmodified VEGFR2 siRNA and C3 modified VEGFR2 siRNA at 1, 5 and 25 micrograms per mouse per day. Figure 8b shows controls, C3 modified VEGFR2 siRNA and of C3-MOE VEGFR2 siRNA at 0.2, 1 and 5 micrograms per mouse per day. In each case pools of 2 anti-VEGFR2 siRNAs were given daily intraperitoneally for three days.

Figure 9: *In vivo* testing of anti-VEGFR2 C3-MOE siRNA given intraperitoneally (i.p.) in a B16 homograft melanoma tumor mouse model at 5 and 20 micrograms per mouse per day. Figure 9a shows that i.p. treatment with modified VEGFR2 siRNA significantly reduces tumour development. Figure 9b also shows that i.p. injection of VEGFR2 siRNA at 20 ug per mouse results in significant inhibition of tumour growth.

Figure 10: *In vivo* testing of C3-MOE siRNA in a growth factor induced angiogenesis mouse model. anti-VEGFR2 siRNAs were given daily orally for three days at 20 micrograms per mouse per day.

Figure 11: *In vivo* testing of C3-MOE siRNA in a growth factor induced angiogenesis mouse model. anti-Tie2 siRNAs were given daily intraperitoneally (1 and 0.2 micrograms per mouse per day) or orally (20 and 5 micrograms per mouse per day) for three days. Figure 11a: weight of excised tissue; Figure 11b: Tie2 protein knock-down

Detailed Disclosure of the Invention:

The present invention relates to compositions and methods for treating angiogenic disorders in a mammal. Specifically, the invention relates to small-interfering RNA ("siRNA") which may be used to treat angiogenic disorders upon oral administration to a mammal.

Angiogenesis targets in vascular endothelial cells include the following targets/genes: VEGFR-1 (GenBank Accession # AF06365); VEGFR-2 (GenBank Accession # AF063658); VEGFR-3 (GenBank Accession # (NM_002020); Tie2 (TEK) (GenBank Accession # NM_000459); bFGFR (GenBank Accession # M60485); IL8RA (GenBank Accession # L19591); IL8RB (GenBank Accession # L19593); Fas (GenBank Accession # X89101); IGFR (GenBank Accession # NM_000876).

The siRNA molecules according to the present invention mediate RNA interference ("RNAi"). The term "RNAi" is well known in the art and is commonly understood to mean the inhibition of one or more target genes in a cell by siRNA with a region which is complementary to the target gene. Various assays are known in the art to test siRNA for its ability to mediate RNAi (see for instance Elbashir et al., Methods 26 (2002), 199-213). The effect of the siRNA according to the present invention on gene expression will typically result in expression of the target gene being inhibited by at least 10%, 33%, 50%, 90%, 95% or 99% when compared to a cell not treated with the RNA molecules according to the present invention.

"siRNA" or "small-interfering ribonucleic acid" according to the invention has the meanings known in the art, including the following aspects. The siRNA consists of two strands of ribonucleotides which hybridize along a complementary region under physiological conditions. The strands are separate but they may be joined by a molecular linker in certain embodiments. The individual ribonucleotides may be unmodified naturally occurring ribonucleotides, unmodified naturally occurring deoxyribonucleotides or they may be chemically modified or synthetic as described elsewhere herein.

The siRNA molecules in accordance with the present invention comprise a double-stranded region which is substantially identical to a region of the mRNA of the target gene. A region with 100% identity to the corresponding sequence of the target gene is suitable. This

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state is referred to as "fully complementary". However, the region may also contain one, two or three mismatches as compared to the corresponding region of the target gene, depending on the length of the region of the mRNA that is targeted, and as such may be not fully complementary. In an embodiment, the RNA molecules of the present invention specifically target one given gene. In order to only target the desired mRNA, the siRNA reagent may have 100% homology to the target mRNA and at least 2 mismatched nucleotides to all other genes present in the cell or organism. Methods to analyze and identify siRNAs with sufficient sequence identity in order to effectively inhibit expression of a specific target sequence are known in the art. Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group).

Another factor affecting the efficiency of the RNAi reagent is the target region of the target gene. The region of a target gene effective for inhibition by the RNAi reagent may be determined by experimentation. A suitable mRNA target region would be the coding region. Also suitable are untranslated regions, such as the 5'-UTR, the 3'-UTR, and splice junctions. For instance, transfection assays as described in Elbashir S.M. et al, 2001 EMBO J., 20, 6877-6888 may be performed for this purpose. A number of other suitable assays and methods exist in the art which are well known to the skilled person.

The length of the region of the siRNA complementary to the target, in accordance with the present invention, may be from 10 to 100 nucleotides, 12 to 25 nucleotides, 14 to 22 nucleotides or 15, 16, 17 or 18 nucleotides. Where there are mismatches to the corresponding target region, the length of the complementary region is generally required to be somewhat longer.

Because the siRNA may carry overhanging ends (which may or may not be complementary to the target), or additional nucleotides complementary to itself but not the target gene, the total length of each separate strand of siRNA may be 10 to 100 nucleotides, 15 to 49 nucleotides, 17 to 30 nucleotides or 19 to 25 nucleotides.

The phrase "each strand is 49 nucleotides or less" means the total number of consecutive nucleotides in the strand, including all modified or unmodified nucleotides, but

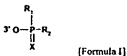
not including any chemical moieties which may be added to the 3' or 5' end of the strand. Short chemical moieties inserted into the strand are not counted, but a chemical linker designed to join two separate strands is not considered to create consecutive nucleotides.

The phrase "a 1 to 6 nucleotide overhang on at least one of the 3' end or 3' end" refers to the architecture of the complementary siRNA that forms from two separate strands under physiological conditions. If the terminal nucleotides are part of the double-stranded region of the siRNA, the siRNA is considered blunt ended. If one or more nucleotides are unpaired on an end, an overhang is created. The overhang length is measured by the number of overhanging nucleotides. The overhanging nucleotides can be either on the 5' end or 3' end of either strand.

The siRNA according to the present invention confer a high in vivo stability suitable for oral delivery by including at least one modified nucleotide in at least one of the strands. Thus the siRNA according to the present invention contains at least one modified or non-natural ribonucleotide. A lengthy description of many known chemical modifications are set out in published PCT patent application WO 2003/0918 and will not be repeated here. Suitable modifications for oral delivery are more specifically set out in the Examples and description herein. Suitable modifications include, but are not limited to modifications to the sugar moiety (i.e. the 2' position of the sugar moiety, such as for instance 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group) or the base moiety (i.e. a non-natural or modified base which maintains ability to pair with another specific base in an alternate nucleotide chain). Other modifications include so-called 'backbone' modifications including, but not limited to, replacing the phosphoester group (connecting adjacent ribonucleotides with for instance phosphorothioates, chiral phosphorothioates or phosphorodithioates). Finally, end modifications sometimes referred to herein as 3' caps or 5' caps may be of significance. As illustrated in Table 1, caps may consist of simply adding additional nucleotides, such as "T-T" which has been found to confer stability on an siRNA. Caps may consist of more complex chemistries which are known to those skilled in the art.

In an embodiment used in the Examples below, the 3' cap is a chemical moiety conjugated to the 3' end via the 3' carbon and is selected from among compounds of Formula I:

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wherein

X is O or S

R₁ and R₂ are independently OH, NH₂, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH₂, SH, carboxylic acid or ester;

or R₁ and R₂ may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S.

Examples of modifications on the sugar moiety include 2'-alkoxyribonucleotide, 2'-alkoxyalkoxy ribonucleotide, locked nucleic acid ribonucleotide (LNA), 2'-fluoro ribonucleotide, morpholino nucleotide.

The internucleotide linkage may also be modified. Examples of internucleotide linkage include phosphorothioate, phosphorodithioate, phosphoramidate, and amide linkages.

R₁ may be OH.

R₁ and R₂ together may comprise from 1 to 24 C-atoms, from 1 to 12 C-atoms, from 2 to 10 C-atoms, from 1 to 8 or from 2 to 6 C-atoms. In another embodiment, R₁ and R₂ are independently OH, lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups as defined above. In another embodiment, R₁ and R₂ are not both OH.

The term "lower" in connection with organic radicals or compounds means a compound or radical which may be branched or unbranched with up to and including 7 carbon

atoms, preferably 1-4 carbon atoms. Lower alkyl represents, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, n-pentyl and branched pentyl, n-hexyl and branched hexyl.

Examples of alkoxy include O-Met, O-Eth, O-prop, O-but, O-pent, O-hex.

Methods for the synthesis of siRNA, including siRNA containing at least one modified or non-natural ribonucleotides are well known and readily available to those of skill in the art. For example, a variety of synthetic chemistries are set out in published PCT patent applications WO2005021749 and WO200370918, both incorporated herein by reference. The reaction may be carried out in solution or, preferably, on solid phase or by using polymer supported reagents, followed by combining the synthesized RNA strands under conditions, wherein a siRNA molecule is formed, which is capable of mediating RNAi.

The present invention provides an siRNA containing at least one modified nucleotide which is suitable for oral delivery. In functional terms this means siRNA will have suitable pharmacokinetics and biodistribution upon oral administration to achieve delivery to the target tissue of concern. In particular this requires serum stability, lack of immune response, and drug like behaviour. Many of these features of siRNA can be anticipated based on the standard gastric acid assays and standard serum assays disclosed elsewhere herein.

In another aspect, the present invention provides methods for the inhibition of a target gene comprising introducing into a cell and siRNA according to the present invention, which is capable of inhibiting at least one target gene by RNAi. Also, more than one species of siRNA, which are each specific for another target region, may be introduced into a cell at the same time or sequentially.

The present invention is not limited to any type of target gene or nucleotide sequence. For example, the target gene can be a cellular gene, an endogenous gene, a pathogen-associated gene, a viral gene or an oncogene. Angiogenic genes are of particular importance to the invention because some of the Examples highlight that the orally delivered siRNA of the invention may accumulate at sites of vasculogenesis, neovascularization or angiogenesis. An updated listing of angiogenic genes at these sites of particular interest for the invention are listed in AngioDB: database of angiogenesis and angiogenesis-related molecules Tse-Kwon

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Sohn, Eun-Joung Moon¹, Seok-Ki Lee¹, Hwan-Gue Cho² and Kyu-Won Kim³, Nucleic Acids Research, 2002, Vol. 30, No. 1 369-371 and online at <http://angiodb.ana.ac.kr/>. Genes of particular significance have been analyzed in detail and are set out elsewhere herein.

In another aspect, the invention also provides a kit comprising reagents for inhibiting expression of a target gene in a cell, wherein said kit comprises dsRNA according to the present invention. The kit comprises at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of the dsRNA according to the present invention to test samples or subjects. In a preferred embodiment, such kits also comprise instructions detailing the procedures by which the kit components are to be used.

"Treatment of an angiogenic disorder" as used in this disclosure means use of a modified siRNA of the invention in a pharmaceutical composition for the treatment of diseases involving the physiological and pathological processes of neovascularization, vasculogenesis and/or angiogenesis. As such, these pharmaceutical compositions are useful for treating diseases, conditions and disorders that require inhibition of neovascularization, vasculogenesis or angiogenesis, including but not limited to cancer tumour growth and metastasis, neoplasm, ocular neovascularization (including macular degeneration, diabetic retinopathy, ischemic retinopathy, retinopathy of prematurity, choroidal neovascularization), rheumatoid arthritis, osteoarthritis, chronic asthma, specific shock, inflammatory diseases, synovitis, bone and cartilage destruction, pannus growth, osteophyte formation, osteomyelitis, psoriasis, obesity, haemangiomas, Kaposi's sarcoma, atherosclerosis (including atherosclerotic plaque rupture), endometriosis, warts, excess hair growth, scar keloids, allergic oedema, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, osteomyelitis, inflammatory and infectious processes (hepatitis, pneumonia, glomerulonephritis), asthma, nasal polyps, transplantation, liver regeneration, leukomalacia, thyroiditis, thyroid enlargement, lymphoproliferative disorders, haematologic malignancies, vascular malformations, and pre-eclampsia.

As used herein, "treatment" means an action taken to inhibit or reduce a process of a disease, disorder or condition, to inhibit or reduce a symptom of a disease, disorder or condition, or to prophylactically prevent the onset or further development of a disease, disorder or condition. "Treat" is the cognitive verb thereof.

An effective dose of the therapeutic agent of the invention is that dose required to treat a disease state. The effective dose depends on the type of disease, the composition used, the

route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of siRNA is administered dependent upon potency. The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, intraperitoneal, or intrathecal injection, or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil. Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions.

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Oral administration of the compositions of the invention include all standard techniques for administering substances directly to the stomach or gut, most importantly by patient controlled swallowing of the dosage form, but also by other mechanical and assisted means of such delivery.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above- indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient. It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

Therapeutic effect of the therapeutic agents of the invention may be enhanced by combination with other agents. Typically such other agents will include agents known for use in treating similar diseases, such as angiogenic disorders. Alternatively, such agents may be used to reduce side-effects or unwanted effects caused by the therapeutic agents of the invention.

The siRNA of the invention also have important research uses. One such study includes research into an angiogenic process *in vitro*. By "angiogenic process *in vitro*" is meant any process for studying angiogenesis or vasculogenesis which does not employ a whole animal. As such, *in vitro* or *ex vivo* methods and assays which study the steps of the angiogenic process using markers or indicators of angiogenesis are included hereby.

RNA Strand Nucleotide Sequences

The siRNA strand sequences identified in Table 1 have been identified as suitable siRNA sequences against the following targets: VEGFR-1 (GenBank Accession # AF06365); VEGFR-2 (GenBank Accession # AF063658); VEGFR-3 (GenBank Accession # NM_002020); Tie2 (TEK) (GenBank Accession # NM_000459); bFGFR (GenBank Accession # M60485); IL8RA (GenBank Accession # L19591); IL8RB (GenBank Accession

L19593); Fas (GenBank Accession # X89101); IGF2R (GenBank Accession # NM_000876).

Table 1: siRNAs against human VEGFR-1, VEGFR-2, VEGFR-3, Tie2, bFGFR, IL8RA, IL8RB, Fas, IGF1R

Target Name	SEQ ID NO	siRNA guide sequence	SEQ ID NO	siRNA complement	SEQ ID NO
VEGFR-1	1731	UUAUAGAAACUUGUUAUCUGTG	1	CAGUUAACAAAGUUCUUAUATT	451
VEGFR-1	1021	UACGGUUUCAAAGCACCUCCTG	2	GCAGGUGCUUGAAACCGUAAT	452
VEGFR-1	1209	UUUUAUUCUCAGCAAGAUUGTA	3	CAAUUCUUCUGAGCAUAAATT	453
VEGFR-1	2904	UUUUCUUCUUGAAAGCCGGAG	4	CCGGCUUUCAGGAAGAUAAATT	454
VEGFR-1	1363	UUUAGGGUUAACCAUUGCGGT	5	CCGAAUUGGUUACCCUCAATT	455
VEGFR-1	1158	UUUGAUUAUUAACGAGUAGCCA	6	GCUACUGUUUAUUAUCAATT	456
VEGFR-1	1091	UUUAGCAUUAACUUCGCGCG	7	CCGSAAGUGUUAUUGGUAAATT	457
VEGFR-1	471	UUAGGUGAGCUAAACCGGCAG	8	CCCGGGUUAUCGUACCUAAATT	458
VEGFR-1	2751	UUUGCUUGAGGUUAGUUGGAG	9	CCAAUACCUCAAGACCAATT	459
VEGFR-1	836	UUUUGCUUUAUACAAGUCCCA	10	GGCAUUGUUAUAGACAAATT	460
VEGFR-1	1254	UUUGACAUUAGAGUGGCGAGT	11	CUGCCACUUAUUGUCAATT	461
VEGFR-1	2375	UUUUAUUGAUAGGUAGUGAG	12	GACUACCUUAUUAUUAATT	462
VEGFR-1	3536	UUUGAGUUGUAAACCCAGUAT	13	AGUGGGUUAUUAUUAUUAATT	463
VEGFR-1	2671	UUCCAUUGUAGUGGCGCCTT	14	GGAGCCCAUUAUUAUUAATT	464
VEGFR-1	1774	UCUUGUUAUUAACUUGCCGAG	15	CGGGACAGUUAUUAUUAATT	465
VEGFR-1	3484	UUUGGGAUGUAGUUAUACCAT	16	GGUAAAGACUUAUUAUUAATT	466
VEGFR-1	2269	UUUUGAGUGUAGUAGUCCAG	17	GGAGCUUUAUUAUUAUUAATT	467
VEGFR-1	525	UUUCCAUAGGGGAUCAAAGT	18	CUUUGAUCCCUAUGGAATT	468
VEGFR-1	789	UUGAACUUCUGUUGUCAAAGG	19	CUUGAACACGAGAGUUAATT	469
VEGFR-1	2246	UAGAGUUGUCCGAGGUUCCTT	20	GGAAUCCUGGAGAAUUAATT	470
VEGFR-1	732	UUUGAGGACAAAGAUUGGCCT	21	GCCAUACUUGUUCUCAATT	471
VEGFR-1	3813	UUACUGUUAUUCUUCAGUCA	22	ACUUGAGAGUUAUUAUUAATT	472
VEGFR-1	3925	UUCCAGCUCAGCGUGGUGTA	23	CGACCACCGUAGUGGUAATT	473

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VEGFR-1	1414	UGCUUCGGAAGUAUAUGGTT	24	CCAUAAUCAUCCGAGCATT	474
VEGFR-1	815	UUGACUCUUUGCUUCACAGTC	25	CCUGUGAAGCAACAGUCAATT	475
VEGFR-1	3300	UCAUCACUUUGUACUCUGGG	26	CAGGAGUACAAUUGGAUATT	476
VEGFR-1	2845	UGGUUUCUUGGCCUUGUUCGAG	27	GGAAACAGGCAAGAACGATT	477
VEGFR-1	2802	UUGGGUCUCAUGUGUAGUGCT	28	CACUACACAUUGAGCCUAATT	478
VEGFR-1	1584	UCUAGAGUACAGCCACACCAA	29	GGUUGUGGUGACUCUAGATT	479
VEGFR-1	1154	UAUUUAACGAGUAGCCACGAG	30	CGUGGCUACUCGUUAUAUATT	480
VEGFR-1	1090	UAACCAUACACUUCGCGCA	31	GCCGGAAGUUUAUGGUATT	481
VEGFR-1	1260	UUCACAUUGACAUUAGAGTG	32	CUCUAUUGUCAUUGAATT	482
VEGFR-1	3530	AUGUAAACCCACUAUUCCTG	33	GGAAUAGUGGUUUAUATT	483
VEGFR-1	1177	AUCCUCUUCAGUAGGUCCCTT	34	GGAGUAUCUGAAGAGUATT	484
VEGFR-1	1183	UUGUAUAUUCGCCUAGUCCCT	35	GAUGCAGGGAUUAUACATT	485
VEGFR-1	1092	UUUAACCAUACAAUUCGGC	36	CGGAAGUUGUAUGUAUATT	486
VEGFR-1	627	UAGAAUAGCCAUUGAGUCTT	37	CAGUCAUUGGCAUUGUATT	487
VEGFR-1	474	AUGUUAUGUGACGUAACCCGG	38	GGGUUACGUACGUACUATT	488
VEGFR-1	2751	UAGUACAGGUUUGCUUGAG	39	CAAGACACUUGAGUUAATT	489
VEGFR-1	2752	UUUGCUUUGAGGUAGUUGGA	40	CAACUACCUACAGAGCAATT	490
VEGFR-1	3516	UUUCCUGUCAGUAUUGCAUTG	41	AUGCCAUUCGACAGGAUATT	491
VEGFR-1	1790	UAUCUGUAGUCAUUGUUGCT	42	AGAACAUAGCUACAGUATT	492
VEGFR-1	1155	AUAUAUACGAGUAGCCACGA	43	GUGGCUACUGGUUAUAUATT	493
VEGFR-1	1370	UUUGAAGGUAGGGGAUACCAT	44	GGUAUCCUCUACCUCAATT	494
VEGFR-1	2227	UUGAACAGUGAGGUAGUCGA	45	AQCAUACCUCAUGUUAATT	495
VEGFR-1	3481	UUUAACCAUCCUGUUGUACATT	46	UGUAACAACAGGAUGUAUATT	496
VEGFR-1	1261	UUUCACAUUGACAAUUGAGT	47	UCUUAUUGUCAUUGAAUATT	497
VEGFR-1	1791	AUACUGUAGUGCAUUGUUCTG	48	GAACAAGUCUACUAGUATT	498
VEGFR-1	3805	UAUCUCUACAGUACAUCUUGAG	49	CAACAUUGACUUGAGUATT	499
VEGFR-1	2764	AAAUAGUCACGUUUGUUCTT	50	GAGCAACGUGACUUAUUAATT	500
VEGFR-2	617	UAUAAGACUGGAACUUUCAT	51	GAAGAGUACCAUGCUUAUATT	501

VEGFR-2	2889	UAGAAGGUUAGCACCAUUGAG	52	CAUUGUGGUCAACCUUUAATT	502
VEGFR-2	561	UAGGUUUAUUGUAGGUGGGA	53	CCAGCUACAUUGACGUUATT	503
VEGFR-2	525	UUGCUGUCCCAAGAAUUGTG	54	GAUUUUCUGGGACAGCAATT	504
VEGFR-2	2277	AUGAUUCCAAUUGCUUUTT	55	AGACGAACUUGGAAUUAATT	505
VEGFR-2	395	UAUUGUACACGAGUCCAUATT	56	CAUGGAGUCUGUAUUAUATT	506
VEGFR-2	2410	UUCAUCUGGUAUCCAUUGATT	57	CGUCAUGGAUCCAGAUUAATT	507
VEGFR-2	2067	UGAUUCCUCCAGGUUUCUGTG	58	CAGGAACCUUGGAGAUUAATT	508
VEGFR-2	1323	UAGACCUUACUUGUACGCTT	59	CGUGACAUUGUACGGUUAATT	509
VEGFR-2	3382	UUUCUGUGUAGUAUAUACAGG	60	UGAUUAUUAUACACCGUAATT	510
VEGFR-2	3078	UUUCUGUGCGCCAGGUCCCTG	61	GGGACCUUGGGCCAGCAUUAATT	511
VEGFR-2	1432	UUUUCACAAAGGUUUGGTT	62	CCCAUACCUUUGGUAUUAATT	512
VEGFR-2	1817	UCAAUUUCCAAAGUAUCCA	63	GAUACUUCUUGGAAUUAUATT	513
VEGFR-2	688	UAGUCAAUUCUUGUACAGCG	64	GUUCUAGGAUUAUUAUUAATT	514
VEGFR-2	2310	AACAUGGCAUACCGCGCTG	65	CGCGCGUUAUUGCAUUAUATT	515
VEGFR-2	2130	UCCUUCAAUUAAGGCUAG	66	CAGGCAUUGUAUUAAGGATT	516
VEGFR-2	799	UACAUGUUUUAUUGGAGTG	67	UCAGCAUUAAGAAUUAUUAATT	517
VEGFR-2	3523	UGAUUCCGAGAGACAUUGTA	68	CAUUGUUCUUGGAAUUAUUAATT	518
VEGFR-2	1843	UGUGCUUAUUAAGAGAUUGTT	69	CAUUCUUCUUAUUAAGCAATT	519
VEGFR-2	2841	UUUCAUACUAGUAGGACTT	70	GUCCUUCAGUAGUUAUUAATT	520
VEGFR-2	2088	UCUUUAACCAUUAUUAUUGT	71	AGAUCAUUGGUUUAUAUUAATT	521
VEGFR-2	472	UCLUUGCACAAGUGACAGCTT	72	CGUGUCAUUUGUGCAAGATT	522
VEGFR-2	180	UGAUUAUUGGCCAAAGCCAG	73	GGCUUUGGCCCAUUAUUAATT	523
VEGFR-2	1588	AUUUUAACAAGCUGACACAT	74	GUGUACGUUUUGUACAUAUUAATT	524
VEGFR-2	3141	UAAUAUCCCGGGCCAGCCA	75	GUUGGCCCGGGUUAUUAUUAATT	525
VEGFR-2	3789	AMCCAUACCAUUGUCCUGCTG	76	GACGACAGUUGUUAUUGUUAATT	526
VEGFR-2	3920	UGUCAUCCGAGUGUAUUCGG	77	GGAUUAUACUUCUUGAUAUUAATT	527
VEGFR-2	1718	UCUCAACGUAUUCUGUCTG	78	GACAGAUUACUUGUUGAUAUUAATT	528
VEGFR-2	2919	UCUUCACAAUCCAGAGCTG	79	GCUCUGAUUUGUGAGGUAUUAATT	529

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VEGFR-2	324	UAAUUGACCGAGGGCGAAGUCA	80	ACUUGGCCUGCGUACUUAUATT	530
VEGFR-2	1050	UAACCAAGGUACUUCGCGAGG	81	CUGCGAAGUACCUUGGUUAATT	531
VEGFR-2	56	UAGGCAAAACCCACAGAGCGGG	82	GCUCUCUGUGGUUUGGCUAATT	532
VEGFR-2	2453	UGGCAUCAUAAGGCGAGUGCTT	83	CGACUGCGCUUAUGAUGCCATT	533
VEGFR-2	1303	UUGAGUGGUUCCCGUAUUGTA	84	CCAGUACGGCCACCAACUCAATT	534
VEGFR-2	1813	UUUCCAAAGAGUAUCCAGTT	85	CUUGGUAUCUCUUGGAAATT	535
VEGFR-2	2015	UUGUCGUCUGAUUCCUCCAGGT	86	CUGGASAAUCAGACGACAATT	536
VEGFR-2	3088	UAAGAGGAAUUAUUCGUCCGCG	87	GGCACGAAUAUUCUCUUAATT	537
VEGFR-2	625	UAUGUACAUUAUAGACUGTA	88	CCAGUCUAUUAUGUACAUAATT	538
VEGFR-2	800	UUACAAGUUUCUUAUGCUGAT	89	CAGCAUAAGAAACUUGUAATT	539
VEGFR-2	811	UAGGUUCUGGUUAUACAAGUTT	90	ACUUGUAAACCGAGACCUAATT	540
VEGFR-2	812	UUAGGUCUCGGUUUACAAGTT	91	CUUGUAAACCGAGACCUAATT	541
VEGFR-2	3093	UCCGAUAGAGGAAUUAUUCGT	92	GAAUAUUCUCUUAUUGCAATT	542
VEGFR-2	801	UUUACAAGUUUCUUAUGUGGA	93	AGCAUUAAGAAACUUGUAATT	543
VEGFR-2	2009	UCUGAUUCUCGAGGUUUCCTG	94	GGAAACCUUGGAGAAUACAATT	544
VEGFR-2	2127	UUCAUAACAAGCCUCUAGUCT	95	ACUACGGCAUUGUUAUGAATT	545
VEGFR-2	1585	UUUGUUGACCGCUUACAUTT	96	AUGUGAAGCGGCUACAATAATT	546
VEGFR-2	562	AUAGCGAUCAUGUAGCUGGG	97	CAGCUACAUGAUCAGCUAATT	547
VEGFR-2	3906	UAUCCGGACUUGGUAAGCGCTT	98	GGCGGUACGAGUCGCGUAATT	548
VEGFR-2	1316	UACAUUGACGCGUUAUGUGG	99	ACUCAACCGCUGACAUUAATT	549
VEGFR-2	3520	UAUCGAGAGAAUUAUUGATC	100	CUACAUGUUAUUCUGUAATT	550
VEGFR-3	453	UUCCUGUUGACCAAGAGCGTG	101	CGCUCUUGGUCAACAGGAATT	551
VEGFR-3	2694	UUAGGUCUCCGACAUACGCGG	102	CGCUGAUUCGGAGCUCAATT	552
VEGFR-3	1689	UUUGAUUCGUAUGGUAAGCGG	103	GCUUACCAUCCGAUUCGAATT	553
VEGFR-3	988	UUCAUUGCAUUAUAGCUCGGT	104	CGAGGUCAUUGUGCAUGAATT	554
VEGFR-3	4374	UUACCAAGGAUUAUUCGCGGG	105	GCCGAUUAUUCUUGUAATT	555
VEGFR-3	2142	UCUUUGUACCAACAGGAGCTG	106	GCAUCGUGUGUAACAAGATT	556
VEGFR-3	1833	UUUGGAGUCGAGCAAGCGGG	107	CGCUUCUGCUCGACUGCAATT	557

VEGFR-3	3903	UUCAGGUACCCUGAAGCCGCTT	108	GCGGCUUCAGGUAGCUGAATT	558
VEGFR-3	3273	UACACCUUUGCGAAGUGCTT	109	GCAUUCUUGCAAGGUGUAATT	559
VEGFR-3	1107	UACCAUCUGAGUCCGCGCGGG	110	CGCCCGAGUUCACAGUGUAATT	560
VEGFR-3	336	UAGCAGAGGUAGCUCUCUGTG	111	CAGGCAGCUAGCUCUUAATT	561
VEGFR-3	2607	UUUGGUAUCCGGAAGCGGAG	112	CCGCUAUKGGCAUCCACAATT	562
VEGFR-3	1556	UCACAGUUAUUAUUCCTT	113	BGAAGAAUUAAGCUGUAATT	563
VEGFR-3	108	UCCGUAUGUUAAGGUCGCGG	114	CGACCUUGAAUACUACGGATT	564
VEGFR-3	1954	AUAUGGCGCCUCUGCUCGCG	115	CGAGCAGAGGGGCCAGUAATT	565
VEGFR-3	2100	AAGCACUCUUCUCCAGCAG	116	CGUGGAGAUUCGUGCUAATT	566
VEGFR-3	693	UACUAGAGCUCUGCCUGTG	117	CAGGCAACGAGCUCUAUAATT	567
VEGFR-3	2337	AGGAUCAGGAUCCCAUGCTG	118	GCAUGGAGAUUGUAUCUATT	568
VEGFR-3	2054	UCAAGUUCUGCGUGAGCCGAG	119	CGGCUACGCGAGAAUUGATT	569
VEGFR-3	860	UCUGUUGGGAGCGUCUCGCG	120	GAGCGACGCGCCCAACAGATT	570
VEGFR-3	2436	UAGCCCGUCUUGAUGUCUGCG	121	CAGCAUACAAGCGGGCUAATT	571
VEGFR-3	3759	UUCAUCCUGGAGGAACCAAGG	122	GUUGUCCUCCAGGAUGAATT	572
VEGFR-3	288	AACACCUUGCAGUAGGGCGCTG	123	GGCCCUAUGCAAGGUGUAATT	573
VEGFR-3	1485	UCCGUGGUAACCGCCGCCAG	124	GGAGCGCGGUGACCAAGATT	574
VEGFR-3	2502	UCGUAAGACAGUUAUUCGAT	125	CGGAUAACCUUCUUAAGATT	575
VEGFR-3	925	AUACGAGCCCGAGUCCGCTG	126	GCACGACUGGGCUCUUAATT	576
VEGFR-3	426	UUUUUAGUAGUAGGCUUGCUA	127	AGCAGCCAUUUAUACAATAATT	577
VEGFR-3	3189	UAGAUGUCCCGGCAAGGCCA	128	GCCUUGCCCGGCAUUAATT	578
VEGFR-3	2274	UUAGGCGAGCGCCUUGGUGCTG	129	GACCCAGGGCGUCCGUAATT	579
VEGFR-3	2186	UUUCGGUUGGAGUCCGCCAAG	130	UAGCGGACUCCCAACCAATAATT	580
VEGFR-3	2019	UGCAACGACAGGUACUUCUTG	131	AGAAGUCCUUGCUGGUGATT	581
VEGFR-3	360	AUGCGUGCUUUGAUGUAUTG	132	AGUACAUAAGGCACGCUAATT	582
VEGFR-3	1755	UACUUGUAGCUGUCGCGUUG	133	AAGCCGACAGCUACAAGUAATT	583
VEGFR-3	3037	UUCCAUUGGUCAGCGGGCUCAG	134	GAGCCCGCUGACCAUGAATT	584

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VEGFR-3	1018	UUUGAGGOCACUCGAGCGUGAT	135	CAGCGUCGAGGUGCUAAATT	585
VEGFR-3	1684	UUCGAUGGUGAGGCGGUCGGG	136	CGACGGGCUUACCAUCGAATT	586
VEGFR-3	2373	UACCAAGGAUAUAUCGGCGGG	137	CGCGAUUAUUCUUGGUATT	587
VEGFR-3	987	UCAUGGCACAAGACCCUGGTG	138	CCGAGGUCUUGUGUCAATT	588
VEGFR-3	3267	UUGUCGAAAGUUGUUCAGGG	139	CUGAAAGCAUUCUGACAATT	589
VEGFR-3	4387	UGUAUUCUCAUAUAUACAAG	140	UGGUAUUGUGUAUAUACATT	590
VEGFR-3	3883	UUUUGUGUUAUGGCGUCCTG	141	GAGCAGGCAUAGACAAGATT	591
VEGFR-3	4376	UAUUAACCAAGGAUAUAUCGG	142	CGAUUAUUCUUGUUAUATT	592
VEGFR-3	2140	UUUGUACCAACAGCAUCUGGG	143	CAGCAUCGUGUGUACAATT	593
VEGFR-3	978	AUGACCUUGGUGCUUCCCGA	144	GGGAGGCAACGAGGUAUATT	594
VEGFR-3	2427	UUGAUGUCUGGUGGCGCGCGG	145	CGGCGCAGCGAGCAUACATT	595
VEGFR-3	1109	UGUACDACUGGAUCUGCGCGG	146	CCCGAUUCCAGUGGUACATT	596
VEGFR-3	319	UGUGUCGUUGGCAUUGACCTC	147	GGUACAUGGCAACGACACATT	597
VEGFR-3	1843	AUGCACGUUCUGGCGAGUCGAG	148	CGAGUGCAAGAGUGCAUATT	598
VEGFR-3	317	UGUGUUGGUGCAUUAUCCGT	149	GAGGUACAUGCCAAAGCAATT	599
VEGFR-3	700	CUUGAUGUCAUAUAGGCUCTT	150	CGAGGCUUAUGAUCCAGTT	600
Tie-2 (TEK)	1223	UAAGCUUAACAUCUGGCCCGT	151	GGGCGAGAUUGUAAGCUUATT	601
Tie-2 (TEK)	2359	UAUCUUCACAUACAACGCGTG	152	GCACGUUGAGUGAGUAUATT	602
Tie-2 (TEK)	706	UAUGUUCACGUUAUUCDCCTT	153	GGGAGUAUACGUGAAUACATT	603
Tie-2 (TEK)	3561	UUUAAGGACACCAUAUUCUGG	154	AGAUAUUGUGUCCUUAUAAATT	604
Tie-2 (TEK)	2763	UGAAAUUUGAUUUAUCCAG	155	CGAAUAGCAUAUAUUAUACATT	605
Tie-2 (TEK)	174	UUGUUAACAAGUUAAGGCAA	156	GCCCUUAUCUUGUAUAACATT	606
Tie-2 (TEK)	1183	UUCAUUGCACUGCAGACCCCTT	157	GGGUCUGGAGUGCAUUAATT	607
Tie-2 (TEK)	805	UAAGAUAUCAGGUUAUCATG	158	UGAAGUACCUUAUAUUCUATT	608
Tie-2 (TEK)	2601	UUCAAUUGCAUAUAUUCAGA	159	UGAUCAUAUUGCAUAUUAATT	609
Tie-2 (TEK)	2277	UAGCCAUCCAAUAUUGUCCAA	160	GGACAAUAUUGGAUGGCUATT	610
Tie-2 (TEK)	1366	UAUUCUUAUAUUAUUGGCAA	161	GCCAGCAUAUAUAGAGUATT	611
Tie-2 (TEK)	32	UUUGGUUAUCAGCAGGGGUGGG	162	CAGCCUCUGCAUACCAUATT	612

Tie-2 (TEK)	4085	UGUAUUAUCAGGGUUAUUGTT	163	CAAUAGCCCUUAUUAUUAATT	613
Tie-2 (TEK)	3881	UUCUGAUUUUACGCCAUUUCTT	164	GAUUGGGGUGAAUAUACAATT	614
Tie-2 (TEK)	648	UUGUUGAGGCAUCUUAUUGTT	165	CAUGAAGAUUGGUGCAACAATT	615
Tie-2 (TEK)	4021	AUAGCAUUAACAUAAGGTA	166	CCUUUAUUGUUAUAUUAUATT	616
Tie-2 (TEK)	209	UUUGUGACAUUCCAUUAAGCAT	167	GCUAUUGGAAAGUACAATAATT	617
Tie-2 (TEK)	4223	UAAAUAGAAACGGGACUGGCTG	168	GCCAGUCCCGUAUUAUUAUATT	618
Tie-2 (TEK)	3961	UAUUAUUGUAUCUACGCCCTT	169	GGCGUGAGUAUAUAUUAUATT	619
Tie-2 (TEK)	1771	UUGGAUAUUGUCCAAAGCCTC	170	GGCUUGGCCAACAUUAUUAATT	620
Tie-2 (TEK)	3909	UUUAUGCAUAUUAAGCAACA	171	UUGGUUAUUAUUAUUAUUAATT	621
Tie-2 (TEK)	3606	UAAAGCGUGGUUAUUCAGUAG	172	ACGUAUAUACCAACGCUUAUATT	622
Tie-2 (TEK)	477	AUUUAGGCUUCAAGUCCCTT	173	GGGACUUUGAAGCGCUUAUATT	623
Tie-2 (TEK)	3421	UUUGGACAAAGUACUCCCGCA	174	CGGGAUGACUUGUGGAGAATT	624
Tie-2 (TEK)	2730	UAAAUUGUAUGAUUUGGUGGTG	175	ACCCAGAUCCUACAUAUUAUATT	625
Tie-2 (TEK)	1800	UAUUAUGAUGUAACAACUACA	176	AGAUUUGUUAACAUAUUAUATT	626
Tie-2 (TEK)	3385	UAAGUUAACAUAUCCCAUAG	177	AUUGGAGAUUUGUUAUUAUATT	627
Tie-2 (TEK)	1692	UAAGGCUACAGGUGUUAUGTG	178	ACAUCAGCUCUGAGCCUUAATT	628
Tie-2 (TEK)	1657	AUUGCCAGUGUAUAUCCGTT	179	CGUGAUUAGACACUGGCAUATT	629
Tie-2 (TEK)	3665	UUUCUUCUUAAGGCCUUCUCTT	180	GAAGCGGCGUAAGGACAAATT	630
Tie-2 (TEK)	2091	UUUAUAGCACCCGAAGUCAAG	181	UGACUUCGUGUGCUUAUUAATT	631
Tie-2 (TEK)	2827	UAACCGAUUCUUAUUAUUGCG	182	CAUCAAGAAGGAUUGGUUAATT	632
Tie-2 (TEK)	1979	UUGGUUGCCAGGUAUAUAUUA	183	AUAUUAAGCCUGGCAUUAUATT	633
Tie-2 (TEK)	87	UAGAUUUGGAUUGGAAAGGCT	184	CCUUUCCCAUUAUUAUUAUATT	634
Tie-2 (TEK)	3459	UUUCCAGGUGUUAUUAUUGCG	185	AGGCGUAUUAUUAUUAUUAUATT	635
Tie-2 (TEK)	2764	UUUUAUAUUGAUUUAUUAUUA	186	GAUUAUUAUUAUUAUUAUUAATT	636
Tie-2 (TEK)	3560	UUUAAGGACACCAUAUUAUUGG	187	CAGAUUAUUGGUUAUUAUUAATT	637
Tie-2 (TEK)	715	UUUUAUAUAUUAUUAUUAUUA	188	CGUUAUAUAUUAUUAUUAUUAATT	638
Tie-2 (TEK)	1368	UUUAUUAUUAUUAUUAUUAUUA	189	CAGAUUAUAUUAUUAUUAUUAATT	639
Tie-2 (TEK)	2351	UUUAUUAUUAUUAUUAUUAUUA	190	CACGUUAUUAUUAUUAUUAUUAATT	640

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Tie-2 (TEK)	205	UGACUUCUUAUAGCAUCGTC	191	CGAUGCUAAUGGAAUGUATT	641
Tie-2 (TEK)	3957	AUUUGUACUCACGGCUUCCTA	192	GGAGGCGUGAGUACAUUUTT	642
Tie-2 (TEK)	3962	AUACUAAUUGUACUCACGCT	193	CGGUGAGUACCAUAGUUAUTT	643
Tie-2 (TEK)	2352	UUUAUUCUACACUACACGUGC	194	ACGUUGAUUGAAGAUAUAATT	644
Tie-2 (TEK)	3983	UAUACUAAUUGUACUACGCC	195	CGUGAGUACAUUAGUAUAATT	645
Tie-2 (TEK)	1777	UGUCACUUGAAUUGUUGCCA	196	GCAACUAAUUCAGUGACATT	646
Tie-2 (TEK)	3388	UCCUAMGCUUACAUUCCCA	197	GGAGAUUGUUGGUUAGGATT	647
Tie-2 (TEK)	636	AUCUUGUUGUUGUUGUUGCTG	198	GGAUACGAACCAUUAUAUUTT	648
Tie-2 (TEK)	74	UCCUUGUUGAGAUUAGGAUGGG	199	CAUCCUAAUUCUACAAAGATT	649
Tie-2 (TEK)	707	AUAUGUUCACGUUUAUUCGCT	200	GGAGUAACGUGUACAUUUTT	650
hFGFR	3814	UAAAUUCUGGUUACGACCT	201	GGUCCUUAACGAGAUUAUUTT	651
hFGFR	1478	UUACACAUAAUUCGACGUTG	202	ACGUGGAGUUAUGUUAUATT	652
hFGFR	3773	UAUACUCAGAUUUAUACACTT	203	GUUGAAUUAUCGAGUUAUATT	653
hFGFR	715	UAGCGGUGCAGAGUGUGGCTG	204	GCCACACUCGACACGCUAATT	654
hFGFR	675	UUCAAACUGACUUCGUGCGG	205	GAGCGAGGGUACGUUUGAATT	655
hFGFR	846	UUCUGCAGUUAAGUGUUGTG	206	CCAACCUUAUCGACGAATT	656
hFGFR	3625	AUCGGAAUUAUAAGGCCACTG	207	GUUGGUUUAUUAUUCGUAUTT	657
hFGFR	2318	UACAAGGGACCAUCCUGGCTG	208	CQCAGGAUGGUCCUUGUAUTT	658
hFGFR	1439	UUGUGGCGGGCAACCCUGCT	209	CAGGUAUGCCCGCAACAATT	659
hFGFR	3860	AUAGCAACUUAUGCCUCCAG	210	GGGAGGCAUGUUGCUUAUTT	660
hFGFR	3183	UGAGGGUUAACGUCAGCGTG	211	CCGUCAGCUGUAACCCUUAATT	661
hFGFR	2600	UCGAUGUGGUGAAUGUCCCTG	212	GGGACAUUACCAUUGGATT	662
hFGFR	2513	UCUGGUGUUAUCACUUCUTG	213	AGAAUGUGCAUACCCGAGATT	663
hFGFR	2214	UUUCUCUGUUGGUGGCCACTT	214	GUCCGACGCAACAGAAUATT	664
hFGFR	1346	UUCUCCACAAGCCAGUGUAG	215	ACACCUCAUUGUGGAUAATT	665
hFGFR	1556	UUGUCUGGSCCAUUCUUGCTC	216	GCAAGAUUGGCCGACAAATT	666
hFGFR	2671	UCGGGUCAAAUAUUGCCUCGG	217	GAGCGAUUUAUUGCCGATT	667
hFGFR	3105	UUUSAGUCCGCCAUUGGCAAG	218	UGCCAUUGGCGGACUCAAATT	668

hFGFR	2991	UUUGCCUAAAGACCAGUCUGTC	219	CAGACUGGUCUUAAGGCAAAATT	669
hFGFR	1590	UCCAGCAGUCUUCAGGAUCTG	220	GAUCUUGAAGACUUGGUAATT	670
hFGFR	1689	UCCGAUAGAGUUAACCGCCAA	221	GCGGGUUAUCUUAUCGGAATT	671
hFGFR	1319	UUGUCAGAGGGGACCAAGAG	222	CUGUGGUGCCCUUGACAAATT	672
hFGFR	2342	UUGGAGGCAUACUCCAGATG	223	UCGUGGAGUUAUGCCUCAAATT	673
hFGFR	107	UUCUGGUCGCCGACCGACGTG	224	CGUCCGUGCGGACCGGAGATT	674
hFGFR	3662	UCUGGUACGAGGCAUUGGTC	225	CCAAUUGCCUGUACCCAGATT	675
hFGFR	2150	UUUGUCCAGCCCGAUAGCCUCT	226	AGGCUAUCGGGCUUGACAATT	676
hFGFR	1517	UUUAGCCACUGGAUGUGCGGC	227	CGCACAUCCAGUGGCUAAATT	677
hFGFR	1264	UGUAGCCUCCAAUUCUGUGGT	228	CACAGAAUGGAGGCUUAATT	678
hFGFR	2576	UUCAAUUGGCGUAGGACAC	229	GCUUCGAGCCAGAUUGAATT	679
hFGFR	613	AUCUCCAUUGGAUACCCACAG	230	GUUGAGUUAUCCAUUGGAUUTT	680
hFGFR	1221	UUUACACCCAGCGCAGUUGGG	231	CACACUCCGUGUUGAAUATT	681
hFGFR	3004	UAGAGCUCCGGUUGUGGGA	232	CCGACACCCCGAGCUUAATT	682
hFGFR	3625	UUACCSAUUGGCUAAUUCUGTC	233	GAGAUUUAUCCAUUGGUUAATT	683
hFGFR	3813	AAAUUCUGUUAACGACCTT	234	GGGUCCUUAACAGAGUAUUTT	684
hFGFR	3861	UUUAGCAACUUAUGCCUCCA	235	GGAGGCAUACGUUGCUUAUTT	685
hFGFR	576	UUUCAAACUAGCCUCCUGUGG	236	AGCGAGGUGUAGUUGAAUATT	686
hFGFR	3772	UAUACAGAUUUAUACAUUTT	237	AGUUGAAUAAUUGAGUAUUTT	687
hFGFR	3624	UACCGAUGGCUAAAUUCUGG	238	AGAGAUUUAUCCAUUGUAUTT	688
hFGFR	2319	AUACAAUGGACCAUUCUGGCT	239	GCAGGAUUGUCCUUGUAUUTT	689
hFGFR	3771	UAUACAGAUUUAUACAUUTT	240	AAGUUGAAUUAUUGAGUAUTT	690
hFGFR	2511	UCGUGUUAUGCAUUCUUGGA	241	CAAGAUGGCAUUAACCCGATT	691
hFGFR	2333	UACUCCAGAGUACAUACAG	242	UGUAUGUUAUCGUGGAGUAUTT	692
hFGFR	3624	UCGGAAUUAUAAGCCACUGG	243	AGUGGCUUUAUUAUUCGATT	693
hFGFR	1304	ACAGAGUCCAUUAUAGUCTC	244	GCACUUAUUAUGGACUUGUUTT	694
hFGFR	1608	UUUGUCUGGUGUUAUUAUCC	245	AGUUAUUAUCCACGACAAATT	695
hFGFR	1301	GACUCCAUUAUUAUUGUCCAG	246	GGAGCAUUAUUAUGGACUUTT	696
hFGFR	3626	UAUCCGAAUUAUUAUGCCACT	247	UGGCUUUAUUAUUCGUAUUTT	697
hFGFR	2672	AUCCGGUCAAAUAUUGCCUGG	248	AGGCAUUAUUAUAGCCGUAUTT	698
hFGFR	2213	UUUCUUCUUGGCGUCCGACUUC	249	AGUGGACGCCAACAGAGAATT	699

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LRRB	219	ACGUUAGCCGAUAAUUCUGT	320	GAAGUUAUACUGUAAAGUUT	773
LRRB	2389	UUGUAGCGUUGUCUGCGUGT	324	ACGAGCAGGACGACUGGAATT	774
LRRB	395	UGUAGGUAGCUUUUUUUGU	325	AGGAGUUAUUUUUUUUGU	775
LRRB	1347	UUGUUGGCGCAUAAAGCGUAG	326	ACGCUUAUUAUUGGCGAATT	776
LRRB	2649	UUUUAAGUGUUAUUAUUAUUA	327	ACGCUUAUUAUUAUUAUUA	777
LRRB	1737	UAGAACAUGAGUGUGCGAUGG	328	AGGAGACUUAUUGUUGUUAU	778
LRRB	455	AUUUUAUUAUUAUUAUUAU	329	GUUGUUAUUAUUAUUAUUA	779
LRRB	955	UACAUUAUUAUUAUUAUUAU	330	GUGUUAUUAUUAUUAUUAU	780
LRRB	1740	UUGUUAUUAUUAUUAUUAU	331	GGCUUAUUAUUAUUAUUAU	781
LRRB	2632	UUGUUAUUAUUAUUAUUAU	332	GUGUUAUUAUUAUUAUUAU	782
LRRB	2755	UAGCGUUGUUAUUUAUUAUUA	333	CGAUUAUUAUUAUUAUUAU	783
LRRB	2183	UUGUUGGUGGUGUAUUAUUAU	334	AGGAGUUAUUAUUAUUAUUA	784
LRRB	2805	UUAUUAUUAUUAUUAUUAUUA	335	GAAGUUAUUAUUAUUAUUA	785
LRRB	2340	UAGCGUUGGUGUAUUAUUAU	336	AGGAGUUAUUAUUAUUAUUA	786
LRRB	2143	UGUGUUAUUAUUAUUAUUAU	337	GAGCAUUAUUAUUAUUAUUA	787
LRRB	998	UUAUUGUGGCUUAUUAUUAUUA	338	GAGCAUUAUUAUUAUUAUUA	788
LRRB	2180	UUGUGGUGUAUUAUUAUUAUUA	339	UAUUAUUAUUAUUAUUAUUA	789
LRRB	2185	UUAUUGUGGUGGUGUAUUAUUA	340	GAUUGUAGCCGCAUUAUUAU	790
LRRB	307	UGUUAUUAUUAUUAUUAUUAU	341	GGUGUUAUUAUUAUUAUUAU	791
LRRB	2481	UGUUAUUAUUAUUAUUAUUAU	342	GAUUGUUAUUAUUAUUAUUA	792
LRRB	2617	UGUGGUGUAUUAUUAUUAUUA	343	AGUUAUUAUUAUUAUUAUUA	793
LRRB	996	UUGUAGGACGCGUUGUUGUGA	344	CGAAGGUGUUAUUAUUAUUA	794
LRRB	456	UAUUUAUUAUUAUUAUUAUUA	345	CGAGUUAUUAUUAUUAUUAU	795
LRRB	1394	UUGUAGCGGUAUUAUUAUUAUUA	347	GUUAUUAUUAUUAUUAUUAU	796
LRRB	458	UUUUUAUUAUUAUUAUUAUUA	348	GAAGUUAUUAUUAUUAUUAU	797
LRRB	2327	UAGCUUUGUUAUUAUUAUUAUUA	350	UAUUUAUUAUUAUUAUUAUUA	800
Fas	109	UUGUUAUUAUUAUUAUUAUUA	351	GAGGAGUUAUUAUUAUUAUUA	801
Fas	41	UUGGAGUUAUUAUUAUUAUUA	352	GUUGUUAUUAUUAUUAUUAU	802
Fas	161	UAUUGGAGUUAUUAUUAUUAUUA	353	GAUGGUGUUAUUAUUAUUAU	803
Fas	182	UUGUAGCGUUAUUAUUAUUAUUA	354	CCGUGUUAUUAUUAUUAUUA	804
Fas	62	UACAUUGGCGUUAUUAUUAUUA	355	AGUUAUUAUUAUUAUUAUUA	805
Fas	377	ACUUCUUAUUAUUAUUAUUAUUA	356	CGAUUGUUAUUAUUAUUAUUA	806
Fas	349	UUGUUAUUAUUAUUAUUAUUA	357	AUUAAGUUAUUAUUAUUAUUA	807
Fas	245	UUCUUCUUAUUAUUAUUAUUAU	358	GUUGGUGUUAUUAUUAUUAU	808
Fas	205	GACUGUUAUUAUUAUUAUUAUUA	359	AGUGGUGUUAUUAUUAUUAU	809

Fas	145	AUGCAUGAUGAGGCGCUGCAACA	350	GGAGGCGGCGCAACGAUGATT	810
Fas	138	UUAGCAUGGACUUAAGUGUATT	351	CUACAGCGGCGGCGCAUGATT	811
Fas	34	UAUUGUACGCAACAGAGGUAAG	352	UAGCUGCGGCGGCGCAUGAATT	812
Fas	114	UACUUGGUAUGCAACAGUCUCC	353	AGGUUUGUUAUUGCAUGUATT	813
Fas	115	CUCAACUGUUAUGAAGUUGATT	354	CGAGUUAUUGUUAUGAUGATT	814
Fas	29	AGCAGCAAGCAAGGAGGCACTG	355	GGUUGUUAUUGUUAUGUATT	815
Fas	122	UUGUAGGUGUACCAACUGGUAUA	356	ACUUAUGUUAUGGCAUGACATT	816
Fas	186	UGUUCUUAACUGGAGAGCAAC	357	UGUCCUUGGAGUUAAGGAATT	817
Fas	42	UGGAGCAAGUAUUGAAGCAACA	358	UGUUGUUAUUGAUAUGUCAATT	818
Fas	111	ACUUAUGUUAACAGUUGUCCUC	359	GGAGGCAUGUUAUGGCAUATT	819
Fas	144	UCUAGUAGCAAGCGCUUCCAAG	370	UGGAGGAGUUAUGCAUGAATT	820
Fas	92	UCAAUUGCAUUGUUGUUGGATT	371	UGAACGGGUAUUGAGUUGATT	821
Fas	291	GGUAGGAGUUGGAGUUGUUDTT	372	GGAAUUGGUGGAGGUAUGATT	822
Fas	128	CCAGUUGUUAUGGACUACACTG	373	GUUGGAGUACAGUACAGUATT	823
Fas	36	UAUUAUUGUUAUGUACAGACATT	374	CGUCUUGUUGUUAUGAUUETT	824
Fas	152	UAUUGGCAAGUAUUGGCGCAUCA	375	AGUGGCGAAUUGGCGCAUAAT	825
Fas	127	CAAGUUGUUAUGGCAUGCAUGT	376	AGUUGGCAUGGCAAGUUGATT	826
Fas	202	UGGAGCAAGGCCUAGUUGUUGU	377	GAAGUUGAAGGGGCAUGCAATT	827
Fas	82	UGUUGUUAUGGAGUUGAGUGATT	378	UGUUGUUAUGGCGUACAGGAATT	828
Fas	190	UGGCGAAGUUGGUGGCAUGCACT	379	UGUUGGCGAAUUGUUGCAUATT	829
Fas	150	UGGCGCAUAUGGAGGCGGCGCT	380	GGGCGUUGUUAUGGAGGCGCAT	830
Fas	63	GUCAUUGCAUGGUAUUAACATT	381	GUUUGUUAUUGGCGAGUGATT	831
Fas	164	UCUUAUGGCGAUAUUGGGCCATT	382	GGGCGAAUUGGCGGAUUGATT	832
Fas	37	CGAAUUAUUGGCAACAGACGCT	383	UGUUGUUGUUGGUAUUGUGGTT	833
Fas	116	UGUUCAGUUGUUAUGGAGUUGATT	384	ACUUGUUAUGGCAUGUGUAGATT	834
Fas	32	AGUCAGCAACAGACAGUAGUAAT	385	CUUAGCGUUAUGUUGUAGUAAT	835
Fas	64	AGUUGUUAUGGCGAUAUGCACT	386	UUGUUAUUGGCGCAUGGCAUATT	836
Fas	167	AGGCGUUAUUGGCGAAGUUGCGT	387	CAUUAUGUUAUGGAGGCGCCU	837
Fas	150	UGGUGUUAUUGGAGUUGGAGUUG	388	UUGUUAUUGGCGUAGGAGUATT	838
Fas	43	UUUUGGCGAAUUAUUGGCGUAG	389	CAAGUUGGAGCGACAGUAGATT	839
Fas	94	UUUUGGCGAAUUAUUGGCGAAG	390	UGUUGGAAUUAUUGGUCAATT	840
Fas	159	UGGCGAUAUUGGCGCAUGAUGA	391	CAAGGAGUUAUUGGCGAUGATT	841
Fas	110	CUUUGAUAUUGGCGAUAUGCAUA	392	UGGUGGCGCAUUAUGGCCAATT	842
Fas	31	UUGUUAUUGGCGAUAUGGAGUATT	393	AGGAGUUAUUGGAGUUGACATT	843
Fas	65	AGCAUUAUUGGCGCAACAGCACT	394	UUGUUAUUGGCGUAGGAGUATT	844
Fas	35	AGGUAUUAUUGGCGAUAUGGCACT	395	UGGUGUUGUUAUUGUUAUGUATT	845

IGF2R	5000	UAGUUGUACAGUUCGUCGCG	431	CGGACGAGCAUUAUCCAUUAT	881
IGF2R	6702	UUGGUGUCAGAGGACGCGGGG	432	CGGCGUGUGUGAGCGGCAAT	882
IGF2R	8479	UUGGUGUGAGGUGUACACUACGA	433	GAGUGAGUGUGUGUCAGAGAT	883
IGF2R	4723	UAGGAGGUGGAGUUAACAGAG	434	GAGGAGGUGUGUGUGUGUAT	884
IGF2R	4237	UAGGACGAGGUGUGGAGUAGCA	435	CGAGUAGGUGUGAUGUUGUAT	885
IGF2R	6703	UAUAGAGGAGGAGGAGGCGUAT	436	AGGAGGUGUGUGUAUUAUAT	886
IGF2R	753	UUGAAGGAGGAGGAGGUGUCGA	437	CGGACGACUUGUGUAUUAUAT	887
IGF2R	8554	UAGGACGAGUAGGUGUGUGUAT	438	CCACCAUUGUGUGUGUGUAT	888
IGF2R	5482	UUGACGUGUAGGUGUGUGUCCCA	439	GAGACGCGGUGUGUGUUAAT	889
IGF2R	1460	UUGGUGUUGGUGUGUAGGUGCTG	440	GUGUAGGUGUGGAAAGGAT	890
IGF2R	5206	UAGGAGGUGAGGAAUACGAGGTT	441	CCGUGAGUAGUUAUUAUAT	891
IGF2R	2559	UAGUAGUUGUUGUGCCAGCTG	442	GUGGUGAGAAUUAUUAUAT	892
IGF2R	8605	UUGAGGUGUGUAGUUAUGUGGA	443	GAGCAUUAAGAGGUGUUAAT	893
IGF2R	4245	UAGGAGGAGGUGUGUUCGCG	444	GAGGAGGUGUGUUAUUAUAT	894
IGF2R	1187	AAUUAUAGGAGGAGGAGCGCG	445	GAGGUGUUAUUAUUAUUAAT	895
IGF2R	1184	UAGGAGGAGGAGGAGGCGGCTG	446	AGGAGGUGUGUUAUUAUUAAT	896
IGF2R	7190	UUGAGUUAUUGGAGGAGGACGT	447	GUGUGUGUACAAUUAUUAAT	897
IGF2R	7192	UUGAGUUAUUGGAGGAGGACGT	448	GUGUCCAGGUGUUAUUAUAT	898
IGF2R	2941	UACUUAUUAAGAGGACGAGCTG	449	CGGACGUGUUAUUAUGGAT	899
IGF2R	3693	UACCGGAGGAGGAGGUGUGGAG	450	CCGACGACGAGCGUUGUAT	900

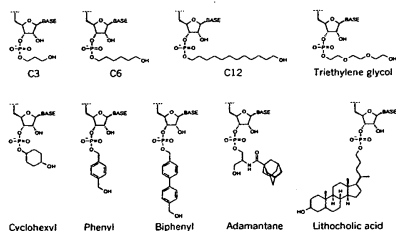
The siRNA according to the invention may comprise at least one modified nucleotide in at least one of the RNA strands. A range of potential modified nucleotides are disclosed elsewhere herein. Useful modifications and combinations of modifications for use according to the invention are shown in Table 2:

Table 2: Chemical Modifications and Sequence Architecture[illegible]

4	Blunt-ended	NNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN
5	2'-OMe o/h	NNNNNNNNNNNNNNNNNNNNN ^{NP} N ^{NP} NNNNNNNNNNNNNNNNNNNN
6	2'-OMe 2'F	NNNNNNNNNNNNNNNNNNNNN ^{NP} N ^{NP} NNNNNNNNNNNNNNNNNNNN
7	LNA (3-7 incorporations)	NNNNNNNNNNNNNNNNNNNNssnn nssnnNNNNNNNNNNNNNNNNNN

N = any unmodified RNA nucleotide
n = unmodified DNA nucleotide
N^p = modified RNA nucleotide
s = identifies phosphorothioate internucleoside linkage
o/h = overhang

The following modifications added to the 3' position of the 3'-terminus of the siRNA strands, sometimes referred to as a '3' end cap' are also recognized as useful embodiments of the invention and may be used with any of the siRNA according to the invention:



Specific compounds with activity according to the invention include the following, shown in Table 3:

Table 3: Sequences and Chemistries of siRNA used in Examples

Name	strand	Sequence (5' RNA, 3' DNA; Z: 5' RNA; A: phosphothioate)	SEQ ID NO
pC3-sRNA	guide strand	UCG AAG UAC UUA CGG UAA GATtt	901
	complement strand	CUU ACG CUG AGU ACU UCG AATtt	902
pC3-3' MDE oh sRNA	guide strand	CUU ACG CUG AGU ACU UCG ASH	903
	complement strand	UGG AGU UUC UUA CGG UAA GSH	904
pC3-C3-sRNA	guide strand	UCG AAG UAC UUA CGG UAA G-C3	905
	complement strand	CUU ACG CUG AGU ACU UCG A-C3	908
pC3-C3-MDE-sRNA	guide strand	UCG AAG UAC CUG CUG A-C3	907
	complement strand	CUU ACG CUG AGU ACU UCG A-C3	908
VEGF ₂ -sRNA1	guide strand	UUU AGG UUU GAA AUC GAC C-G3	909
	complement strand	GGU CGA UUU CAA ACC UUA A-C3	910
VEGF ₂ -sRNA2	guide strand	UUA UUG UUU CCU GUC UUC C-G3	911
	complement strand	CGA AGC CAG GAA GAA UUG AATtt	912
sRNA control	guide strand	ACG UGA CAC GUU CGG AGA AATtt	913
	complement strand	UUC UCC GAA CGU GCG AGC UATtt	914
VEGF ₂ -C3-sRNA1	guide strand	UUU AGG UUU GAA AUC GAC C-C3	915
	complement strand	GGU CGA UUU CAA ACC UUA A-C3	916
VEGF ₂ -C3-sRNA2	guide strand	UUA UUG UUU CCU GUC UUC C-C3	917
	complement strand	CGA AGC CAG GAA GAA AUU A-C3	918

C3-siRNA control	guide strand	ACG UGA CAC GUU CGG AGA A-C3	919
	complement strand	UUC UCC GAA CGU GUC ACG U-C3	920
VEGF162-C3-MOE-siRNA1	guide strand	UUC AGG UUU GAA AUC GAc c-C3	921
	complement strand	GGU CGA UUU CAA ACC UCA p-C3	922
VEGF162-C3-MOE-siRNA2	guide strand	UUA UUA GUU CCU GUC UUC c-C3	923
	complement strand	CGA AGA CAG GAA CAA AUU a-C3	924
Tie2-C3-MOE-siRNA1	guide strand	UUC UUC UUU AAU UAA CAc c-C3	925
	complement strand	GUU GUU AAU UAA AGA AGa p-C3	926
Tie2-C3-MOE-siRNA2	guide strand	UCU GAG UAU GUA AAU AUc g-C3	927
	complement strand	CGA UUU UUA CAA AGU CAg p-C3	928
C3-MOE-siRNA control	guide strand	ACG UGA CAC GUU CGG AGa p-C3	929
	complement strand	UUC UCC GAA CGU GUC ACg t-C3	930

Examples:

The following Examples illustrate aspects of the invention, and are not intended to limit the embodiments included in the claims recited below. The results and discussion section further below refers to experiments conducted according to the following protocols and employing the following materials. Materials and protocols that are not specifically described are considered to be routinely available to those skilled in the art.

Example 1

Preparation of siRNAs.

Single strand siRNA derivatives were synthesized by standard 2'-O-TOM phosphoramidite technology and purified by Oasis® HLB Extraction Plates (Waters). Sense- and antisense

stranded siRNA were mixed in hybridization buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM Hepes, pH 7.6) heat-denatured at 90° C for 3 min and annealed at 37° C for 60 min. 100 µM stock solutions of siRNA duplexes were stored at -20° C.

Example 2

Incubation in Serum and analysis by IE-HPLC (LC-MS).

In a standard serum assay, 6 µL 20 µM of each siRNA were mixed with 54 µL serum or CSF and heated at 37° C in an incubator. 50 µL of the cooled mixture was loaded on an analytical DNA-pac PA-100 Column (Dionex) and analyzed with a NaCl gradient (0 – 0.6 M in 30 min) in a 1:10 Acetonitrile:Buffer (20 mM sodium acetate, 1 mM magnesium acetate, pH 6.5) solution.

For LC-MS analysis 100 µL (20 µM or 50 µM) each siRNA was mixed with 900 µL sterile fetal bovine serum (GIBCO) incubated at 37° C and separated by HPLC as indicated previously (except of the NaCl gradient: 0M - 0.36M in 9' / 0.36M - 0.6M in 12'). Degradation products were desalted on NAP columns and analyzed by LC-ESI-MS.

Example 3

Incubation in gastric acid

To prepare a standard gastric acid assay, FVB and C57BL6 mice, weighing 18 to 20 g (6 to 8 weeks old), were obtained from Charles River Laboratories (Les Oncins, France). Animals were sacrificed using CO₂, and then stomachs were quickly recovered. Gastric fluid as well as stomach contents were collected and pooled, then loaded on centrifugal filter devices (Ultrafree MC, Millipores). Filter units were spun for 10 minutes according to manufacturer's recommendations. The filtrate, corresponding to mouse gastric fluid, was recovered, aliquoted and frozen prior further experiments.

For each assay, 20 µM of siRNA solutions were diluted in 9x volume of gastric acid as above described and incubated at 37°C for 0, 5, 10, 15, 30, 60 and 120 min.

Example 4

Incubation in intestinal lavage

To prepare a standard intestinal lavage assay, Male Wistar rat were fasted, anesthetized with isoflurane. Intestinal lavage was obtained by in situ perfusion of the small intestine (duodenum, jejunum, ileum) with 10 mL saline (0.5 mL/min) followed by 20 mL water (1

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mL/min). Outlet collected was centrifuged (3000 x g, 15 min, 22°C), and supernatant passed through a 1.2-µm filter and stored at -20°C.
For each assay, 20 µM siRNA solutions were diluted in 9x volume of intestinal lavage and incubated at 37°C for 0, 15, 30, 60, 180 and 360 min.

Example 5
Incubation in mouse liver microsomes

In a standard liver microsome assay, to 10 µl of a 250 µM solution of siRNA were added 25 µl of mouse liver microsomes (GEtest 452701 Charge 11) at 20 mg protein /ml, 365 µl of 100 mM phosphate buffer (pH 7.4), 50 µl of UDPGA cofactor (24 mM in water), 50 µl of NADPH. Incubation was quenched by freezing at t=0 min and t= 60 min.

Example 6
Incubation in rat S12 supernatant

For a standard rat S12 supernatant assay, 10 µl of a 250 µM solution of siRNA were added to 17 µl of rat liver S12 at 29.9 mg protein /ml, 373 µl of 100 mM phosphate buffer (pH 7.4), 50 µl of UDPGA cofactor (24 mM in water), 50 µl of NADPH. Incubation was quenched by freezing at t=0 min and t= 60 min.

Example 7
Incubation in mouse serum

For a standard incubation in mouse serum, 20 µM siRNA solutions were diluted in 9x volume of murine serum (Harlan nude mouse) and incubated at 37°C for 0, 15, 30, 60, 180 and 360 min.

Example 8
Gel electrophoresis stability assay.

A 10 µL aliquot of incubation solution was taken immediately after shaking and shock-frozen on dry ice, the mixtures were incubated at 37° C and aliquots were shock frozen at various time points. Aliquots were thawed in 30 µL (15 µL respectively) Loading Buffer (Elchrom Sc., Cham, Switzerland) and separated on a SF30 gels (Elchrom Sc., Cham, Switzerland) at 120 V, 8° C for 240 min. Bands were stained with SYBR Gold (Molecular Probes) and picture were taken with a BIORAD ChemiDoc™ XRS system.

Example 9
Cell culture

The mouse immortalized endothelial cell line MSI (ATCC CRL-22179) was grown in DMEM high glucose (4.5g/l) supplemented with L-Glutamine and 10% heat-inactivated FCS (AMIMED, Switzerland) on 1.5% Gelatine-coated culture dishes. MSI cells were transfected in 24 well-format with siRNA using HiPerfect (QIAGEN) according to manufacturer procedure (tetraplicate, final siRNA concentration was 10nM or as indicated).

Example 10
FACS analysis

Non-transfected and siRNA transfected MSI cells were analyzed by FACS for VEGFR2 levels. Briefly, cells were trypsinized from duplicate or triplicate wells, pooled for each conditions, then washed twice with PBS+10% FCS and incubated 10 minutes on ice prior addition of RPE-conjugated anti-VEGFR2 Ab (1µg/10⁶ cells; Avas 12e1, BD Pharmingen). RPE-labeled isotype IgG2α were used as FACS control (BD Pharmingen). FACS acquisition and analysis were performed on a FACScalibur using Cell Quest Software (Becton-Dickinson).

Example 11
Animal studies

Female FVB mice (6 to 8 weeks old), were obtained from Charles River Laboratories (Les Oncins, France). Mice were identified via ear markings and kept in groups (6 animals per cage) under normal conditions and observed daily. Six mice were used per treatment group and all animal experiments were performed in strict adherence to the Swiss law for animal protection.

The reference chamber model has been described in publications (e.g. Wood J, Bold G, Buchdunger E, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res 2000;60:2178-89). In brief, porous tissue chambers made of perfluoro-alkoxy-Teflon (Teflon®-PFA, 21 mm x 8 mm diameter, 550 µl volume) were filled with 0.8% agar (BBL® Nr. 11849, Becton Dickinson, Meylan, France) and 20U/ml heparin, (Novo Nordisk A/S, Bagsvaerd, Denmark) supplemented with or without 3 µg/ml recombinant human VEGF and siRNAs as indicated.

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Solutions were maintained at 42 °C prior the filling procedure. Mice were anesthetized using 3% Isoflurane (Forane®, Abbott AG, Cham, Switzerland) inhalation. For subcutaneous implantation, a small skin incision was made at the base of the tail to allow the insertion of an implant trocar. The chamber was implanted under aseptic conditions through the small incision onto the back of the animal. The skin incision was closed by wound clips (Autoclip 9 mm Clay Adams). Depending on the required dose, siRNAs were diluted in "injectable quality grade" 0.9% saline solution then delivered to animals either i.p. (200µL /dose) or p.o. by gavage (100µL /dose). The mice were receiving the first dose 2 to 4 hours before implanting chambers; then treated daily for 2 days. If not otherwise indicated, mice were sacrificed three days after implantation, chambers excized and the vascularized fibrous tissue formed around each implant carefully removed. Body weight was used to monitor the general condition of the mice. Statistical analysis was done using one-way ANOVA followed by Dunnett test.

Example 12

B16 melanoma xenograft model

The syngeneic B16/BL6 murine melanoma model, previously identified to be responsive to antiangiogenic therapy (e.g. LaMontagne K, Littlewood-Evans A, Schnell C, O'Reilly T, Wyder L, Sanchez T, Probst B, Butler J, Wood A, Liu G, Billy E, Theuer A, Hla T, Wood J. Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. Cancer Res. 2006 Jan 1;66(1):221-31), was used to evaluate the antitumor activity of standard or modified siRNAs. Tumor cells (1 µL, 5 x 10⁵µL) were injected intradermally into the dorsal pinna of both ears of syngeneic female C57BL/6 mice. Measurements of primary tumor area (mm²) were carried out on days 7, 14, and 21 after tumor cell inoculation using computer-assisted image analysis software (KS-400 3.0 imaging system, Zeiss) and a specifically designed macro. From days 7 to 21, mice were receiving siRNAs diluted in "injectable quality grade" 0.9% saline solution either i.p. (200µL /dose) or p.o. by gavage (100µL /dose) once a day. Mice were sacrificed on day 21, and cranial lymph node metastases were weighed and then frozen.

In these results, actual siRNA sequences and chemistries employed may be determined by reference to Table 3.

Wild-type siRNAs are degraded in mouse serum from both 3'-ends

Oligonucleotide degradation by nucleases is predominantly 3'-exonucleolytic. Modification of antisense oligonucleotides at their termini by the introduction of aromatic or lipophilic residues delays their nucleolytic degradation¹⁷. To verify whether this metabolic pathway would also be dominant for siRNA, we incubated at 37°C a unmodified siRNA (wild-type siRNA) in mouse serum for up to 3 hours.

The unmodified siRNA sequence employed was pG13-siRNA (see Table 3)

The mixtures were analyzed with Strong Anion Exchange HPLC at t=0 min., t=30 min, t= 180 min.

As shown in Figure 1a, 1b and 1c, at t= 30 min, a well defined peak corresponding to blunt ended siRNA was observed. By t=3h substantial degradation is observed. Figure 1d and 1e illustrate the metabolites identified by HPLC-ESI-MS analysis. This analysis revealed the presence of several metabolites corresponding to the loss of the 3' overhangs and of the 3'-terminal first base pairing ribonucleotide on both strands. Digestion of the 5'-terminal ribonucleotide of the guide strand was also observed.

Figure 1 suggests the degradation pathway of unmodified siRNAs in serum. DNA overhangs are first digested, possibly by 3'-exonucleases. In the LC-MS, additional metabolites were also detected which correspond to the loss of the first base-pairing 3'-ribonucleotide of both strands and also the first 5'-base-pairing ribonucleotide of the guide strand.

3'-modified siRNAs are stable through the GI tract

siRNAs with 2'-methoxyethyl ribonucleotides overhangs (MOE on siRNA), blunt-ended siRNAs 3'-capped with a hydroxypropoxy phosphodiester moiety (C3-siRNA), and hydroxypropoxy phosphodiester 3'-capped siRNAs where the two first base pairing nucleotide at 3'-end of each strand were modified by 2'-methoxyethyl ribonucleotides residues (C3-MOE siRNA) were synthesized. These compounds are illustrated schematically in Figure 2.

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First siRNAs were incubated in mouse gastric acid for 2h (Figure 3). No degradation was observed in the cases of C3 siRNA and C3-MOE siRNA, while degradation of wild-type siRNA was observed after 30 minutes.

Stability in intestinal fluid obtained from intestinal lavage of rats revealed almost complete degradation of wild-type siRNA after 15 minutes whereas parent compound in the MOE o/h siRNA, C3-siRNA and C3-Moe siRNA were observed for 60 minutes. (Figure 4)

Stability in liver was evaluated using a liver microsome assay and a S12 assay (representative of liver cytosolic enzymatic activity). Results are shown in Figure 5. In both cases, no degradation was observed after 60 minutes of incubation.

Finally, siRNAs were tested in mouse serum by incubation at 2 micromolar for up to 6 hours at 37°C (results in Figure 6). Parent compound stability was followed by gel electrophoresis. In the cases of modified siRNAs (C3 siRNA, C3-MOE siRNA of MOE o/h siRNA), no significant degradation was observed while the wild-type siRNA

This study indicate that wild type (unmodified) siRNAs are metabolized in mouse gastric acid and in mouse serum. In case of 3'-ends modified siRNAs, no degradation was observed in the GI tract. Therefore it is likely that 3'-modified siRNAs will have a higher oral bioavailability than wild-type siRNAs

Systemically delivered 3'-modified siRNAs are more active in an *in vivo* growth factor induced angiogenesis model¹⁸.

Firstly, the ability of modified siRNAs (C3-siRNA and CE-MOE siRNA) to down regulate a target gene was checked in cellulo by measuring VEGFR2 surface level of MS1 cells transfected with anti-VEGFR2 siRNAs.

Pools of 2 anti VEGFR2 siRNAs as wild-type siRNAs, C3-siRNAs and C3-MOE siRNAs were administered intraperitoneally. Results are shown in Figure 7. Pooled Wild type siRNAs reduced significantly the VEGF induced vascularization at the higher dose of 25 micrograms per mice per day. The same level of inhibition was observed at a 5-fold lower dose with C3-siRNA. In the case of the C3-MOE siRNAs pool, significant reduction of vascularized tissue weight was observed at all tested doses including the lowest 0.2 microgram per mouse per day.

Figure 8a and 8b show that, when given intraperitoneally, both VEGFR2 - C3 and C3-MOE siRNAs were active at below 1 microgram per mouse per day dose.

In vivo testing of anti-VEGFR2 C3-MOE siRNA given intraperitoneally (i.p.) in a B16 homograph melanoma tumor mouse model. Figure 9a shows that i.p. treatment with modified VEGFR2-C3-MOE-siRNA significantly reduces tumour development. Figure 9b also shows that i.p. injection of VEGFR2-C3-MOE-siRNA at 20 ug per mouse results in significant inhibition of tumour growth.

Oral Delivery of siRNA for Treatment of Angiogenic Disorders

Figure 10 shows that given orally, at a dose of 20 micrograms per mouse per day, the VEGFR2-C3-MOE-siRNA1 reduced vascularization weight down to basal level (e.g. weight without growth factor induction). Actual siRNA sequences used are referred to in Table 3.

Anti Tic2 C3-MOE siRNAs were also tested in the growth factor induced angiogenesis model under both intraperitoneal and oral deliveries. Figure 11a and 11b show that given orally, both C3-MOE siRNAs directed at Tic2 were active at 20 microgram per mouse per day. Actual siRNA sequences used may be determined by reference to Table 3.

The data shows that 3'-end modified siRNAs with or without additional internal modifications are able to demonstrate therapeutic effect at reasonable doses upon oral administration.

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We Claim:

1. Short interfering ribonucleic acid (siRNA) for oral administration, said siRNA comprising two separate RNA strands that are complementary to each other over at least 15 nucleotides, wherein each strand is 49 nucleotides or less, and wherein at least one of which strands contains at least one chemical modification.
2. siRNA according to claim 1, comprising at least one modified nucleotide.
3. siRNA according to claim 1 or 2 wherein said siRNA comprises at least one 3' end cap.
4. siRNA according to claim 2 or 3, wherein said modified nucleotide is selected from among 2' alkoxyribonucleotide, 2' alkoxyalkoxy ribonucleotide, a locked nucleic acid ribonucleotide (LNA), 2'-fluoro ribonucleotide, morpholino nucleotide.
5. siRNA according to any one of claims 2-4 wherein said modified nucleotide is selected from among nucleotides having a modified internucleoside linkage selected from among phosphorothioate, phosphorodithioate, phosphoramidate, boranophosphonate, and amide linkages.
6. siRNA according to any one of claims 1-5 wherein said two RNA strands are fully complementary to each other.
7. siRNA according to claim 1-6 comprising a 1 to 6 nucleotide overhang on at least one of the 5' end or 3' end.
8. siRNA according to any one of claims 3-7 wherein said at least one 3' cap, when present, is a chemical moiety conjugated to the 3' end via the 3' carbon and is selected from among compounds of Formula I:



[Formula I]

wherein
X is O or S
R₁ and R₂ are independently OH, NH₂, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and

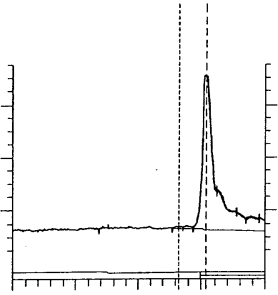
functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH₂, SH, carboxylic acid or ester;

or R₁ and R₂ may be of formula Y-Z, where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S.

- 9. siRNA according to any one of claims 1-8 wherein at least one strand is complementary over at least 15 nucleotides to the mRNA or pre-mRNA of VEGFR-1, VEGFR-2, VEGFR3, Tie2, bFGFR, IL8RA, IL8RB, Fas, or IGF2R.
- 10. siRNA according to any one of claims 1-9 wherein at least one strand comprises a sequence selected from SEQ ID NO 1 - 900.
- 11. siRNA chosen from the group consisting of SEQ ID NO 901-930.
- 12. siRNA according to any one of claims 1 to 11 having stability in a standard gastric acid assay that is greater than an unmodified siRNA with the same nucleotide sequence.
- 13. siRNA according to any one of claims 1 to 11 having stability in a standard gastric acid assay that is greater than or equal to 50% after 30 minutes exposure.
- 14. siRNA according to any one of claims 1 to 11 having stability in a standard serum assay is greater than unmodified siRNA.
- 15. siRNA according to any one of claims 1 to 11 having stability in a standard serum assay is greater than or equal to 50% after 30 minutes exposure.
- 16. siRNA of claim 1 to 11 having stability in a standard intestinal lavage assay that is greater than unmodified siRNA.
- 17. siRNA according to any one of claims 1 to 11 having an enhanced oral bioavailability compared to an unmodified siRNA of the same nucleotide sequence.
- 18. Pharmaceutical composition comprising an siRNA according to any one of claims 1-17.
- 19. siRNA according to any one of claims 1 to 17 for use as a medicament.

- 20. Use of an siRNA according to any one of claims 1 to 17 in the preparation of a medicament for treating an angiogenic disorder.
- 21. Use according to claim 20, wherein the gene targeted by the siRNA is expressed on endothelial cells.
- 22. Use according to claim 21, wherein the gene targeted by the siRNA is chosen from the group consisting of: VEGFR-1 (GenBank Accession # AF063653); VEGFR-2 (GenBank Accession # AF063658); VEGFR-3 (GenBank Accession # NM_002020); Tie2 (TEK) (GenBank Accession # NM_000459); bFGFR (GenBank Accession # M60485); IL8RA (GenBank Accession # L19591); IL8RB (GenBank Accession # L19593); Fas (GenBank Accession # X89101); IGF2R (GenBank Accession # NM_000876).
- 23. Use of an siRNA according to any one of claims 1 to 22 to inhibit an angiogenic process *in vitro*.

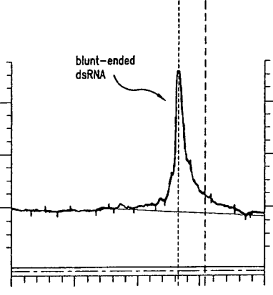
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t=0'

FIG. 1a

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t=30'

FIG. 1b

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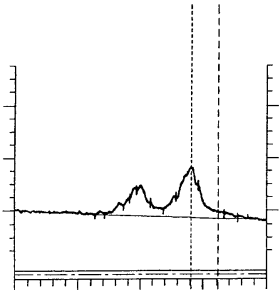
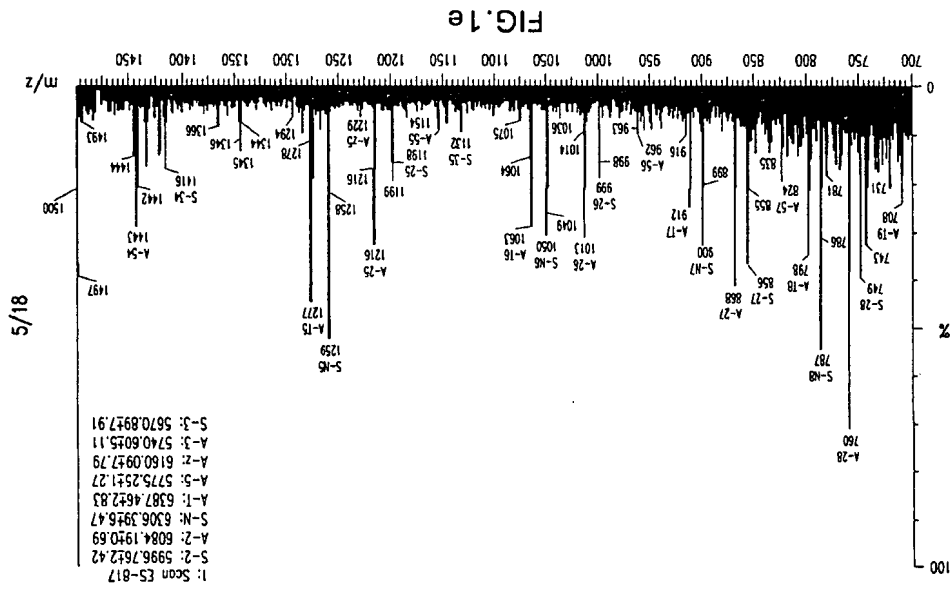
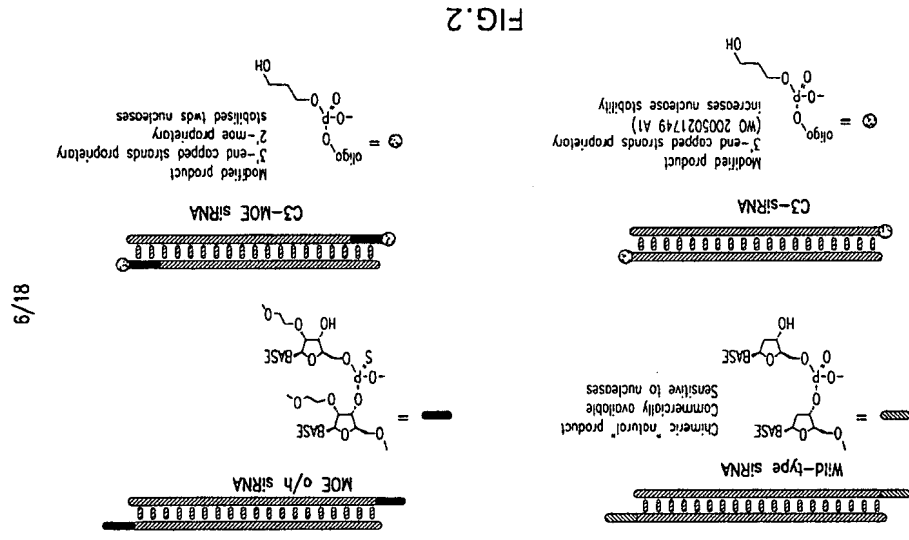


FIG.1c

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LC-MS analysis			
Compound	Sequence	Mh	found
parent_compound	QIAGDQIAGIAGIAGIAGI	6807.32	
compound -1p	QIAGDQIAGIAGIAGIAGI	6302.9	6308.4
compound -1p1p	QIAGDQIAGIAGIAGIAGI	5988.7	5986.8
compound -1p1p -1p	QIAGDQIAGIAGIAGIAGI	5655.5	5670.9
parent_guide	UCIAGIAGIAGIAGIAGI	6893.37	
guide -1p	UCIAGIAGIAGIAGIAGI	6385.1	6387.3
guide -1p1p	UCIAGIAGIAGIAGIAGI	6084.8	6084.2
guide -1p1p -1p	UCIAGIAGIAGIAGIAGI	5739.6	5740.6
guide -1p1p -1p1p	UCIAGIAGIAGIAGIAGI	5778.7	5775.2

FIG.1d



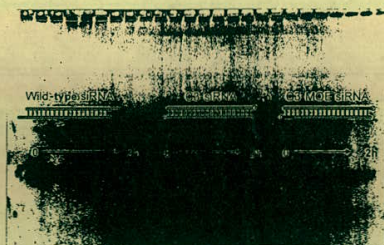


FIG.3

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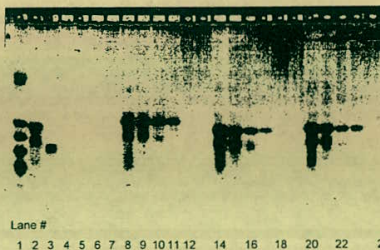
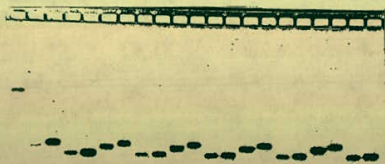


FIG.4

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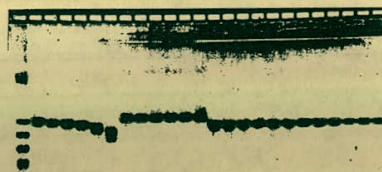


Lane #
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 21

FIG.5

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Lane #
1 2 3 4 5 6 7 8 9 10 11 12 14 16 18 20 22 25

FIG.6

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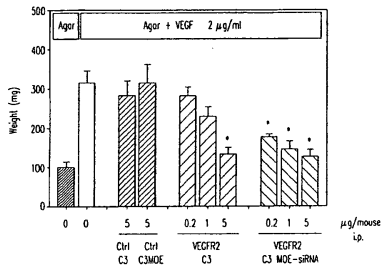


FIG.8b

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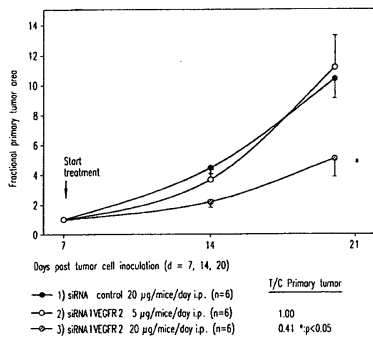


FIG.9a

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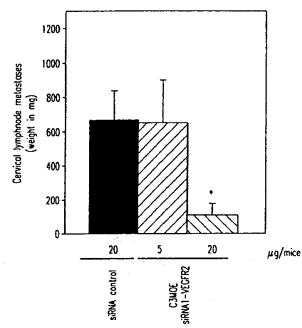


FIG.9b

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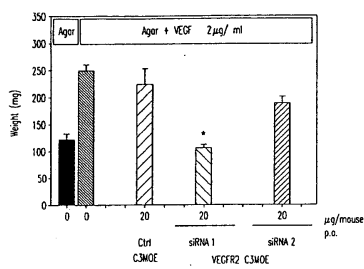


FIG.10

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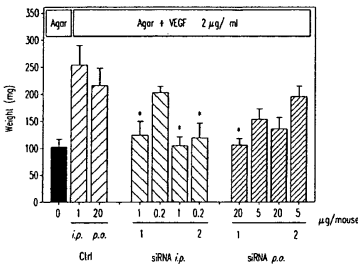


FIG.11a

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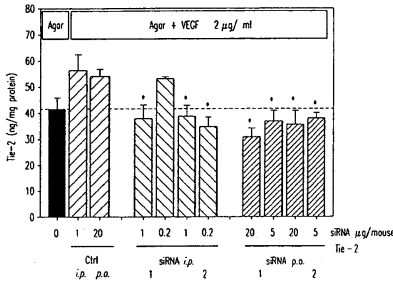


FIG.11b

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ANEXO 2

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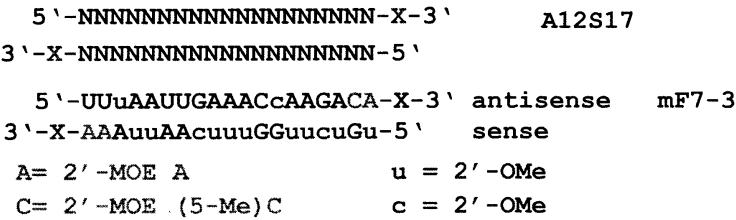
*Serum stability of siRNAs
with chemically capped 3'-terminal overhangs*

1 | Jan Weller | 17-June-08 | Business Use Only

 NOVARTIS

Experimental design

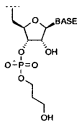
- ▣ 10 siRNAs with identical sequence (mF7-III, 19-mer blunt, A12S17)
- ▣ 10 different non-nucleotidic 3'-terminal caps
- ▣ Tested in mouse and human sera at 4 time points
- ▣ Parent mF7-III in A6S11 format and wt luc siRNAs as controls



X = C3 REDACTED

000047

Structures non-nucleotidic overhangs



REDACTED

C3

Materials & Methods

- RNA samples were incubated in 100% mouse serum and human serum at 37°C, withdrawn at 0, 5', 6h and 24 h time points and snap-frozen. Oligos were separated by precast hydrogels (Elchrom Scientific) and visualized with SYBR gold (Biorad, Chemidoc XRS).

siRNAs

Sample ID	siRNA ID	Project	Strain	ID	Format	siRNA passenger sequence	ID	Format	siRNA guide sequence
S1	144033	mFVII 3'-caps	Mouse	144037	S17	uGu cuu GUu uuc AAu uA5 5' C3	144033	A12	UUu AAU UGA AAC cAA GA5 5' C3

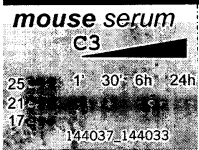
REDACTED

000050

Enzymatic stability

Pascal Basselet, Jan Weiler
NBC/siRNA Therapeutics

REDACTED



REDACTED

000051

Conclusions

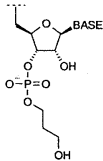
- in mouse serum all 3'-capped A12S17 siRNAs display high resistance up to 24h

REDACTED

Profiling of 3'-Ligand Set in F7 In Vivo Model

3'-Conjugates of 19-bp blunt-ended siRNAs:

- 3'-Conjugates increase nuclease resistance in vitro, may bind to serum proteins
- In vitro potency of conjugates very similar
- Biochemical assay with recombinant Ago2 shows differential cleavage activities

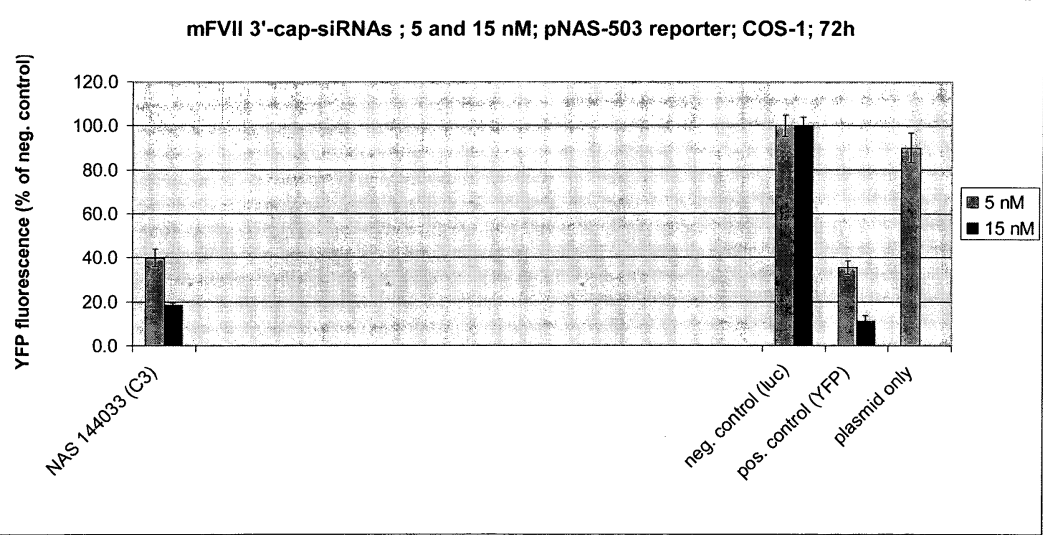


C3

000053

In Vitro Potency of 3'-Conjugated siRNAs

mm_F7_1841 siRNA in reporter gene assay:



Antisense strand: UUu AAU UGA AAC cAA GA6 5X
Sense strand: uGu cuu GGu uuc AAu uA5 5X

u,c = 2'-OMe-U and -C
5,6 = 2'-MOE-A and -meC; X = cap

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ANEXO 3

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Compound	R	R'	R''	Reference
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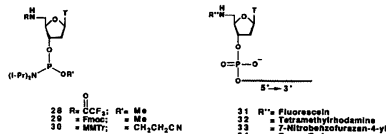
DMTr = di-(p-anisyl)phenylmethyl
MMTr = (p-anisyl)diphenylmethyl
Tr = triphenylmethyl
Pz = (8-phenyl)zanthene-8-yl
Fmoc = 9-fluorenylmethoxycarbonyl

The facile 5'-phosphorylation of oligonucleotides covalently linked to a solid support provided the impetus for the development of specific phosphoramidites that would enable the direct or indirect incorporation of a number of ligands at the 5'-terminus of oligonucleotides.

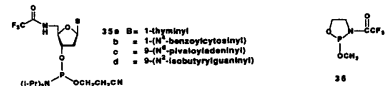
000057

1.2. Amination, Aminoalkylation, and Ligand Incorporation

The synthetic challenge involved with the derivatization of oligonucleotides with non-radioactive markers has led to the development of methods for the generation of aliphatic amino groups at the 5'-terminus of these oligonucleotides. One strategy in achieving this goal has been published by Smith *et al.*²² The approach involved the preparation of 5'-amino-5'-deoxyribosides and its conversion into the deoxyribonucleoside phosphoramidites 28-30. These were incorporated at the 5'-end of oligonucleotides by standard solid-phase synthesis. Once deprotected, the 5'-aminated oligomers were reacted with either fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, 4-fluoro-7-nitrobenzofuran or Tama Red sulfonyl chloride yielding the conjugates 31-34. These oligomers have been used as primers in DNA sequence analysis according to the Sanger approach.²³ The hybridization of these primers to their respective templates was unaltered by the presence of the fluorophores. Furthermore, the facile detection of fluorescent DNA bands on polyacrylamide gels stressed the potential application of oligomers such as 31-34 in automated DNA sequence analysis.^{24,25}



Synthetic routes for the preparation of the protected 5'-amino-2',5'-dideoxyribonucleoside-3'-O-phosphoramidites 35a-d have been developed.²⁴ Specifically, the phosphoramidite 35b has been applied to the synthesis of 5'-amino-d(CGATATCGG) which was, subsequently, reacted with an excess of the pentachlorophenyl ester of a tetraimidium cluster. Co-crystals of the complex formed between the metallated self-complementary decamer and EcoRV were obtained. These crystals were suitable for electron microscopy studies and X-ray crystallography.²⁴ Alternatively, the incorporation of the phosphoramidite 30 into oligonucleotides generated 5'-aminated oligomers which were condensed with the *N*-hydroxysuccinimide ester of biotophenanthroline-ruthenium (II) complexes. These metallated oligomers can be detected by time-resolved fluorescence techniques.²⁶



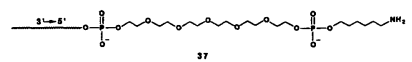
To avoid the preparation of 5'-amino-2',5'-dideoxynucleosides, the phosphoramidites 12, 26, 27, 13b, 28-30, 14, 3-5, 15a-b, 20b, 16, 17, 17a-b, 36-41, 18, 39, 19, 42 and 36/44 have been synthesized and applied to the aminoalkylation of synthetic oligodeoxyribonucleotides at the 5'-terminus. The reaction of deprotected 5'-aminoalkylated oligonucleotides with excess biotin *N*-hydroxysuccinimide ester^{26,27} or biotin *p*-nitrophenyl ester²⁸ produced the corresponding 5'-biotinylated oligomers in yields exceeding 85% with no detectable side reaction.³⁶ The purified 5'-biotinylated oligomers were used as hybridization probes for the detection of specific gene sequences.^{26,28} The strong and noncovalent interaction between biotin and avidin or streptavidin leading to the formation of a high affinity complex

($K_d = ca. 10^{-15}$ M) that is easily detectable by enzymatic methods, has offered an alternative to conventional ³²P radiolabelling. In addition to hybridization probes, 5'-biotinylated oligonucleotides have found application as primers in experiments involving the Polymerase Chain Reaction (PCR).^{29,30,43,44} to facilitate the sequencing of amplified DNA.^{45,46}

The versatility of the phosphoramidite 20 has been exemplified by the solid-phase synthesis of an oligonucleotide carrying five primary amine functions attached to the 5'-terminus. The multiple incorporation of the phosphoramidite 20 occurred with a coupling efficiency of 95%.⁴⁸ Upon removal of the protecting groups with ammonium hydrosulfide, the aminoalkylated oligonucleotide was biotinylated and then hybridized to complementary M13mp18 single-stranded DNA immobilized on nitrocellulose filters. Colorimetric detection showed specific hybridization with a sensitivity of 0.5 ng.⁴⁸

In an attempt to facilitate the detection of PCR-amplified DNA sequences through an improved immobilization of sequence-specific oligonucleotide probes to nylon membranes, Zhang *et al.*⁴⁹ described the reaction of a hexaethylene glycol-based 8-cyanoethyl-*N,N*-diisopropylphosphoramidite with the 5'-OH of an oligonucleotide linked to a solid support. The resulting 'spacer-armed' oligonucleotide was aminoalkylated with the trifluoroacetylated phosphoramidite 13b yielding, after deprotection, the oligomer 37. The purified aminoalkylated oligonucleotide was then efficiently coupled to nylon membranes activated with 1-ethyl-3-(*N,N*-dimethylaminopropyl)carbodiimide hydrochloride.⁴⁹

Alternatively, 5'-aminoalkylated oligomers generated from the incorporation of the phosphoramidite 17b were activated with cyanuric chloride and then coupled to a nylon support covalently coated with poly(ethyleneimine). This support may also find application in the capture of specific RNA and DNA targets.⁴⁹



The conjugation of 5'-aminoalkylated oligomers to fluorophores has allowed the classification of *Flavobacter* strains by fluorescence microscopy on the basis of complementarity to partial 16S rRNA sequences.⁴⁴ In addition to enhancing the detection of specific DNA sequences by fluorescence amplification,²¹ fluorescently labelled oligodeoxyribonucleosides have served in fluorescence energy transfer experiments⁵⁰ and as primers in automated DNA sequence analysis.^{43,50}

The phosphoramidite 17b has been applied to the attachment of a 5'-amino linker to a ribavirin phosphate homopolymer. The amino linker could permit the potential conjugation of the ribavirin oligomer to a monoclonal antibody.³⁸

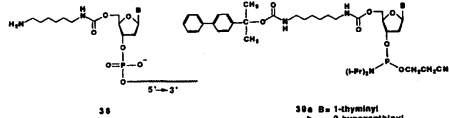
5'-Aminoalkylated oligodeoxyribonucleosides resulting from the incorporation of the phosphoramidite 17b have also found application in the preparation of oligonucleotide-quinone conjugates.⁴¹ When hybridized to their complementary sequences, these conjugates were activated by cytochrome *c* reductase in the presence of NADH and triggered the formation of cross-linked strands by reductive alkylation.^{41b} These oligonucleotide conjugates may find interesting applications in antisense experiments. Similar applications are anticipated for those 5'-aminoalkylated oligomers which have been reacted with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate or *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate and conjugated to interleukin-18 as a means to deliver such conjugates to cells harboring the interleukin-1 receptor.²⁹

While the synthesis of oligodeoxyribonucleoside methylphosphonates from deoxyribonucleoside methylphosphonates has been reported in the mid-eighties, only recently has the 5'-aminoalkylation of a methylphosphonate oligomer been effected by the methylphosphonamidite 21 (Table 1).⁵¹ The deprotected oligomer was biotinylated and, after purification, was immobilized on streptavidin-agarose. Given the stability of the internucleotide methylphosphonate linkages to the nucleolytic activity of nucleases, the biotinyl-oligonucleoside methylphosphonate was employed in the capture of maxi U2

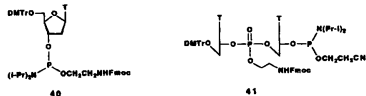
RNA/ribonucleoprotein complexes from nuclear extracts of cultured human cells.⁵¹ It is to be noted that solid-phase syntheses of 5'-aminoalkylated oligonucleotides and oligodeoxyribonucleotides containing a 5'-aminoalkylphosphate function have also been achieved by the phosphotriester approach.^{52,53}

Wächter *et al.*⁵⁴ further investigated the 5'-aminoalkylation of oligodeoxyribonucleotides. Their approach involved the reaction of the 5'-OH function of solid-phase linked oligomers with carbonylimidazole followed by hexamethylenediamine. The deprotected 5'-aminoalkylated deoxyribonucleotide 38 was biotinylated with the *N*-hydroxysuccinimide ester of biotin at pH 7.7. Interestingly, the carbamate linkage in 38 survived the usual basic conditions required for the removal of the base protecting group.^{54,55} This methodology was also employed by Beck *et al.*⁵⁶ toward the development of a chemiluminescent detection of DNA and by others^{56b} to evaluate the cellular uptake and trace the intracellular localization of fluorescently-labelled oligonucleotide-neoglycoprotein conjugates.

Along similar lines, De Vos *et al.*⁵⁵ described the synthesis of the deoxyribonucleoside phosphoramidite 39a-b to facilitate the incorporation of an aminoalkyl function at the 5'-end of oligonucleotides. The (*p*-biphenyl)isopropoxyoxycarbonyl group was conveniently removed under the acidic conditions required for the cleavage of a DMT group. The resulting 5'-aminoalkylated oligomer was biotinylated while the oligonucleotide was still attached to the solid support or, alternatively, in solution phase after complete deprotection.⁵⁵



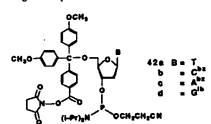
Seliger *et al.*⁵⁷ reported the synthesis of the deoxyribonucleoside phosphoramidite 40 and the dinucleoside phosphoramidite 41 as a means of inserting aminoalkyl functions in oligonucleotides. The monomeric phosphoramidite 40 led to coupling yields of ca. 99% whereas the coupling efficiency of the dimeric phosphoramidite 41 was ca. 87%. The purified aminoalkylated oligomers were biotinylated in yields up to 90% with the *N*-hydroxysuccinimide ester of biotin at pH 7.5.⁵⁷ A biotinylated hairpin loop was immobilized on an avidin-coated solid support to demonstrate the accessibility to double-stranded DNA manipulations.⁵⁷



A versatile linker for the modification of synthetic biomolecules has been reported by Gildea *et al.*⁵⁸ The linker was incorporated into oligonucleotides via the phosphoramidites 42a-d. The solid-phase synthesis of a pentadecamer was performed and the oligomer was reacted with 1,6-diaminohexane followed with the *N*-hydroxysuccinimide ester of biotin. The crude biotinylated oligonucleotide was purified by binding to streptavidin-agarose and washing away non-biotinylated

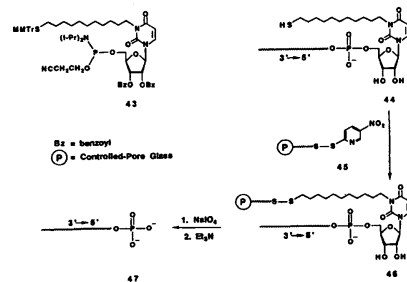
oligonucleosidic sequences. Treatment of the agarose with aqueous acetic acid released the captured oligomer in 85% yield.⁵⁸

In addition to this specific application, the phosphoramidites 42a-d could potentially lead to the attachment of a variety of ligands at the 5'-end of oligonucleotides with the unique possibility of recovering the unmodified oligomers upon mild acidic conditions.

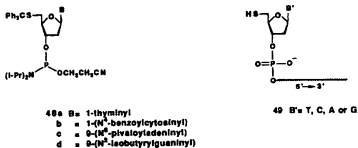


1.3. Thiolation, Mercaptoalkylation, and Ligand Incorporation

A strategy for solid-phase purification of oligonucleotides has also been proposed by Bamnash and Wippler.⁵⁹ The approach relied on the incorporation of the ribonucleoside phosphoramidite 43 at the 5'-end of an hexadecanucleotide linked to a solid support. The derivatized oligonucleotide was then deprotected with concentrated ammonium hydroxide and treated with aqueous silver nitrate. The thiol-containing oligonucleotide (44) along with the underivatized oligonucleosidic sequences were reacted with the activated disulfide support 45 at pH 7.0. The resulting support 46 was freed from underivatized sequences and the pure 5'-phosphorylated oligonucleotide 47 was released from 46 upon treatment with aqueous sodium metaperiodate and 5% aqueous triethylamine, respectively.⁵⁹ The yield of pure 47 has not, however, been reported.



The synthesis of protected 5'-mercapto-2'-5'-dideoxyribonucleoside-3'-O-phosphoramidites (48a-d) and their application toward the preparation of 5'-mercapto-oligonucleotides have been reported.⁶⁰ Like 5'-amino-oligonucleotides, 5'-mercapto-oligonucleotides, represented by the structure 49, can react with a variety of electrophiles, heavy metals and fluorescent markers.⁶¹



The mercaptoalkylated phosphoramidites 112L⁶² and 223AL63a,64a⁶³ provided an alternate route to the synthesis of 5'-thiolated oligonucleotides. It has been shown that 5'-mercapto-oligonucleotides can be derivatized with tetramethylrhodamine iodoacetamide or fluorescein iodoacetamide to yield fluorescent M13 primers suitable for automated DNA sequencing.⁶⁴

Furthermore, Mori *et al.*⁶⁵ described the synthesis of the phosphoramidite 53 and its incorporation at the 5'-end of synthetic oligonucleotides. The 5'-mercaptoalkylated oligonucleotides were then coupled with *N*-6-acetylthymine, affording fluorescent oligonucleotides which were used in cellular uptake experiments.⁶⁶

5'-Mercaptoalkylated oligodeoxyribonucleotides have also been applied to the synthesis of sequence-defined polypeptide-oligonucleotide conjugates.⁶⁵ Specific polypeptides were prepared by standard solid-phase methodology and were reacted with purified 5'-mercaptoalkylated oligonucleotides at pH 6-7 to yield the desired polypeptide-oligonucleotide conjugates. It is noteworthy that the conjugation of 5'-thiolated oligonucleotides with a maleimido-poly-L-lysine derivative⁶⁶ led to the formation of conjugates that were particularly effective in the selective inhibition of gene expression in intact cells.⁶⁷

Of interest, 5'-thiolated oligomers have been synthesized from the incorporation of a tritylated mercaptoethylethylene glycol phosphoramidite derivative by solid-phase techniques.⁶⁸ These thiolated oligomers were condensed with horseradish peroxidase derivatized with maleimide functions to generate valuable conjugates for the sequence-specific detection of PCR-amplified DNA.⁶⁹

The reaction of 5'-mercaptoalkylated oligodeoxyribonucleotides with Trypt-activated Sepharose 4B or Epoxy-activated Sepharose 6B resulted in the covalent attachment of specific oligomers to Sepharose. The addition of complementary unmodified oligodeoxyribonucleotides yielded DNA duplexes as affinity ligands for the isolation of specific DNA binding proteins.⁶³

It is important to mention that 5'-aminoalkylated oligonucleotides can easily be converted into oligonucleotides carrying a 5'-mercapto function upon reaction with dithiobis(*N*-succinimidyl)propionate at pH 7.7 followed by treatment with dithioerythritol at pH 8.5.⁷⁰ 5'-Mercaptoalkylated oligonucleotides were obtained in 70-80% yield. Similarly, Clair *et al.*⁶⁹ reported the reaction of a 5'-aminoalkylated oligomer with *N*-succinimidyl-3-(2-pyridyldithio)propionate⁷¹ instead of dithiobis(*N*-succinimidyl)propionate. The activated disulfide-containing oligonucleotide produced the 5'-mercaptoalkylated oligonucleotide upon treatment with dithioerythritol (DTT) at pH 9.0.^{72,69} Furthermore, the reaction of a 5'-aminoalkylated oligomer with *N*-acetyl-DL-homocysteine thioacetate at pH 8.0⁷⁰ or with *N*-succinimidyl-5-acetyl thioacetate followed by hydroxylamine

hydrochloride⁷¹ generated the corresponding oligonucleotide carrying a free thiol group in yields greater than 95%. The thiolated oligomer was then derivatized with *N*-(4-dimethylaminobenzene-4')iodoacetamide or *N*-(3-pyrenyl)maleimide.^{70,71}

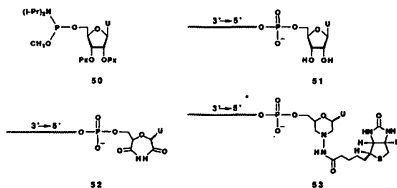
In this context, the reaction of the 5'-hydroxy function of an oligonucleotide linked to a solid support with, successively, carbonyl diimidazole and 5-DMT-lysine yielded, after deprotection and purification, an oligomer derivatized with both mercapto and carboxylic acid functionalities.⁷²

Thoung and Chassagnol⁷³ described the synthesis of oligonucleotides carrying a 5'-terminal thiophosphate monoester function. Their approach was based on the phosphorylation of the 5'-OH function of oligomers anchored to a solid support with the phosphoramidite 1a followed by sulfuration of the phosphite triester intermediate with elemental sulfur. The purified 5'-thiophosphorylated oligonucleotides were reacted with various electrophiles in aqueous solution, affording the efficient incorporation of photoactivatable cross-linkers,⁷⁴ intercalators and chelators^{75,76} in addition to fluorescent and radioactive markers.^{73,75,76} This strategy has been particularly useful for the introduction of proflavin at the 5'-end of α- or β-oligonucleotides as a means to direct photochemical reactions to the complementary DNA sequences.^{77,78} In a similar manner, the phosphoramidite 24 (Table I) enabled the incorporation of a 5'-hexamethylene thiophosphate group into oligonucleotides and the subsequent functionalization of the thiolated oligomers with a phenanthroline-copper chelate.⁷⁴

Recently, the coupling of a specific 5'-thiophosphorylated oligomer with *p*-azidophenacyl bromide was performed to demonstrate that the inhibition of SV40 DNA replication in CV-1 cells resulted from the formation of a triple helix between the viral DNA and the oligonucleotide carrying the cross-linking agent.⁷⁹ Poralen-oligonucleotide conjugates have also been similarly prepared by reacting 5-(6-iodohexyloxy)poralens with oligodeoxyribonucleotides carrying a 5'-thiophosphate group.⁸⁰ The sequence-specific photoinduced cross-linking of the two DNA strands by the triple helix-forming poralen-oligonucleotide conjugates occurred with efficiencies greater than 80%.^{80a} Thus, poralen-oligonucleotide conjugates can be designed to recognize and irreversibly modify specific DNA sequences.⁸⁰ Such modifications are expected to block replication and transcription by preventing the opening of the double helix.

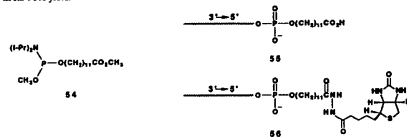
1.4. Oxidation, Esterification, and Ligand Incorporation

Alternate methods for linking non-radioactive labels to the 5'-end of synthetic oligodeoxyribonucleotides employing phosphoramidite intermediates have been reported. Specifically, the ribonucleoside phosphoramidite 50 was used to generate a (5'-5')-internucleotide link with an oligomer covalently bound to a solid support.⁴⁰ The deprotected oligomer 51 was oxidized to 52 with sodium metaperiodate. The reaction of 52 with biotin hydrazide followed by reduction with sodium



borohydride afforded the biotinylated oligonucleotide 53 which has been applied to the detection of M13 DNA immobilized on nitrocellulose filters.⁵⁶

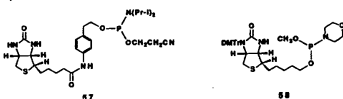
The phosphoramidite 54 has been recommended by Krensky *et al.*⁵⁴ for the phosphorylation of the 5'-OH function of an oligonucleotide anchored to a solid support. Iodine oxidation followed by complete deprotection yielded the oligomer 55 which upon condensation with biotin hydrazide in the presence of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DEC) afforded the biotinylated oligomer 56 in ca. 90% yield.



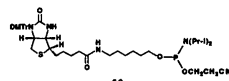
Noteworthy procedures for the 5'-biotinylation of synthetic oligonucleotides have also been reported by Kempe *et al.*,⁵⁵ Chollet *et al.*,⁵³ and Chu *et al.*⁵⁴

1.5. Direct Ligand Incorporation.

1.5.1. Biotinyl Phosphoramidites. 5'-Biotinylated oligonucleotides were easily prepared by reacting the phosphoramidite 57 with the 5'-OH group of solid-phase bound oligomers under standard conditions.⁵⁵ The synthesis of 57 was accomplished by coupling biotin *N*-hydrazinylsuccinimide with *p*-*O*-hydroxyethylamine. The resulting ethanamide was then phosphorylated with bis-(*N,N*-diisopropylamino)-5-cyanoethylphosphine and *N,N*-diisopropylammonium tetrazolidine.⁵⁵ Alternatively, the phosphorylation of a biotin derivative with chloro-(methoxy)-*N*-morpholinophosphine led to the biotinylated phosphoramidite 58.⁵⁶ The coupling efficiency of 58 with the 5'-OH function of solid-phase bound oligonucleotides can potentially be measured during the removal of the *N*⁴-DMTr group of the biotin moiety.

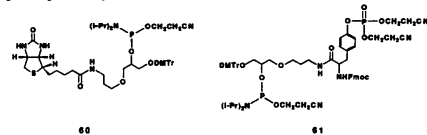


In a recent report, Foa described the preparation of [1-*N*-(4,4'-dimethoxytrityl)-biotinyl-6-aminoheptyl]-5-cyanoethyl-*N,N*-diisopropylphosphoramidite (59) for the automated synthesis of 5'-biotinylated oligonucleotides.⁵⁷ The *N*⁴-DMTr group provided solubility and purification advantages while the 6-aminoheptyl linker facilitated the interaction of biotin with large proteins. The biotinylated phosphoramidite 59 was incorporated into oligonucleotides with an efficiency of ca. 85-95%. The biotinylated oligonucleotides could be heated in concentrated ammonium hydroxide for 24 h at 50 °C without significant loss of biotin.⁵⁷

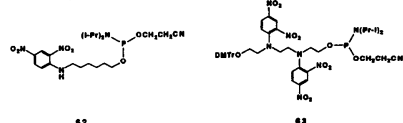


Phosphoramidite linkers have been designed for the direct insertion of biotin at multiple sites in synthetic oligonucleotides.⁵⁸ For example, the biotinyl phosphoramidite 60 was prepared in six steps from solketal and was repeatedly incorporated at the 5'-end of a universal M13 sequencing primer with a coupling efficiency of ca. 99%. Oligonucleotides having up to eight biotin residues were synthesized. Depending on the assay used, it was found that an oligomer having eight biotin residues was detected at a concentration 10 fold lower than that of a singly biotinylated oligomer.⁵⁸

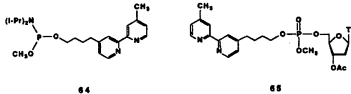
1.5.2. Phosphotyrosinyl Phosphoramidites. The phosphotyrosinyl phosphoramidite 61 was synthesized in nine steps from the *p*-toluenesulfonate salt of L-tyrosine benzyl ester and was also repeatedly inserted in the sequence of the universal M13 sequencing primer (*vide supra*) with a coupling efficiency of ca. 96%. Phosphotyrosinyl oligonucleotides enabled the detection of M13 DNA with a signal strength increasing with the number of phosphotyrosinyl residues.⁵⁹



1.5.3. 2,4-Dinitrophenyl Phosphoramidites. Will *et al.*⁶⁰ reported the synthesis of the phosphoramidites 62 and 63 for the single or multiple incorporation of dinitrophenyl (DNP) at the 5'-terminus of oligonucleotides. The insertion of 63 in oligonucleotides by solid-phase techniques occurred with a coupling efficiency of ca. 97% and afforded oligonucleotides having up to ten DNP groups.⁶⁰ Due to the sensitivity of the multiple DNP labels to concentrated ammonium hydroxide under the usual deprotection conditions, FOD-amidites⁶⁰ carrying base protecting groups that can be removed under milder conditions have been used in the synthesis of DNP-labelled oligonucleotides. The facile detection of such oligonucleotides was effected with anti-DNP monoclonal or polyclonal antibodies harboring suitable markers.⁶⁰



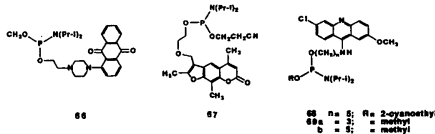
1.5.4. Bipyridinyl Phosphoramidite. The phosphoramidite **64** has recently been prepared by Modak *et al.*⁹¹ and employed in the phosphorylation of 3'-O-acetyl-2'-deoxythymidine. After oxidation and purification, the bipyridinyl phosphotriester **65** was complexed with Cu(II) and incubated with poly rA₁₂₋₁₈ at 37 °C, pH 7.1, for 24 or 48 h. The RNA substrate was degraded to the extent of 21% within 48 h. Adenosine (2'-3'-cyclic monophosphate) was identified as the major hydrolysis product. No degradation of the RNA homopolymer was detected in the absence of Cu(II) under similar conditions.⁹¹ The phosphoramidite **64** may, therefore, be useful toward the incorporation of a bipyridinyl group at the 5'-end of oligonucleotides and the development of sequence-specific RNA cleaving reagents.



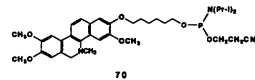
1.5.5. Anthraquinonyl Phosphoramidite. Mori *et al.*⁹² developed the phosphoramidite **66** for the derivatization of oligonucleotides at the 5'-end with an anthraquinone function. Anthraquinones are potential radical producing entities which do not necessarily require photoactivation or the presence of metal ions. Consequently, anthraquinone-linked oligonucleotides may find application in the delivery of radical-producing anti-cancer drugs to cellular nucleic acid targets.⁹²

1.5.6. Psoralenyl Phosphoramidite. The direct incorporation of psoralen derivatives at the 5'-terminus of oligodeoxynucleotides was effected by the phosphoramidite **67** via solid-phase methods.^{93a} Psoralen derivatives can intercalate between the base pairs of double-stranded nucleic acids and may become covalently attached to thymine residues upon exposure to long-wavelength UV irradiation. Under these conditions, each psoralen-DNA adduct is generated through the formation of a cyclobutane ring. These adducts have been applied to the elucidation of nucleic acid structures and functions.^{80,95,96}

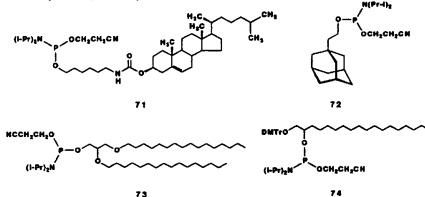
1.5.7. Acridinyl and Fagaroninyl Phosphoramidites. The synthesis of the acridinyl phosphoramidites **68**^{97a} and **69a-b**⁹⁸ and their introduction at the 5'-terminus of modified and unmodified oligodeoxynucleotides has been documented.^{74c,97b} In addition to providing fluorescence to these oligonucleotides, the acridinyl group stabilized the formation of double- and triple-helical structures with complementary nucleic acid targets by virtue of its intercalating properties.^{74c,75,97b,99,100} Such fluorescent oligonucleotides have been particularly useful in kinetic experiments pertaining to the cellular uptake of these biomolecules.⁹⁸



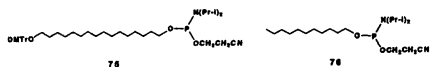
It has been demonstrated that the benzophenanthridine alkaloid fagaronine chloride, isolated from *Fagaria zanthoxyloides* inhibited the reverse transcriptase activity of several viruses.¹⁰¹ These findings provided the impetus to determine whether antisense oligonucleotide-fagaronine conjugates would enhance the inhibition of retroviral reverse transcriptases. Fagaronine chloride was converted to the phosphoramidite **70** which was then reacted with the 5'-hydroxy function of oligonucleotides.¹⁰¹ Relative to unmodified oligomers, oligonucleotide-fagaronine conjugates hybridized better to complementary RNA sequences presumably because of the intercalating abilities of the fagaronine entity. The conjugates were ca. 3 times more potent than unmodified oligomers at inhibiting HIV reverse transcriptase but were ca. 3 times less inhibitory than fagaronine chloride itself.¹⁰¹



1.5.8. Cholesteryl and Other Lipophilic Phosphoramidites. The application of oligonucleotides as potential therapeutics has been hampered by the low efficiency with which these polyanionic molecules permeated intact cells. It has been rationalized that the attachment of lipophilic groups known to interact with cell membranes, to oligonucleotides, may increase the therapeutic potency of these biopolymers.¹⁰² To test this rationale, the cholesteryl phosphoramidite **71** along with the adamantylethyl phosphoramidite **72** and the polysilyl phosphoramidites **73-75** were synthesized and incorporated at the 5'-terminus of an oligonucleotide phosphorothioate complementary to a region of the HIV-1 *rev* gene.^{102a} It has been shown, so far, that the denaturation temperatures of duplexes composed of the lipophilic oligonucleotide phosphorothioates and complementary DNA strands were identical to that of the duplex resulting from an equivalent underivatized phosphorothioate oligomer and its complementary DNA sequence.^{102a}

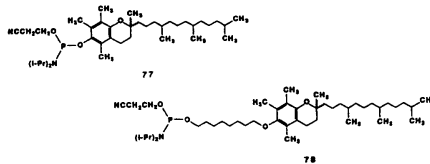


Along similar lines, Kabanov *et al.*^{103b} reported the synthesis the phosphoramidite **76** as a means to introduce an hydrophobic group at the 5'-end of oligonucleotides. Targeting an underacylated oligonucleotide against a loop forming site of the influenza A/PR8/34 viral RNA, considerably suppressed the development of the virus in permissive MDCK cells relative to that observed with unmodified oligonucleotides under identical conditions.^{103b} The attachment of hydrophobic groups to

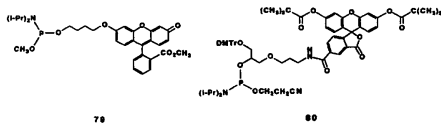


oligonucleotides has therefore demonstrated its efficacy at improving the biological activity of these biomolecules.

In an additional effort to impart lipophilicity to oligonucleotides and improve their cellular uptake and cellular distribution, the synthesis of the DL- α -tocopherol (Vitamin E) phosphoramidites 77 and 78 has been described.¹⁰² Similar to the phosphoramidite 76, the incorporation of 77 and 78 into oligonucleotides occurred in yields greater than 95% according to HPLC analysis of the conjugates.^{102d} The anti-HIV activity of these modified oligonucleotides are currently being evaluated in cell cultures.



1.5.9. Fluoresceinyl Phosphoramidites. The preparation of fluorescently labelled oligonucleotides for automated dideoxy-DNA sequencing has been further simplified by Schubert *et al.*¹⁰³ who reported the facile integration of the fluoresceinyl phosphoramidite 79 into synthetic oligonucleotides. The phosphoramidite was prepared from the alkylation of fluorescein methyl ester with 4-chloro-1-(4,4'-dimethoxytrityl)butane followed by detritylation and phosphorylation with bis-(*N,N*-diisopropylamino)methylphosphine. The phosphoramidite 79 has been used in the solid-phase synthesis of various M13mp18 sequencing primers. Coupling yields were equivalent as those recorded with the standard deoxyribonucleoside phosphoramidites.^{103a}



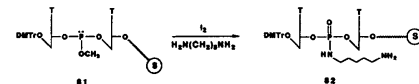
The multistep synthesis of the 5-carboxyfluoresceinyl phosphoramidite 80 from 5-carboxyfluorescein and solketal has recently been reported by Thelsen *et al.*^{102b} It was argued that because of the steric hindrance imparted by the trityl group, the coupling of 80 at the 5'-OH function of

a solid-phase bound oligonucleotide necessitated a coupling time of 120 sec and proceeded with an efficiency of only 70%. The fluorophore and its linkage to the oligomer were stable during chain assembly and to the conditions required for complete oligonucleotide deprotection.^{102b} Like the biotinyl- and phosphotyrosinyl phosphoramidites (60 and 61), the fluoresceinyl phosphoramidite 80 can be incorporated at any site within a DNA sequence or can serve in the derivatization of solid supports to yield oligonucleotides labelled at the 3'-terminus.

Aside from the 5'-OH group of oligonucleotides, the internucleotide phosphodiester function of these biomolecules could also be functionalized. This subject will be dealt with in the forthcoming section.

2. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE INTERNUCLEOTIDIC LINKAGES

Jäger *et al.*¹⁰⁴ demonstrated that aminoalkylated phosphoramidate oligonucleotides can be prepared by routine solid-phase synthesis using deoxyribonucleoside 3'-*O*-(*N,N*-diisopropylamino)methoxyphosphine monomers. For example, the oxidation of the dinucleoside phosphate triester 81 with iodine and 1,5-diaminopentane afforded the aminoalkylated phosphoramidate dimer 82 which was subsequently coupled to 5-chloro-4-(*p*-chlorophenyl)-2-methylpyridine.¹⁰⁴



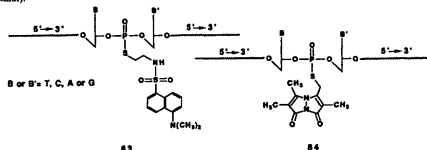
Due to the stability of the phosphoramidate linkage to the conditions used in solid-phase oligonucleotides synthesis by the phosphoramidite method, the combination of deoxyribonucleoside phosphoramidite and *H*-phosphonate monomers has allowed the insertion of cholesteryl,¹⁰⁵ tetramethylpyridine-*N*-oxyl¹⁰⁶ or phenazinyi di-*N*-oxide¹⁰⁷ phosphoramidate links at predetermined sites. The validity of this approach has been previously demonstrated by Letsinger *et al.*¹⁰⁷ in a report delineating the preparation of cationic oligonucleotides.

In a similar manner, Agrawal *et al.*¹⁰⁸ and others¹⁰⁹ introduced either (*N*-1-trifluoroacetyl)hexanediamine arms at specific sites along the oligonucleotide chains¹⁰⁸ or ethylenediamine and hexamethylenediamine tethers at the 5'-end of oligonucleotides.¹⁰⁹ After complete deprotection, the aminoalkylated phosphoramidate oligomers were coupled to ligands such as fluorescein isothiocyanate, rhodamine isothiocyanate or activated esters of biotin. This approach provided multiple labelling capabilities and thereby increased sensitivity for diagnostic purposes.¹⁰⁸

The specific functionalization of oligonucleotides with two different reporter groups has been described by Agrawal and Zamecnik.¹¹⁰ Oligonucleotide assembly began with the incorporation of a nucleoside *H*-phosphonate monomer followed by oxidation with carbon tetrachloride and (*N*-1-trifluoroacetyl)hexanediamine to generate a phosphoramidate link. Standard deoxyribonucleoside phosphoramidite monomers were then employed until completion of the synthesis. The last oxidation step was effected with a sulfuring reagent^{111,112} to generate a phosphorothioate link which, after oligonucleotide deprotection, was reacted with monobromobimane at pH 7.2. The aminoalkyl function of the purified bimane-labelled oligomer was then reacted with fluorescein isothiocyanate and the resulting bifunctionalized oligomer was isolated in 60% yield. Such bifunctionalized oligomers may find application in cellular uptake experiments or toward the targeting of specific messenger RNAs.

Numerous reporter groups have been introduced at specific sites in oligonucleotides carrying phosphorothioate diester links.¹¹³ For example, the reaction of an oligonucleoside phosphorothioate with *N*-dansylhydrazine afforded the dansylated phosphorothioate oligomer 83 in yields greater than 85%.¹¹⁴ Substituting monobromobimane for *N*-dansylhydrazine at pH 7 led to the complete formation

of the bimane-labelled phosphorothioate oligomer 84.¹¹⁵ Interestingly, bimane labels have been incorporated at multiple sites into oligonucleotide phosphorothioates while the oligomers were still embedded in the polyacrylamide gel matrix after electrophoresis. Oligonucleotides containing two hundred to four hundred labels can be detected with the naked eye at the low femtomole level (10-20 fmol).^{115,116}

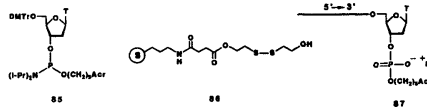


The nucleophilicity of phosphorothioate diesters has been exploited in the sequence determination of DNA and RNA.^{117,118} Specifically, the reaction of oligodeoxy- or oligoribonucleotides having randomly distributed phosphorothioate links with iodoethanol or 2,3-epoxy-1-propanol generated hydrolytically labile phosphorothioate triesters which promoted the subsequent cleavage of the oligonucleotide chains.¹¹⁷ This approach represents an alternative to the current DNA sequencing procedures.

The following section will assess the recent developments toward the derivatization of oligonucleotides at the 3'-end.

3. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 3'-TERMINUS

The incorporation of a fluorescent marker at the 3'-terminus of oligonucleotides was reported by Asseline and Thuong.¹¹⁹ Their approach involved the phosphorylation of the solid support 86 with the fluorescent deoxyribonucleoside phosphoramidite 85 in the presence of *1H*-tetrahydrofuran. The labelled oligomers (87) were subsequently released from the support by treatment with DTT under alkaline conditions. This methodology also permitted the multiple insertion of fluorescent labels within an oligonucleotide chain.



Ac(CH₂)₆OH = 2-methoxy-4-chloro-6-hydroxyheptan-1-amine
 (●) = Fructose 608

In recent years, solid supports have been engineered to facilitate the derivatization of synthetic oligonucleotides with functional groups including masked^{90,91,92,120} or unmasked 3'-sulfhydryl.^{90,121}

terminal 3'-phosphate,^{92,119,120,121-123} 3'-thiophosphate,^{92,100,119,124} and 3'-aminoalkyl.^{92,119,125-129} These functional groups can then be further derivatized with various ligands to serve as tools in molecular biology experiments. Solid supports functionalized with acridine,¹³⁰ cholesterol,^{130a,b} DL-α-tocopherol^{130c} or fluorescein¹³⁰ have also been described for the synthesis of oligonucleotides bearing these ligands at the 3'-terminus.

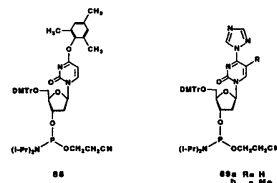
Aside from the internucleotide linkage and both termini, the functionalization of oligonucleotides at the nucleobases has been thoroughly investigated. The results of these investigations will be summarized in the next section.

4. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE NUCLEOBASES

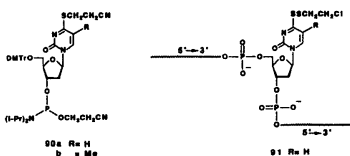
4.1. Derivatization of Activated Purines and Pyrimidines.

It is well-known that the triazole function of 4-triazolopyrimidinone nucleosides are susceptible to nucleophilic displacement by amines.^{131,132} This feature has been exploited by Le Brun *et al.*¹³³ who inserted 4-triazolopyrimidinones at predetermined sites in oligonucleotides according to the phosphoramidite methodology. The triazole groups were then displaced with 1,10-diaminodecane and the corresponding aminoalkylated oligomers were subsequently biotinylated. The specificity of these biotinylated oligomers was tested in hybridization assays with plasmid DNA immobilized on nitrocellulose filters. It was found that the stability of the resulting hybrids was not affected by biotinylated arms located at the 5'-end of the oligomers. Conversely, the stability of the hybrids was strongly impaired by the location of the biotinylated linkers within the oligonucleotide chain. The denaturation temperature (*T*_m) of these hybrids was at least 10 °C lower than that measured with unmodified oligomers.¹³⁴ Consequently, the 5'-terminus appears to be the ideal position for the incorporation of these modified nucleobases into oligonucleotides.

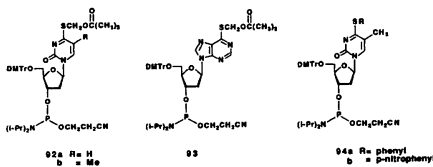
MacMillan and Verdine^{134a,b} reported the synthesis of functionally tethered oligodeoxynucleotides by incorporating the deoxyribonucleoside phosphoramidite 88 as a preselected site during oligonucleotide assembly. The use of deoxyribonucleoside phosphoramidites having base-labile protecting groups (*N*⁴-isobutyl cytosine, *N*⁶-phenylacetyl adenine and *N*⁶-phenylacetyl guanine)¹³⁵ permitted rapid oligonucleotide deprotection with concentrated ammonium hydroxide without affecting the *O*⁶-arylated uracil residue. Treatment of the modified oligonucleotides with amines such as *N*-methylamine, 1,2-diaminoethane, 1,4-diaminobutane, glycine or bis-(aminoethyl)disulfide at 65 °C resulted in functionally tethered oligonucleotides with a conversion efficiency of 89-100%.^{134a,b} A similar postsynthetic generation of oligomers containing *O*⁶-alkylthymine, 5-methylcytosine, *N*⁶-(dimethylamino)-5-methylcytosine and 4-thiobenzene residues stemmed from the insertion of the triazole phosphoramidite derivative 89b in oligonucleotides.^{134c}



It has been recently shown that 4-thio-2'-deoxyuridine-containing oligonucleotides led to tether attachment by postsynthetic S-alkylation or mixed disulfide formation.¹³⁴ The 4-thio-2'-deoxyuridine phosphoramidite 90a has been synthesized and incorporated into oligothymidylates by solid-phase synthesis.

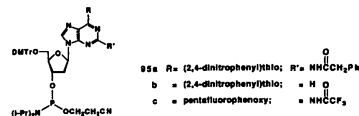


Quantitative 5-deprotection of each oligonucleotide was effected by treatment of the solid support with 1 M DBU in acetonitrile. The modified oligothymidylate was then released from the stationary phase and reacted with *N*-(2-chloroethylthio)phthalimide in phosphate buffer (pH 8) to yield the oligonucleotide 91 which can be further derivatized with selected reporter groups.¹³⁴ The phosphoramidites 90b and 92-94 have analogously been applied to the synthesis of oligonucleotides containing thioated nucleobases.^{134b}



To investigate the synthesis of oligonucleotides containing purine modified at C-6 with 5-, *N*- and *O*-derivatives, the 6-(2,4-dinitrophenyl)thiopurine phosphoramidites 95a-b were prepared in several steps from 3',5'-di-*O*-acetyl-*N*⁶-phenylacetyl-2'-deoxyguanosine and 3',5'-di-*O*-acetyl-2'-deoxyinosine, respectively.^{134ab} The insertion of 95a into oligonucleotides occurred within 3 min with an efficiency of 98%. A dodecamer was synthesized and fractions of the solid support carrying the fully protected 98%. A dodecamer was synthesized and fractions of the solid support carrying the fully protected 98%. A dodecamer was synthesized and fractions of the solid support carrying the fully protected 98%. Alternatively, the treatment of similar oligonucleotide fractions with either concentrated ammonium hydroxide containing *N*¹,*N*²,*N*³,*N*⁴-tetramethylguanidine (65 mM) and *o*-nitrobenzaldehyde (75 mM), 40% aqueous

methylaniline, methanol/DBU (9:1) or 0.5 M aqueous sodium hydroxide at 25 °C, afforded deprotected dodecamers having either 2,6-diaminopurine, 2-amino-6-methylaminopurine, *O*⁶-methylguanine or guanine residues.^{134a} This general procedure also allowed the introduction of NMR sensitive groups such as ¹⁵N, ¹³C, and ¹⁷O derived from (15N)-ammonia, (13C)-methanol and (17O)-water, at defined locations into synthetic oligonucleotides.^{134a} Like 95a-b, the incorporation of the phosphoramidite 96c into oligonucleotides enabled the postsynthetic modification of these molecules and yielded oligomers containing *O*⁶-methylguanine, 2,6-diaminopurines and their *N*⁶-aminoalkyl analogues, or fluorescent 6-(*N,N*-dimethylamino)pyridinium-2'-amino-purine residues.¹³⁶



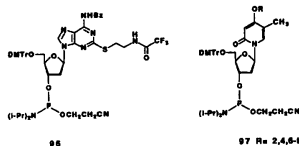
Along similar lines, *O*⁶-phenyl-2'-deoxyinosine was converted to a suitably protected phosphoramidite intermediate and inserted in a self-complementary dodecamer. The deprotected and purified oligomer was treated with either bis(aminoethyl)disulfide or bis-(aminopropyl)dimethylurea yielding a DNA duplex having an aminoalkyl disulfide tether at a specific location in consecutive base pairs on opposite strands. Upon reduction with dithiothreitol, the free thiol function formed an interstrand disulfide linkage during aerobic dialysis.¹³⁷ Such cross-linked oligonucleotides should facilitate the study of enzyme-mediated unpairing during either transcription, replication or recombination.

The sequence-specific cross-linking of oligodeoxyribonucleotides has also been investigated by Webb *et al.*^{138,139} Their approach relied on the incorporation of the modified deoxyribonucleoside phosphoramidites 89a-b into oligonucleotides by solid-phase methods. The triazoly nucleobases were converted to their corresponding ethyleneimine derivatives upon treatment with ethyleneimine. The resulting oligomers cross-linked with their complementary oligodeoxyribonucleotides albeit at a slow rate ($t_{1/2} = 30$ h at 22 °C). It was argued that the ethyleneimine function did not provide optimum "interstrand reach" to efficiently cross-link with target sequences.¹³⁹

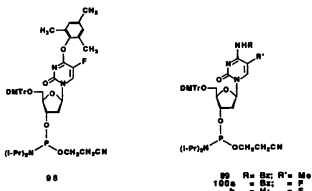
Kido *et al.*¹⁴⁰ described the multistep synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 96 from 2-mercapto-2'-deoxyadenosine. The single insertion of 96 into oligonucleotides by solid-phase synthesis required a coupling time of 300 sec. Following deprotection and purification, the aminoalkylated oligomers were iodouracilylated and characterized by enzymatic degradation to the nucleosides.¹⁴⁰ Oligonucleotides bearing a 2-(*N*-iodoacetylaminomethyl)thiodenine residue were shown to crosslink and cleave complementary strands at adenines and guanines. It is believed that alkylation occurred at the *N*-3 of adenine from the minor groove or with reactive groups of guanine in the major and minor grooves of the duplex. The efficiency of alkylation was low probably because of the free rotation of the reactive linker around the sulfur atom.¹⁴⁰ More rigid tethers may provide higher alkylation selectivity.

Of interest, the deoxyribonucleoside phosphoramidite 97 has been synthesized to enable the preparation of oligonucleotides containing 3-deazathymine residues.¹⁴¹ It is anticipated that these modified oligonucleotides will be applied to experiments aimed at estimating the specificity of replication catalyzed by DNA polymerase I. Using nucleosides as models, it has been demonstrated

that 3-deaza-4-*O*-(2,4,6-trisopropylbenzenesulfonyl)thymidine was easily converted to 3-deaza-thymidine upon treatment with concentrated ammonium hydroxide at 55 °C.¹⁴¹



In an effort to determine the active-site nucleophile of DNA (cytosine-5)-methyltransferase *M.HaeIII*, an enzyme that recognizes dyad-symmetric sites (d[CGCC]) and carries out two sequential methylations of specific cytosine nucleobases, the construction of a suicide DNA duplex was undertaken.^{142a} One strand of the DNA duplex had a 5-methylcytosine residue at the expected *M.HaeIII* methylation site (3'-d[GGGTATC(5-MedC)GGTACTGC]-5'). The complementary strand carried a 5-fluorocytosine nucleobase also at the expected *M.HaeIII* methylation site (5'-(CGCATAGG(5-FC)CATGACC)-3'). These hexadecanucleotides were prepared by the incorporation of the 4-*O*-(2,4,6-trimethylphenyl)-5-fluoro-2'-deoxyuridine phosphoramidite 98 and the 5-methyl-2'-deoxyuridine phosphoramidite 99 by conventional solid-phase synthesis. Upon removal of the protecting groups with concentrated ammonium hydroxide, the 4-*O*-(2,4,6-trimethylphenyl)-5-fluorouracil residue was converted into a 5-fluorocytosine nucleobase in quantitative yield.^{142b} The purified hexadecanucleotides were annealed and then mixed with *M.HaeIII* along with the cofactor *S*-adenosyl-L-methionine. The covalent DNA-*M.HaeIII* complex was purified and submitted to proteolysis. Sequencing of the proteolytic fragments revealed that Cyt₇₁ of *M.HaeIII* formed a covalent bond with DNA during catalytic methyl transfer.^{142c}

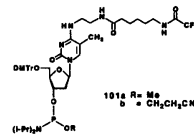


Schmidt *et al.*^{143a} independently described the preparation and the incorporation of the 5-fluoro-2'-deoxyuridine phosphoramidite 100a into synthetic oligonucleotides to investigate the catalytic mechanism whereby the DNA cytosine methyltransferase of *E. coli* K-12 methylates cytosine residues at

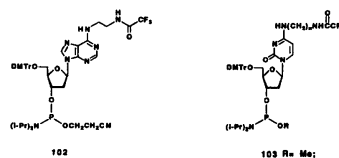
C-5 in a sequence-specific manner. It is important to mention that the 5-fluoro-2'-deoxyuridine phosphoramidite 100b can be inserted in oligodeoxyribonucleotides by solid-phase techniques without protecting its exocyclic amino function.^{143b}

4.2. Ligand Incorporation from Aminoalkylated Purines and Pyrimidines

Urdea *et al.*^{144a} reported the synthesis of the deoxyribonucleoside phosphoramidites 101a-b from the corresponding 4-triazolopyrimidinone nucleosides. These derivatives led to the solid-phase synthesis of oligonucleotides having aminoalkyl functions at various locations within the oligonucleotide chains. Reporter groups such as biotin, fluorescein, and isoluminol were coupled to the purified aminoalkylated oligomers and their detection limits were assessed.^{144a-b} Furthermore, the reaction of the aminoalkylated oligomers with *p*-phenylene diisothiocyanate followed by incubation with either horseradish peroxidase or alkaline phosphatase afforded the corresponding enzyme-DNA conjugates.^{144c}



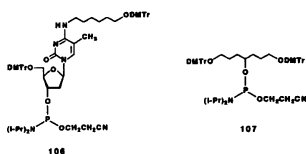
Cossick and Douglas¹⁴⁵ described the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 102 and its incorporation into a dimer as a model to evaluate the formation of specific interstrand cross-links. It was argued that the hybridization of an oligonucleotide having a 5'-*O*-aminoethyladenine nucleobase with a complementary target DNA or RNA sequence would position the nucleophilic aminoethyl function in the vicinity of a pyrimidine residue located on the complementary strand.¹⁴⁵ Activation of the 5,6-double bond of pyrimidines with bisulfites would trigger a nucleophilic attack at C-4 by the aminoethyl group and would result in the cross-linking of the two strands. No data supporting this concept have, as yet, been reported.



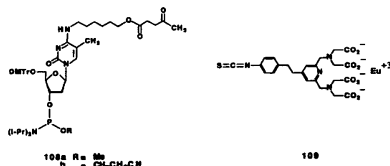
The aminoalkylated phosphoramidites 103 and 104 originated from either the transamination of 2'-deoxyuridine with 1,3-diaminopropane in the presence of sodium metabisulfite^{146a,b} or the facile

aminoalkylation of *N*⁶-*p*-toluenesulfonyl 2'-deoxycytidine derivatives with 1,6-diaminohexane.^{146d} The deoxyribonucleoside phosphoramidite 104 was incorporated at the 5'-end of an oligonucleotide anchored to a solid support and, after deprotection, the aminoalkylated oligonucleotide was biotinylated.^{146d} Along similar lines, the phosphoramidite 105 was prepared in a multistep synthesis from 3',5'-di-*O*-tert-butyl(dimethylsilyl)-2'-deoxycytidine and methanesulfonyl chloride.¹⁴⁷ The aminoalkylated phosphoramidite was repeatedly inserted at the 5'-terminus of a 2'-*O*-alkyl oligonucleotide and the resulting aminoalkylated oligonucleotide was tetrabiotinylated in an attempt to facilitate the purification of RNA-protein complexes.^{147,148}

In an alternate approach, Babin *et al.*¹⁴⁹ described the synthesis of the deoxyribonucleoside phosphoramidite 106 from the corresponding 4-thiothymidine and its incorporation into oligonucleotides toward the preparation of polybiotinylated probes. The synthesis of the phosphoramidite 107 was also reported for the same purpose. Incidentally, the coupling of 107 to solid-phase linked oligonucleotides occurred with an efficiency of 94-97%.¹⁴⁹



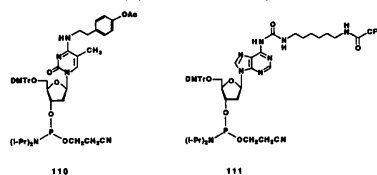
The synthesis of "branched" DNA molecules as amplification multimers in bioassays has been reported by Horn *et al.*¹⁵⁰ and by Chang *et al.*¹⁵¹ This type of branched DNA molecules are composed of two distinctive domains of oligonucleotide sequences. One domain consists of a target specific segment contiguous to an arrangement of branching nucleobases generated by the multiple incorporation of the levulinylated phosphoramidite 108a-b by standard solid-phase synthesis. The selective removal of the levulinyl groups unmasked hydroxy functions for the solid-phase synthesis of the second domain of oligonucleotides. Labelled probes were hybridized to this second set of DNA sequences thereby providing enhanced detection sensitivity.^{150,151}



In an effort to prepare Eu³⁺-labelled DNA probes, Sund *et al.*¹⁵² inserted the deoxyribonucleoside phosphoramidite 105 fifty times at the 5'-end of a synthetic oligomer (50-mer). The purified oligonucleotide (100-mer) was then reacted with the Eu³⁺ chelate 109 at pH 10.5. The Eu³⁺-labelled probe permitted the detection of 200 picograms of phage lambda DNA by time-resolved fluorometry.¹⁵² It must be noted that a bathophenanthroline-Ru(II) complex derivatized as a 8-quinolyl-*N,N'*-diisopropylphosphoramidite derivative, has also been applied to the solid-phase labelling of oligonucleotides.¹⁵³ Like Eu³⁺-labelled oligonucleotides, oligomers labelled with bathophenanthroline-Ru(II) complexes can be detected by time-resolved fluorometry.¹⁵³

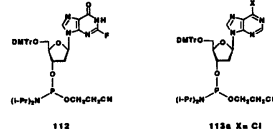
The deoxyribonucleoside phosphoramidite 110 was synthesized from 4-biotinimidine and tyramine¹⁵⁴ and inserted at specific locations within oligonucleotides by solid-phase synthesis. The tyramine residues of the deprotected oligonucleotides were radioiodinated by the standard "chloramine T" oxidation. The resulting oligomers have been useful as primers for PCR amplification and for the detection of viral DNA sequences.¹⁵⁴

The deoxyribonucleoside phosphoramidite 111 has been prepared from *N*⁶-phenoxycarbonyl-2'-deoxyadenosine derivatives¹⁵⁵ and represents an addition to the repertoire of deoxyribonucleoside phosphoramidites dedicated to the preparation of aminoalkylated oligonucleotides.



4.3. Ligand Incorporation via Halogenated Purines

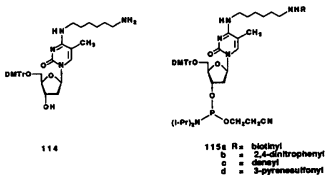
Harris *et al.*¹⁵⁶ reported the synthesis and application of the halogenated deoxyribonucleoside phosphoramidites 112 and 113a-b in the solid-phase synthesis of modified oligonucleotides. Typically, the treatment of these solid-phase bound oligonucleotides with either D-(+)-phenylglycine or with amines derived from (*z*)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-8,9,10,11-tetrahydro-*a*-pyrene and (*z*)-*trans*-8,9-dihydroxy-*anti*-10,11-epoxy-8,9,10,11-tetrahydro-*a*-anthracene, under the recommended conditions, afforded the corresponding adducted oligomers.¹⁵⁶ This synthetic method is quite attractive for the preparation of oligonucleotides having structurally defined adducts in the quantities required for structural studies.



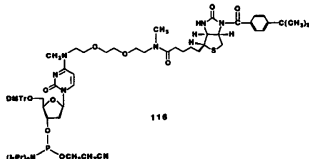
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4.4. Modified Nucleoside Phosphoramidites and Direct Ligand Incorporation

Protected deoxyribonucleoside phosphoramidites carrying various markers such as biotinyl, dinitrophenyl, dansyl and pyrenyl have been synthesized by Roget *et al.*¹⁵⁷ as a means to label synthetic oligonucleotides. Specifically, 114 was prepared in 85% yields from the aminoalkylation of the corresponding 4-thiodenylthymidine with 1,6-diaminohexane. The reaction of 114 with either biotin *N*-hydroxysuccinimide ester, 1-fluoro-2,4-dinitrobenzene, dansyl chloride or pyrenesulfonyl chloride, followed by phosphorylation, yielded the labelled phosphoramidites 115a-d. These were activated with 1H-tetrazole and incorporated into oligonucleotides with high coupling yields (>97%) by standard solid-phase synthesis. Multiple labeling can be performed on the same oligonucleotide to provide increased detection sensitivity in *in situ* hybridization experiments.¹⁵⁷



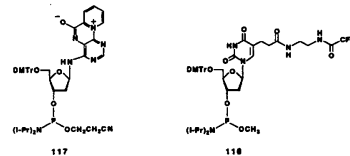
This approach has been further refined by the synthesis of the biotinylated deoxyribonucleoside phosphoramidite 116.¹⁵⁸ Relative to 115a, the phosphoramidite 116 carries a larger and more polar spacer arm to facilitate biological applications. Furthermore, 116 is readily soluble in acetonitrile and has been singly or repeatedly inserted at either the 3', 5', or at both termini of 2'-O-alkyloligonucleotides.¹⁵⁹ The deprotection of DMT-containing oligonucleotides with concentrated ammonium hydroxide under standard conditions, proceeded without significant loss of biotin. It has been shown that purified tetra-biotinylated oligomers prepared from the incorporation of 116 had reduced non-specific binding to streptavidin-agarose. The tandem incorporation of four biotin residues gave reproducibly superior binding when compared to oligonucleotides containing only one or two biotin residues.¹⁵⁸



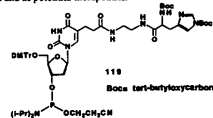
Milewicz *et al.*¹⁵⁹ reported the synthesis of the deoxyluminarosine phosphoramidite 117 as a means to generate fluorescent oligonucleotides that could be applied to the non-isotopic detection of specific DNA sequences or the study of nucleic acids stereodynamics. Due to the sensitivity of the luminarosine residue to nucleophilic bases, nucleobase protecting groups different than the benzoyl and isobutyryl groups had to be used to allow complete oligonucleotide deprotection with concentrated ammonium hydroxide at ambient temperature. The solid-phase synthesis of hexadecanucleotides having a single deoxyluminarosine residue has been achieved and deoxyluminarosine-containing oligomers were detected as strong fluorescent bands by polyacrylamide gel electrophoresis analysis.¹⁵⁹

4.5. Ligand Incorporation at C-5 of Pyrimidines

The reaction of 5-chloromercury-2'-deoxyuridine with methyl acrylate led to the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 118 and its subsequent incorporation into oligonucleotides.^{160-c} Following deprotection and purification, the oligomers were then derivatized with either *N*-succinimidyl-1-pyrenebutyrate, 1-pyrenesulfonyl chloride, fluorescein-5-isothiocyanate, sulfosuccinimidyl-6-(biotinamido)hexanoate, *N*-hydroxysuccinimidyl-anthraquinone-2-carboxylate or the *N*-hydroxysuccinimide ester of 4-carboxy-4'-methyl-3,2'-bipyridine.^{160-c} Incidentally, bipyridine-labelled oligomers reacted with Ru(bpy)₃(H₂O)₆³⁺ to yield oligonucleotides covalently attached to Ru(bpy)₃²⁺ derivatives.¹⁶⁰ These modified oligonucleotides displayed normal hybridization behavior and ruthenium(II) has been known to effect light-induced DNA strand scission.¹⁶⁰

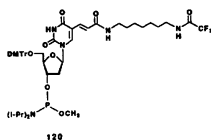


It is generally accepted that RNA hydrolysis, catalyzed by bovine pancreatic ribonuclease A, requires the participation of two imidazole groups located on histidine residues 12 and 119 in the active site.¹⁶⁰ In this context, Basile *et al.*¹⁶⁰ proposed the synthesis of ribonuclease mimics by combining the RNA hydrolysis activity of imidazole with the ability of oligonucleotides to bind to RNA with sequence specificity. The deoxyribonucleoside phosphoramidite 119 has been prepared and incorporated at one specific location into oligonucleotides via standard solid-phase phosphoramidite methodology. These modified oligonucleotides are now being evaluated as antisense molecules in the control of gene expression and as potential therapeutics.

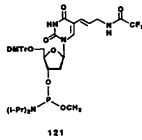


The synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 120 and its insertion in synthetic oligonucleotides has been reported by Jablonski *et al.*^{161a} and others.^{161b} These modified oligomers were then reacted with di-*N*-succinimidylsuccinate and coupled with alkaline phosphatase.^{161a,162} The resulting conjugates efficiently hybridized to complementary target sequences and retained the full enzymatic activity associated with the alkaline phosphatase entity. Hybrids were detected with a dye precipitation assay at a level of 10⁶ molecules (2x10⁻¹⁸ M) of target DNA. Purified oligonucleotides carrying two aminoalkylated nucleobases could alternatively be reacted with fluorescein isothiocyanate and used as primers in DNA sequencing according to Brumbaugh *et al.*¹⁶³

It is to be noted that the incorporation of the phosphoramidite 121 into oligonucleotides during solid-phase synthesis also produced aminoalkylated oligomers which, upon deprotection, were suitable for reaction with the *N*-hydroxysuccinimide ester of *N*-biotinyl-6-aminocaproic acid.¹⁶⁴ Following purification, the biotinylated oligonucleotides were hybridized to target DNA immobilized on microtiter plates and were detected with a streptavidin-biotinylated horseradish peroxidase complex. Oligonucleotides carrying biotin labels near or at the end of the sequences were more effective probes than those oligomers bearing internal biotin labels.¹⁶⁴

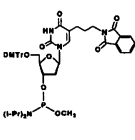


120

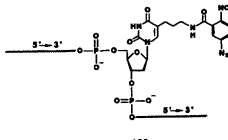


121

In a strategy to investigate protein-DNA interactions Gibson and Benkovic¹⁶⁵ described the synthesis of the deoxyribonucleoside phosphoramidite 122 from 5-iododeoxyuridine and its integration at the terminus of two undecamers. The deprotected oligomers were treated with the cross-linker (*N*-hydroxysuccinimidyl)-5-azido-2-nitrobenzoate yielding the adduct 123. Oligomers carrying the photolabile group adjacent to the 3'-end have served as primers for template-directed DNA synthesis with either the Klenow fragment of *E. coli* DNA polymerase I, bacteriophage T4 DNA polymerase, or AMV reverse transcriptase. Irradiation of the primer elongation mixture with 302 nm light produced covalent complexes between DNA and the polymerases. Cross-linking with AMV reverse transcriptase was shown to occur predominantly in the 5'-subunit.^{165a}



122



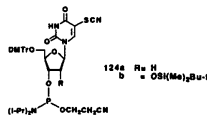
123

Along similar lines, the incorporation of the deoxyribonucleoside phosphoramidite 122 into oligomers has been achieved as a means to generate DNA duplexes biotinylated at specific locations and determine the structural requirements for the exonuclease and polymerase activities of prokaryotic and phage DNA polymerases.¹⁶⁶ It was demonstrated that, in the presence of avidin, the exonuclease and polymerase activities of the Klenow fragment required the primer terminus to be at least fifteen and six base pairs downstream from the biotin-avidin complex, respectively. These results showed that the polymerase and exonuclease sites of the Klenow fragment were physically distinct in solution and displayed different substrate structural requirements for activity.^{166b}

The insertion of 122 in oligonucleotides also allowed the derivatization of aminoalkylated oligomers with manyl or danyl chloride.^{165c} Annealing the fluorescent oligomers with complementary oligonucleotides generated duplexes exhibiting fluorescence emissions that increased in intensity upon binding with the Klenow fragment of DNA Polymerase I. By varying the position of the fluorescent label within the DNA duplexes and observing fluorescence emissions, strong enzyme-DNA contact points were identified.^{165c}

Bradley and Hanna¹⁶⁶ described the synthesis of 5-thiocyano-2'-deoxyuridine and uridine phosphoramidites (124a-b) for the functionalization of oligonucleotides without altering Watson-Crick base-pairing. Modified oligonucleotides resulting from the incorporation of 124a were deprotected under standard conditions without affecting the 5-thiocyano function. Upon treatment with DTT at 55 °C, the thiocyano group was reduced to a 5-mercapto function which was then reacted with either *p*-azidophenacyl bromide or 5-iodoacetamidofluorescein to yield functionalized oligonucleotides suitable for the study of protein-nucleic acid interactions.^{166a} It must be understood that a variety of functional groups could also be attached to such modified oligonucleotides.

In a different context, dodecamers containing 4-thiothymine residues and the recognition site d(GATATC) of the endonuclease *EcoRV* or *EcoRV* methyltransferase were found to photochemically cross-link to either enzymes.^{166b}



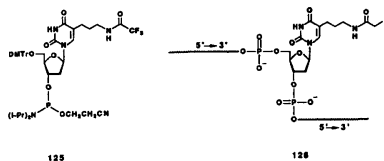
124a

b

c

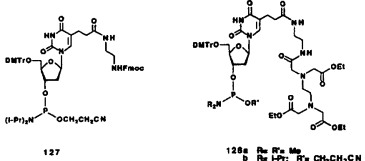
The protected aminoalkylphosphoramidite 125 was synthesized by Meyer *et al.*^{167a} and applied to the solid-phase synthesis of an oligonucleotide carrying an internal 5-(3-aminopropyl)uracil residue. The deprotected oligomer was iodocoupled with *N*-hydroxysuccinimidyl iodacetate affording 126. The incubation of 126 with its complementary DNA target generated observable interstrand cross-linking within 48 h at 37 °C. It was shown that the cross-linking reaction occurred by alkylation of a guanine residue of the complementary strand. Heating the DNA duplex at 51 °C resulted in the depurination of the alkylated guanine residue and scission of the target strand at the depurination site.^{167a} In addition, the modified oligonucleotide selectively alkylated the target strand without random alkylation of non-complementary nucleic acids. This feature is quite attractive for the application of these oligonucleotides as chemotherapeutic agents.

Similarly, Fovsic *et al.*^{167b} reported the synthesis of the deoxyribonucleoside phosphoramidite 127 and its incorporation at the 5'-end of an oligonucleotide by solid-phase synthesis. The deprotected and modified oligonucleotide was treated with *N*-hydroxysuccinimidyl bromoacetate to yield the corresponding *N*-bromoacetylated oligodeoxyribonucleotide. This oligomer hybridized to adjacent



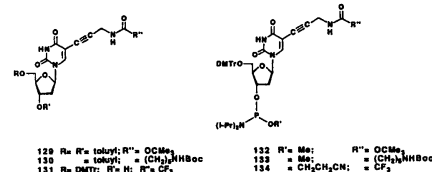
inverted purine tracts on double-helical DNA by triple-helix formation and alkylated single guanine residues on opposite strands at 37 °C (pH 7.4). After depurination, double-strand cleavage at a single site within plasmid DNA occurred in yields greater than 85%.^{167b} This methodology may become a valuable tool for the sequence-specific cleavage of large DNA molecules.

In their efforts to develop sequence-specific DNA cleaving agents, Dreyer and Dervan^{168a} reported the synthesis of a DNA hybridization probe carrying an EDTA-functionalized derivative of thymidine. In the presence of Fe(II), O₂ and DTT the modified probe led to the cleavage of a complementary single-stranded DNA segment.^{168a} The EDTA-functionalized deoxyribonucleoside was incorporated into the hybridization probe via the phosphoramidite 128a during routine solid-phase synthesis.^{168a,b} The phosphoramidites 128a-b were similarly inserted in triple-helix forming oligodeoxyribonucleotides which mediated the single site-specific cleavage of double-helical DNA *in vitro*.^{168c,d}



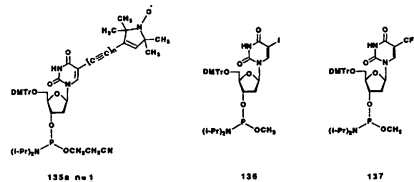
The derivatization of deoxyuridines at C-5 with masked primary aliphatic amino groups has also been exploited by Cruickshank *et al.*¹⁶⁹ and Hamambidi *et al.*¹⁷⁰ Their strategies involved the reaction of a suitably protected 5-iododeoxyuridine with a *N*-blocked propargylamine, bis-(triphenylphosphine)palladium chloride, and cuprous iodide to yield the corresponding alkynyl nucleoside 129¹⁷⁰ or 131.¹⁶⁹ Additionally, the alkynyl nucleoside 130 was prepared by reacting the deprotected amino function of 129 with the *p*-nitrophenyl ester of 6-tert-butylisopropylcarbamoylaminobenzoic acid and DBU.¹⁷⁰ The alkynyl nucleosides 129-131 were converted to their corresponding phosphoramidites (132-134) and inserted in oligonucleotides by standard solid-phase synthesis.^{169,170} Following the removal of the phosphate protecting groups, the support-linked oligomers were treated with trifluoroacetic acid/ethanedithiol (5:1) for 5 min to cleave the *tert*-butylisopropylcarbamoyl protecting group(s) without extensive depurination.¹⁷⁰ The purified aminoalkylated oligonucleotides were

conjugated to fluorescein.^{169,170} and the resulting conjugates were hybridized with complementary polyadenylated mRNAs.¹⁷⁰ Probes having short linker arms (derived from 132) did not hybridize as efficiently as the probes carrying larger linker arms (derived from 133). Furthermore, 5'-singly labeled probes hybridized better than the multilabeled ones.¹⁷⁰



Boc = *tert*-butyloxycarbonyl

Using a similar synthetic methodology, Spaltenstein *et al.*¹⁷¹ prepared the deoxyribonucleoside phosphoramidite 135a for the site-specific incorporation of a paramagnetic probe into a self-complementary oligonucleotide. The bulky and hydrophobic nitroxide moiety did not induce disruption of DNA secondary structures and was sufficiently constrained to permit the correlation of its motion with that of the carrier DNA. However, the length of the alkynyl arm (from the incorporation of 135b) affected the rotation of the nitroxide moiety about the alkynyl axis and should be taken into consideration when monitoring sequence-dependent structural features.¹⁷² These probes may, nonetheless, be useful in investigations pertaining to the dynamics of unusual DNA structures and/or sequences of biological relevance.



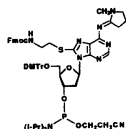
The study of macromolecular structures can be performed by X-ray scattering methods both in solution and in the solid state. The presence of a heavy atom label facilitates the elucidation of the geometric and dynamic structure of the molecule. In this context, Sheardy and Seeman¹⁷³ reported the

synthesis of the 5-iododeoxyuridine phosphoramidite 136 and its incorporation into a DNA segment corresponding to an analogue of strand 2 of the immobile nucleic acid junction 11.¹⁷⁴ To minimize the loss of iodine, the oligonucleotide analogue was completely deprotected by treatment with concentrated ammonium hydroxide at ambient temperature.¹⁷⁵ In an effort to unravel the detailed chemistry of genetic recombination, oligonucleotides required for the additional construction and analysis of immobile¹⁷⁶ and monomobile DNA junctions have also been synthesized from deoxyribonucleoside phosphoramidite derivatives.¹⁷⁸

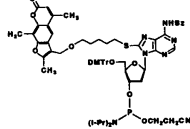
The trifluorothymidine phosphoramidite 137 has been synthesized and incorporated with a coupling efficiency of ca. 94% at defined locations into an oligonucleotide designed to inhibit the expression of gene sequences encoding serine proteases in T-lymphocytes.¹⁷⁸ The modified oligomer d(GAGAT⁺CT⁺T⁺CAT⁺CT⁺T⁺CCCCGG), where T⁺ represents trifluorothymidine, was detected by ¹⁹F NMR spectroscopy at a concentration of 10 μ M with a signal to noise ratio of 10:1.¹⁷⁸ It has been postulated that such limits of detection should be satisfactory for the *in vivo* NMR imaging of the cellular uptake and intracellular distribution of the oligonucleotide analogue.

4.6. Ligand Incorporation at C-8 of Purines

Roduit *et al.*¹⁷⁷ described a different approach for the introduction of an amino linker into synthetic DNA. The method entailed the reaction of 8-bromodeoxyadenosine with a protected aminoalkylthiolate salt. The deoxyribonucleoside phosphoramidite 138 was prepared in seven steps from 2'-deoxyadenosine and was inserted in oligonucleotides with high coupling yields (96-99%). The purified aminoalkylated oligonucleotides were biotinylated upon reaction with either the *N*-hydroxysuccinimide ester of biotin or caproylamidobiotin in a neutral buffer.¹⁷⁷



138



139

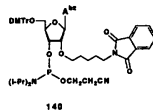
Using a similar concept, Piesles *et al.*¹⁷⁸ described the attachment of 4,5,8-trimethylpsoralen at C-8 of deoxyadenosine through a sulfur atom and a five carbon atom linker. The modified nucleoside was converted to the phosphoramidite 139 and incorporated into oligonucleotides. The hybrid formed between a modified oligomer and its unmodified complementary strand was exposed to near UV light (345 nm) for 50 min. It was shown that the extent of cross-linking in this experiment was greater than 90%.¹⁷⁸ The potential biological relevance of this *in vivo* cross-linking experiment should provide the impetus to define the parameters required for such events to occur *in vivo*.

5. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE CARBOHYDRATES

The derivatization of oligonucleotides, particularly at the internucleotide phosphodiester linkages or at the nucleosides has inherent limitations. Functional groups attached to these entities may interfere with base pairing and/or stacking interactions. The functionalization of oligonucleotides

by incorporating 2'-O-modified ribonucleosides may provide an alternate approach to the multiple labeling of nucleic acids in the minor groove.

Manoharan *et al.*¹⁷⁹ reported the synthesis of the ribonucleoside phosphoramidite 140 in 21% overall yield from adenosine. The synthetic method consisted of the preferential 2'-O-alkylation of the nucleoside with an alkyl halide followed by nucleoside/5'-O-protection and 3'-O-phosphitylation.



140

The insertion of 140 in oligonucleotides (20-mers) by solid-phase synthesis proceeded within 10-15 min with a coupling efficiency better than 95%. Standard deprotection yielded 2'-O-aminoalkylated oligonucleotides which, after purification, were reacted with fluorescein isothiocyanate or with the *N*-hydroxysuccinimide ester of either biotin, cholic acid, or digoxigenin-3-O-methylcarboxyl- ϵ -aminocaproic acid in aqueous buffers at pH 8-9. This methodology also led to the functionalization of oligodeoxyribonucleoside phosphorothioates and 2'-O-methyl oligoribonucleosides.¹⁷⁹ It must be noted that the length of the 2'-aminoalkylated linker can be selected to facilitate minor groove modification for biological applications.

CONCLUDING REMARKS

Inasmuch as nucleoside phosphoramidites have had a tremendous impact on the synthesis of oligonucleotides,¹ this Report further delineates applications of phosphoramidite derivatives to the functionalization of oligonucleotides for, in most cases, biomedical purposes. The incorporation of nucleoside phosphoramidites with modified nucleobases into oligonucleotides has also contributed to a better understanding of the dynamics and thermal stability of DNA duplexes in solution^{180,181} and their recognition by proteins. Furthermore, such modified oligonucleotides have recently served as models in mutagenesis experiments and as inhibitors of gene expression by interfering with the transcription of specific genes or with the translation of specific messenger RNAs. These applications will be addressed in detail in a future Report.¹⁸²

Acknowledgements: We are indebted to Judith B. Regan for her assistance in drafting and proofreading the manuscript.

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ANEXO 4

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Predicting the efficacy of short oligonucleotides in antisense and RNAi experiments with boosted genetic programming

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Received on March 26, 2004; revised and accepted on June 10, 2004
Advance Access publication June 17, 2004

ABSTRACT

Motivation: Both small interfering RNAs (siRNAs) and antisense oligonucleotides can selectively block gene expression. Although the two methods rely on different cellular mechanisms, these methods share the common property that not all oligonucleotides (oligos) are equally effective. That is, if mRNA target sites are picked at random, many of the antisense or siRNA oligos will not be effective. Algorithms that can reliably predict the efficacy of candidate oligos can greatly reduce the cost of knockdown experiments, but previous attempts to predict the efficacy of antisense oligos have had limited success. Machine learning has not previously been used to predict siRNA efficacy.

Results: We develop a genetic programming based prediction system that shows promising results on both antisense and siRNA efficacy prediction. We train and evaluate our system on a previously published database of antisense efficacies and our own database of siRNA efficacies collected from the literature. The best models gave an overall correlation between predicted and observed efficacy of 0.46 on both antisense and siRNA data. As a comparison, the best correlations of support vector machine classifiers trained on the same data were 0.40 and 0.30, respectively.

Availability: The prediction system uses proprietary hardware and is available for both commercial and strategic academic collaborations. The siRNA database is available upon request.

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INTRODUCTION

Antisense oligonucleotides and RNA interference are the two technologies that are available for sequence-specific knockdown of mRNA. Antisense oligonucleotides (ODNs) typically consist of 15–20 nt that inhibit gene expression by complementary base-pairing to target mRNA. The result is a simple mechanistic blocking of the ribosome thereby inhibiting protein translation, or activation of the RNase H enzyme that subsequently induces cleavage and degradation of mRNA (Kurreck, 2003). Modern ODNs have been chemically modified in various ways to increase their potency.

More recently, RNA interference (RNAi)—a natural biological pathway for mRNA depletion—was discovered: 21–23 nt long double-stranded RNA with characteristic 3' overhangs is incorporated into a ribonucleoprotein complex called RNA-induced silencing complex, which cleaves mRNA with complementarity to its RNA component (McManus and Sharp, 2002). The RNA agent is called short interfering RNA (siRNA).

Scherer and Rossi (2003) reviews the relative strengths and weaknesses of ODNs and siRNAs as well as catalytically active classes of oligonucleotides (oligos) for mRNA knockdown that will not be discussed here.

The question of oligo efficacy arises because not all oligos are equal; common to both ODNs and siRNAs are that only a fraction of the oligos are effective in biological assays. Methods that can predict the efficacy of potential oligos are important since these would reduce the cost of carrying out antisense and siRNA knockdown experiments.

There has been some previous work on *in silico* methods that predicts oligo efficacy. The work range from considering thermodynamic properties (Matveeva *et al.*, 2003; Khvorova *et al.*, 2003; Schwarz *et al.*, 2003), to using sequence motifs (Matveeva *et al.*, 2000) and artificial neural networks (Giddings *et al.*, 2002; Chalk and Sonnhammer, 2002) for predicting efficacy. Since the RNAi pathway is a more recent discovery, however, most of this work has been done on ODNs. Here, we bridge the gap by (i) collecting a database of publicly available siRNA efficacy data and (ii) developing and evaluating a machine learning method that can predict both ODN and siRNA efficacy.

Our main contribution is a machine learning system that predicts ODN and siRNA efficacy and is consistently better than previously published methods. Furthermore, we present a uniform analysis of the thermodynamic properties of ODNs and siRNAs.

METHODS

In the following sections, we will describe the databases used in our experiments and present the algorithms used in

Production	Semantic Rule
(1) $R \rightarrow \{C : p \geq N\}$	$R.hit := C.count \geq N.cutoff$
(2) $C \rightarrow (C_1)(C_2)$	$C.count := C_1.count + C_2.count$
(3) $C \rightarrow A$	$C.count := A.count$
(4) $A \rightarrow A_1 \mid A_2$	$A.count := A_1.count + A_2.count$
(5) $A \rightarrow L$	$A.count := L.count$
(6) $L \rightarrow a$, for some $a \in \{a, c, g, t\}$	$L.count := match(a)$
(7) $N \rightarrow n$, for some $n \in \{1, 2, \dots\}$	$N.cutoff := n$

(a)

(b)

Fig. 1. Our genetic programming solution language. (a) Grammar. The grammar is shown in Backus–Naur form, with non-terminals represented by uppercase letters and terminals represented by boldface letters. Syntactical elements in the language, such as parentheses and operators, are in normal typeface. Alternatives are represented as separate productions. Adjacent symbols are concatenated. (b) Semantics. An expression matches a string if *R.hit* is true. *match*(a) returns 1 if a is identical to the character it is compared with, and 0 otherwise.

our classification system. This includes sections on genetic programming (GP), boosting and boosted GP. We also briefly discuss the inherent stochastic properties of GP, and describe how support vector machines (SVMs) can be used to predict ODN and siRNA efficacy. SVMs are often regarded as state-of-the-art among the different machine learning methods, and will be used as a benchmark throughout this work. Finally, we describe a procedure for estimating the predictive accuracy of classifiers, and detail the methods used to compute oligo thermodynamics.

Databases

A collection of experimentally evaluated siRNAs were collected from different sources (Vickers *et al.*, 2003; Kawasaki *et al.*, 2003; Harborth *et al.*, 2003; Holen *et al.*, 2002; Khvorova *et al.*, 2003). We cross-referenced the siRNAs with their corresponding GenBank sequences when possible, and otherwise used the sequences as given in the articles.

We assigned an efficacy score based on the reported mRNA or protein knockdown levels to each siRNA. For the human cyclophilin sequences in Khvorova *et al.* (2003), where individual efficacies were missing, we assigned 0.05 and 0.75 knockdown to the effective and ineffective sequences. The efficacy score measures the fraction of mRNA or protein that remains after the knockdown experiment. Hence, effective and ineffective siRNAs are assigned low and high efficacy scores. The same measure was used in Giddings *et al.* (2000).

For our antisense experiments, we used the set of antisense sequences collected by Giddings *et al.* (2000).

We partitioned both the siRNA database and the antisense database into a set of positive and negative sequences. We used a cut-off score of 0.5; i.e. the sequences that scored above the threshold were classified as negative and vice versa. After duplicates were removed, a total of 101 positive and 103 negative siRNAs, and 125 positive and 186 negative ODNs remained.

Genetic programming

Genetic programming (Koza, 1992), as the genetic algorithms of Holland (1975), use evolution in a population of candidate solutions to solve problems. The main difference between genetic algorithms and GP is that the former operate on a population of (bit) strings, whereas the GP populations usually consist of syntax trees in some solution language. The solution language defines the structure of the possible solutions. Other solution representations such as linear or graph-based GP exist (see Banzhaf *et al.*, 1997 for an overview and a good introduction).

Our GP algorithm is designed to solve two-class classification problems where the positive and negative data consists of strings. It uses subtree swapping crossover, tree generating mutation and reproduction as generic operators. Individuals are chosen for participation in new generations using tournament selection.

Each individual in the population is a syntax tree in a formal query language (Interagon AS, 2002, <http://www.interagon.com/pub/whitepapers/IQL.reference-latest.pdf>). To ensure that all individuals are legal expressions, we use a set of production rules when generating and combining the individuals, as Montana (1995). These production rules correspond to the grammar of the query language.

In the experiments described in this paper, only a subset of the full query language is used. Figure 1 gives a grammar for this subset along with a formal definition of its semantics.

The semantics can be explained as follows: the numeral *N* in the $p \geq N$ part indicates the minimum number of terminals in the *C*-production that must match. This means that if the fourth production is removed from the language, the expressions search for strings with Hamming distance less than $|C| - N$, where $|C|$ is the number of characters in the expression. For example, the expression $\{gcggtt : p \geq 4\}$ will match the strings *gcgatt* and *acgatt*, but not *acgata* and *actatt*.

Alternatives (production 4) enables, in addition to alternative bases at the same string position, the construction of

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expressions where some positions in the string are considered more important than others. To illustrate this, consider the expression $\{gc(g|g)gtt : p \geq 4\}$, where $(g|g)$ matches g in the same string position twice (we will in the following use the shorthand $(g|g) = g_2$ for weighted positions). This expression now matches the string *acgata*, but still misses *actatt*.

Note that if the string is longer than the expression, the expression reports a match if it matches any of the string's possible substrings. Thus, the expression $\{gcggtt : p \geq 4\}$ will match strings *aagcaatt* and *gcgcgtt*, but will miss *agcaaat* and *aattgcgg*.

The Interagon pattern matching chip (PMC) (Halaas *et al.*, 2004) is an application specific integrated circuit designed to provide orders of magnitude higher performance than comparable regular expression matchers. In our GP system, we use the PMC to evaluate the individual expressions in the GP population, thus reducing the GP system's total computation time. This increased performance becomes important when the datasets are large, or when many GP runs must be done, as for instance in cross-validation experiments or when GP is used as the base learner in a boosting algorithm.

Given an oligo sequence, the expressions generated by our GP system will only be able to answer 'yes' or 'no' as to whether the sequence is effective or not. The following section describes a method that creates classifiers with a more refined output.

Boosting

Boosting algorithms combine a set of simple rules to form a single model. The algorithms construct the combined model so that the performance of the model is increased compared to each of the single rules. Given a learning algorithm that generates a set of hypotheses $h_1, h_2, \dots, h_T$, the boosting algorithms construct a combined hypothesis f on the form

$$f(x) = \sum_{t=1}^T \alpha_t \cdot h_t(x). \quad (1)$$

α_t is the weight of hypothesis h_t , and both weights and hypotheses are learned by the boosting algorithm.

The AdaBoost algorithm, introduced by Freund and Schapire (1997), was the first step towards practical boosting algorithms. AdaBoost constructs classifiers that maximize the minimal margin in the training data (Meir and Rätsch, 2003), but as a result, the algorithm does not handle noise or outliers in the dataset very well. To solve this, regularized boosting algorithms, such as AdaBoost_{Reg} (Rätsch *et al.*, 2001) and v -Arc (Rätsch *et al.*, 2000), have been developed. Boosting algorithms are related to other large margin classifiers such as SVMs (Vapnik, 1995; Müller *et al.*, 2001); for instance, Rätsch *et al.* (2002) constructed a boosting algorithm for detecting outliers from a corresponding support vector algorithm.

Generally, boosting algorithms works as follows (Meir and Rätsch, 2003): given a training set S of m examples (x, y) , the algorithms start by assigning weights $d_{i=1, \dots, m}$ to each element in the set. Then the algorithms iteratively construct the T hypothesis in (1), so that for each iteration t the algorithms:

- (1) Train a basic hypothesis h_t on the weighted data.
- (2) Find the hypothesis weight α_t , which is found by minimizing the loss function $G(f_t + \alpha \cdot h_t, S)$.
- (3) Set $f_{t+1} = f_t + \alpha_t \cdot h_t$.
- (4) Update each data weight so that $d_i^{(t+1)} = \partial / [\partial f_{t+1}(x_i)] G(f_{t+1}, S), i \in \{1, \dots, |S|\}$.

See Meir and Rätsch (2003) for further information on the loss functions used by different boosting algorithms.

Boosted GP

The idea of combining boosting algorithms with GP was first presented by Iba (1999). Here the training set for the GP algorithm was constructed by sampling from S , using the weight distribution D . Paris *et al.* (2001) extended this, by training on the complete set S , and including the data weights in the fitness function

$$\varepsilon(h, D, S) = \sum_{i=1}^{|S|} d_i \cdot |h(x_i) - y_i|. \quad (2)$$

The GPboost algorithm presented by Paris *et al.* (2001) is based on AdaBoost, and was used to solve simple benchmark regression problems without noise. Since GPboost is based on the AdaBoost algorithm, it is expected that GPboost will have difficulties on datasets that contain noise and outliers. To alleviate this, we propose a regularized algorithm, GPboost_{Reg}, which is based on the AdaBoost_{Reg} algorithm of Rätsch *et al.* (2001).

Our implementation uses the GP system described in the Genetic programming section as the base learner, with (2) as the fitness function. It produces classifiers of the form given in (1).

Variance in GP

Genetic programming is inherently a stochastic method. Consequently, two separate GP runs are not guaranteed to produce identical results. This also goes for boosted GP, hence there will be some variance in the predictions of different GPboost classifiers. This inherent variance can, however, be used to create even better classifiers. Hansen and Salamon (1990) showed that diverse and accurate classifiers can be combined to produce a more accurate classifier, which also has the added benefit of a reduced variance compared with the individual classifiers.

In our experiments, we create combined classifiers by taking the simple average of the individual classifiers. That is, for a

set of classifiers $f_1, f_2, \dots, f_n$, the combined classifier F is given by

$$F(x) = \frac{1}{n} \sum_{i=1}^n f_i(x). \quad (3)$$

Support vector machines

The classic SVM algorithm (C-SVM) was introduced by Vapnik (1995). In our experiments, we compare the performance of this algorithm and the ν -SVM algorithm by Schölkopf *et al.* (2000) with the performance of our GP system. Both SVM algorithms use radial basis functions as kernels. The implementation used in this study is part of the LIBSVM library (available at <http://www.csie.ntu.edu.tw/~cjlin/libsvm/>).

While our GP system uses the oligo sequences as input, the input to the SVMs must be numerical data. In our experiments we solve this by using three different encodings, where the first encoding simply transforms the sequences into numerical data by mapping *as* to 0, *cs* to 0.33, *gs* to 0.67 and *ts* to 1. This encoding ensures that the letters are evenly distributed in the [0, 1] attribute range suggested in the LIBSVM documentation.

The second encoding uses the relative number of each nucleotide and dinucleotide in the sequence. That is, the input is a 20-dimensional vector where the first element in the input is the relative number of *as* in the sequence, the fifth element is the relative number of *aas*, and so forth.

Our third encoding uses the relative number of tetramers in each sequence. We use this representation because Matveeva *et al.* (2000) showed that certain tetramers correlate with antisense efficacy. In addition, Giddings *et al.* (2002) found that using the relative number of tetramers in the sequence as input to an artificial neural network classifier gave better results than using dimers or trimers.

Classifier accuracy

The predictive accuracy (or generalization accuracy) of a classifier is important, because it gives a measure of how well the classifier will perform on unseen data. That is, it estimates the probability that the classifier will correctly predict the class of unseen data. The classifier accuracy is given by

$$\text{Acc} = \frac{\text{Tp} + \text{Tn}}{\text{Tp} + \text{Tn} + \text{Fp} + \text{Fn}}, \quad (4)$$

where Tp, Fp, Tn and Fn are the number of true positive, false positive, true negative and false negative predictions.

A 10-fold cross-validation is known to create a good estimate of the predictive accuracy of classification methods (Martin and Hirschberg, 1996). The average of the 10 testing accuracies from the cross-validation procedure estimates the predictive accuracy of a classifier trained on the original dataset.

In our experiments, we use cross-validation for both accuracy and parameter estimation. That is, we do a separate

10-fold cross-validation experiment on each of the 10 training sets to determine the optimal parameters of the classification algorithm. This is done to ensure that the training process—both parameter estimation and model generation—is completely independent of the test data.

An alternative to using the accuracy as the 10-fold estimate is to use the Matthews correlation:

$$M = \frac{\text{Tp} \cdot \text{Tn} - \text{Fp} \cdot \text{Fn}}{\sqrt{(\text{Tn} + \text{Fn})(\text{Tn} + \text{Fp})(\text{Tp} + \text{Fn})(\text{Tp} + \text{Fp})}}. \quad (5)$$

Matthews (1975) points out that this correlation measure is often a more informative quality measure than the accuracy.

The receiver operating characteristic (ROC) is an even more general quality measure (Hanley and McNeil, 1982). A ROC curve is a plot of the sensitivity Se versus the specificity Sp of a given classifier, where

$$\text{Sp} = \frac{\text{Tn}}{\text{Tn} + \text{Fp}}, \quad \text{and} \quad (6)$$

$$\text{Se} = \frac{\text{Tp}}{\text{Tp} + \text{Fn}}. \quad (7)$$

A ROC curve can be characterized by the area under its curve: perfect classification gives an area of 1.0, and random classification gives an area of 0.5. The ROC area or ROC score gives a good indication of whether one classifier has a higher overall performance than another classifier. One can also use sign tests on the classifiers's output (Salzberg, 1997) for varying specificity levels to test whether one classifier is significantly better on the given specificity level. In this work, we focus these tests on the high specificity levels, since in practical applications, users will in most cases only be interested in the oligos that are highest ranked. That is, they want few false positives among the highest ranked oligos (on high specificity levels), and do not care if there are some false negatives among the oligos of low rank (on low specificity levels).

All the above measures assume that the examples to be classified have been characterized as being either positive or negative sequences. The correlation coefficient R between the predicted and the observed efficacy is an alternative quality measure that does not depend on such a characterization. R^2 represents the proportion of variation in the observed efficacy that can be explained by the classifiers. A Student's t -test gives the statistical significance of a given correlation.

Oligo thermodynamics

We use the nearest-neighbour model (Xia *et al.*, 1998) to compute the oligo/mRNA duplex stability. The model is used in several of our custom applications that in addition to the overall duplex stability, also compute the oligo 5' stability and the difference in oligo 5' and oligo 3' stability.

The oligo 5' stability is computed from the first five nucleotides in the oligo (antisense) sequence. When computing the difference in 5' and 3' stability, we compute the stability of

Table 1. The average results from 10 different 10-fold cross-validation experiments using the genetic programming algorithms on the antisense data

Algorithm	ROC	<i>R</i>	<i>P</i>	<i>M</i>	Sc	Sp
GP	N/A	−0.18 ± 0.06	1 × 10 ^{−3}	0.15 ± 0.05	0.34 ± 0.05	0.79 ± 0.04
GPboost	0.71 ± 0.01	−0.37 ± 0.02	2 × 10 ^{−11}	0.31 ± 0.03	0.61 ± 0.03	0.70 ± 0.03
GPboost _{Reg}	0.71 ± 0.02	−0.38 ± 0.04	4 × 10 ^{−12}	0.29 ± 0.05	0.59 ± 0.05	0.71 ± 0.02

R is the correlation and *P* is its statistical significance. *M*, Sc and Sp are the Matthews correlation, sensitivity and specificity when using the the standard cutoff for the algorithms (≥0 for the boosted GP algorithms). Note that because the standard GP classifiers are binary, no ROC score is given for this algorithm.

the first four (5′) and the last four (3′) nucleotides in the oligo sequence. The difference is given by the 5′ stability minus the 3′ stability, and a negative value means that the 5′ end of the antisense strand is more stable than the 3′ end.

We use the parameters of Xia *et al.* (1998) in all our RNA/RNA nearest-neighbour model computations, except for the 5′ stability calculations where we use the same parameters as that of Khvorova *et al.* (2003). For DNA/RNA interactions, we use the parameters of Sugimoto *et al.* (1995).

The Mfold web server of Zuker (2003) is used to compute the oligo secondary structure stability.

RESULTS AND DISCUSSION

The following sections detail the results from our experiments at predicting siRNA and antisense efficacy. Furthermore, we show how different thermodynamic properties correlate with the observed efficacies sequences, and that effective antisense and siRNA oligos have different thermodynamic properties. We also show that the genetic programming classifiers for the antisense and siRNA oligos differ. Finally, we discuss the possibilities of using our predictors in the genomewide oligo design.

Antisense predictions

We analyzed the antisense data using the GP, GPboost and GPboost_{Reg} algorithms, and Table 1 shows the average results of 10 different 10-fold cross-validation experiments for these algorithms. The GP solutions were binary classifiers of the form given in Figure 1 and the boosted GP classifiers had the form given in (1). Although all three methods have a significant correlation between the predicted and observed efficacy, the boosted GP algorithms get better results than the standard algorithm.

Table 2 compares the accuracy of the three input encodings for the two SVM algorithms with the accuracy of the GP-based algorithms. The GP-based algorithms use the average of 10 different models as output (3); i.e. the basic GP models are the average of 10 binary classifiers of the form given in Figure 1, and the boosted GP classifiers are the average of 10 classifiers of the form given in (1). ROC curves for the GP-based algorithms were generated by measuring the sensitivity and specificity for different thresholds of the average classifiers (the average GP classifier has 10 different thresholds; the

Table 2. Antisense results

Algorithm	ROC	<i>R</i>	<i>P</i>	<i>M</i>	Sc	Sp
GP	0.61	−0.28	5.2 × 10 ^{−7}	0.22 ± 0.20	0.35	0.83
GPboost	0.75	−0.45	8.1 × 10 ^{−17}	0.38 ± 0.20	0.64	0.75
GPboost _{Reg}	0.76	−0.46	1.7 × 10 ^{−17}	0.43 ± 0.18	0.66	0.77
ν-SVM(seq)	0.54	−0.04	0.45	0.01 ± 0.14	0.09	0.93
C-SVM(seq)	0.52	−0.01	0.86	0.03 ± 0.13	0.10	0.94
ν-SVM(1–2)	0.68	−0.35	5.6 × 10 ^{−10}	0.28 ± 0.15	0.45	0.82
C-SVM(1–2)	0.67	−0.37	1.6 × 10 ^{−11}	0.16 ± 0.24	0.26	0.89
ν-SVM(4)	0.66	−0.31	2.8 × 10 ^{−8}	0.23 ± 0.17	0.50	0.72
C-SVM(4)	0.69	−0.40	2.2 × 10 ^{−13}	0.26 ± 0.13	0.36	0.85

See Table 1 for table header explanations.

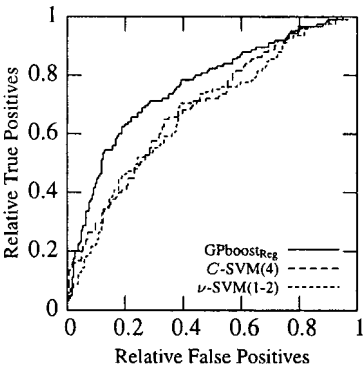


Fig. 2. ROC graphs for the GPboost_{Reg} and best SVM classifiers on the antisense data. The GPboost_{Reg} algorithm has the highest sensitivity for almost all specificity levels.

average boosted GP classifiers have more). This table shows that the two boosting algorithms have the best overall results in terms of ROC score and correlation. They also get the best Matthews correlation. Figure 2 shows the ROC graphs for the GPboost_{Reg} algorithm and the input-encodings to the ν-SVM and C-SVM algorithms that have the highest ROC-score. To test whether the GPboost_{Reg} algorithm had a significant better performance than the other two classifiers, we used a sign test (Salzberg, 1997) on specificities 0.95, 0.9, 0.8 and 0.6 (for a motivation see Classifier Accuracy section). The GPboost_{Reg} algorithm was significantly better (*P* ≤ 0.05) than the ν-SVM

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Table 3. The average results of 10 different runs of the genetic programming based algorithms on the siRNA data

Algorithm	ROC	<i>R</i>	<i>P</i>	<i>M</i>	Se	Sp
GP	N/A	−0.19 ± 0.05	7 × 10 ^{−3}	0.16 ± 0.05	0.47 ± 0.05	0.68 ± 0.05
GPboost	0.65 ± 0.03	−0.31 ± 0.03	6 × 10 ^{−6}	0.21 ± 0.07	0.58 ± 0.04	0.63 ± 0.06
GPboost _{Reg}	0.67 ± 0.03	−0.33 ± 0.05	1 × 10 ^{−6}	0.22 ± 0.08	0.59 ± 0.04	0.63 ± 0.05

See Table 1 for table header explanations.

algorithm on specificities 0.95 and 0.9, and significantly better than the C-SVM algorithm on specificities 0.9 and 0.8.

Another point worth considering is that using (3) to combine several GP-based classifiers do result in better classifiers. This is evident from comparing the results in Tables 1 and 2: the results for the GP-based classifiers in Table 2 are consistently better than the corresponding results in Table 1; the GP-based classifiers in Table 2 use the algorithm from (3).

Giddings *et al.* (2002) observed that using the complete tetramer vector gave poor results. To reduce the risk of overfitting, they went on to consider only the 40 most significant tetramers in the dataset. However, the cross-validation procedure does not remain independent when the complete dataset is used to select the input. Consequently, one would expect that the cross-validation accuracy estimate becomes positively biased; i.e. the estimates are better than they would be if tested on a truly independent test set. Indeed, when subsequently testing their classifiers on an independent test set, Giddings *et al.* found that the accuracy was lower than the reported 10-fold cross-validation accuracy.

To test this hypothesis of a possible bias, we did an experiment where we first estimated the 10-fold cross-validation accuracy using the 40 most significant tetramers in the global dataset, as measured by the Matthews correlation coefficient (5). This is analogous to the experiments of Giddings *et al.* (2000). We then did a 10-fold cross-validation experiment where we used the 40 most significant tetramers in each local training set as input to the SVM classifiers. When we compared the results of these two experiments, we found that although the local top 40 estimates gave results similar to using the complete tetramer input, the global top 40 estimate gave much higher accuracy estimates (ROC score, correlation and Matthews correlation of 0.77, −0.47 and 0.39). Hence, using the 40 most significant tetramers in the dataset does give a biased estimate of the classifier accuracy.

siRNA predictions

We did the same analysis on the siRNA data as we did on the antisense data. Table 3 shows the average results of 10 different runs of the GP, GPboost and GPboost_{Reg} algorithms (please confer the relevant Methods sections and the comments to Tables 1 and 2 for details on the different GP classifiers). The results are similar to the antisense results (confer Table 1).

Table 4. siRNA results

Algorithm	ROC	<i>R</i>	<i>P</i>	<i>M</i>	Se	Sp
GP	0.62	−0.27	9.4 × 10 ^{−5}	0.19 ± 0.09	0.45	0.73
GPboost	0.70	−0.42	4.0 × 10 ^{−10}	0.27 ± 0.18	0.56	0.70
GPboost _{Reg}	0.72	−0.46	4.5 × 10 ^{−12}	0.24 ± 0.24	0.50	0.73
ν-SVM(seq)	0.57	−0.05	0.48	0.09 ± 0.23	0.52	0.56
C-SVM(seq)	0.53	−0.02	0.78	0.05 ± 0.20	0.35	0.70
ν-SVM(1–2)	0.70	−0.21	2.6 × 10 ^{−3}	0.31 ± 0.19	0.61	0.68
C-SVM(1–2)	0.64	−0.30	1.3 × 10 ^{−5}	0.26 ± 0.14	0.45	0.80
ν-SVM(4)	0.62	−0.14	0.046	0.25 ± 0.16	0.49	0.73
C-SVM(4)	0.65	−0.30	1.3 × 10 ^{−5}	0.19 ± 0.20	0.35	0.82

See Table 1 for table header explanations.

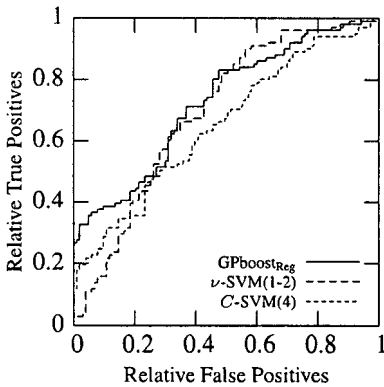


Fig. 3. ROC graphs for the GPboost_{Reg}, and best SVM classifiers on the siRNA data.

Table 4 summarizes the performance of the SVM algorithms and GP-based algorithms. Just as in the antisense analysis, the GP-based algorithms use the average of 10 different models as the final output. We also used the same input to the SVM algorithms here as previously. The two boosting algorithms achieve the best overall results in terms of ROC score and correlation, which was also the case for the antisense experiments. But in terms of the Matthews correlation, the ν-SVM classifiers that use the nucleotide and dinucleotide percentage input are now the highest. Figure 3 shows the ROC graphs for the GPboost_{Reg} algorithm and the input-encodings to the ν-SVM and C-SVM algorithms that have the highest

Table 5. The correlation of structural features with siRNA and antisense efficacy (see Methods section for details on thermodynamic computations)

Structural feature	siRNA sequences			Antisense sequences		
	oligos	<i>R</i>	<i>P</i>	oligos	<i>R</i>	<i>P</i>
oligo 5' stability	204	−0.21	2.6×10^{-3}	311	0.11	0.053
oligo 5' – oligo 3' stability	204	−0.28	5.0×10^{-5}	311	0.051	0.37
ΔG_{37}^o oligo 2D structure	203	−0.26	1.8×10^{-4}	302	−0.10	0.083
ΔG_{37}^o oligo/target duplex	204	−0.14	0.046	311	0.20	3.9×10^{-4}
Oligos with oligo/target duplex stability $\Delta G_{37}^o \leq -30$ kcal/mol						
ΔG_{37}^o oligo 2D structure	176	−0.28	1.7×10^{-4}	84	−0.44	2.8×10^{-5}
ΔG_{37}^o oligo/target duplex	177	−0.23	2.1×10^{-3}	89	−0.011	0.92
Oligos with oligo/target duplex stability $\Delta G_{37}^o > -30$ kcal/mol						
ΔG_{37}^o oligo 2D structure	27	−0.35	0.073	218	0.055	0.42
ΔG_{37}^o oligo/target duplex	27	−0.26	0.19	222	0.37	1.3×10^{-8}

Significant correlations *R* (*P* = 0.05) are marked in boldface.

ROC score. A sign test on specificities 0.95, 0.9, 0.8 and 0.6 showed that the GPboost_{Reg} algorithm was significantly better (*P* ≤ 0.05) than the *ν*-SVM algorithm on specificities 0.95 and 0.9, and significantly better than the *C*-SVM algorithm on specificity 0.95.

As a final note, comparing Tables 3 and 4 again shows that combining several GP-based classifiers results in better classifiers.

Structural feature predictions

Several researchers have pointed at correlations between different structural features of an oligo and its efficacy. For antisense oligos, Matveeva *et al.* (2003) have identified a significant correlation between the antisense efficacy and the free energy ΔG_{37}^o in the antisense and RNA target duplex formation. They also found a significant correlation between the antisense efficacy and the free energy in the predicted antisense secondary structure. Finally, they found that oligos that form less stable duplexes with target RNA depend more on high duplex stability to be effective. Conversely, ODNs that form highly stable duplexes depend on smaller self-interaction potentials to be effective.

For siRNA oligos, Khvorova *et al.* (2003) found that the 5' antisense region is significantly less stable in effective siRNAs than in ineffective siRNAs. Moreover, they observed different patterns in the internal stability profile for effective and ineffective siRNAs. At the same time, Schwarz *et al.* (2003) found that the 5' antisense region was less stable than the 5' sense region in functional siRNAs.

We tested these different structural features on both the antisense and siRNA data sets. Table 5 lists the correlation between each structural feature and the antisense and siRNA efficacy as well as its statistical significance. The details on the different thermodynamic computations are given in the Methods section.

Table 5 shows that siRNA and antisense oligos share some physical characteristics: both become less active when the self-interaction potential of an oligo is large. However, this seems to be the only common feature. The antisense and siRNA oligos seem to have complete opposite characteristics, both regarding 5' and overall duplex stability. To determine whether the opposite characteristics could be explained by the differences in oligo length for the antisense data, we did a separate analysis on the antisense sequences of length 20 in the database (178 out of 311 sequences). But this analysis gave the same overall results as reported in Table 5 (data not shown).

In addition, we investigated the relationship between duplex stability and self interaction potential, found by Matveeva *et al.* (2003). Table 5 support these results, since it shows that the efficacy of antisense oligos that are more stable ($\Delta G_{37}^o \leq -30$) is negatively correlated with the free energy in the predicted secondary structure of oligos. Likewise, the efficacy of less stable oligos ($\Delta G_{37}^o > -30$) is positively correlated to the free energy in the oligo/mRNA duplex. We found a similar trend in the smaller set of antisense sequences of length 20. For the more stable duplexes, the *P*-values were 0.75 for the duplex stability and 0.085 for the self-interaction potential; for the less stable duplexes, the corresponding *P*-values were 2.4×10^{-5} and 0.83. We did not, however, find a similar trend in the siRNA sequences.

We note that a possible extension to the method presented here is to try to combine our sequence-based, boosted GP predictors with the thermodynamic characteristics. This is, however, left as an extension for further work.

Antisense and siRNA classifiers differ

As the analysis in the previous chapter showed, effective antisense and siRNA oligos have very different thermodynamic characteristics. To determine whether this difference also was reflected in the classifiers produced by our algorithm, we examined the binary classifiers created by the standard GP

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Table 6. Examples of GP classifiers generated on the antisense and siRNA database

Data	Classifier	Example matches
ODNs	$\{c(g_6 a_5 c_8)(g_4 a_2 c_6)(g_3 a_2 c_8)(a_6 c_2)lc : p \geq 25\}$	.ccca., .gccca., .acca., .cgca.
siRNAs	$\{gcct(c_1 t_1)ct(a_1 t_1)ct(t_2)(c_4 t_1)(c_3 t_1)(c_3 t_1) : p \geq 13\}$	a..tccc,c.....tccc

The example matches list some of the least complex subsequences matched by the corresponding expression (. represents any letter). Thus, we have not listed matches involving the characters that have the least weight, as for instance *cccaate* for the ODN expression or *gcctctaattt...* for the siRNA expression. These longer matches are more likely to be specific to the training set than to represent general characteristics of ODNs or siRNAs. Note that the expressions use the subscript operator to denote the weight of each letter in alternatives (see production 4 in Fig. 1 and the accompanying examples in the text).

algorithm (Fig. 1). Because of the complexity of the boosted GP classifiers [these classifiers are a weighted combination of the basic expressions—see (1)], a detailed analysis of these classifiers was not done.

Table 6 shows two typical examples of the expressions generated on the antisense and siRNA data, along with some of the subsequences matched by each expression. The two expressions illustrate the main difference between the antisense and siRNA expressions: the subsequences matched by the antisense expressions are shorter than the subsequences matched by the siRNA expressions. Indeed, in an experiment where we greatly increased the number of generations that the GP system was run, the length of the subsequences matched by the siRNA expressions increased to 19. This equals the length of the siRNA oligos. The length of the subsequences matched by the antisense expressions, however, remained the same (data not shown).

This difference in motif length between the antisense and siRNA expressions suggests that for effective siRNAs, the base composition at different positions in the oligo is important. This dependence on base composition is also reflected in the 5' and 3' thermodynamics of the siRNA oligos (Table 5). For effective antisense oligos, on the other hand, the occurrence of specific, short motifs is important. This was also observed by Matveeva *et al.* (2000).

Genomewide oligo design

One of the important questions in genomewide oligo design is the tradeoff between oligo efficacy and oligo uniqueness. That is, for each gene in a genome one would like to have at least one oligo that is sufficiently unique so that it does not target any other genes, and has a high probability of being effective. As not all oligos predicted to be effective will be effective, one will need a set of candidate oligos for each gene.

To test how many potential candidate oligos our boosted GP classifiers predict to be effective, we downloaded 10 human mRNAs at random from the NCBI RefSeq database. We then

tested all candidate 19mer oligos on the antisense and siRNA efficacy predictors, and counted the number of oligos where the predictor output were above a threshold corresponding to 90% specificity. This resulted in that on average 22% of the candidate siRNAs and 16% of the candidate antisense oligos for each mRNA were predicted to be effective. The SD in both cases were 4%.

We also determined the number of candidate oligos that were unique in the human transcriptome. To do this we computed the Hamming distance between each candidate oligo and every 19mer in the Ensembl cDNA database, NCBI build 34. Oligos that had a Hamming distance greater than two to all other 19mers in the database were considered unique. The relative number of unique oligos for each mRNA varied between 0.5 and 55%, with an average of 24%.

Each mRNA had, however, candidate oligos that were both unique and predicted to be effective, both in RNAi and antisense experiments. The number ranged from one candidate to 11% of all possible oligos. The number of candidate oligos can be increased by decreasing the specificity threshold, but as the result of the uniqueness screening shows, the limiting factor on the number of candidate oligos is the uniqueness screening. One should expect that this would also be the case for true, genomewide oligo design.

CONCLUSION

We have developed a GP-based machine learning system that successfully predicts the efficacy of oligos used in antisense and siRNA experiments. The system is consistently better than both SVMs and physical property-based classifiers at predicting the efficacy of both antisense and siRNA oligos.

We have also showed that siRNA and antisense sequences do not seem to share the same physical characteristics. The only common feature is a sensitivity to a high self-interaction potential of the oligo. That is, oligos that form more stable secondary structures are less likely to be effective when used in both antisense and RNAi experiments.

Antisense and RNAi are very different technologies, and, as shown here, antisense and siRNA oligos have very different physical characteristics. Despite this, our novel, sequence-based machine learning system can successfully predict the efficacy of both antisense and siRNA oligos. This suggests that the oligo efficacy for both technologies is in a large part determined by the oligo sequence itself.

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REVIEW

ANTISENSE OLIGONUCLEOTIDES: A NEW TOOL IN
NEUROSCIENCE

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SUMMARY

1. Recent studies have shown that gene expression can be selectively attenuated by administration of short sequences of nucleotides (oligonucleotides) that are complementary to a portion of messenger RNA coding for a particular gene product.

2. This technique is known as ANTISENSE, because the oligonucleotides are complementary to the mRNA which has the same sequence as the SENSE strand of DNA.

3. In the present review we focus, after a brief discussion of gene expression and mechanisms of action of ANTISENSE, on the methodological aspects of ANTISENSE experiments in neuroscience. In particular, we address the advantages, disadvantages and controls for the ANTISENSE technique, as well as the choice, design, mode of delivery, dose and storage of ANTISENSE oligonucleotides.

Key words: antisense, Fos, gene, nervous system, oligonucleotides, phosphorothioate.

INTRODUCTION

Recent developments in molecular biology are revolutionizing our approach to the study of the nervous system. Transgenic animals (Mullins *et al.* 1990), site-directed mutagenesis (Kuhse *et al.* 1990), *in situ* hybridization (Foster *et al.* 1992) and the use of antisense oligonucleotides (ANTISENSE) to block gene expression (Caceres & Kosik 1990; Listerud *et al.* 1991; Harrison 1993; Wahlestedt *et al.* 1993a,b) are a few examples of the techniques now available to the modern physiologist and neuroscientist. The most accessible of these developments, and the topic of this review, is ANTISENSE technology: a method that allows the expression of many genes, whose sequence

is known, to be attenuated. Even for those of us whose molecular education stopped with the Hardy-Weinberg equation and recessive genes, ANTISENSE technology is a powerful molecular technique that is simple to grasp and easy to use. In this review we will first provide a brief background about gene expression, and then discuss the use of ANTISENSE technology in physiological studies of the nervous system (McCarthy *et al.* 1993a; Wahlestedt 1994). Many excellent reviews on the use of ANTISENSE in other areas are available elsewhere (Dolnick 1990; Cazenave & Hélène 1991; Mirabelli *et al.* 1991; Crooke 1993; Milligan *et al.* 1993; Neckers & Whitesell 1993; Stein & Cheng 1993). The use of ANTISENSE oligonucleotides in other situations such as *in situ* hybridization is not addressed here.

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Received 29 March 1994; revision 22 June 1994; accepted 24 June 1994.

NORMAL GENE EXPRESSION

The process of normal gene expression is illustrated in Fig. 1. A gene is a specific sequence of bases. Four

List of abbreviations:

A	Adenine	HPLC	High Performance Liquid Chromatography
AMPA	α -amino-3-hydroxy-5-methylisoxazolepropionic acid	LTP	Long-term potentiation
AT	Angiotensin	mRNA	Messenger RNA
ANTISENSE	Antisense oligonucleotides	NMDA	N-methyl-D-aspartate
CSF	Cerebrospinal fluid	NPY	Neuropeptide Y
C	Cytosine	AChR	Nicotinic acetylcholine receptor
DNA	Deoxyribonucleic acid	RNaseH	Ribonuclease H
GABA	γ -aminobutyric acid	RNA	Ribonucleic acid
G	Guanine	SENSE	Sense oligonucleotides
hnRNA	Heterologous nuclear RNA	T	Thymine
		U	Uracil

bases are present in DNA: Adenine (A), thymine (T), guanine (G) and cytosine (C). Any of the bases can be covalently bound to each other to form a strand of any length using any combination of bases. In addition, two strands can *hybridize* into a double-stranded form, where base A can form weak bonds with base T, while base G can form weak bonds with base C, resulting in A-T or G-C 'Watson-Crick' (Watson & Crick 1953) base pairs. The weak Watson-Crick base-pair bonds allow DNA to form hybrids in the nucleus with complementary strands of DNA. The strength with which two strands of DNA or RNA will form a hybrid is determined mainly by the length of the strands and the degree of complementarity of the two strands. A mismatch of even one base will result in a change in affinity of about 500-fold in short lengths of DNA (Crooke 1993). When a gene is expressed, an RNA copy of the strand of DNA containing the code for that gene is transcribed. As DNA is double stranded, one strand provides the genetic code or SENSE strand, while the other side provides the complementary ANTISENSE strand. Therefore, RNA is copied from, and complementary to, the ANTISENSE strand and thus has the same sequence as the SENSE DNA strand, except that in RNA the base uracil (U) takes the place of base T. Not all of the DNA in a gene codes for protein; coding segments are called exons (expressed portions), non-coding segments between exons are called introns (Gilbert 1978). Non-coding DNA between genes may be involved in regulatory functions. After transcription, SENSE RNA is known as heterologous nuclear RNA (hnRNA). It undergoes a series of enzymatic reactions that remove the introns and add a cap (Shatkin 1976) and a long tail of adenine bases (a poly-A tail; Lim & Canellakis 1970; Proudfoot & Brownlee 1976). Strands of RNA with introns removed and poly-A tails are known as messenger RNA (mRNA). Variations in splicing of hnRNA usually result in different protein

products from the same gene (e.g. calcitonin and calcitonin gene related peptide; Amara *et al.* 1982). mRNA is then bound to ribosomes and proteins are 'translated'. Each triplet of bases (codon) in the mRNA codes for one amino acid. The first codon translated in every mRNA is AUG which codes for methionine (Baralle & Brownlee 1978; Kozak 1986). This sequence is known as the 'initiation codon', and is a common target for ANTISENSE technology.

BLOCKING GENE EXPRESSION WITH ANTISENSE OLIGONUCLEOTIDES

The principle underlying ANTISENSE technology is illustrated in Fig. 1. The aim of ANTISENSE technology is to interfere with gene expression by preventing the translation of proteins from mRNA. This is achieved by using ANTISENSE oligonucleotides. The simplest ANTISENSE oligonucleotide is a short (around 15 bases) sequence of deoxynucleotides that have a sequence complementary to a portion of the mRNA that is being targeted. The ANTISENSE oligonucleotide then hybridizes with the mRNA and prevents it from being translated into protein (Fig. 1). The mechanism of action of ANTISENSE oligonucleotides described above is termed *translational arrest*. Another mechanism that may be equally or more important is destruction of ANTISENSE-mRNA hybrids by the enzyme RNase H (Fig. 1) (Cazenave & Hélène 1991; Crooke 1993; Ramanathan *et al.* 1993).

Other mechanisms for ANTISENSE to attenuate gene expression are also possible, but their relative importance has not been established. If the ANTISENSE oligonucleotide is designed to hybridize at a splice (intron-exon) junction it could affect the process that converts hnRNA into mRNA (Smith *et al.* 1986). Another mechanism would be to hybridize with DNA in the nucleus and block transcription. These other

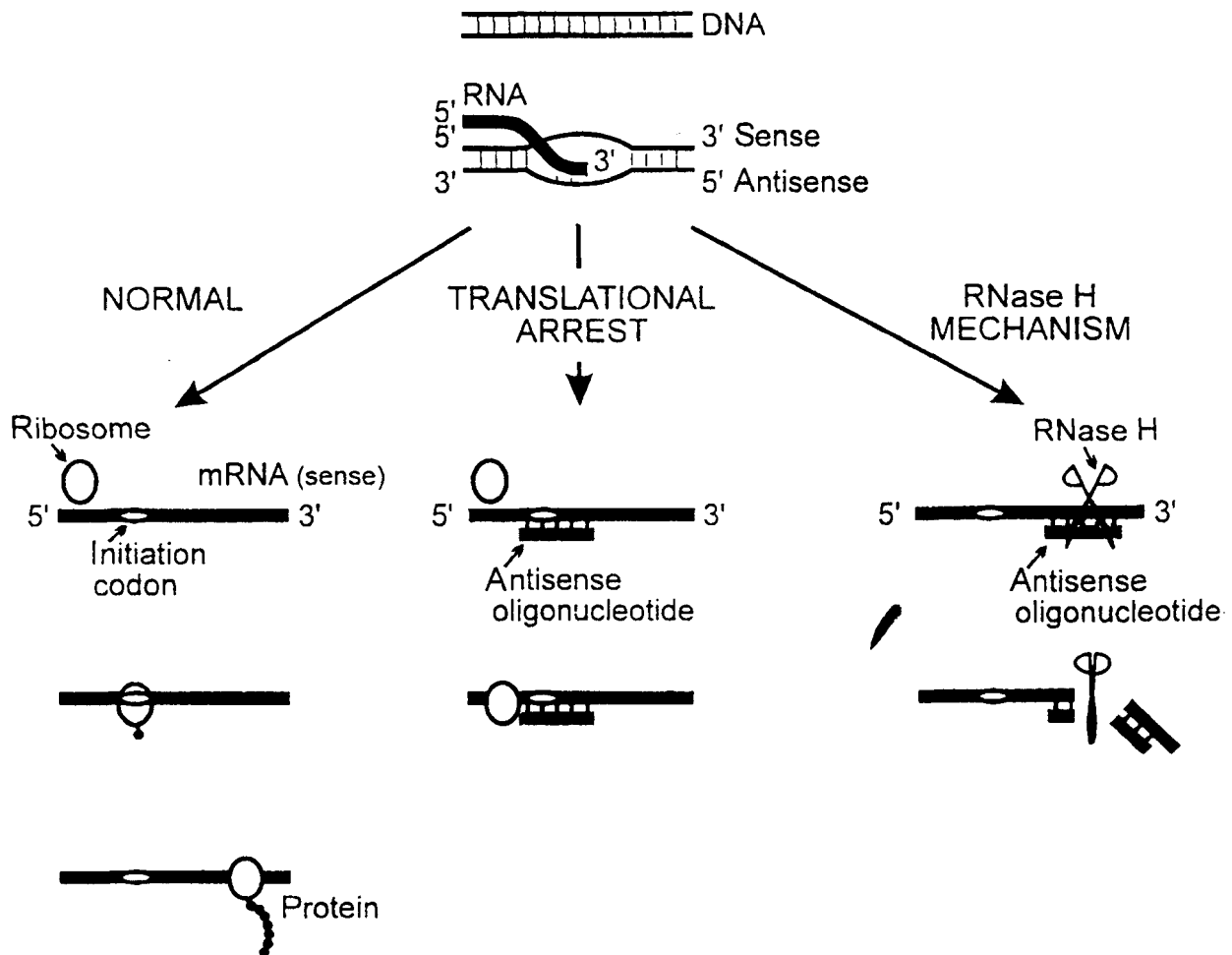


Fig. 1. A schematic representation of the process of normal gene expression and of two possible mechanisms by which ANTISENSE oligonucleotides might attenuate translation of protein from mRNA. DNA has two strands; a SENSE strand and an ANTISENSE strand. The two strands are complementary. Messenger RNA is copied from the ANTISENSE strand, so that its sequence is the same as the corresponding SENSE sequence of the DNA (except for conversion of base T in DNA to base U in RNA). ANTISENSE oligonucleotides have the same sequence as a portion of the ANTISENSE strand of DNA, so that they bind to the messenger RNA preventing its translation by ribosomes (TRANSLATIONAL ARREST). Alternatively, hybrids of ANTISENSE and mRNA might be more rapidly degraded in the cytoplasm by the enzyme RNase H (RNase H MECHANISM).

mechanisms are reviewed elsewhere (van der Krol *et al.* 1988; Cazenave & Hélène 1991; Crooke 1993; Ramanathan *et al.* 1993).

In fact, ANTISENSE sequences can be RNA as well as DNA (van der Krol *et al.* 1988) and by using various molecular biological techniques it is even possible to insert a gene into cells whose product is an ANTISENSE mRNA that will turn off another gene. This method of producing ANTISENSE sequences was the first demonstration of its use to block gene expression (Izant & Weintraub 1984, 1985). Regulation of gene expression by ANTISENSE RNA is used physiologically by some prokaryotic cells such as

bacteria (Green *et al.* 1986) and it was the observation of this phenomenon that triggered experiments into the use of ANTISENSE oligonucleotides to intervene experimentally (and ultimately therapeutically) with gene expression.

DESIGN OF ANTISENSE

Since the first experiments into the use of ANTISENSE oligonucleotides much experience has been acquired about the optimal characteristics needed. A major problem that must be overcome for *in vivo*

experiments is the stability of the oligonucleotides. Conventional wisdom has it that unmodified DNA strands are rapidly degraded by various enzymes. They are therefore not easily accumulated in high concentrations in cells and cannot block mRNA translation. Despite this, unmodified ANTISENSE oligonucleotides have been used to good effect in an *in vivo* study on the neuropeptide Y-Y1 (Wahlestedt *et al.* 1993b) and N-methyl-D-aspartate (NMDA)-R1 (Wahlestedt *et al.* 1993a) receptor genes. In other studies the oligonucleotides have been modified in order to prevent degradation and yet retain biological activity. Various techniques are available, the most popular of which is to construct the oligonucleotides with a phosphorothioate (or phosphothioate) 'backbone'. ANTISENSE oligonucleotides prepared in this way are much more resistant to cellular nucleases (van der Krol *et al.* 1988; Cazenave & Hélène 1991; Milligan *et al.* 1993). Other modifications include chemical changes to the 3' end (van der Krol *et al.* 1988; Khan & Coulson 1993; Tang *et al.* 1993) or biotinylation of the 3' end followed by conjugation with avidin (Boado & Pardridge 1992). Another method is *lipofection*, where the ANTISENSE oligonucleotides are packaged inside a lipid 'Trojan Horse' that is taken into the cell while protected from nuclease enzymes (Thierry *et al.* 1993). This method has been used in several studies in the central nervous system (McCarthy *et al.* 1993b, 1994; Ogawa *et al.* 1994). Attachment of ANTISENSE oligonucleotides to substances such as acridine or glycoproteins that can bind to cell membranes may also improve uptake (Stein *et al.* 1988; Bonfils *et al.* 1992; Budker *et al.* 1992). Clearly, the design (Burger 1993) and delivery (Barry *et al.* 1993) of ANTISENSE oligonucleotide probes is in its infancy, and a close monitoring of developments over the next few years will be well repaid.

CHOICE OF ANTISENSE

Having decided that you want to do an experiment using ANTISENSE oligonucleotides the next step is to decide which part of the gene sequence to target, and how long it ought to be. A number of factors are important. The easiest way is to use the same sequence that someone else has had success with. If this is not possible then you will have to design the oligonucleotide yourself. At this stage assistance from a 'card-carrying' molecular biologist can be useful. Simple problems to avoid include choosing a sequence that is common to more than one gene. It has been estimated that the human genome contains 100 000 genes, and that to avoid the possibility of coincidental hybridiz-

ation an oligonucleotide probe should be at least 15 bases long (Cazenave & Hélène 1991). Coincidentally, this is also an optimal size for uptake into cells. Remember that you pay for each additional base! Such calculations would be fine if genes were random collections of bases. Unfortunately, genes often come in families (Hollenberg 1991) and it is important to choose an ANTISENSE sequence that is appropriate to your experiment. Thus, if you wish to knock out an entire family of genes you might choose to make an ANTISENSE oligonucleotide directed against a sequence that was common to all members of that family (Holopainen & Wojcik 1992). Alternatively, if you wanted to examine a single gene in a family you would have to be careful to choose a part of the gene that is unique. Another problem to be wary of is the possibility that the ANTISENSE oligonucleotides will self-hybridize. Self-hybridization can occur when parts of the oligonucleotide are complementary to other parts of the oligonucleotide.

A common strategy in ANTISENSE oligonucleotide design is to target the region around the AUG initiation codon (van der Krol *et al.* 1988). Many of the successful experiments with ANTISENSE oligonucleotides published to date have used this approach (Fagarasan *et al.* 1990; Listerud *et al.* 1991; Chiasson *et al.* 1992; Gyurko *et al.* 1993; Heilig *et al.* 1993; Lucas *et al.* 1993; McCarthy *et al.* 1993b; Wahlestedt *et al.* 1993a,b; Zhang & Creese 1993; Suzuki *et al.* 1994).

DELIVERY OF ANTISENSE OLIGONUCLEOTIDES

For neuroscience experiments there are several options. Administration may be by any of the standard methods that have been devised for delivery of neuropharmacological agents. The possibility of delivering ANTISENSE oligonucleotides by microdialysis has not been assessed. Intracerebroventricular injection (Whitesell *et al.* 1993) and injection directly into brain tissue (Chiasson *et al.* 1992; McCarthy *et al.* 1993b; Akabayashi *et al.* 1994; Suzuki *et al.* 1994) have been used. In addition to the mode of delivery, the duration of administration is also important. *In vitro* studies have shown that phosphorothioated oligonucleotides are taken up by cells and retained at high concentrations. The intracellular oligonucleotide concentration reaches a maximum after 2–16 h in most cases. Uptake of oligonucleotides into cells, and possible mechanisms involved, have been investigated in many recent studies, (Stein *et al.* 1988; Loke *et al.* 1989; Yakubov *et al.* 1989; Shoji *et al.* 1991; Bonfils *et al.* 1992; Budker *et al.* 1992; Iversen *et al.* 1992; Barry *et*

al. 1993; Burger 1993). If the gene being examined has a low basal expression, and turns over rapidly, then one injection 6–8 h (Heilig *et al.* 1993; Suzuki *et al.* 1994) prior to study may be sufficient. On the other hand, if the basal expression is high and the turnover low, then longer pre-treatments may be required (Ramanathan *et al.* 1993). Experience to date suggests that 2 to 3 days are needed to have a substantial effect on receptors (Wahlestedt *et al.* 1993a,b; Zhang & Creese 1993). Unmodified oligonucleotides will survive in cerebrospinal fluid (CSF) *in vitro* for 24 h, but in *in vivo* studies, degradation as well as clearance from CSF should be considered (Whitesell *et al.* 1993). Empirically, injections twice daily seem to work well and may be necessary (Wahlestedt *et al.* 1993a,b). This dosing regimen also appears to be necessary for phosphorothioated ANTISENSE oligonucleotides (Campbell *et al.* 1990; Weiss *et al.* 1993; Whitesell *et al.* 1993). This is usually done by repeated intracerebroventricular injection. An alternative is to use osmotic mini-pumps (ALZET corporation). Mini-pumps can be loaded with ANTISENSE oligonucleotides enabling a continuous infusion for at least 7 days (Whitesell *et al.* 1993; Zhang & Creese 1993).

DOSE OF ANTISENSE

As with any pharmacological experiment, the best dose must be worked out by trial and error. Remem-

ber, too high a dose may be toxic (Krieg & Hawkins 1991). Dosages that have been used in recent experiments are listed in Tables 1 and 2.

CONTROLS FOR ANTISENSE

ANTISENSE in high concentrations can display toxicity or have non-specific effects. It is therefore important, as in any physiological experiment, to have the appropriate controls (Krieg & Hawkins 1991). One possible solution is to use a SENSE oligonucleotide as the control. A SENSE oligonucleotide is the same length as an ANTISENSE oligonucleotide, but has the same sequence as the homologous segment of mRNA and therefore cannot bind to it. One major potential risk with this strategy is that the SENSE oligonucleotide might have a very different base composition to the ANTISENSE oligonucleotide, resulting in different intracellular base concentrations when the oligonucleotides are degraded. Since different bases might cause different metabolic effects it is important to be aware of this possibility. For example, the ANTISENSE oligonucleotide might have a sequence 5'-TTCCCTTCTCTCTTC-3' while the corresponding SENSE oligonucleotide has the sequence 3'-AAGGGAAGAGAGAAG-5'. It may therefore be better to use a jumbled sequence that has the same base composition as the ANTISENSE oligonucleotide such as 5'-CTCCTTCCTCTCTTT-3'. Alternatively, a

Table 1. Dosages of ANTISENSE in recent experiments using direct tissue micro-injection

Dose	Target gene	Site of injection	Reference
2 nmol (2 µl of 1 mmol/L)	<i>c-fos</i>	Striatum	Chiasson <i>et al.</i> 1992
0.2 nmol (0.2 µl of 1 mmol/L)	<i>c-fos</i>	Rostral ventral medulla oblongata*	Suzuki <i>et al.</i> 1994
5 nmol (1 µl of 5 mmol/L)	<i>c-fos</i>	Nucleus accumbens	Heilig <i>et al.</i> 1993
0.05–0.1 nmol (in 0.5 µl sesame oil)	Glutamic acid decarboxylase	Hypothalamus	McCarthy <i>et al.</i> 1994
0.45 nmol (0.3 µl of 1.5 mmol/L; Neuropeptide Y 1 injection/day for 4 days		Hypothalamus	Akabayashi <i>et al.</i> 1994
0.07 nmol (1 µl of 0.07 mmol/L)	Progesterone receptor	Hypothalamus	Pollio <i>et al.</i> 1993

* Further discussion in text and Fig. 2.

Table 2. Dosages of ANTISENSE in recent experiments using intracerebroventricular injection

Dose	Frequency	Gene Target	Reference
Injection			
11 nmol/dose	3 injections, 12 hrly	Angiotensinogen	Gyurko <i>et al.</i> 1993
15 nmol/dose	4 injections, 12 hrly	NMDA-R1 receptor	Wahlestedt <i>et al.</i> 1993a
11 nmol/dose	4 injections, 12 hrly	NPY-Y1 receptor	Wahlestedt <i>et al.</i> 1993b
2.5 nmol/dose	Twice daily, up to 7 days	Dopamine D2 receptor	Weiss <i>et al.</i> 1993
Continuous infusion by osmotic mini-pump			
39 nmol/24 µL per day for 3 days	Dopamine D2 receptor	Zhang & Creese 1993	

sequence with only a few mismatches may be used (Erlinge *et al.* 1993; Wahlestedt 1994). A mismatch of only one base will cause a decrease in hybridization efficiency of around 500-fold (Crooke 1993). Sequences containing four consecutive G bases may be more toxic than other sequences (Yaswen *et al.* 1993). Workers should be aware that newly created sequences may hybridize to unintended mRNA. To some extent, this can be prevented by checking control sequences on gene data bases, but because only about 1% of the genome has been sequenced, some risk will remain. Finally, it is essential to examine in some way the protein product of the gene to see if the ANTISENSE has been effective. Several methods have been used to do this, including receptor binding assays (Wahlestedt *et al.* 1993a,b; Zhang & Creese 1993), receptor activity assays (Wahlestedt *et al.* 1993a), immunocytochemistry (Chiasson *et al.* 1992; Ogawa *et al.* 1994; Skutella *et al.* 1994; Suzuki *et al.* 1994), and radioimmunoassay (Akabayashi *et al.* 1994).

OBTAINING ANTISENSE OLIGONUCLEOTIDES

Several local and international companies provide an oligonucleotide synthesizing service. These companies will usually offer oligonucleotides at a variety of different synthesis scales and purities. Most workers use HPLC purity but the need for this level of purity in every situation is not established.

STORING ANTISENSE OLIGONUCLEOTIDES

Oligonucleotides should be kept lyophilized, and then aliquoted and stored at -20 — 80°C . They are much more stable than proteins. Care should be taken not to contaminate the ANTISENSE oligonucleotides with blood, serum or other tissues that may contain nucleases.

ADVANTAGES OF ANTISENSE OLIGONUCLEOTIDES

The main advantage of ANTISENSE oligonucleotides is the ability to block the expression of genes for which no pharmacological agents are available, or where the available pharmacological agents have poor specificity. Thus, ANTISENSE technology provides the ultimate specific antagonist. This use of ANTISENSE technology is likely to be especially useful for looking at receptors that are only known to exist

because they have been cloned, but have no known function. Another advantage of ANTISENSE technology is that it can be used to investigate the role played by genes that code for proteins other than receptors, including nuclear proteins (Fagarasan *et al.* 1990; Lucas *et al.* 1993), various intracellular proteins (Amaratunga *et al.* 1993; Lledo *et al.* 1993), autocoids (Cook *et al.* 1992; Cumin *et al.* 1993), enzymes (Contrera *et al.* 1992; Thomae *et al.* 1993) and oncogenes (Harel-Bellan *et al.* 1988).

DISADVANTAGES OF ANTISENSE TECHNOLOGY

The disadvantages of ANTISENSE technology are significant, and success is not guaranteed. First, it can only be used to examine genes whose sequence is known. At the present time this is believed to be about 1% of the human genome, but this figure is increasing and accelerating as gene-sequencing technology is improved. This raises the small additional risk that an ANTISENSE oligonucleotide might be directed at more than one gene. Second, ANTISENSE oligonucleotide might not produce complete suppression of gene expression. This is particularly likely if constitutive expression and protein turnover are low. If an enzyme under investigation has a high activity and is non rate-limiting, ANTISENSE technology as is presently available might not be a useful tool (Ramanathan *et al.* 1993). Third, in order to provide adequate suppression, long treatment regimens may be required. Several recent studies illustrate this point. Zhang and Creese (1993) used an implanted mini-pump to deliver an ANTISENSE oligonucleotide to the dopamine D2 receptor intracerebroventricularly. After 3 days, there was a 50–70% reduction in D2 receptor binding. In spite of this relatively modest reduction, the authors were able to demonstrate marked changes in behaviour and changes in responses to a D2 receptor agonist in rats treated with ANTISENSE oligonucleotides. Similarly, Wahlestedt *et al.* (1993a) used ANTISENSE oligonucleotides to the NMDA-R1 receptor, and achieved a reduction in NMDA binding sites of around 50%. Again, this reduction was physiologically significant in that it attenuated the size of cerebral infarctions induced by occlusion of the middle cerebral artery (Wahlestedt *et al.* 1993a). Finally, although it can be used to block the expression of an entire gene product it provides no information of the role of different sites on that gene product. However, if a receptor consists of a complex of proteins that are coded for by different genes then selective deletion of the different subunits

may provide some useful information about the role of these different subunits. This has been achieved in a recent study on the nicotinic acetylcholine receptor (AChR) in chick lumbar sympathetic ganglia *in vitro*. In these experiments pre-existing receptors were eliminated pharmacologically. Treatment with ANTISENSE oligonucleotides to different AChR subunits affected different characteristics of the newly formed receptors (Listerud *et al.* 1991).

ANTISENSE OLIGONUCLEOTIDES IN THE NERVOUS SYSTEM

A growing number of studies have used ANTISENSE oligonucleotides to investigate the role of various genes in the nervous system both *in vitro* and *in vivo*. Recently, we studied the effect of administering ANTISENSE to the *c-fos* gene into the rostral ventrolateral medulla. Unilateral administration of ANTI-

SENSE markedly attenuated the usual increase in Fos production seen after inhibition of the caudal ventrolateral medulla (Fig. 2). After bilateral administration of *c-fos* ANTISENSE we found a fall in basal arterial blood pressure and a decrease in the pressor response to inhibition of the caudal ventrolateral medulla (Suzuki *et al.* 1994). *C-fos* is a particularly good candidate for the ANTISENSE approach because it has a low basal expression and a rapid and transient expression after stimulation. Other workers have used ANTISENSE to *c-fos* in studies on behaviour (Heilig *et al.* 1993) and on endorphin release *in vitro* (Fagarasan *et al.* 1990).

Since the first reported use of ANTISENSE to attenuate gene expression in the nervous system *in vivo* (Chiasson *et al.* 1992) there have been numerous reports, both *in vivo* and *in vitro*. We do not intend to provide an exhaustive list of ANTISENSE studies in the nervous system, but a few examples, illustrating the scope of the approach, are given in Table 3.

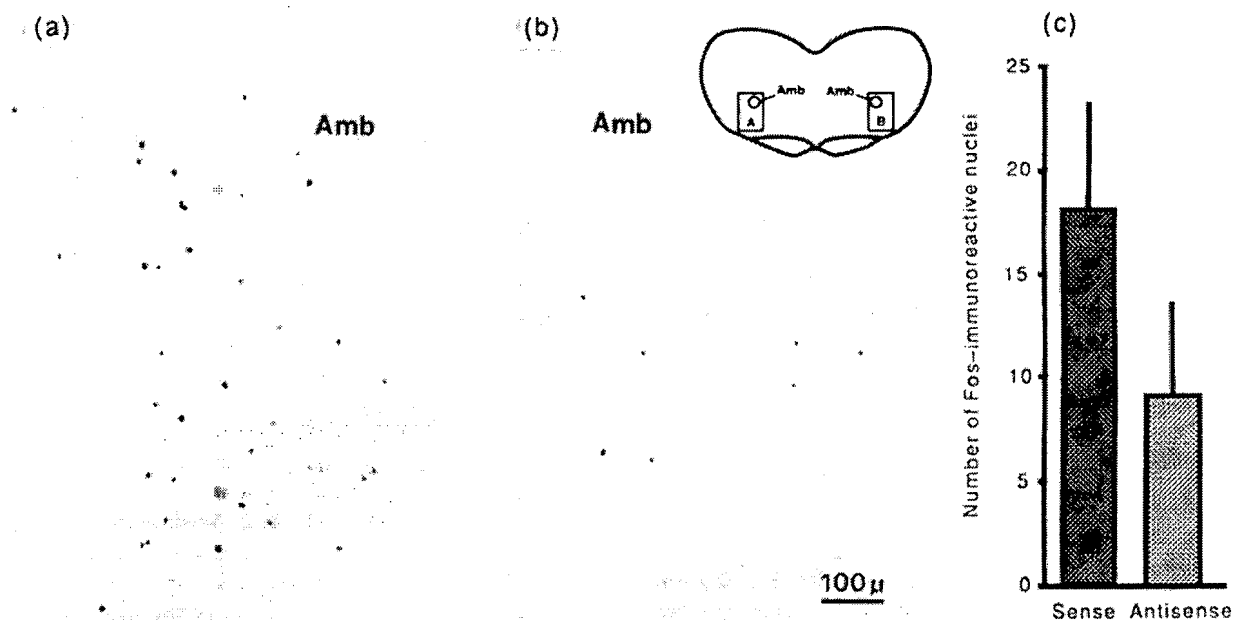


Fig. 2. An illustration of the effect of ANTISENSE administration *in vivo*. Panels (a) and (b) show photomicrographs of the left and right hand sides of the medulla oblongata, as illustrated in the inset in (b). Six hours prior to injection of muscimol into the caudal ventrolateral medulla, a micro-injection of phosphorothioated oligonucleotides (0.2 nmol in 200 nL) that correspond to a portion of the SENSE sequence of the *c-fos* gene were injected into the region of the left hand side rostral ventrolateral medulla. After injection of muscimol, *c-fos* expression in the rostral ventrolateral medulla is high as indicated by the large number of Fos-immunoreactive nuclei (a). On the right hand side of the medulla which had been pre-treated with ANTISENSE oligonucleotides, *c-fos* expression is markedly reduced (b). The average number of Fos-immunoreactive nuclei on each side (SENSE and ANTISENSE treated; mean $\pm$ s.e.m.) of the medulla in 12 sections (50 μ thick) is shown in (c). ANTISENSE treatment has caused a large reduction in Fos-immunoreactivity. Amb indicates the location of the nucleus ambiguus pars compacta. These data are reproduced with permission of the American Physiological Society from Suzuki *et al.* 1994.

Table 3. Examples of ANTISENSE studies of the nervous system

Target gene	Mode of administration	Effect of ANTISENSE	Reference
NMDA-R1 receptor	Cultured cortical cells and intracerebroventricular	Decreased cell death	Wahlestedt <i>et al.</i> 1993a
Dopamine D2 receptor	Intracerebroventricular	Decreased locomotor activity and catalepsy	Zhang & Creese 1993
Muscarinic M2 & GABA-B receptor	Cultured cerebellar granule cells	Decreased binding	Holopainen & Wojcik 1992
AMPA receptor	Hippocampal slices	Decreased LTP	Vanderklish <i>et al.</i> 1992
Neuropeptide Y-Y1 receptor	Intracerebroventricular	Increased anxiety	Wahlestedt <i>et al.</i> 1993b
<i>c-fos</i>	Micro-injection into brain tissue <i>in vivo</i>	Decreased blood pressure; Decreased locomotion after cocaine	Suzuki <i>et al.</i> 1994 Heilig <i>et al.</i> 1993
SNAP-25	Cultured PC12 cells	Decreased neurite elongation	Osen-Sand <i>et al.</i> 1993
Angiotensin, AT1 receptors	Intracerebroventricular	Decreased blood pressure	Gyurko <i>et al.</i> 1993

CONCLUSION

ANTISENSE technology is a powerful method with a large and growing number of applications. With improvements in stability, delivery and oligonucleotide design, and with the rapidly increasing number of genes cloned, ANTISENSE technology is certain to become one of the most important tools in the neuroscientists' armamentarium.

ACKNOWLEDGEMENTS

Work in our laboratories is supported by a programme grant from the National Health and Medical Research Council of Australia and by Grants-in-Aid from the National Sudden Infant Death Syndrome Council of Australia and the National Heart Foundation of Australia.

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ANEXO 6

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ogy), respectively. Staining specificity was controlled by single staining, as well as by using secondary antibodies in the absence of the primary stain.

Generation of target cells

Target cells displaying a membrane-integral version of either wild-type HEL or a mutant¹⁰ exhibiting reduced affinity for HyHEL10 ([R²¹, D¹⁰¹, G¹⁰², N¹⁰³] designated HEL*) were generated by transfecting mouse J558L plasmacytoma cells with constructs analogous to those used¹⁰ for expression of soluble HEL/HEL*, except that 14 Ser/Gly codons, the H2K^b transmembrane region, and a 23-codon cytoplasmic domain were inserted immediately upstream of the termination codon by polymerase chain reaction. For mHEL–GFP, we included the EGFP coding domain in the Ser/Gly linker. Abundance of surface HEL was monitored by flow cytometry and radiolabelled-antibody binding using HyHEL5 and D1.3 HEL-specific monoclonal antibodies, for which the mutant HELs used in this work show unaltered affinities¹⁰.

Interaction assays

For B-cell/target interaction assays, splenic B cells from 3–83 or MD4 transgenic mice^{28,29} carrying (IgM + IgD) BCRs specific for HEL or H2K^b/H2K^b were freshly purified on Lympholyte and incubated with a twofold excess of target cells in RPMI, 50 mM HEPES pH 7.4, for the appropriate time at 37 °C before being applied to polylysine-coated slides. Cells were fixed in 4% paraformaldehyde/PBS or methanol and permeabilized with PBS/0.1% Triton X-100 before immunofluorescence. We acquired confocal images using a Nikon E800 microscope attached to BioRad Radiance Plus scanning system equipped with 488-nm and 543-nm lasers, as well as differential interference contrast for transmitted light. GFP fluorescence in living cells in real time was visualized using a Radiance 2000 and Nikon E300 inverted microscope. Images were processed using BioRad Lasersharp 1024 or 2000 software to provide single plane images, confocal projections or slicing.

Antigen presentation

Presentation of HEL epitopes to T-cell hybridomas 2G7 (specific for I-E^k[HEL^{1–18}]) and 1E5 (specific for I-E^d[HEL^{108–116}]) by transfectants of the LK35.2 B-cell hybridoma expressing an HEL-specific IgM BCR was monitored as described¹⁰.

Received 12 December 2000; accepted 30 March 2001.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

Acknowledgements

We thank B. Amos and S. Reichelt for help and advice with confocal microscopy, and S. Munro for helpful discussions. We are indebted those who provided antibodies, transgenic mice and cell lines. F.D.B. and D.I. were supported by the Arthritis Research Campaign and Studienstiftung des deutschen Volkes, respectively.

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Duplexes of 21–nucleotide RNAs mediate RNA interference in cultured mammalian cells

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RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene^{1–4}. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs^{5–9}. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.

Uptake of dsRNA by insect cell lines has previously been shown to ‘knock-down’ the expression of specific proteins, owing to sequence-specific, dsRNA-mediated mRNA degradation^{6,10–12}. However, it has not been possible to detect potent and specific RNA interference in commonly used mammalian cell culture systems, including 293 (human embryonic kidney), NIH/3T3 (mouse fibroblast), BHK-21 (Syrian baby hamster kidney), and CHO-K1 (Chinese hamster ovary) cells, applying dsRNA that varies in size between 38 and 1,662 base pairs (bp)^{10,12}. This apparent lack of RNAi in mammalian cell culture was unexpected, because RNAi exists in mouse oocytes and early embryos^{13,14}, and because RNAi-related, transgene-mediated co-suppression was also observed in cultured Rat-1 fibroblasts¹⁵. But it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound physiological

reactions that lead to the induction of interferon synthesis¹⁶. In the interferon response, dsRNA > 30 bp binds and activates the protein kinase PKR¹⁷ and 2',5'-oligoadenylate synthetase (2',5'-AS)¹⁸. Activated PKR stalls translation by phosphorylation of the translation initiation factors eIF2 α , and activated 2',5'-AS causes mRNA degradation by 2',5'-oligoadenylate-activated ribonuclease L. These responses are intrinsically sequence-nonspecific to the inducing dsRNA.

Base-paired 21- and 22-nucleotide (nt) siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos⁹. To test whether siRNAs are also capable of mediating RNAi in cell culture, we synthesized 21-nt siRNA duplexes with symmetric 2-nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*, RL) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 1a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *Drosophila* S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In *Drosophila* S2 cells (Fig. 2a and b), the specific inhibition of luciferases was complete and similar to results previously obtained for longer dsRNAs^{6,10,12,19}. In mammalian cells, where the reporter genes were 50- to 100-fold more strongly expressed, the specific suppression was less complete (Fig. 2c-j). In NIH/3T3, monkey COS-7 and HeLa S3 cells (Fig. 2c-h), GL2 expression was reduced 3-

to 12-fold, GL3 expression 9- to 25-fold, and RL expression 2- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 2i and j). The lack of reduction of RL expression in 293 cells may be because of its expression, 5- to 20-fold higher than any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2-nucleotide 3' overhang in all siRNA duplexes was composed of (2'-deoxy) thymidine, except for uGL2, which contained

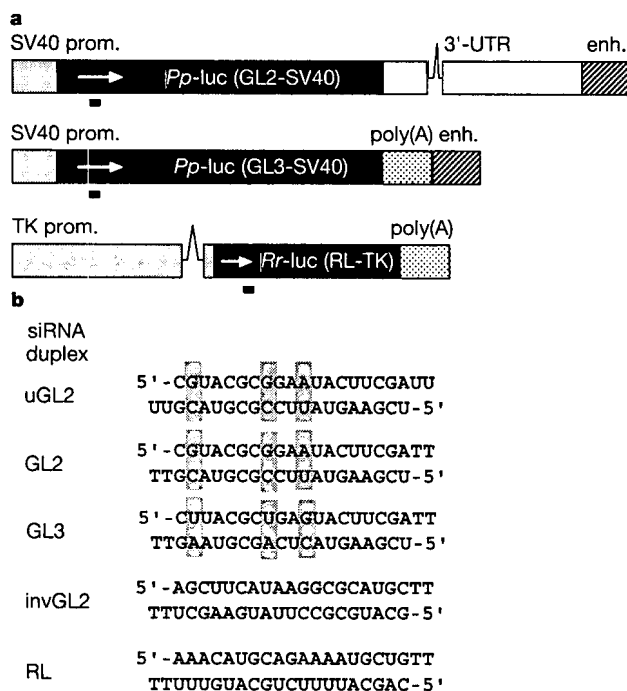


Figure 1 Reporter constructs and siRNA duplexes. **a**, The firefly (*Pp-luc*) and sea pansy (*Rr-luc*) luciferase reporter-gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega) are illustrated; simian virus 40 (SV40) promoter (prom.); SV40 enhancer element (enh.); SV40 late polyadenylation signal (poly(A)); herpes simplex virus (HSV) thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. **b**, The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3, and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only three single-nucleotide substitutions (boxed in grey). As nonspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2-nucleotide 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

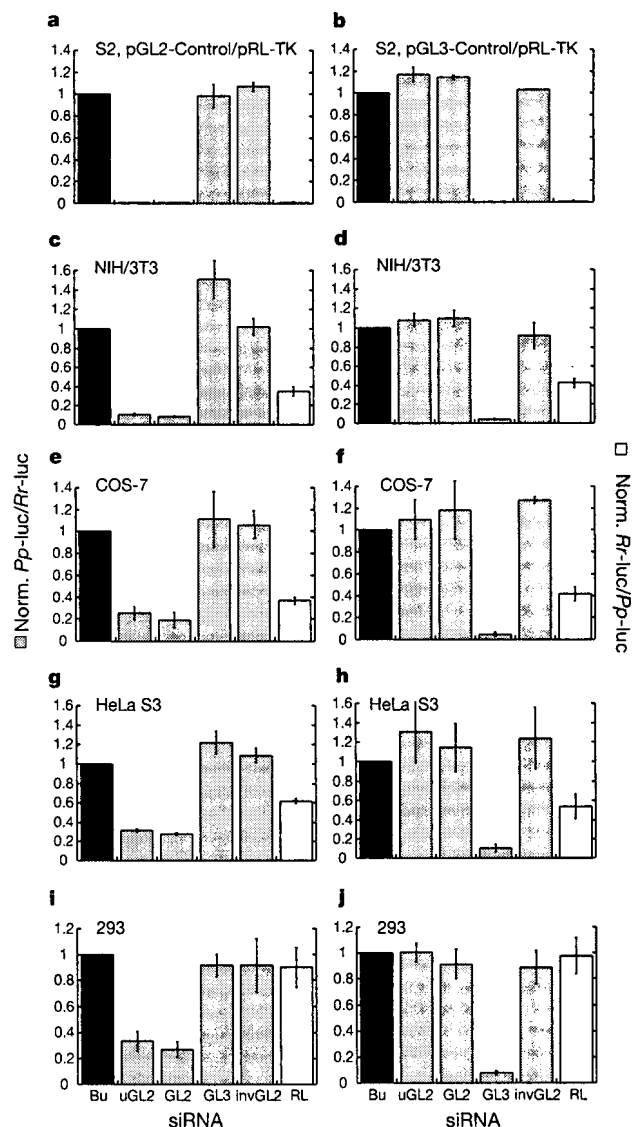


Figure 2 RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (Bu, black bars); grey bars indicate ratios of *Photinus pyralis* (*Pp-luc*) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr-luc*) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). **a, c, e, g** and **i**, Experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids; **b, d, f, h** and **j**, experiments performed with the combination of pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp-luc/Rr-luc* for the buffer control (Bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments $\pm$ s.d.

uridine residues. The thymidine overhang was chosen because it reduces costs of RNA synthesis and may enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells. As in the *Drosophila in vitro* system (data not shown), substitution of uridine by thymidine in the 3' overhang was well tolerated in cultured mammalian cells (Fig. 2a, c, e, g and i), and the sequence of the overhang appears not to contribute to target recognition⁹.

In co-transfection experiments, 25 nM siRNA duplexes were used (Figs 2 and 3; concentration is in respect to the final volume of tissue culture medium). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies, perhaps due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids; the silencing effect only vanishes completely if the siRNA concentration was dropped below 0.05 nM. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene-targeting experiments²⁰.

To monitor the effect of longer dsRNAs on mammalian cells, 50- and 500-bp dsRNAs that are cognate to the reporter genes were prepared. As a control for nonspecific inhibition, dsRNAs from humanized GFP (hG)²¹ was used. In these experiments, the reporter plasmids were co-transfected with either 0.21 µg siRNA duplexes or 0.21 µg longer dsRNAs. The siRNA duplexes only reduced the expression of their cognate reporter gene, while the longer dsRNAs strongly and nonspecifically reduced reporter-gene expression. The effects are illustrated for HeLa S3 cells as a representative example (Fig. 3a and b). The absolute luciferase activities were decreased nonspecifically 10- to 20-fold by 50-bp dsRNA, and 20- to 200-fold by 500-bp dsRNA co-transfection, respectively. Similar nonspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold nonspecific reduction was observed only for 500-bp dsRNAs. Nonspecific reduction in reporter-gene expression by dsRNA > 30 bp was expected as part of the interferon response¹⁶. Interestingly, superimposed on the nonspecific interferon response, we detect additional sequence-specific, dsRNA-mediated silencing. The sequence-specific silencing effect of long dsRNAs, however, became apparent only when the relative reporter-gene activities were normalized to the hG dsRNA controls (Fig. 3c). Sequence-specific silencing by 50- or 500-bp dsRNAs reduced the targeted reporter-gene expression by an additional 2- to 5-fold. Similar effects were also detected in the other three mammalian cell lines tested (data not shown). Specific silencing effects with dsRNAs (356–1,662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments¹². Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/β-galactosidase (lacZ) reporter combinations and 829-bp specific lacZ or 717-bp nonspecific green fluorescent protein (GFP) dsRNA¹⁰. The lack of detected RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA > 30 bp.

To test for silencing of endogenous genes, we chose four genes coding for cytoskeletal proteins: lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA) and vimentin²⁷. The selection was based on the availability of antibodies needed to quantitate the silencing effect. Silencing was monitored 40 to 45 h after transfection to allow for turnover of the protein of the targeted genes. As

shown in Fig. 4, the expression of lamin A/C was specifically reduced by the cognate siRNA duplex (Fig. 4a), but not when nonspecific siRNA directed against firefly luciferase (Fig. 4b) or buffer (Fig. 4c) was used. The expression of a non-targeted gene, NuMA, was unaffected in all treated cells (Fig. 4d–f), demonstrating the integrity of the targeted cells. The reduction in lamin A/C proteins was more than 90% complete as quantified by western blotting (Fig. 4j, k). We note that lamin A/C 'knock-out' mice are

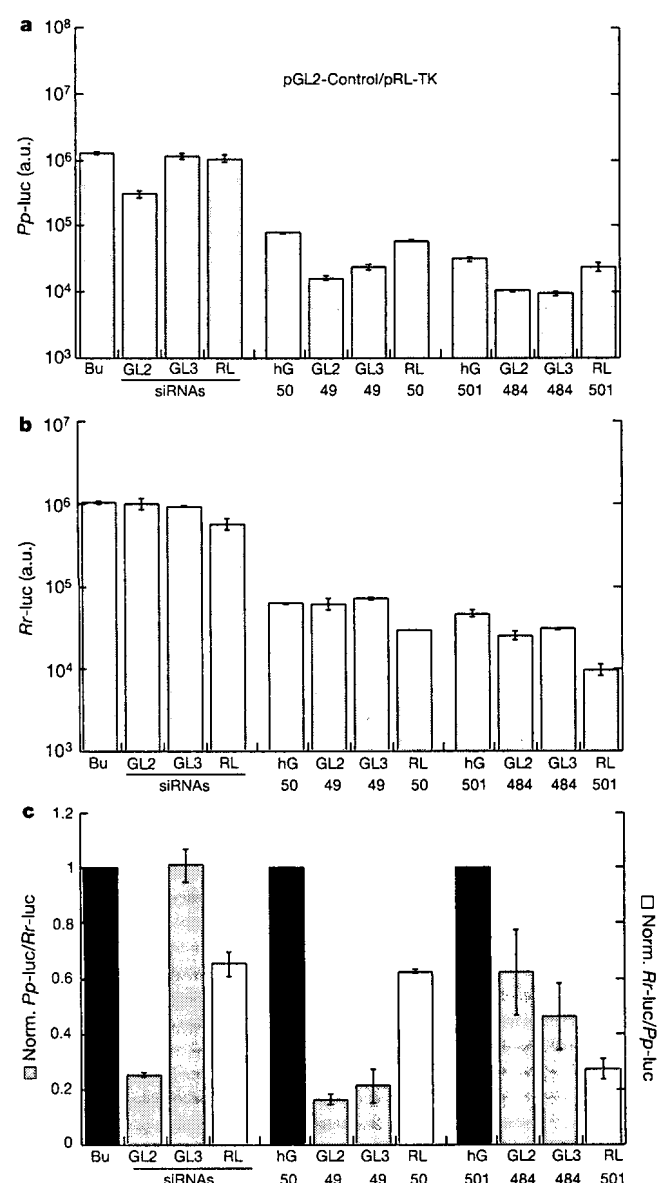


Figure 3 Effects of 21-nucleotide siRNAs, 50-bp, and 500-bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs in base pairs is indicated below the bars. Experiments were performed with pGL2-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments $\pm$ s.d. **a**, Absolute Pp-luc expression, plotted in arbitrary luminescence units (a.u.). **b**, Rr-luc expression, plotted in arbitrary luminescence units. **c**, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (Bu, black bars); the luminescence ratios for 50- or 500-bp dsRNAs were normalized to the respective ratios observed for 50- and 500-bp dsRNAs from humanized GFP (hG, black bars). We note that the overall differences in sequence between the 49- and 484-bp GL2 and GL3 dsRNAs are not sufficient to confer specificity for targeting GL2 and GL3 targets (43-nucleotide uninterrupted identity in 49-bp segment, 239-nucleotide longest uninterrupted identity in 484-bp segment)³⁰.

viable for a few weeks after birth²³ and that the lamin A/C knock-down in cultured cells was not expected to cause cell death. Lamin A and C are produced by alternative splicing in the 3' region and are present in equal amounts in the lamina of mammalian cells (Fig. 4j, k). Transfection of siRNA duplexes targeting lamin B1 and NuMA reduced the expression of these proteins to low levels (data not shown), but we were not able to observe a reduction in vimentin expression. This could be due to the high abundance of vimentin in the cells (several per cent of total cell mass) or because the siRNA sequence chosen was not optimal for targeting of vimentin.

The mechanism of the 21-nucleotide siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing might occur post-transcriptionally and/or transcriptionally. In *Drosophila* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of siRNA-protein complexes (siRNPs), which guide mRNA recognition and targeted cleavage^{6,7,9}. In plants, dsRNA-mediated post-transcriptional silencing has also been linked to DNA methylation, which may also be directed by 21-

nucleotide siRNAs²⁴. Methylation of promoter regions can lead to transcriptional silencing²⁵, but methylation in coding sequences does not²⁶. DNA methylation and transcriptional silencing in mammals are well documented processes²⁷, yet their mechanisms have not been linked to that of post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG dinucleotide sequences. There is no CpG sequence in the RL or lamin A/C siRNA, although both siRNAs mediate specific silencing in mammalian cell culture, so it is unlikely that DNA methylation is essential for the silencing process.

Thus we have shown, for the first time, siRNA-mediated gene silencing in mammalian cells. The use of exogenous 21-nucleotide siRNAs holds great promise for analysis of gene function in human cell culture and the development of gene-specific therapeutics. It will also be of interest in understanding the potential role of endogenous siRNAs in the regulation of mammalian gene function. □

Methods

RNA preparation

21-nucleotide RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified⁹. The accession numbers given below are from GenBank. The siRNA sequences targeting GL2 (Acc. No. X65324) and GL3 luciferase (Acc. No. U47296) corresponded to the coding regions 153–173 relative to the first nucleotide of the start codon; siRNAs targeting RL (Acc. No. AF025846) corresponded to region 119–139 after the start codon. The siRNA sequence targeting lamin A/C (Acc. No. X03444) was from position 608–630 relative to the start codon; lamin B1 (Acc. No. NM_005573) siRNA was from position 672–694; NuMA (Acc. No. Z11583) siRNA from position 3,988–4,010, and vimentin (Acc. No. NM_003380) from position 346–368 relative to the start codon. Longer RNAs were transcribed with T7 RNA polymerase from polymerase chain reaction (PCR) products, followed by gel purification. The 49- and 484-bp GL2 or GL3 dsRNAs corresponded to positions 113–161 and 113–596, respectively, relative to the start of translation; the 50- and 501-bp RL dsRNAs corresponded to position 118–167 and 118–618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (ref. 21), whereby 50- and 501-bp hG dsRNA corresponded to positions 121–170 and 121–621, respectively, to the start codon.

For annealing of siRNAs, 20 μ M single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50- and 500-bp dsRNAs, and these annealing reactions were performed at 8.4 μ M and 0.84 μ M strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) 100 units ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, HeLa S6, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four h before transfection at 50–80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 $\times$ 10⁵ cells ml⁻¹) and transferred to 24-well plates (500 μ l per well). S2 cells were not trypsinized before splitting. Co-transfection of reporter plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μ g pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μ g pRL-TK (Promega), and 0.21 μ g siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μ l per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μ g hGFP-encoding pAD3 (ref. 21) and 0.21 μ g inverted GL2 siRNA, and were 70–90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Transfection of siRNAs for targeting endogenous genes was carried out using Oligofectamine (Life Technologies) and 0.84 μ g siRNA duplex per well, but it was recently found that as little as 0.01 μ g siRNAs per well are sufficient to mediate silencing. HeLa S6 cells were transfected one to three times in approximately 15 h intervals and were assayed 40 to 45 h after the first transfection. It appears, however, that a single transfection is as efficient as multiple transfections. Transfection efficiencies as determined by immunofluorescence of targeted cells were in the range of 90%. Specific silencing of targeted genes was confirmed by at least three independent experiments.

Western blotting and immunofluorescence microscopy

Monoclonal 636 lamin A/C specific antibody²⁸ was used as undiluted hybridoma supernatant for immunofluorescence and 1/100 dilution for western blotting. Affinity-purified polyclonal NuMA protein 705 antibody²⁹ was used at a concentration of 10 μ g ml⁻¹ for

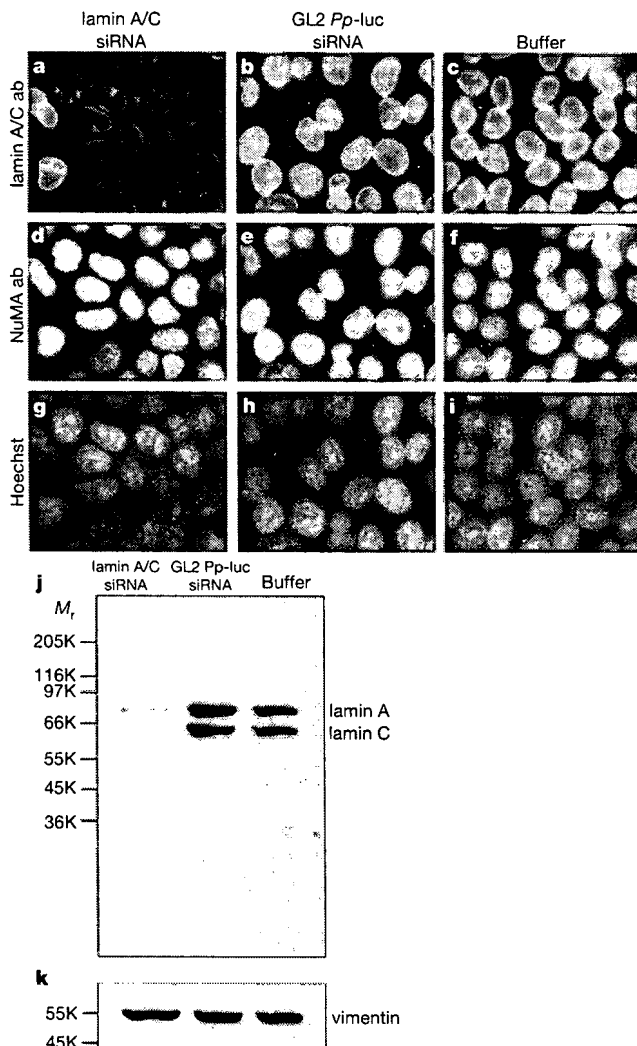


Figure 4 Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining of cells transfected with lamin A/C siRNA duplex (a, d, g), with GL2 luciferase siRNA duplex (nonspecific siRNA control) (b, e, h), and with buffer only (c, f, i). a–c, Staining with lamin A/C specific antibody; d–f, staining with NuMA-specific antibody; g–i, Hoechst staining of nuclear chromatin. Bright fluorescent nuclei in a represent untransfected cells. j, k, Western blots of transfected cells using lamin A/C- (j) or vimentin-specific (k) antibodies. The Western blot was stripped and re-probed with vimentin antibody to check for equal loading of total protein.

immunofluorescence. Monoclonal V9 vimentin-specific antibody was used at 1/2,000 dilution. For western blotting, transfected cells grown in 24-well plates were trypsinized and harvested in SDS sample buffer. Equal amounts of total protein were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose. Standard immunostaining was carried out using ECL enhanced chemiluminescence technique (Amersham Pharmacia).

For immunofluorescence, transfected cells grown on glass coverslips in 24-well plates were fixed in methanol for 6 min at -10°C . Target gene specific and control primary antibody were added and incubated for 80 min at 37°C . After washing in phosphate buffered saline (PBS), Alexa 488-conjugated anti-rabbit (Molecular Probes) and Cy3-conjugated anti-mouse (Dianova) antibodies were added and incubated for 60 min at 37°C . Finally, cells were stained for 4 min at room temperature with Hoechst 33342 ($1\text{ }\mu\text{M}$ in PBS) and embedded in Mowiol 488 (Hoechst). Pictures were taken using a Zeiss Axiophot camera with a Fluor 40/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation) with equal exposure times for the specific antibodies.

Received 20 February; accepted 26 April 2001.

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Acknowledgements

We thank J. Martinez, J. Ludwig and D. Bartel for comments on the manuscript; L. Fredel for help with image processing; H.-J. Dehne for technical assistance; F. Döring, R. Nehring,

D. Ingelfinger and C. Schneider for supplying cell lines; A. Dickmanns for the gift of the plasmid pAD3; and R. Lührmann for support. This work was funded by a Biofuture grant of the Bundesministerium für Bildung und Forschung.

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Ribosomal peptidyl transferase can withstand mutations at the putative catalytic nucleotide

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Peptide bond formation is the principal reaction of protein synthesis. It takes place in the peptidyl transferase centre of the large (50S) ribosomal subunit. In the course of the reaction, the polypeptide is transferred from peptidyl transfer RNA to the α -amino group of amino acyl-tRNA. The crystallographic structure of the 50S subunit showed no proteins within 18 Å from the active site, revealing peptidyl transferase as an RNA enzyme¹. Reported unique structural and biochemical features of the universally conserved adenine residue A2451 in 23S ribosomal RNA (*Escherichia coli* numbering) led to the proposal of a mechanism of rRNA catalysis that implicates this nucleotide as the principal catalytic residue^{2,3}. *In vitro* genetics allowed us to test the importance of A2451 for the overall rate of peptide bond formation. Here we report that large ribosomal subunits with mutated A2451 showed significant peptidyl transferase activity in several independent assays. Mutations at another nucleotide, G2447, which is essential to render catalytic properties to A2451 (refs 2, 3), also did not dramatically change the transpeptidation activity. As alterations of the putative catalytic residues do not severely affect the rate of peptidyl transfer the ribosome apparently promotes transpeptidation not through chemical catalysis, but by properly positioning the substrates of protein synthesis. The proposed role of A2451 in the peptidyl transfer reaction is

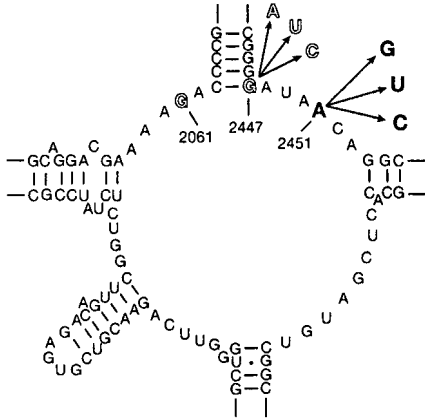


Figure 1 The secondary structure of the central loop of domain V of *T. aquaticus* 23S rRNA. Position A2451 (*E. coli* 23S rRNA numeration), the principal catalytic nucleotide in the proposed general acid–base catalytic mechanism of peptide bond formation^{2,3}, is shown in bold. Its tertiary interaction partners, guanine residues 2061 and 2447, suggested to be essential for rendering catalytic properties to A2451, are outlined. Arrows indicate the mutations engineered in 23S rRNA.

Clarke, Modet & Cº

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ANEXO 7

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Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi

Scott M. Hammond, *et al.*
Science **293**, 1146 (2001);
DOI: 10.1126/science.1064023

ANEXO 7

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that is a previously known transcription-associated kinase, Snf1, and demonstrate that histone H3 Ser-10 phosphorylation by Snf1 leads to Gcn5-mediated acetylation at the *INO1* promoter. The linked and sequential modifications are required for full *INO1* transcriptional induction. The finding that Snf1 functions as a histone kinase suggests that other transcription-linked kinases may target histones for phosphorylation. The data support a targeting model where Snf1 is recruited to certain promoters, perhaps through the function of associated subunits. We have shown that Snf1 cofractionates in an apparent multisubunit complex, which may include the previously identified activating component Snf4 and substrate targeting subunits Sip2, Sip4, and Gal83 (27). These latter proteins may promote association of Snf1 with activators to direct catalysis to promoter-bound histones.

The experiments reported here demonstrate an interdependent pattern of histone modifications during gene activation. There is also evidence for multiple linked modifications (deacetylation leading to methylation) occurring during heterochromatic silencing in *Schizosaccharomyces pombe* and mammalian cells (28, 29). The relation between histone modifications for silencing and those required for gene activation remains to be determined, although the balance between Lys-9 methylation and Ser-10 phosphorylation could represent a critical switch.

Why are there multiple modifications? Two general mechanisms are possible for the role of modifications. In the first, the electrostatic charge alterations may serve to directly alter nucleosome structure (30), and dual acetylation/phosphorylation would increase negative charge beyond either modification alone. In the second model, modifications provide an interaction surface for binding of proteins, an idea advanced as the "histone code" hypothesis (2, 31). Phosphorylation of serine may provide an intermediate step, as discussed above, for binding of proteins in a sequence, or in tandem with acetylation, could provide a stronger or more selective binding surface. In either mechanism, multiple modifications could provide increased combinatorial and synergistic control during gene regulation.

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32. We thank F. Winston, M. Grunstein, C. D. Allis, H. Goto, and M. Inagaki for plasmids, strains, and antibodies, and N. Keiser for assistance with construction of the K14A yeast strain. We thank P. Lieberman, G. Moore, D. Sterner, K. Wang, and members of the Berger laboratory for discussions. Supported by National Institute of General Medical Sciences (S.L.B. and R.S.) and NSF (S.L.B.).

7 May 2001; accepted 11 July 2001

Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi

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Double-stranded RNA induces potent and specific gene silencing through a process referred to as RNA interference (RNAi) or posttranscriptional gene silencing (PTGS). RNAi is mediated by RNA-induced silencing complex (RISC), a sequence-specific, multicomponent nuclease that destroys messenger RNAs homologous to the silencing trigger. RISC is known to contain short RNAs (~22 nucleotides) derived from the double-stranded RNA trigger, but the protein components of this activity are unknown. Here, we report the biochemical purification of the RNAi effector nuclease from cultured *Drosophila* cells. The active fraction contains a ribonucleoprotein complex of ~500 kilodaltons. Protein microsequencing reveals that one constituent of this complex is a member of the Argonaute family of proteins, which are essential for gene silencing in *Caenorhabditis elegans*, *Neurospora*, and *Arabidopsis*. This observation begins the process of forging links between genetic analysis of RNAi from diverse organisms and the biochemical model of RNAi that is emerging from *Drosophila* in vitro systems.

RNA interference is a process whereby double-stranded RNA (dsRNA) induces the silencing of cognate genes (1). Posttranscriptional silencing phenomena have also been observed in plants (e.g., PTGS) and fungi (e.g., quelling), and genetic studies indicate that these are likely to be mechanistically related to RNAi. Moreover, RNAi per se has been demonstrated in a variety of experimental systems, including insects, protozoans, and mammals (2).

A synthesis of in vivo and in vitro experiments has led to a mechanistic model for RNAi/PTGS. Silencing is initiated by exposure of a cell to dsRNA. This "trigger" may

be introduced experimentally or may derive from endogenous sources such as viruses, transgenes, or cellular genes (e.g., transposons). Double-stranded RNAs are processed into discrete ~21- to 25-nucleotide (nt) RNA fragments known as siRNAs (small interfering RNAs) (3, 4). These small RNAs join a multicomponent nuclease complex, RISC, and guide that enzyme to its substrates through conventional base-pairing interactions (5). Recognition of mRNAs by RISC leads to their destruction.

To date, mechanistic studies have approached RNAi/PTGS from two standpoints. Genetic studies have identified nearly a dozen genes that affect the dsRNA response. These include genes that encode putative nucleases [*mut-7* (6)], helicases [*qde-3* (7), *SDE3* (8), *mut-6* (9)], RNA-dependent RNA polymerases [e.g., *ego-1* (10), *qde-1* (11), *SDE1* (12)/*SGS2* (13)], and members of the Argonaute family [*rde-1* (14), *qde-2* (15),

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AGO1 (16). Biochemical studies, carried out exclusively in extracts from *Drosophila* embryos and cultured cells, have identified enzymatic activities that are proposed to contribute to the interference process (4, 5, 17, 18). However, links between biochemical and genetic studies of RNAi have yet to be made.

We have sought to identify the proteins and RNAs that carry out RNAi in vitro as a step toward unifying biochemical and genetic data into a single mechanistic model. Previously, we identified a ribonuclease III family enzyme, Dicer, as a candidate for processing long dsRNA silencing triggers into ~22-nt siRNAs (18). Recently, a requirement for Dicer in RNAi in vivo has been demonstrated in *C. elegans* (19). Here we report the biochemical purification of RISC, the effector nuclease of RNAi, and the identification of one subunit of this enzyme. This protein is a member of the Argonaute family, which has been linked to RNAi through genetic studies in several experimental systems.

RNA interference can be provoked in cultured *Drosophila* S2 cells by transfection with dsRNA, or indeed by simply adding dsRNA to the culture media (5, 20). Extracts from such cells contain a nuclease complex, RISC, that specifically degrades mRNAs that are homologous to the dsRNA trigger. The hypothesis that this nuclease constitutes the effector activity of RNAi is strengthened by the observation that RISC cofractionates with ~22-nt RNAs that are derived directly from the silencing trigger (5). Furthermore, this nuclease contains an essential nucleic acid subunit, which is presumably a siRNA.

We have developed a biochemical fractionation protocol that permits the purification of RISC to near-homogeneity. Our previous studies have shown that RISC is bound to ribosomes in cell-free extracts; however, the biological relevance of this association remains to be established (5). Ribosomes can be concentrated from S2 lysates by high-speed centrifugation, and soluble RISC can be recovered from the ribosome pellet by extraction with high concentrations of salt.

Size fractionation of soluble RISC yielded a single peak of sequence-specific nuclease activity (Fig. 1A) (21). Thus, a single complex contains all the activities and information needed to identify and degrade cognate mRNAs. The large size of this complex (~500 kD) is consistent with its being composed of several subunits, which, according to our previous studies, comprise both RNA and protein. We developed a series of additional chromatographic steps that yielded a fraction with a sequence-specific nuclease activity, which was purified ~1:10,000 from the crude extract. These are outlined in Fig. 1B, and representative activity profiles from several columns are shown in Fig. 1A and Fig. 2, A to C (21).

Analysis of fractions from the hydroxy-

apatite column by SDS-polyacrylamide gel electrophoresis (PAGE) indicated that the complex had not been purified to complete homogeneity; however, several proteins clearly cofractionated with the active RISC fraction. Candidate proteins were excised from the gel and

microsequenced using tandem mass spectroscopy (22). Two of four bands failed to produce protein sequence. However, numerous peptides were obtained from bands of ~87 and ~130 kD, which matched a single *Drosophila* gene. Database and domain searches identified this as

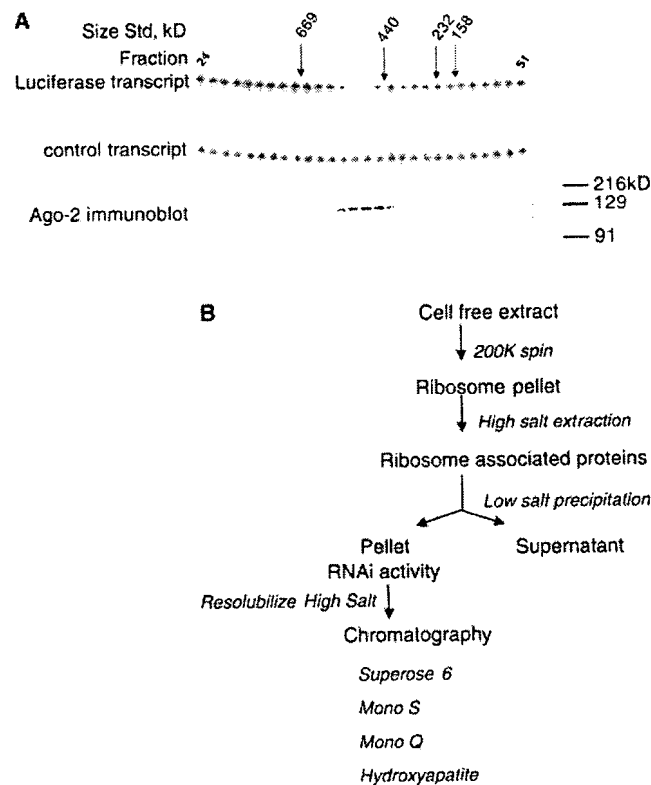


Fig. 1. (A) Soluble extracts were prepared from luciferase dsRNA-transfected S2 cells and fractionated on a Superose-6 sizing column. Fractions were assayed for RISC activity toward cognate luciferase mRNA and a control (cyclin E) mRNA. Fraction numbers are indicated. Size standards were used to calibrate the column; peaks of standards are indicated. Fractions were subjected to Western blotting using anti-AGO2. Western blot standards are indicated at right. **(B)** Strategy used for purification of RISC.

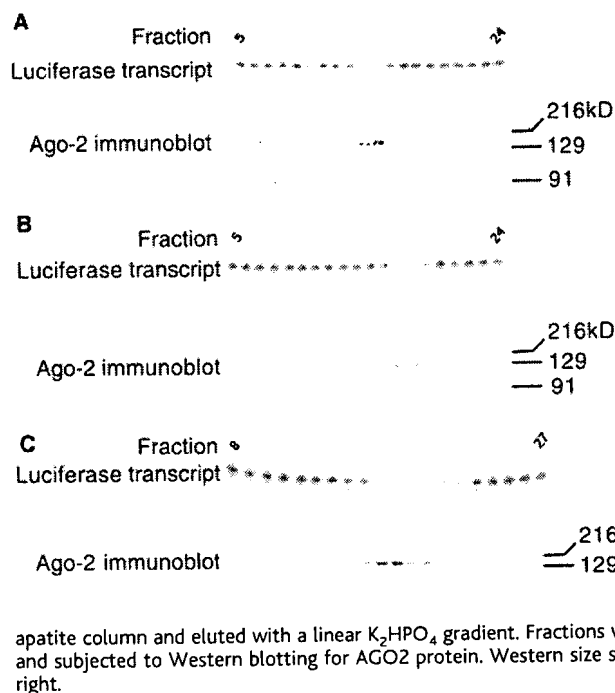


Fig. 2. (A) The fraction with peak activity from the Superose-6 column (Fig. 1A) was loaded on a Mono S column and eluted with a linear KCl gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right. **(B)** The fraction with peak activity from the Mono S column in (A) was loaded on a Mono Q column and eluted with a linear KCl gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right. **(C)** The activity peak from the Superose 6 column (Fig. 1A) was loaded on a hydroxy-

apatite column and eluted with a linear K_2HPO_4 gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right.

Sequencing revealed several discrepancies with the predicted *AGO2* gene. Sequences that

The 63-nt direct repeats translate into a region composed largely of glutamine residues (Fig. 3A). Similar glutamine-rich repeats are found in a number of proteins, including an

To verify the presence of AGO2 in RISC, we generated AGO2-specific antibodies (26). Western blotting of chromatography column fractions with affinity-purified anti-AGO2 showed precise cofractionation of a ~130-kD AGO2 protein and the active RISC fraction through each purification step (Fig. 1A and Fig. 2, A to C). In addition, we tested the association between AGO2 and another component of RISC, the siRNAs. We constructed a version of AGO2 that was tagged at its NH₂-terminus with both a T7 epitope and polyhistidine. This was expressed in cells in which RNAi had been induced against firefly luciferase (27). Tagged AGO2 protein cofractionated with endogenous AGO2, and with the active RISC fraction, in the 500-kD size range (28). RISC was affinity-purified from cell extracts on a polyhistidine-binding resin (Talon, Clontech). Analysis of the imidazole elution profile from this column

A

MGKKDKNNKKGGDSAAAPQPPQQQKQQQQRQQQPQQLQQPQQLQQPQQLQQPQQQQQKQ
QPHQQQQSSRRQPPSTSSGSSRASGFGQQGQQKQSQDAEAGTAKKGGKKGQVGTGKQ
QGGGGHQQGGQGGGQGGVQQRPPQQGGGGHQQGGQGGEGGYQQRPPQQGGGGHQQGGKQ
QEGGYQQRPSGQQGGHQQGRQCGEGGYQQRPPQQGGGGHQQGRQGGGGGGQQRPSGQ
QQGGHQQGRQGGEGGYQQRPSGQQGGHQQGRQGGEGGYQQRGGGGHQQGRPSGQ
EGGYQQRPPGQQPNQTOSGGYQSRGPQQQQAAAPLPLPPQAGSIKRGTIGKPGQGV
INYLDDLKMPSEVAYHYDVKIMPERAPRYQFAEQFGRDVLGGAVLADYGKASCYS
VDKPLNSQNPVTVTRDNRGTLRYTIEIKETGSDTDLKSLVYTMNDRTIDRPFMRAM
QCEVVVLSPCHNKAI RVRGSFTFKMSDPNNRHLEDDGYEALVGLQAFLMGDRPFLNV
DISHKSFVINSMPMIELERFSLKAKINTNTLLVYSRRFLPEFLRGINVLVYPPQSFS
APRVYRVNGLSRAPASSETEFHDGKKVTVSYAFHSRNPYLPKFQHLNVLGSSISIL
LPIELCSIEEGLANKRKGATQVANMIKYAATSNTVNRKRKIMNLLQYFQHNLDPTISR
FGIRIANDLIVYSTRVLSQYHSGKAVTMRVNGSSWRMDGMKFLPEPKPAKHCAVLY
CDPRSGRKNMYQLNDFGNLLIPEQHKAVNLSLDSDVTRTDEERSLDTIFADLRKS
QHDLAIVIPQFYSYDITKQKAELOHGILTCQIKQFTVERKCNQQTIGNLLKINSK
LGNINKIKDDPLPMKMTMYIGADVTRHPSDQREIPSVGVAAASHDPYGASYNMQ
LRQGALEIEDMFSSITLHLYRVKERNAYPNHTIYYRDGSDGDFQFKIKNELRKL
QKACDKVGCKPKICCVIVVKRHHTREFPSGDVTTNKFNNMDFGTCVVDRTIVHPNEMQ
FFHVSQAIGTAKPTRYNVIENTGNLIDLQQLTYNLNCHMFPDGCNRSVYPAPAYL
AFLVAARGVYLLGTNRFLDLKSGYAKRTIVEFPMKNPMYFV



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by Western blotting with a T7 antiserum and by Northern blotting with a luciferase probe indicated cofractionation of the tagged AGO2 and 22-nt siRNAs (Fig. 4A) (27). Considered together, our data strongly support the hypothesis that AGO2 is a component of RISC.

To test whether AGO2 is essential for RNAi in *Drosophila* S2 cells, we used RNA interference to suppress endogenous AGO2, much as we had previously done to establish a role for Dicer in RNAi (18). Treatment of S2 cells with either of two different ~1000-nt dsRNAs homologous to *AGO2* reduced the levels of this protein by a factor of >10 (Fig. 4B) (29). We assessed the ability of these cells to carry out RNAi by transfection with a mixture of firefly and *Renilla* luciferase (as an internal control) expression plasmids in combination with either a control dsRNA (green fluorescent protein, GFP) or a firefly luciferase dsRNA (Fig. 4C) (29). Suppression of *AGO2* expression correlated with a pronounced reduction in the ability of cells to silence an exogenous reporter by RNAi.

The biochemical function of Argonaute family members is completely unknown. However, one domain of this protein, the PAZ domain, is shared with Dicer, which initiates RNAi by processing dsRNA silencing triggers into siRNAs (18). We therefore considered the possibility that Dicer and AGO2 might physically interact, perhaps through their shared PAZ domains. Indeed, endogenous AGO2 can be coimmunoprecipitated with an epitope-tagged version of Dicer protein from transfected S2

cells (Fig. 4D) (30). We have previously shown that Dicer and RISC are biochemically separable, and none of our purified RISC fractions is able to process dsRNA into 22-nt fragments. One possibility is that Dicer is indeed a component of RISC but fails to process dsRNA when present in this complex. However, our current model is that the interaction between AGO2 and Dicer facilitates the incorporation of siRNAs into RISC complexes, which ultimately dissociate from Dicer and target cognate mRNAs for destruction.

Previous genetic studies in three organisms have indicated that Argonaute family members are essential for RNAi/PTGS. The first link between Argonaute proteins and RNAi was shown by the isolation of *C. elegans rde-1* in a screen for RNAi-deficient mutants (14). In *Neurospora*, another member of the Argonaute family, QDE-2, emerged from a selection of mutants that were defective in a transgene co-suppression phenomenon, termed quelling (15). The founding member of this family (AGO1) was first identified in *Arabidopsis* in a screen for mutants with aberrant leaf morphology (31). Subsequently, *ago1* was re-isolated in a screen for plants that were defective in transgene co-suppression (16).

Argonaute proteins are typically members of multigene families. In *Drosophila* there are four annotated genes: *Sting*, *Piwi*, *AGO1*, and *AGO2*. Mutations in three family members (*ago1*, *piwi*, and *sting*) have previously been studied. *Piwi* is required for maintenance of cell proliferation in both the male

and female germ line, and *sting* mutations lead to spermatid defects and male sterility. *Ago1* was identified in a screen for mutations in the *wingless* pathway, and null mutations in this gene cause defects in neurological development (32).

Thus, Argonaute family members have been linked both to gene silencing phenomena and to the control of development in diverse species. The critical question is whether these two roles of Argonaute proteins are mechanistically related. It is already clear that RNAi-related silencing pathways can control the activity of endogenous genetic elements (e.g., transposons). The possibility also exists that these same pathways may control the expression of endogenous protein-coding genes that regulate development. An answer to this question is likely to emerge both from further genetic studies of RNAi pathways and from a search for endogenous targets of RISC that may be identified via its internal RNA guides to substrate selection.

Note added in proof: Recent data from Mello and colleagues (33) have also demonstrated a role for Dicer in RNAi. Furthermore, these investigators and Zamore and colleagues (34) have implicated Dicer and other components of the RNAi machinery in the regulation of developmental timing via the processing of small temporal RNAs. A role for RNAi, and in particular the Argonaute family member *Sting*, in control of the *Stellate* locus has also been described since the submission of this report (35).

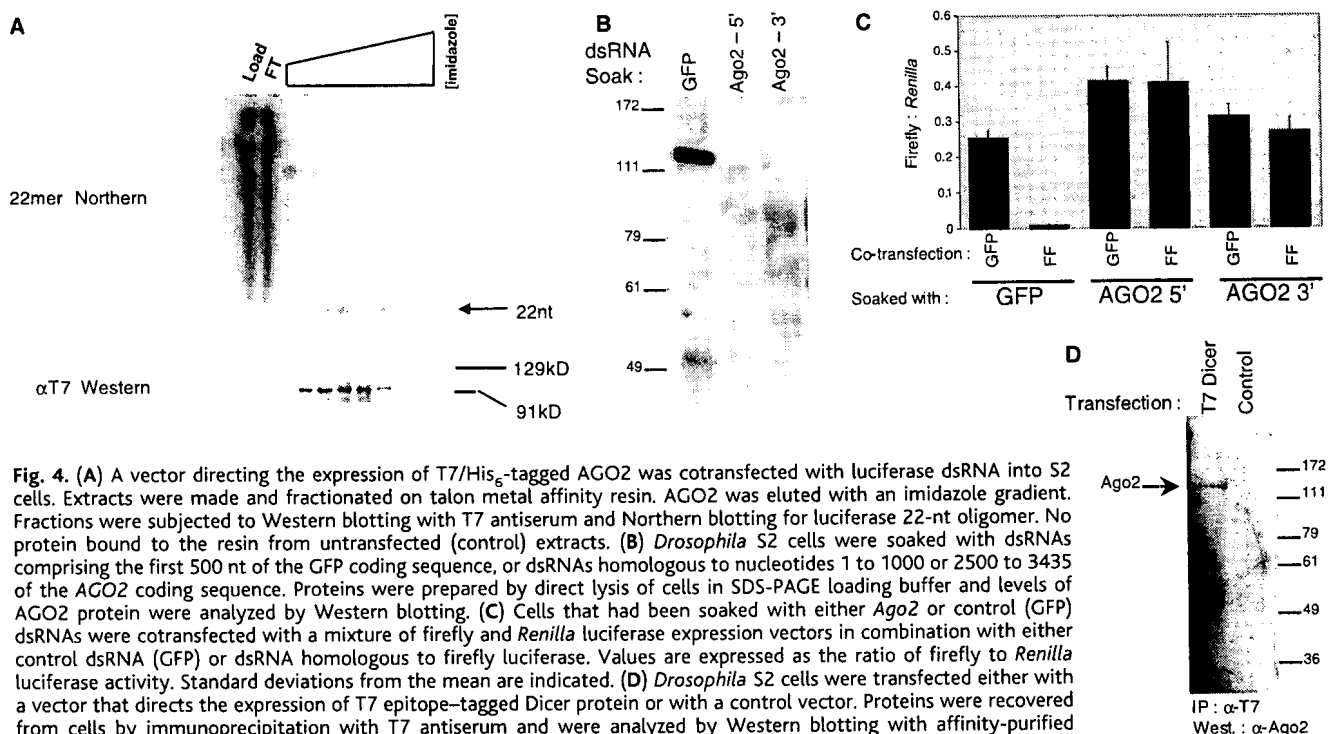


Fig. 4. (A) A vector directing the expression of T7/His₆-tagged AGO2 was cotransfected with luciferase dsRNA into S2 cells. Extracts were made and fractionated on talon metal affinity resin. AGO2 was eluted with an imidazole gradient. Fractions were subjected to Western blotting with T7 antiserum and Northern blotting for luciferase 22-nt oligomer. No protein bound to the resin from untransfected (control) extracts. (B) *Drosophila* S2 cells were soaked with dsRNAs comprising the first 500 nt of the GFP coding sequence, or dsRNAs homologous to nucleotides 1 to 1000 or 2500 to 3435 of the AGO2 coding sequence. Proteins were prepared by direct lysis of cells in SDS-PAGE loading buffer and levels of AGO2 protein were analyzed by Western blotting. (C) Cells that had been soaked with either *Ago2* or control (GFP) dsRNAs were cotransfected with a mixture of firefly and *Renilla* luciferase expression vectors in combination with either control dsRNA (GFP) or dsRNA homologous to firefly luciferase. Values are expressed as the ratio of firefly to *Renilla* luciferase activity. Standard deviations from the mean are indicated. (D) *Drosophila* S2 cells were transfected either with a vector that directs the expression of T7 epitope-tagged Dicer protein or with a control vector. Proteins were recovered from cells by immunoprecipitation with T7 antiserum and were analyzed by Western blotting with affinity-purified anti-AGO2.

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21. S2 cells were soaked with luciferase dsRNA at 3 mg/liter and harvested after 7 days. Hypotonic extracts were spun at 200,000g for 3 hours to pellet ribosomes, which were resuspended and extracted in 400 mM potassium acetate (KOAc). Resultant soluble protein was precipitated by 1:4 dilution in hypotonic buffer and redissolved in buffer A [20 mM Hepes (pH 7.0), 2 mM MgCl₂, 2 mM dithiothreitol, and 0.5% octyl glucoside] with 400 mM KOAc. This protein was then fractionated on Superose-6 HR10/10 (Pharmacia). Fractions were assayed for RISC activity as described (5). The fraction showing peak activity was diluted 1:5 in buffer A and fractionated over a Mono S HRS/5 column (Pharmacia) with a linear gradient elution of buffer A with 0 to 500 mM KCl. The RISC fraction with peak activity from the Mono S column was then fractionated on a Mono Q HRS/5 column (Pharmacia); in turn, the active RISC fraction from this column was fractionated on hydroxyapatite HT (Bio-Rad) in a HRS/5 column (Pharmacia). Elution was in buffer A with 400 mM KCl, with a gradient of KH₂PO₄ from 0 to 500 mM. Fractions were analyzed on a 10% polyacrylamide gel. Four bands appeared to cofractionate with the active RISC fraction and were excised for microsequencing. Total protein was reduced by a factor of 10⁶ during the entire purification. The estimated purification was 1:10,000.
22. Protein bands were digested with modified trypsin (Roche) and analyzed by liquid chromatography-mass spectrometry (LCQ MS/MS; Hewlett-Packard HP1100 connected to a Thermo-Finnigan LCQdecade electrospray ionization ion trap mass spectrometer). The acquired MS data were analyzed by SEQUEST software (Thermo-Finnigan) against the National Center for Biotechnology Information (NCBI) database.
23. Polyadenylated RNA was obtained from S2 cells and *Drosophila* 0- to 12-hour embryos using Trizol (Gibco) followed by magnetic oligo(dT) bead selection (Dyna); 2.5 µg of RNA from each source was used to construct a λZAP cDNA library using a commercial kit (Stratagene). This library was screened using a PCR probe corresponding to nucleotides 811 to 1069 in the GenBank predicted coding sequence. Two of four clones were full length; however, one had four missing NH₂-terminal polyglutamine repeats.
24. Total RNA from S2 cells, *Drosophila* 0- to 12-hour embryos, and adult whole body was fractionated on 1% agarose formaldehyde gel and transferred to Hybond N+ (Pharmacia). The membrane was hybridized with a ³²P-labeled oligonucleotide, CTGAGGCG-GTAATGGTAATGGGCGAGCCTGCTGTGCTGAGGTGGTCCAC.
25. PCR was done using 1 µg of S2 genomic DNA, first-strand cDNA prepared using the C. *thermus* kit (Roche), 0.001 ng of the full-length λZAP clone, or 0.001 ng of ago2/piZ expression construct as templates. Primers were AACAGCAGGTACAAGGGTGGA and GATTGGTATTGGCCTTGGCTC. Roche taq conditions were used for 45 cycles.
26. A peptide corresponding to the eight COOH-terminal amino acids of AGO2 was conjugated to KLH and used for inoculation into rabbits for polyclonal antibody production (Covance). Antibodies were affinity-purified on a peptide-conjugated resin (Sulfolink, Pierce Biochemicals).
27. Ago2 was amplified with the primers ACCGATATC-ACCATGGCTAGTATGACTGGTGGTCAACAAATGGGT-CACCATCACCATCATCATCATGGCTTCTGTGGCATTAC-CAC and ACCCTCGAGTCAGACAAAGTACATGGGGT. This initiates at the second methionine codon, truncating the polyglutamine tract. The product was cloned into the expression vector pIZ (Invitrogen). S2 cells were transfected with 15 µg of luciferase dsRNA and 45 µg of ago2/piZ per 15-cm plate, as described. Hypotonic extracts were made, adjusted to 400 mM KOAc, 0.5% octyl glucoside, and loaded on 1 ml of talon resin (Clontech) in a hi trap column (Pharmacia). Protein was eluted with an imidazole gradient, 0 to 100 mM, 10 column volumes. Protein was analyzed by Western blotting using T7 antiserum (Novagen). RNA was extracted with trizol and run on 15% acrylamide/urea/tris-acetate-EDTA gel. RNA was electroblotted as described and membranes probed with luciferase sense riboprobe (5).
28. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, data not shown.
29. *Drosophila* S2 cells were incubated with dsRNA (30 mg/liter) corresponding to either the first 500 nt of GFP or the first or last ~1000 nt of AGO2 for 30 min in the absence of serum (20). Serum was added to a final concentration of 10%, and cells were cultured in suspension for 48 hours at 27°C. Cells were then divided into six-well dishes and cotransfected with a combination of *Renilla* and firefly luciferase expression vectors (3 µg total) and with dsRNA corresponding either to a control (GFP) or to firefly luciferase (75 ng). Assays for Firefly and *Renilla* luciferase were performed 36 hours after transfection. All analyses were performed on triplicate transfections. Identical experiments were also performed with *Renilla* dsRNA with a qualitatively similar outcome. For Western blotting, aliquots of soaked cells were lysed directly in SDS sample loading buffer.
30. S2 cells were transfected with a vector that directs the expression of a T7 epitope-tagged Dicer protein (18) or with a control vector. Cells were lysed and Dicer protein was immunoprecipitated as described (18). Proteins were released from Protein A-Sepharose beads by boiling in SDS sample loading buffer and were analyzed by Western blotting with the affinity-purified AGO2 antibody.
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37. We thank E. Bernstein, A. Denli, and D. Conklin for critical reading of the manuscript. S.M.H. is a visiting scientist at CSHL from Genetics Inc. A.A.C. is a George A. and Marjorie H. Anderson Fellow of the Watson School of Biological Sciences and is a predoctoral fellow of the Howard Hughes Medical Institute. Supported in part by NIH grant RO1-GM62534 (G.J.H.) and by a grant from Genetics Inc. G.J.H. is a Rita Allen Foundation scholar.

3 July 2001; accepted 11 July 2001

Transitions in Distinct Histone H3 Methylation Patterns at the Heterochromatin Domain Boundaries

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Eukaryotic genomes are organized into discrete structural and functional chromatin domains. Here, we show that distinct site-specific histone H3 methylation patterns define euchromatic and heterochromatic chromosomal domains within a 47-kilobase region of the mating-type locus in fission yeast. H3 methylated at lysine 9 (H3 Lys⁹), and its interacting Swi6 protein, are strictly localized to a 20-kilobase silent heterochromatic interval. In contrast, H3 methylated at lysine 4 (H3 Lys⁴) is specific to the surrounding euchromatic regions. Two inverted repeats flanking the silent interval serve as boundary elements to mark the borders between heterochromatin and euchromatin. Deletions of these boundary elements lead to spreading of H3 Lys⁹ methylation and Swi6 into neighboring sequences. Furthermore, the H3 Lys⁹ methylation and corresponding heterochromatin-associated complexes prevent H3 Lys⁴ methylation in the silent domain.

In eukaryotes, chromosomes are partitioned into structurally and functionally distinct regions that help to separate independently regulated parts of the genome (1, 2). Specialized DNA elements, known as insulators or boundary elements, have been suggested to

mark the borders between adjacent chromatin domains and to act as barriers against the effects of enhancer and silencer elements from neighboring regions (3–5). The best characterized example of such long-range chromatin effects comes from studies of po-

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COLOMBIA

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ANEXO 8

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The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides

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Received April 22, 2003; Revised and Accepted May 29, 2003

ABSTRACT

The biological activity of siRNA seems to be influenced by local characteristics of the target RNA, including local RNA folding. Here, we investigated quantitatively the relationship between local target accessibility and the extent of inhibition of the target gene by siRNA. Target accessibility was assessed by a computational approach that had been shown earlier to be consistent with experimental probing of target RNA. Two sites of ICAM-1 mRNA predicted to serve as accessible motifs and one site predicted to adopt an inaccessible structure were chosen to test siRNA constructs for suppression of ICAM-1 gene expression in ECV304 cells. The local target-dependent effectiveness of siRNA was compared with antisense oligonucleotides (asON). The concentration dependency of siRNA-mediated suppression indicates a >1000-fold difference between active siRNAs ($IC_{50} \approx 0.2\text{--}0.5\text{ nM}$) versus an inactive siRNA ($IC_{50} \geq 1\text{ }\mu\text{M}$) which is consistent with the activity pattern of asON when relating target suppression to predicted local target accessibility. The extremely high activity of the siRNA si2B ($IC_{50} = 0.24\text{ nM}$) indicates that not all siRNAs shown to be active at the usual concentrations of >10–100 nM belong to this highly active species. The observations described here suggest an option to assess target accessibility for siRNA and, thus, support the design of active siRNA constructs. This approach can be automated, work at high throughput and is open to include additional parameters relevant to the biological activity of siRNA.

INTRODUCTION

The increasing use of siRNA to specifically suppress target gene expression in mammalian cells progressively reveals an

influence of local target characteristics on the biological effectiveness (1–3). In major part, this is assigned to characteristics of the local folding of target RNA (1–3) and a recent study indicates to some extent a similarity between the site-dependent biological activity of phosphorothioate-derived antisense oligonucleotides (asON) and siRNA (3). When taken together, these findings on target structure-related biological activity of siRNA in mammalian cells, raise two increasingly relevant questions. First, what are the rules governing the structure–function relationship of siRNA and, secondly, can one find ways to distinguish effective and non-effective siRNA constructs on the basis of target structure analysis. The latter issue guides one to the systematic design of effective siRNA.

Since siRNA is regarded to be highly sequence-specific (4), it is reasonable to assume that a recognition step occurs between siRNA and its target sequence. This step might occur between the siRNA double strand and the target or subsequent to a possible preceding dissociation of the siRNA with its antisense strand. In any case it seems to be reasonable to assume that the local target RNA segment has to be accessible to intermolecular nucleotide–nucleotide interactions with siRNA or its antisense strand, respectively. There is a need to find a mammalian test system and experimental conditions to study the influence of target structure on the activity of siRNA.

Here, we used the human gene *ICAM-1/CD54*, which is one of the best studied targets for antisense nucleic acids (5,6), and siRNA (3) to investigate the extent of apparent gene expression in relation to local target accessibility. Target gene suppression in this system is reproducible and robust. Regarding RNA folding of the complete ICAM-1 mRNA, we had access to a highly systematic computational RNA secondary structure analysis (6; R.Kretschmer-Kazemi Far, J.Leppert and G.Sczakiel, manuscript in preparation). Even a less detailed analysis was earlier shown to give rise to results that are highly consistent with structural probing of RNA in the presence of cellular extracts (7), suggesting that the theoretical analysis of target accessibility as performed here and as described recently (6), represents a valid basis to study the relation between accessibility and efficacy of siRNA.

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Dedicated to the memory of Claude Hélène

However, as an additional measure of the reliability of this kind of computational analysis of target accessibility, we included asON in this study. Furthermore, asON were used here to compare their effectiveness with siRNA on a quantitative level.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides were purchased from commercial suppliers. asON were synthesized as phosphorothioate-modified oligodeoxyribonucleotides. All single-stranded oligoribonucleotides constituting siRNA were synthesized with two 3'-deoxythymidine nucleotides. The following phosphorothioate-modified control oligonucleotides with scrambled sequences were used: 5'-GGTACGTGCTGAGGCCTG-3'; for siRNA we used the scrambled control: 5'-CGAACUCA-CUGGUCUGACCtdt-3' (sense strand), 5'-GGUCAGAC-CAGUGAGUUCGtdt-3' (antisense strand). The yield and purity of all oligonucleotides was determined by UV absorption spectroscopy and the integrity was controlled with 20% denaturing polyacrylamide gels followed by staining with Stains-All (Sigma-Aldrich, Deisenhofen, Germany). The sequences of all oligonucleotides are shown in Figure 1.

Formation of double-stranded siRNA

Complementary RNA strands at 20 μ M were annealed in 50 mM potassium acetate, 1 mM magnesium acetate and 15 mM HEPES pH 7.4 by heating at 90°C for 2 min followed by incubation at 37°C for 1 h. The formation of double-stranded RNA was confirmed by non-denaturing (15%) polyacrylamide gel electrophoresis and staining with Stains-All.

RNA secondary structure prediction

To predict secondary structures of target sequences, we used the software mfold version 2.3 (8,9) which is included in the software Wisconsin Package Version 10.0, Genetics Computer Group. Characteristic parameters of oligonucleotide sequences were determined using the software Oligo version 3.4, which is based on the work of Rychlik and Rhoads (10).

Cell lines and cell culture

The cell line ECV304 was described as an endothelial cell line derived from spontaneously immortalized human umbilical vein cells (11). However, the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) showed by DNA fingerprinting that this cell line is a derivative of the human urinary bladder carcinoma cell line T-24 (12), which expresses *ICAM-1* in an inducible manner. ECV304 cells were maintained in medium 199 (Sigma-Aldrich) buffered with 25 mM HEPES pH 7.4 and supplemented with 0.68 mM L-glutamine and 10% FCS (Invitrogen, Karlsruhe, Germany). Cells were routinely split two to three times a week after trypsinization. For stimulation of *ICAM-1* (CD54) 200 U/ml interleukin 1 β (IL-1 β ; PromoCell, Heidelberg, Germany) was added to the medium and cells were incubated overnight (for 16–18 h).

Transfection of cells with asON or siRNA

ECV304 cells were seeded in 12-well culture plates at a density of 1.5×10^5 cells/well, 15 h prior to oligonucleotide treatment. Then, the cells were washed once with pre-warmed (37°C) serum-reduced Opti-MEM I medium (Invitrogen). To deliver asON or siRNA to cells we used Lipofectamine 2000 (Invitrogen). The transfection of asON or siRNA was performed with 0.4 ml of Opti-MEM I medium containing 0.1 μ M asON or siRNA and 10 μ g/ml Lipofectamine 2000 per well. The cells were incubated for 4 h at 37°C, 5% CO₂. Subsequently, the transfection medium was replaced by medium 199 containing 10% FCS. After an incubation of 4 h at 37°C, 5% CO₂, the medium was again substituted with medium 199 containing 10% FCS and 200 U/ml IL-1 β to stimulate *ICAM-1* expression. For immunofluorescence analysis, cells were removed from the culture dish by trypsinization with 100 μ l of 0.25% trypsin/0.02% EDTA in PBS for 5 min at 37°C, 16–18 h after stimulation with IL-1 β . *ICAM-1*-specific gene expression of cells treated with IL-1 β and control asON or siRNA (scrambled sequence for both) was set at 100%, while gene expression of cells treated with control asON or siRNA only was defined as the basal expression level.

Quantification of *ICAM-1*-specific target protein: immunofluorescence staining and flow cytometry

Cells were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated with a phycoerythrin (PE)-conjugated CD54 monoclonal antibody (clone LB-2) or an isotype-identical PE control (both, Becton Dickinson, Heidelberg, Germany) in 50 μ l of PBS containing 1% BSA at 4°C for 30 min. Subsequently, cells were washed with PBS and suspended in PBS containing 1% paraformaldehyde (pH 7.4). The cells were analyzed using a Becton Dickinson FACSCalibur and CellQuest Pro software (Becton Dickinson). After gating out dead cells, the mean (PE) fluorescence intensity was determined and the inhibition of *ICAM-1* gene expression was standardized to the IL-1 β -induced state which was set at 100%. On average, basal gene expression levels were ~35%.

Quantification of *ICAM-1*-specific target RNA: quantitative RT-PCR

Total RNA was extracted from transfected cells using the RNeasy mini kit including treatment with RNase-free DNase I (Qiagen, Hilden, Germany). The yield and purity of RNA were determined by spectrophotometry. Synthesis of cDNA was carried out using random hexamer primers and Superscript II RNase H⁻ reverse transcriptase according to the manufacturer's specifications (Invitrogen). Quantitative PCR was performed using the GeneAmp 5700 sequence detection system (Applied Biosystems, Darmstadt, Germany) and SYBR green PCR core reagents (Eurogentec, Seraing, Belgium). *ICAM-1* cDNA was amplified with the forward primer 5'-GCCACTTCTTCTGTAAAGTCTGTGGG-3' and reverse primer 5'-CTACCGGCCCTGGGACG-3', resulting in a fragment of 300 bp. Samples were standardized using primers specific to cDNA encoding human GAPDH (forward primer 5'-AACAGCGACACCCACTCCTC-3' and reverse primer 5'-GGAGGGGAGATTCACTGTGGT-3') resulting in a product of 258 bp. Standard curves were obtained after

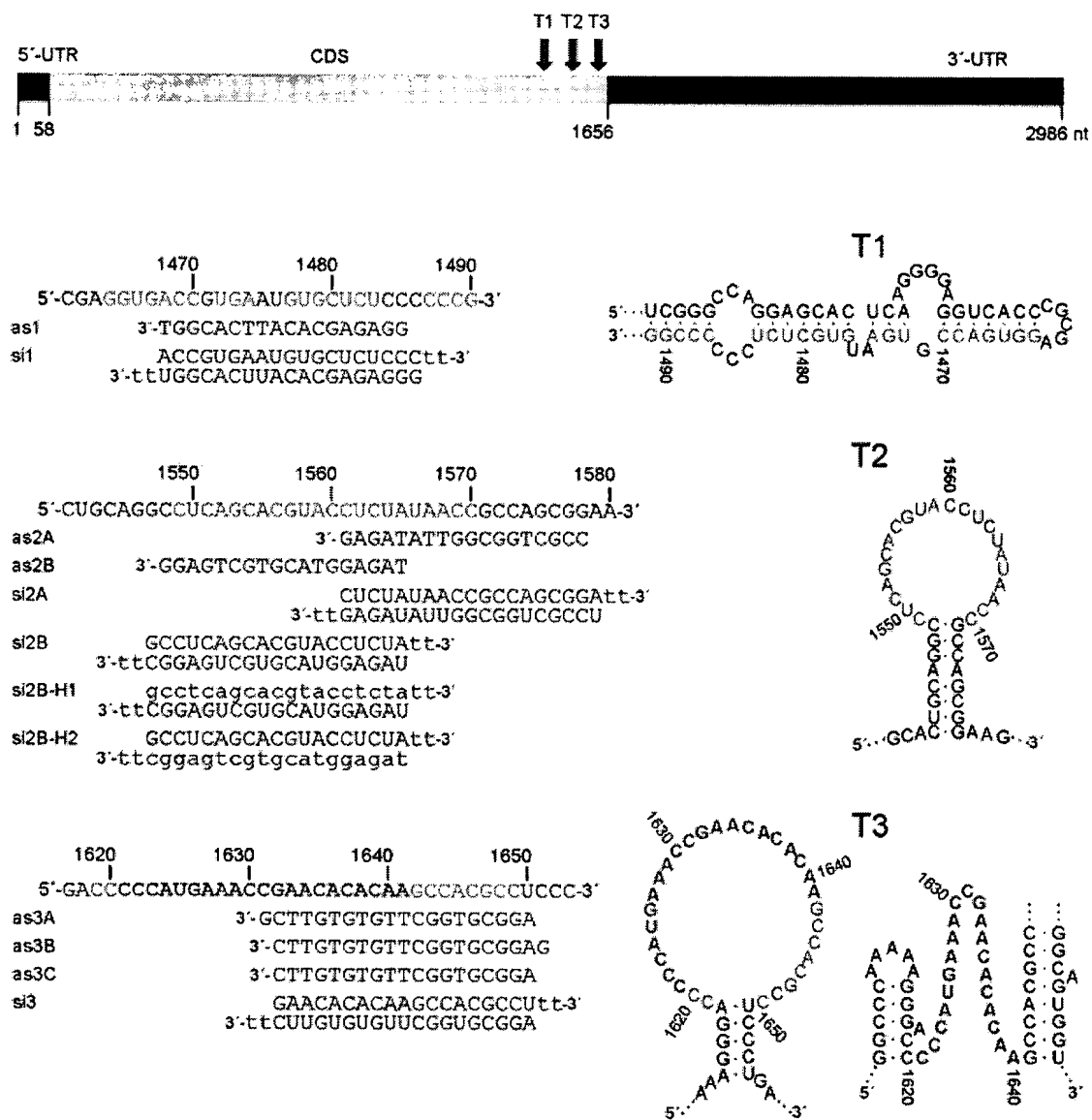


Figure 1. Schematic depiction of the *ICAM-1/CD54* mRNA sequence (top), the local target structures T1, T2 and T3 (top right), and the nucleotide sequences and names of the asON and siRNA used here (top left). The 'closed' target sequences that are predicted to be involved in intramolecular folding of the target T1 are indicated in red. Target segments of T2 and T3 that are predicted not to be involved in intramolecular base-pairing are indicated in green (right). The same color code is used for the complementary sequences of asON and siRNA of the targets T1, T2 and T3 (left). For T1 and T2 all systematic computer foldings predict the structural domains indicated on the right. For T3 all foldings predict either of the two structures shown here. The sequence stretch that is not involved in intramolecular folding in both structures is indicate in dark green (bottom right). The numbers along the sequences refer to nucleotide sequence positions of the *ICAM-1* mRNA. The GeneBank accession number of the human *ICAM-1* sequence is JO3132. UTR, untranslated region; CDS, coding sequence.

amplification of 2.5×10^3 – 2.5×10^7 copies of purified plasmid pP5, a derivative of pEGFP-C1 carrying the amplicon generated with the *ICAM-1* primer set and plasmid pCR-GAPDH, a derivative of pCR 2.1 harboring the GAPDH amplicon sequences.

RESULTS

Local target sites of *ICAM-1* mRNA

Many approaches have been developed in order to obtain insights into the relationship between local target accessibility

and the effectiveness of antisense nucleic acids in living cells (reviewed in 13). Accessibility of a given RNA for intermolecular base-pairing is directly related to RNA structure which can be assessed experimentally (14–18) or theoretically (reviewed in 19). Based on the identified or presumed local accessibility, complementary nucleic acids can be selected. Among the theoretical ways to design effective asON, a specific systematic computational analyses of predicted RNA structures and nucleotide sequence characteristics has been shown to be significantly related to the biological activity of asON in living cells (6,20). This theoretical approach has also been shown to be closely related

to the experimental probing of target RNA in the presence of cellular extracts indicating its significance (7). This methodology (6) was applied here to the sequence of the ICAM-1 mRNA in order to define local folding, i.e. structural accessibility. Window sizes ranging between 600 and 1400 nt were analyzed by mfold (see Materials and Methods), shifted along the target sequence at a step width of 100 nt, and the 10 lowest energy structures of each were recorded and used to select local targets that occurred in all predictions at high abundance (data not shown).

To investigate and to compare the possible role of local target characteristics for the efficacy of asON and siRNA, three local target sites along the ICAM-1 mRNA were chosen. The local sequence segment at position 1430–1490 termed T1 in this work is predicted to adopt a local RNA structure that is not regarded as being accessible to complementary strands and, thus, it should not support their binding and efficacy in living cells (Fig. 1, top right). Conversely, the local sequence segments in the vicinity of positions 1570 and 1640 are predicted to be accessible in structural terms and, thus, are regarded to favor the biological activity of complementary nucleic acids including asON and, possibly, siRNA (Fig. 1, middle and bottom right). In addition to structural characteristics of the target RNA, the design of asON sequences in this work was based on criteria related to their sequences as described recently (20), including the results of nucleotide sequence homology searches, the exclusion of specific motifs such as G-quartets and intramolecular folding, or a high potential of dimer formation. The sequences of siRNA constructs were chosen such that they are directed against the central portion of the target motifs shown in Figure 1. All siRNA constructs were tested for nucleotide sequence homology with unrelated human transcribed sequences and they all share the same GC content and length.

The target gene *ICAM-1* is expressed at low basal levels and at increased levels in the presence of stimulatory cytokines such as IL-1 β . Routinely, inhibitory effects in this system are monitored by the suppression of IL-1 β -induced stimulation of *ICAM-1* expression. Here, we also tested effects on the basal levels of *ICAM-1* expression since this increases the range in which the effects of siRNA and asON can be tested. If one compares the effectiveness of different classes of biologically active molecules such as asON and siRNA, then it is reasonable to attempt to apply optimal experimental conditions for each. Experimental evidence suggests that the transfection step is critical for the extent of apparent target gene suppression by siRNA (21–23). In this work, we used a constant concentration of 10 μ g/ml Lipofectamine 2000 in cell culture with ECV304 cells. At 100 nM asON this is equivalent to a ratio of Lipofectamine 2000 to asON of ~16:1 (w/w) and to siRNA of ~7:1 (w/w). Under these conditions we observed significantly stronger siRNA-mediated inhibition than with the use of the transfection reagent 'lipofectin'. We performed further experiments using 3 μ g/ml Lipofectamine 2000, equivalent to a ratio of Lipofectamine 2000/asON of 5:1 (w:w), which showed a decrease in asON-mediated inhibition. At 3 μ g/ml Lipofectamine 2000, the transfection conditions were appropriate, in principle, although at the higher dose of Lipofectamine 2000, suppressive effects were stronger. For further experiments we used Lipofectamine 2000 at 10 μ g/ml in cell culture because this favored the effectiveness of siRNA

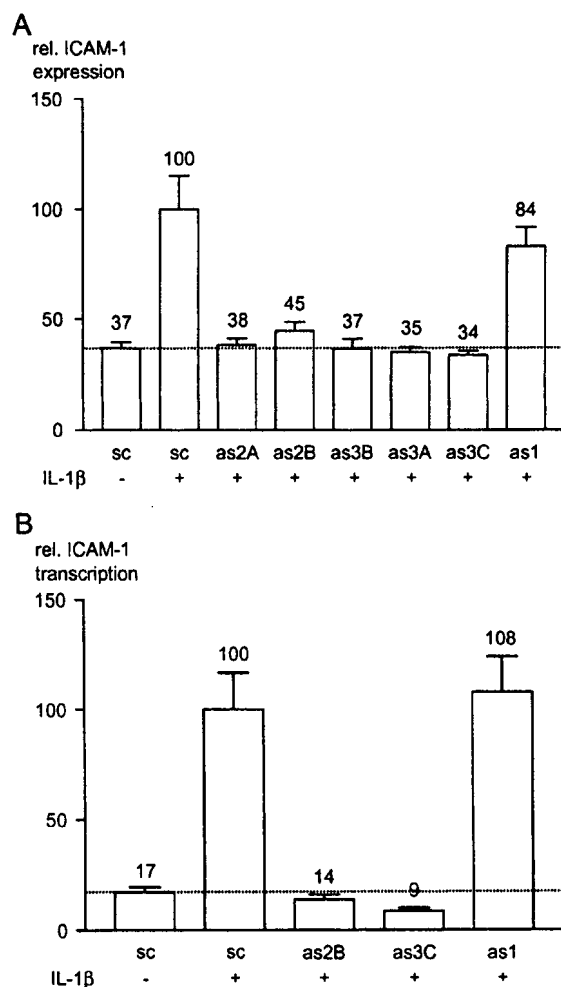


Figure 2. Suppression of the IL-1 β -induced state of *ICAM-1* gene expression by asON at the level of the ICAM-1 protein (A) and target mRNA (B). Basal expression levels of ~37% at the protein level and ~17% at the level of mRNA are indicated by a dotted line. Error bars indicate the standard deviation of two (B) or three (A) individual sets of experiments performed in triplicate. The transfection reagent Lipofectamine 2000 was used at 10 μ g/ml in cell culture according to the supplier's instructions. The final asON concentration was 100 nM.

and, to a minor extent, also that of asON. This allowed a greater dynamic range for measuring suppression of target gene expression.

Suppression of *ICAM-1* by asON

The biological activity of the set of asON schematically depicted in Figure 1 was monitored by suppression of IL-1 β -induced expression of *ICAM-1* in ECV304 cells at the protein level as described (6). Values for asON-mediated inhibition of *ICAM-1* were related to control oligonucleotides with a scrambled order of nucleotides at a comparable base composition. The results clearly show strong inhibition of IL-1 β -induced gene expression of *ICAM-1* by asON directed against the favorable target sites T2 and T3 and even indicates a suppression of gene expression below the levels of constitutive gene expression (Fig. 2). The unfavorable target site T1

(Fig. 1A) does not give rise to significant inhibitory effects by asON (Fig. 2A). The observations described here are consistent with extensive studies on asON-mediated inhibition of gene expression where asON had been selected according to the protocol used in this work. This experiment shows a large dynamic range of suppression of *ICAM-1* gene expression when comparing the unfavorable local target T1 with the favorable targets, T2 and T3, respectively.

A real antisense effect implies that the target gene is suppressed at the level of RNA which gives rise to reduced levels of the protein encoded by the target gene. Levels of target mRNA were measured by quantitative RT-PCR and standardized to the levels of GAPDH mRNA. The inhibition data summarized in Figure 2B show that this is the case for the antisense species used here. When considering the phosphorothioate chemistry of the asON it is important to note that no side effects were observed in the use of the scrambled controls (data not shown). Together, these findings are consistent with the view that the asON-mediated down-regulation of *ICAM-1* gene expression observed in this study is specific and follows an antisense mechanism.

Suppression of *ICAM-1* by siRNA

The effects of siRNA were monitored at the protein level by FACS analysis of transfected cells and standardized to IL-1 β -induced expression of *ICAM-1* in the presence of a scrambled siRNA control (Fig. 3A). The most potent siRNA species were directed against the local targets T2 and T3 which gave rise to the highest inhibition levels in the use of asON. The siRNA directed against the local target T1 which was unfavorable for asON showed no significant influence of *ICAM-1* gene expression. This differential behavior is highly consistent with the inhibition properties of the asON investigated in this work. The biologically active siRNA constructs suppress stimulation of the target completely and even reduce basal levels of *ICAM-1* gene expression, indicating a significantly increased activity when compared with asON (Figs 2 and 3).

Since the action of siRNA is presumed to occur post-transcriptionally and involves the degradation of target RNA, we also measured effects in this system at the level of *ICAM-1* mRNA (Fig. 3B). The results of this experiment are compatible with siRNA-mediated inhibition at the protein level. To further support the presumed siRNA mechanism, we tested whether the antisense strand of the siRNA construct si2B is more sensitive to modifications than the sense strand, as one would believe from the literature (24,25). We transfected DNA-RNA hybrid constructs in which either the RNA sense strand or the RNA antisense strand was substituted by a DNA strand (si2B-H1, si2B-H2; Fig. 1). A substantial loss of activity was observed when the antisense strand was DNA

rather than RNA but the activity remained high when the sense strand was replaced. Moreover, the antisense or sense strand of the active siRNA si2B did not show significant suppression of

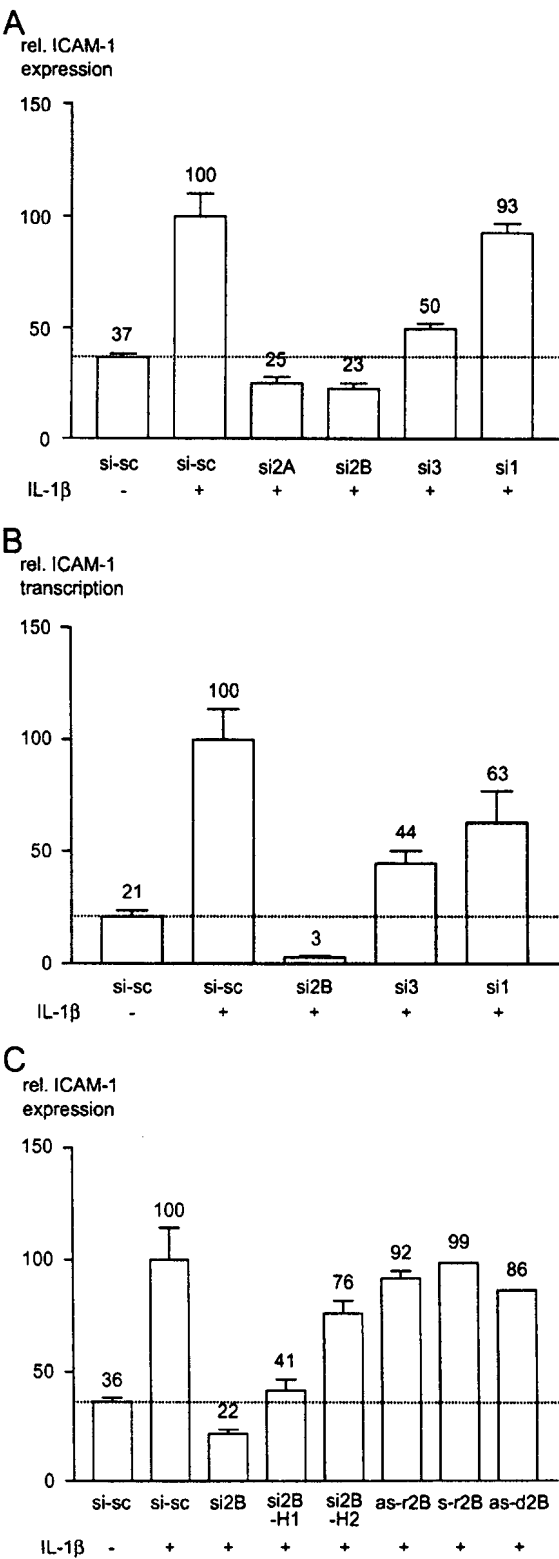


Figure 3. Suppression of the IL-1 β -induced state of *ICAM-1* gene expression by siRNA at the level of the *ICAM-1* protein (A) and target mRNA (B). Effects of derivatives of si2B and single-stranded controls on the IL-1 β -induced state of *ICAM-1* gene expression are shown in (C). The names of the constructs are defined in Figure 1. Basal expression levels are indicated by a dotted line. Error bars indicate the standard deviation of at least two individual sets of experiments performed in triplicate. The transfection reagent Lipofectamine 2000 was used at 10 μ g/ml. The final concentration of siRNA and derivatives thereof was 100 nM.

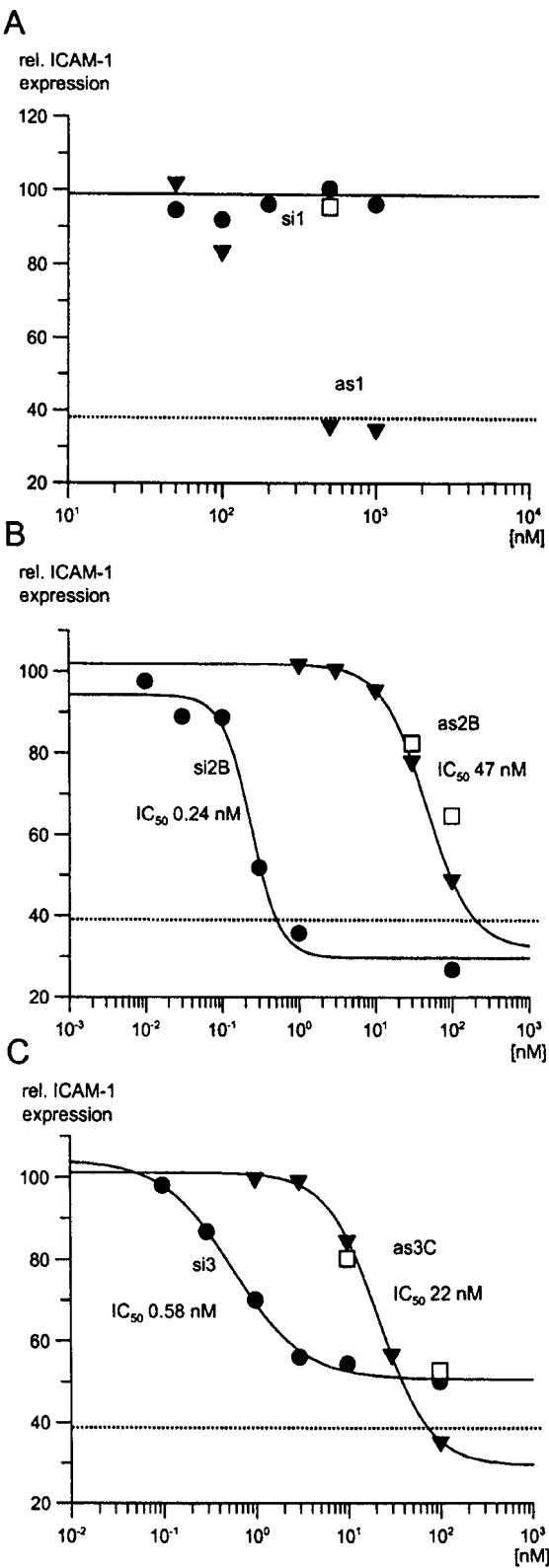
target gene expression at the same concentration (Fig. 3C). These observations suggest that siRNA is the active molecule in the test system used here.

Dose-dependency of suppression of ICAM-1 by asON and siRNA

In order to quantitatively compare the biological activity of asON and siRNA and the influence of the choice of the local target, we determined apparent values of half maximal inhibition (IC_{50} values) and the maximal achievable extent of inhibition (Fig. 4). At the level of the ICAM-1 protein, the values of the apparent IC_{50} for siRNA against T2 and T3 were 0.24 and 0.58 nM, respectively (Fig. 4B and C), whereas si1 did not exert any significant inhibition at concentrations of up to 100 nM (Fig. 4A). For antisense constructs, an IC_{50} value of 47 (as2B) and 22 nM (as3C) was measured, whereas the possible IC_{50} value for as1 against T1 cannot be derived from the experiments. It is reasonable to assume that this value is not <500 nM (Fig. 4A). In the latter case, a substantial cytotoxicity was observed at concentrations >100 nM. The IC_{50} value of the most active siRNA construct si2B is ~100-fold lower than the value for one of the most effective known phosphorothioate-substituted asON, as3C. When assuming that as3C represents the maximal achievable level of target gene suppression in the use of phosphorothioate-substituted asON, and si2B represents the corresponding level in the use of siRNA, then this suggests that under the conditions used here, siRNA is ~100-fold more active in mammalian cells than asON. Furthermore, even though si3 (Fig. 4C; IC_{50} = 0.58 nM) is ~40-fold more potent than the homologous asON as3C (Fig. 4C; IC_{50} = 22 nM), the maximal extent of inhibition is greater with the use of the asON (~30% gene expression) than with the siRNA (~50% gene expression).

At a first look one might hypothesize that observed IC_{50} values monitor the biological activity of the siRNAs used here. When comparing the effectiveness of si1, si2B and si3 at 100 nM (Fig. 3A and B) with their dose-dependency, then it seems that the maximal achievable level of target inhibition rather than the IC_{50} value is more closely related to biological activity. This means that above certain concentrations of siRNA a further increase does not increase the extent of inhibition; this indicates a bottle neck for the effectiveness of siRNA in mammalian cells that cannot be compensated by increased doses. One might deduce that the mode of action of siRNA includes at least two critical parameters: a concentration-dependent and a concentration-independent parameter.

Figure 4. Concentration dependency of the inhibition of ICAM-1 gene expression at the protein level by asON and by siRNA against the local targets T1 (A), T2 (B) and T3 (C). IC_{50} values are indicated at the respective curves. Data points represent the mean of at least two individual experiments with a standard deviation between 5 and 10% for siRNA and between 5 and 20% for asON, respectively. Basal expression levels are indicated by a dotted line. Lipofectamine 2000 was used at a final concentration of 10 μ g/ml. As a control for the ratio of transfection reagent to asON we also tested target suppression at 3 μ g/ml Lipofectamine 2000 (open squares) which gave rise to a reduced level of inhibition. Closed circles, siRNA; closed triangles, asON.



DISCUSSION

In this study, experimental evidence is provided for the view that structural local target characteristics are related to target accessibility and to the biological activity of siRNA. At a first glance this relationship seems to be similar to the presumed structure–function relationship of asON which is compatible between this work and detailed published work in the same biological system (6). The findings described here are based on a computational analysis of target accessibility. The significance of this theoretical RNA structure analysis is supported (i) by its consistency with further detailed studies on the structure–function relationship of asON (7,26,27), (ii) by its close relationship with an experimental approach to monitor target accessibility (7) and (iii) by the results in the use of asON as measured here.

Local RNA structures along the ICAM-1 target mRNA that are predicted to be accessible, serve as favorable targets for siRNA; this suggests the usefulness of the theoretical accessibility approach for the selection of siRNA constructs. This observation is consistent with a recent report in which the oligonucleotide scanning array technology has been used to experimentally monitor target accessibility (2). In principle, this work suggests on a quantitative level that target accessibility is correlated with biological activity of asON and siRNA. It remains to be tested, however, whether the significant effects of single additional or lacking nucleotides of siRNA with respect to complementary nucleotides of the target strand give rise to a heterogeneous pattern of effectiveness within a given local target as observed for asON (7,28,29). At a lower resolution this possibility is supported by a study with overlapping siRNAs shifted along a target sequence by 3 nt (1). Kawasaki *et al.* (30) provide experimental evidence for the view that target structure matters with regard to the biological activity of siRNA. Interestingly, this seems to be overcome with the use of siRNA that has been exposed to recombinant Dicer *in vitro* (30).

The biological activities of si2A and si2B are comparable (Fig. 3A). If one speculates on the importance of the termini of siRNA or its complementary single strands, then one might conclude that there is no difference between the ends with respect to their effectiveness in mammalian cells. When considering the local target for both siRNAs (T2; Fig. 1), then it does not seem to matter whether either terminus of the siRNA is directed against a sequence segment that is involved in intramolecular base-pairing or not. It seems to be reasonable, however, to assume that a closer look at the relationship between local target characteristics and the efficacy of siRNA will reveal new mechanistic insights that can be exploited for the design of highly effective siRNA species.

The extent of inhibition of ICAM-1 gene expression in the use of asON versus siRNA indicates an ~100-fold increase in the apparent IC₅₀ value of siRNA (Fig. 4B). This observation is consistent with a recent report that provides experimental evidence for a similar gap of activity reflected by an ~100-fold increased activity of siRNA versus asON (23). The authors further suggest that the activity of double-stranded RNA is dependent on 'positional effects' of the target RNA.

The ICAM-1 mRNA was used as a target RNA for a large set of asON and siRNA in a recent study (3). We compared the inhibition in this study with local target accessibility using the

approach in our study. For a number of cases we also find a significant relationship between local target accessibility and efficacy. Conversely, most local target sites described in Vickers *et al.* (3) cannot be assessed by the theoretical approach used here and, to some extent, the inhibition data on the level of target RNA and target protein are not consistent. Thus, a systematic analysis of the data in Vickers *et al.* (3) using the concept in our work is not possible.

The work on asON-mediated effects made use of phosphorothioate-derived asON. It might well be that improved second generation chemistries significantly reduce the gap of biological activity between asON and siRNA. In this context, it is also interesting to look at the sequence specificity and side effects of siRNA and asON at the available different chemistries.

One might hypothesize that sequence-specific interactions between siRNA and its target RNA are crucial and are closely related to efficacy. As a consequence, insights into the biochemical properties of this step might help to understand the structure–function relationship of siRNA, thereby supporting the rational improvement of the efficacy. It is likely that the recognition process between double-stranded and single-stranded RNA inside mammalian cells is accompanied by cellular proteins and other endogenous compounds. It remains open, however, as to whether such additional substances influence the molecular mechanism of RNA interactions (e.g. by changing RNA folding and the mode of recognition) or whether they catalyze this reaction in an enzyme-like fashion in order to facilitate this process and increase the kinetics without affecting the mechanism [e.g. as observed in the case of facilitator-promoted annealing of complementary RNA which does not measurably influence the underlying structure–function relationship (31)].

ACKNOWLEDGEMENTS

We thank M. Lehmann and T. Sabelhaus for providing plasmid constructs. This work was supported by a grant from the Faculty of Medicine of the University of Lübeck (FSO) to R.K.K.F. and G.S.

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ANEXO 9

000119

Efficient Reduction of Target RNAs by Small Interfering RNA and RNase H-dependent Antisense Agents

A COMPARATIVE ANALYSIS*

ANEXO 9

Received for publication, October 9, 2002, and in revised form, December 20, 2002
Published, JBC Papers in Press, December 23, 2002, DOI 10.1074/jbc.M210326200

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RNA interference can be considered as an antisense mechanism of action that utilizes a double-stranded RNase to promote hydrolysis of the target RNA. We have performed a comparative study of optimized antisense oligonucleotides designed to work by an RNA interference mechanism to oligonucleotides designed to work by an RNase H-dependent mechanism in human cells. The potency, maximal effectiveness, duration of action, and sequence specificity of optimized RNase H-dependent oligonucleotides and small interfering RNA (siRNA) oligonucleotide duplexes were evaluated and found to be comparable. Effects of base mismatches on activity were determined to be position-dependent for both siRNA oligonucleotides and RNase H-dependent oligonucleotides. In addition, we determined that the activity of both siRNA oligonucleotides and RNase H-dependent oligonucleotides is affected by the secondary structure of the target mRNA. To determine whether positions on target RNA identified as being susceptible for RNase H-mediated degradation would be coincident with siRNA target sites, we evaluated the effectiveness of siRNAs designed to bind the same position on the target mRNA as RNase H-dependent oligonucleotides. Examination of 80 siRNA oligonucleotide duplexes designed to bind to RNA from four distinct human genes revealed that, in general, activity correlated with the activity to RNase H-dependent oligonucleotides designed to the same site, although some exceptions were noted. The one major difference between the two strategies is that RNase H-dependent oligonucleotides were determined to be active when directed against targets in the pre-mRNA, whereas siRNAs were not. These results demonstrate that siRNA oligonucleotide- and RNase H-dependent antisense strategies are both valid strategies for evaluating function of genes in cell-based assays.

RNA interference has become a powerful and widely used tool for the analysis of gene function in invertebrates and plants (1, 2). Introduction of long double-stranded RNA into the cells of these organisms leads to the sequence-specific degradation of homologous gene transcripts. The long double-stranded RNA molecules are metabolized to small 21–23-nu-

cleotide interfering RNAs (siRNAs)¹ by the action of an endogenous ribonuclease, Dicer (3, 4). The siRNA molecules bind to a protein complex, termed RNA-induced silencing complex, which contains a helicase activity that unwinds the two strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule (4, 5) and an endonuclease activity that hydrolyzes the target RNA at the site where the antisense strand is bound. It is unknown whether the antisense RNA molecule is also hydrolyzed or recycles and binds to another RNA molecule. Therefore, RNA interference is an antisense mechanism of action, since ultimately a single-stranded RNA molecule binds to the target RNA molecule by Watson-Crick base pairing rules and recruits a ribonuclease that degrades the target RNA.

In mammalian cells, long double-stranded RNA molecules were found to promote a global change in gene expression, obscuring any gene-specific silencing (6, 7). This reduction in global gene expression is thought to be mediated in part through activation of double-stranded RNA-activated protein kinase which phosphorylates and inactivates the translation factor eukaryotic initiation factor 2 α (8). Recently, it has been shown that transfection of synthetic 21-nucleotide siRNA duplexes into mammalian cells does not elicit the RNA-activated protein kinase response, allowing effective inhibition of endogenous genes in a sequence-specific manner (9, 10). These siRNAs are too short to trigger the nonspecific double-stranded RNA responses, but they still promote degradation of complementary RNA sequences (9, 11).

Multiple mechanisms exist by which short synthetic oligonucleotides can be used to modulate gene expression in mammalian cells (12). A commonly exploited antisense mechanism is RNase H-dependent degradation of the targeted RNA. RNase H is a ubiquitously expressed endonuclease that recognizes a DNA-RNA heteroduplex, hydrolyzing the RNA strand (13, 14). Antisense oligonucleotides that contain at least five consecutive deoxynucleotides are substrates for human RNase H (15, 16). Thus, the RNase H-dependent antisense mechanism differs from the siRNA mechanism by utilizing RNase H, instead of a double-stranded RNase, as the terminating mechanism.

Initial reports in which siRNA was compared with single-stranded antisense approaches to gene knockdown have indicated that the siRNA is more potent and effective than a traditional antisense approach (4, 10). However, the antisense

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¹ The abbreviations used are: siRNA, small interfering RNA; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROC, receiver operating characteristic; TPR, true positive rate; FPR, false positive rate; nt, nucleotide; 2'-MOE, 2'-O-methoxyethyl; ICAM, intercellular adhesion molecule; TNF, tumor necrosis factor.

molecules used in these experiments were single-stranded unmodified RNA, which is rapidly degraded and does not recruit RNase H to cleave the target. Phosphorothioate oligodeoxynucleotides are first generation antisense agents that have been widely used to modulate gene expression in cell-based assays, in animal models, and in the clinic (18). The phosphorothioate modification dramatically increases the nuclease resistance of the oligonucleotide and still supports RNase H activity (19). Further improvements to phosphorothioate oligodeoxynucleotides have been made, resulting in second generation oligonucleotides such as 2'-O-methyl or 2'-O-methoxyethyl modifications (15, 20). The 2'-O-methoxyethyl modification is particularly attractive, since it increases the potency of the oligonucleotide, further increases nuclease resistance, decreases toxicity, and increases oral bioavailability (21–24).

In this report, we compare oligonucleotides that were designed to work by a siRNA mechanism (siRNA oligonucleotides) to optimized first and second generation antisense oligonucleotides that were designed to work by an RNase H-dependent mechanism (RNase H oligonucleotides). Active siRNA oligonucleotides and homologous RNase H-dependent oligonucleotides were evaluated for relative potency, efficacy, duration of action, sequence specificity, and site of action within the cell to determine whether significant advantages could be found for the different antisense strategies in cell-based assays. Our results suggest that in human cell culture assays, double-stranded oligoribonucleotides that work by siRNA mechanism exhibit similar potency, efficacy, specificity, and duration of action as RNase H oligonucleotides. Furthermore, as we have previously found for RNase H oligonucleotides, not all sites on the target RNA are good target sites for siRNA molecules. Like RNase H-dependent oligonucleotides, activity of siRNAs is affected by the secondary structure of the target RNA. Finally, siRNAs and RNase H oligonucleotides appear to work upon the target mRNA at different stages of its processing/metabolism.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis and siRNA Duplex Formation—Synthesis and purification of phosphorothioate-modified oligodeoxynucleotides or chimeric 2'-O-methoxyethyl/deoxyphosphorothioate modified oligonucleotides was performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (22). Sequences of oligonucleotides and placement of 2'-O-methoxyethyl modifications are detailed in Tables I and II. RNA oligonucleotides were synthesized at Dharmacon Research, Inc. (Boulder, CO). siRNA duplexes were formed by combining 30 μ l of each 50 μ M RNA oligonucleotide solution and 15 μ l of 5 $\times$ annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) followed by heating for 1 min at 90 °C and then 1 h at 37 °C. Successful annealing was confirmed by non-denaturing polyacrylamide gel electrophoresis. The melting temperatures (T_m) were experimentally determined for a subset of siRNA tested as described previously (15). In each case, the measured T_m values were greater than 55 °C. The predicted T_m values for all siRNA duplexes used in this paper were >50 °C (100 mM salt, 0.1 μ M oligonucleotide).

Cell Culture—T24 cells, (American Type Tissue Culture Collection, Manassas, VA) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in six-well culture dishes at a density of 250,000 cells/well. Oligonucleotides were administered to cells using Lipofectin reagent (Invitrogen) as described previously (25, 26). Other transfection reagents were evaluated (e.g. Transit TKO, LipofectAMINE 2000, and Oligofectamine) and found to provide similar levels of siRNA-mediated target reduction in T24 cells (data not shown); however, Lipofectin was determined to be superior to the other transfection reagents for RNase H-dependent oligonucleotide administration. In addition, LipofectAMINE was found to be more toxic to the cells than Lipofectin, and Transit TKO failed to provide consistent results for delivery of siRNA molecules. Optimal Lipofectin/oligonucleotide ratios were empirically determined for both siRNAs and RNase H-dependent oligonucleotides. For RNase H antisense oligonucleotides, cells were incubated with a mixture of 3 μ g/ml Lipofectin per 100 nM oligonucleo-

tide in OptiMEM medium (Invitrogen), whereas siRNA duplexes were incubated with a mixture of 6 μ g/ml Lipofectin per 100 nM siRNA duplex. Since concentrations reported in the paper represent concentration of the siRNA duplex, the same weight/Lipofectin ratio was maintained for siRNA duplexes and antisense oligonucleotides. After 4 h, the transfection mixture was aspirated from the cells and replaced with fresh Dulbecco's modified Eagle's medium plus 10% fetal calf serum and incubated at 37 °C, 5% CO₂ until harvest.

To induce CD54 mRNA expression, oligonucleotide-treated cells were incubated overnight and then treated with 5 ng/ml TNF- α (R&D Systems, Minneapolis, MN) for 2–3 h prior to harvest of cells for RNA expression analysis. For analysis of cell surface expression of CD54 protein, cells were induced with 5 ng/ml TNF- α immediately following the transfection and incubated overnight.

RNA Expression Analysis—Total RNA was harvested at the indicated times following the beginning of transfection using an RNeasy Mini preparation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Gene expression was analyzed using quantitative RT-PCR essentially as described elsewhere (27). Briefly, 200 ng of total RNA was analyzed in a final volume of 50 μ l containing 200 nM gene-specific PCR primers, 0.2 mM each dNTP, 75 nM fluorescently labeled oligonucleotide probe, 1 $\times$ RT-PCR buffer, 5 mM MgCl₂, 2 units of Platinum *Taq* DNA polymerase (Invitrogen), and 8 units of ribonuclease inhibitor. Reverse transcription was performed for 30 min at 48 °C followed by PCR: 40 thermal cycles of 30 s at 94 °C and 1 min at 60 °C using an ABI Prism 7700 Sequence Detector (Applied Biosystems; Foster City, CA). All mRNA expression was normalized to levels of GAPDH mRNA, also determined by quantitative RT-PCR, from the same total RNA samples. The following primer/probe sets were used: *c-ras* kinase (accession number X03484), forward primer (AGCTTGGAAGACGATCAGCAA), reverse primer (AACTGCTGAACATTGTAGGAGAGATG), and probe (AGATGCCGTGTTTGATGGCTCCAGCX); CD54 (accession number J03132), forward primer (CATAGAGACCCCGTTGCCTAAA), reverse primer (TGGCTATCTTCTTGACATTGC), and probe (CTCCTGCTGGGAACAACCGAAX); PTEN (accession number U92436), forward primer (AATGGCTAAGTGAAGATGACAAATCAT), reverse primer (TGACATATCATTACACCAGTTCGT), and probe (TTGCAGCAATTCATGTAAAGCTGGAAGGX); Bcl-x (accession number Z23115), forward primer (TGCAGGTATTGGTTCAGTCGG), reverse primer (TCCAAGGCTCTAGTGGTCAAT), and probe (TCGAGCTTGGATGGCCACTTACCTX); GAPDH (accession number X01677), forward primer (GAAGGTGAAGTCCGGAGTC), reverse primer (GAAGATGGTGTGATGGGATTTC), and probe (CAAGCTTCCCGTTCTCAGCCX); COREST (accession number NM_015156), forward primer (ACAATCCCATTTGACATTGAGGTT), reverse primer (TTTGCTCTATTTTAGCTTGTGTGCT), and probe (AAGAGGTTCCCCCTACTGAGACAGTTCCTX); Notch homolog 2 (accession number NM_024408), forward primer (TGGCAACTAACGTAGAACTCAACA), reverse primer (TGCCAAGAGCATGAATACAGAGA), and probe (ACAATATAGACTTGCTCATTGTTTCAGACTGATTGCCX); PAK1 (accession number U51120), forward primer (TGTGATTGAACCACTTCCGTCA), reverse primer (GGAGTGGTGTATTTCAGTAGGTGAA), and probe (TCCAACCTCGGACGTGGCTACAX); CARD-4 (accession number NM_006092), forward primer (GCAGGCGGGACTATCAGGA), reverse primer (AGTTTGCCGACCAGACCTTCT), and probe (TCCAC-TGCCCTCCAT-GATGCAAGCCX).

Flow Cytometry—Following oligonucleotide treatment, cells were detached from the plates with Dulbecco's phosphate-buffered saline (without calcium and magnesium) supplemented with 4 mM EDTA. Cells were transferred to microcentrifuge tubes, pelleted at 5000 rpm for 1 min, and washed in 2% bovine serum albumin, 0.2% sodium azide in Dulbecco's phosphate-buffered saline at 4 °C. PE anti-human CD54 antibody (catalog no. 555511; Pharmingen, San Diego, CA) was then added at 1:20 in 0.1 ml of the above buffer. The antibody was incubated with the cells for 30 min at 4 °C in the dark. Cells were washed again as above and resuspended in 0.3 ml of PBS buffer with 0.5% paraformaldehyde. Cells were analyzed on a Becton Dickinson FACScan. Results are expressed as percentage of control expression based upon the mean fluorescence intensity.

Luciferase Assays—For luciferase-based reporter gene assays, 10 μ g of plasmid pGL3-5132-S0 or pGL3-5132-S20 (26) was introduced into COS-7 cells at 70% confluence in 10-cm dishes using SuperFect Reagent (Qiagen). Following a 2-h treatment, cells were trypsinized and split into 24-well plates. Cells were allowed to adhere for 1 h, and then RNase H or siRNA oligonucleotides were added in the presence of Lipofectin reagent as detailed above. All oligonucleotide treatments were performed in duplicate or triplicate. Following the 4-h oligonucleo-

tide treatment, cells were washed, and fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum was added. The cells were incubated overnight at 37 °C. The following morning, cells were harvested in 150 μ l of passive lysis buffer (Promega, Madison, WI), and 60 μ l of lysate was added to each well of a black 96-well plate followed by 50 μ l of luciferase assay reagent (Promega). Luminescence was measured using a Packard TopCount microplate scintillation counter.

Statistical Analyses of Gene Screen Data—Simple statistical analyses were conducted to examine the association between siRNA and RNase H oligonucleotide screens. Similarity between the two screens for a given gene was measured by using correlation coefficients and average difference. Two different correlation measures were employed: Pearson's product-moment correlation coefficient, which measures a linear relationship between siRNA and RNase H oligonucleotide screens, and Spearman's rank-order correlation coefficient, which measures a linear relationship between the potency of siRNA and RNase H oligonucleotide screens. One-sample one-tailed *t* tests were conducted for observed correlation coefficients to assess whether they are significantly greater than the null hypothesis of no correlation. Statistical inference on observed average difference was conducted by randomizing sample pairs of siRNA and RNase H oligonucleotide screen. Again, one-tailed tests were used to determine whether the observed distances are significantly smaller than those expected from random chance. The association between siRNA and RNase H oligonucleotide screen was further examined by the receiver operating characteristic (ROC) analysis. First, siRNAs were classified as potent when the percentage inhibition rate was smaller than the median value of 67.4% for the CD54 siRNA screen and 57.1% for the PTEN screen. An arbitrary cut-off was then set for RNase H oligonucleotide screens. RNase H oligonucleotides with percentage inhibition rates smaller than this cut-off value were classified as potent. From the classification of siRNAs and RNase H oligonucleotides, a 2 $\times$ 2 contingency table was constructed. Finally, true positive rate (TPR) and false positive rate (FPR) were determined based on this table. For example, TPR is the number of cases where potent RNase H oligonucleotides correspond to potent siRNAs divided by the number of potent siRNAs. Similarly, FPR is the number of cases where potent RNase H oligonucleotides corresponds to nonpotent siRNAs divided by the number of nonpotent siRNAs. For CD54, a cut-off value of 70% gives TPR = 75% and FPR = 45%. For the PTEN gene, a cut-off of 40% gives TPR = 72% and FPR = 44%. By varying these cut-off values, a ROC curve can be drawn on a plane spanned by FPR and TPR. The area under the ROC curve provides a measure of overall accuracy.

RESULTS

Active RNase H-dependent Antisense Oligonucleotide Target Sites Predict siRNA Target Sites—Since both siRNAs and RNase H-dependent oligonucleotides must hybridize to target RNA and subsequently direct specific RNases to bind and cleave the bound RNA (15, 28), we examined whether an active RNase H oligonucleotide site would also be an active siRNA site. Initially, siRNAs were designed and synthesized based upon the target sequences of active RNase H oligonucleotides previously identified. ISIS 5132 is a 20-base phosphorothioate oligodeoxynucleotide that targets the 3'-untranslated region of human *c-ras* mRNA and specifically reduces expression of both mRNA and protein (29). An siRNA duplex (si5132) composed of 21-nt sense and 21-nt antisense strands was designed using the first 19 nucleotides of the target site for ISIS 5132 in the paired region and unpaired 2-nt 3'-dTdT overhangs. T24 cells were treated with oligonucleotides at doses ranging from 3 to 300 nM as detailed under "Experimental Procedures." Total RNA was analyzed for expression of *c-ras* mRNA by quantitative RT-PCR. The results, shown in Fig. 1A, are normalized to GAPDH mRNA expression. Both ISIS 5132 (solid bars) and the corresponding siRNA to the same target site (open bars) were found to inhibit the expression of the *c-ras* kinase mRNA, each with an IC_{50} of ~50 nM. siRNAs targeted to human CD54 and Bcl-X had no effect on the expression of *c-ras* (data not shown).

Chimeric oligonucleotides in which 2'-O-methoxyethyl (2'-MOE) substituted nucleosides flank a central unmodified 2'-oligodeoxynucleotide region that serves as substrate for RNase

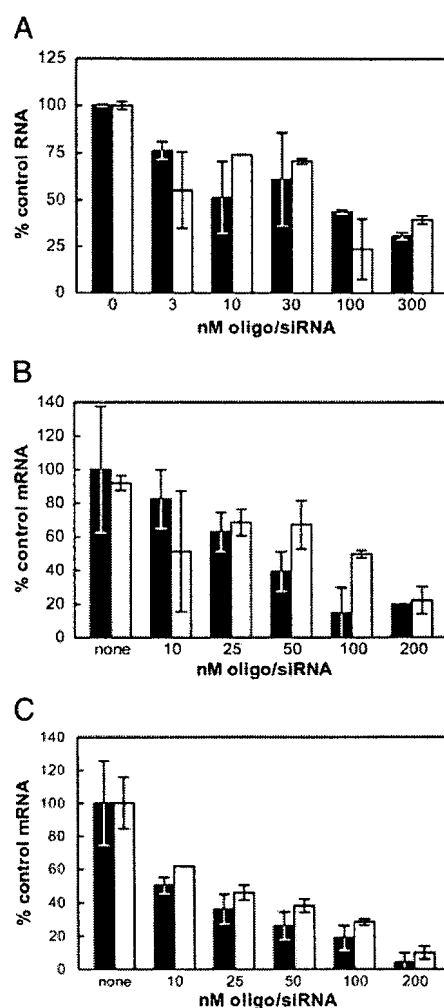


FIG. 1. Comparative activity of RNase H-dependent oligonucleotides and siRNA oligonucleotides. T24 cells were dosed at 3–300 nM with RNase H or siRNA oligonucleotides as detailed under "Experimental Procedures." Total RNA was harvested the following day, and mRNA expression was assessed by quantitative RT-PCR. Results shown represent percentage of untreated control expression. All expression data are normalized to GAPDH mRNA expression. A, *c-ras* kinase; B, *Bcl-X*; C, *PTEN*. Solid bars, RNase H-dependent oligonucleotides; open bars, siRNA.

H region have been shown to have increased potency and duration of action as compared with phosphorothioate oligodeoxynucleotides (22). ISIS 16009 is a 20-base chimeric oligonucleotide that has previously been demonstrated to be an effective inhibitor of human Bcl-X (31). Another 20-base chimeric oligonucleotide, ISIS 116847, has been shown to effectively inhibit expression of the human PTEN gene (32). The siRNA versions, si16009 and si116847, as well as the homologous parent RNase H-dependent oligonucleotides were transfected into T24 cells at doses ranging from 10 to 200 nM. In both cases the 2'-MOE chimeric RNase H-dependent oligonucleotides (solid bars) were slightly more potent inhibitors of mRNA expression than the corresponding siRNA (open bars) (Fig. 1, B and C). In the case of Bcl-X, the RNase H-dependent oligonucleotide has an IC_{50} of ~30 nM, whereas the siRNA version, si16009, has an IC_{50} of ~100 nM. PTEN is more potently inhibited, with IC_{50} values of 10 and 25 nM for the RNase H oligonucleotide and siRNA, respectively.

RNase H-dependent oligonucleotides and siRNAs were also compared for activity in T24 cells against CD54 (ICAM-1), a gene whose expression is induced by cytokine treatment. ISIS

TABLE I
Sequence of CD54 RNase H-dependent oligonucleotides and siRNAs

All oligonucleotides are full phosphorothioate with 2'-O-methoxyethyl substitutions at positions 1–5 and 16–20 (boldface type). Residues 6–15 are unmodified oligodeoxynucleotides, so they can serve as substrates for RNase H. The corresponding siRNAs use the same start position but are 19 rather than 20 nucleotides in length and have dTdT additions at the 3'-end of each strand. The GenBank™ accession number for CD54 is J03132.

ISIS no.	Start position	Sequence	Region
121725	8	AGAGGAGCTCAGCGTCGACT	5'-UTR
121726	33	GGCTGAGGTTGCAACTCTGA	5'-UTR
121727	256	CCAGGCAGGAGCAACTCCTT	Coding
121728	321	TTGAATAGCACATTGGTTGG	Coding
121729	422	GCCCACTGGCTGCCAAGAGG	Coding
121730	571	TCTCTCTCACCAGCACCGT	Coding
121731	674	AAAGGTCTGGAGCTGGTAGG	Coding
121732	732	GCGTGTCCACCTCTAGGACC	Coding
121733	801	CCAGTGCCAGGTGGACCTGG	Coding
121734	921	CCAGTATTACTGCACACGTC	Coding
121735	1002	CCTCTGGCTTCGTCAGAATC	Coding
121736	1121	GGTGGCCTTCAGCAGGAGCT	Coding
121737	1221	CATACAGGACACGAAGCTCC	Coding
121738	1341	CATCCTTTAGACACTTGAGC	Coding
121739	1421	GCTCCTGGCCCGACAGAGGT	Coding
121740	1501	GCTACCACAGTGATGATGAC	Coding
121741	1622	TTGTGTGTTCCGTTTCATGG	Coding
121742	1633	GGAGGCGTGGCTTGTGTGTT	Coding
121743	1654	CCTGTCCCGGGATAGGTTCA	Coding
121744	1666	CGAGGAAGAGGCCCTGTCCC	3'-UTR
121745	1711	TCCACTCTGTTCAGTGTGGC	3'-UTR
121746	1781	TCTGACTGAGGACAATGCC	3'-UTR
121747	1818	TAGGTGTGCAGGTACCATGG	3'-UTR
121748	1924	CCTCTCATCAGGCTAGACTT	3'-UTR
121749	1971	CCAGTTGTATGTCTCATGG	3'-UTR
121750	2012	GGGCCTCAGCATACCCAATA	3'-UTR
121751	2056	ATGCTACACATGTCTATGGA	3'-UTR
121752	2100	GCCCAAGCTGGCATCCGTCA	3'-UTR
121753	2103	AGTGCCCAAGCTGGCATCCG	3'-UTR
121754	2221	GCTCCGTGAGGCCAGAGACC	3'-UTR
121755	2291	CAGGCACTCTCCTGCAGTGT	3'-UTR
121756	2341	GAAAGGCAGGTTGGCCAATG	3'-UTR
121757	2417	GGTAATCTCTGAACCTGTGA	3'-UTR
121758	2531	GTCCAGACATGACCGCTGAG	3'-UTR
121759	2619	CTGGAGCTGCAATAGTGCAA	3'-UTR
121760	2731	TACACATACACACACACA	3'-UTR
121761	2831	GCTGAGGTGGGAGGATCACT	3'-UTR
121762	2871	GGTGTGGTGTGTGAGCCTA	3'-UTR
121763	2944	CTAACACAAAGGAAGTCTGG	3'-UTR
121764	3104	CAGTGCCCAAGCTGGCATCC	3'-UTR

2302, a first generation phosphorothioate oligodeoxynucleotide, hybridizes to the 3'-untranslated region of human CD54 (ICAM-1) and was previously shown to be a potent and specific inhibitor of CD54 expression (33). Whereas ISIS 2302 reduced ICAM-1 expression by 85%, si2302 had no inhibitory effect on message levels at concentrations as high as 200 nM as measured by quantitative RT-PCR (data not shown).

Screening for Optimized RNase H and siRNA Oligonucleotides—In order to identify potent antisense agents, many investigators design and test multiple oligonucleotides that target different sites and regions of the target mRNA (33, 34). To determine if the lack of activity of the CD54 siRNA molecule was due to suboptimal siRNA design or to a blocking activity induced by TNF- α treatment, we designed 40 siRNA and 40 2'-MOE chimeric oligonucleotides to the same sites of the CD54 mRNA (Table I). The siRNA duplexes were composed of 21-nt sense and 21-nt antisense strands, paired in a manner to have a 19-nt duplex region and a 2-nt overhang at each 3' terminus (Table I). The target sites included various regions of the human CD54 message including 5'-untranslated region (5'-UTR), coding region, and 3'-UTR. T24 cells were treated with oligonucleotides at a single concentration of 100 nM as described under "Experimental Procedures." Active sequences were identified in both the RNase H oligonucleotide and siRNA screens (Fig. 2). In the RNase H oligonucleotide screen (*solid bars*), 12 of 40 oligonucleotides were found to inhibit expression of CD54

mRNA by greater than 50% as compared with the untreated control, whereas the siRNA screen (*open bars*) identified 9 of 40 sequences as active by the same criteria. Comparison of the active target sites revealed that five of the nine active siRNA sites were also identified as active sites in the RNase H oligonucleotide screen. Similarly, the majority of sites where the RNase H-dependent oligonucleotide failed to inhibit expression, the siRNA also failed. The data also indicate that regions of greater activity or "hot spots" along the RNA transcript can be identified for both siRNA oligonucleotides and RNase H-dependent oligonucleotides. For example, homologous siRNAs and RNase H-dependent oligonucleotides both show good activity in the ~200 nucleotide span from base 1781 to 1971 of the 3'-untranslated region. These results demonstrate that the initial lack of activity for the CD54 directed siRNA molecules is not due to induction of an inhibitory factor by TNF- α treatment and that not all siRNA molecules designed to hybridize to an RNA transcript are effective.

Cell surface CD54 protein expression was also evaluated by flow cytometry. Comparison of mRNA reduction and protein reduction for the siRNA and RNase H-dependent oligonucleotides screens are shown in Fig. 3A. In general, the results are highly correlated with the same active targets identified by either mRNA or protein reduction. However, several oligonucleotides were identified that appear to produce a more robust reduction of protein compared with the corresponding RNA

FIG. 2. **CD54 antisense screen.** A series of 40 chimeric oligonucleotides designed to work by an RNase H-dependent mechanism and a series of corresponding siRNAs were administered to T24 cells in the presence of Lipofectin transfection reagent. The following day, CD54 expression was induced, and RNA was harvested. CD54 mRNA expression was analyzed by quantitative RT-PCR. Results represent the percentage of induced CD54 mRNA relative to untreated control. *Solid bars*, RNase H oligonucleotides; *open bars*, siRNAs. The target site start position is the 5'-most nucleotide in the mRNA target.

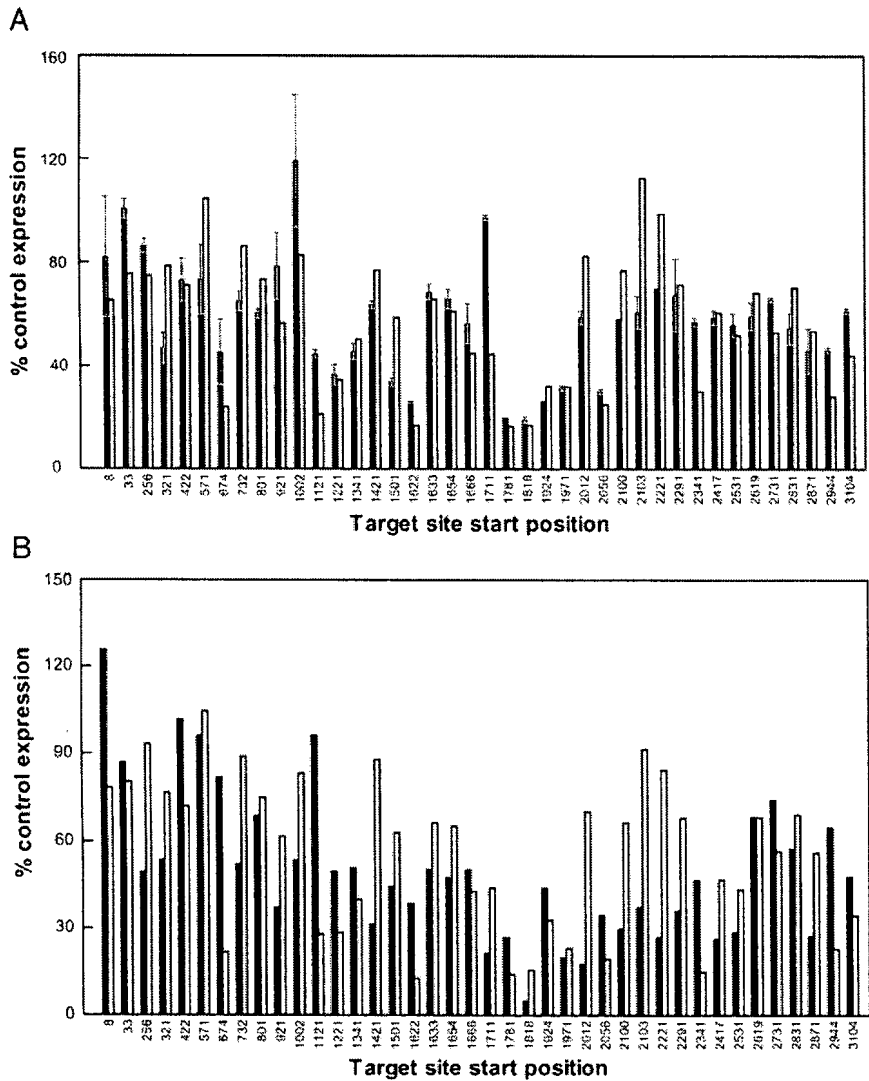
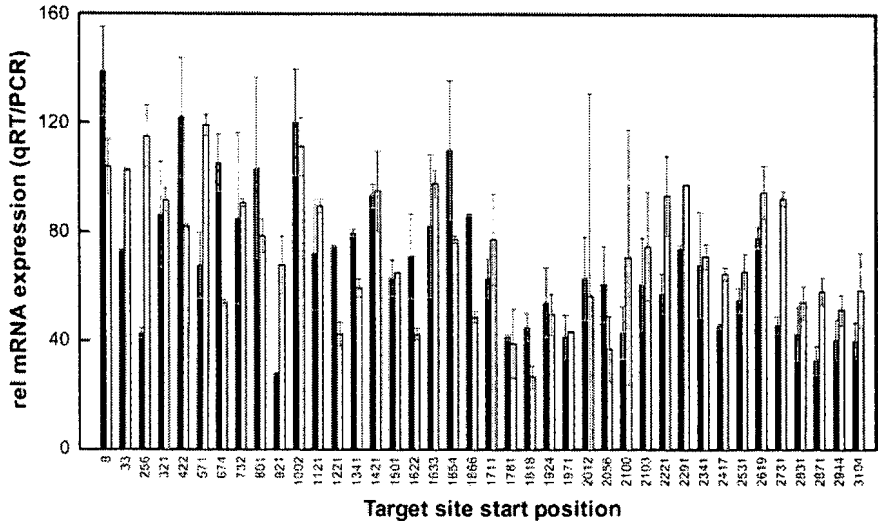


FIG. 3. **Comparison of mRNA and protein reduction in CD54 siRNA oligonucleotide screen.** A, cell surface expression of CD54 was analyzed by flow cytometry following siRNA administration and overnight induction of CD54 as detailed under “Experimental Procedures.” *Solid bars*, mRNA reduction; *striped bars*, protein reduction. B, comparison of RNase H-dependent oligonucleotide and siRNA reduction of CD54 cell surface protein expression. Results are presented as percentage of untreated control expression. *Solid bars*, RNase H-dependent oligonucleotides; *open bars*, siRNAs.

(Fig. 3, A and B). Note, however, that CD54 RNA and protein were measured at different times following TNF- α induction, which may account for the discrepancies. Statistical analyses described under “Experimental Procedures” were applied to siRNA and RNase H oligonucleotide screening data for CD54 mRNA reduction. The data were composed of two independent RNase H oligonucleotide screens and five independent siRNA screens that were averaged to produce composite siRNA/RNase H-dependent oligonucleotide screens. Pearson’s correlation coefficient was determined to be 0.424

TABLE II
Sequence of human PTEN RNase H-dependent oligonucleotides and siRNAs

All oligonucleotides are full phosphorothioate with 2'-O-methoxyethyl substitutions at positions 1–4 and 15–18 (boldface type). Residues 5–14 are unmodified 2'-oligodeoxynucleotides, so they can serve as substrates for RNase H. The corresponding siRNAs use the same start position but are 19 rather than 18 nucleotides in length and have dTdT additions at the 3'-end of each strand. The GenBank™ accession number for PTEN is U92436

ISIS no.	Start position	Sequence	Region
29574	19	CGAGAGGCGGACGGGACC	5'-UTR
29575	57	CGGGCGCCTCGGAAGACC	5'-UTR
29576	197	TGGCTGCAGCTTCCGAGA	5'-UTR
29577	314	CCCGCGGCTGCTCACAGG	5'-UTR
29578	421	CAGGAGAAGCCGAGGAAG	5'-UTR
29579	494	GGGAGGTGCCGCCGCCGC	5'-UTR
29581	671	CCGGGTCCCTGGATGTGC	5'-UTR
29582	757	CCTCCGAACGGCTGCCTC	5'-UTR
29583	817	TCTCCTCAGCAGCCAGAG	5'-UTR
29584	891	CGCTTGGCTCTGGACCGC	5'-UTR
29585	952	TCTTCTGCAGGATGGAAG	5'-UTR
29587	1106	GGATAAATATAGGTCAAG	Coding
29588	1169	TCAATATTGTTCTGTAT	Coding
29589	1262	TAAATTTGGCGGTGTCA	Coding
29590	1342	CAAGATCTTCACAAAAGG	Coding
29591	1418	ATTACACCAGTTCGTCCC	Coding
29592	1504	TGTCTCTGGTCCCTACTT	Coding
29593	1541	ACATAGCGCCTCTGACTG	Coding
29595	1694	GAATATATCTTCACCTTT	Coding
29596	1792	GGAAGAACTCTACTTTGA	Coding
29597	1855	TGAAGAATGTATTACCC	Coding
29599	2020	GGTTGGCTTTGTCTTTAT	Coding
29600	2098	TGCTAGCCTCTGGATTTG	Coding
29601	2180	TCTGGATCAGAGTCAGTG	Coding
29602	2268	TATTTTCATGGTGTTTTA	3'-UTR
29603	2347	TGTTCTCTATAACTGGTAA	3'-UTR
29604	2403	GTGTCAAAACCCTGTGGA	3'-UTR
29605	2523	ACTGGAATAAAACGGGAA	3'-UTR
29606	2598	ACTTCAGTTGGTGACAGA	3'-UTR
29607	2703	TAGCAAAACCTTTCGGAA	3'-UTR
29608	2765	AATTATTTCCCTTCTGAG	3'-UTR
29609	2806	TAAATAGCTGGAGATGGT	3'-UTR
29610	2844	CAGATTAATAACTGTAGC	3'-UTR
29611	2950	CCCAATACAGATTCACT	3'-UTR
29612	3037	ATTGTTGCTGTGTTCTT	3'-UTR
29613	3088	TGTTTCAAGCCCATTCTT	3'-UTR

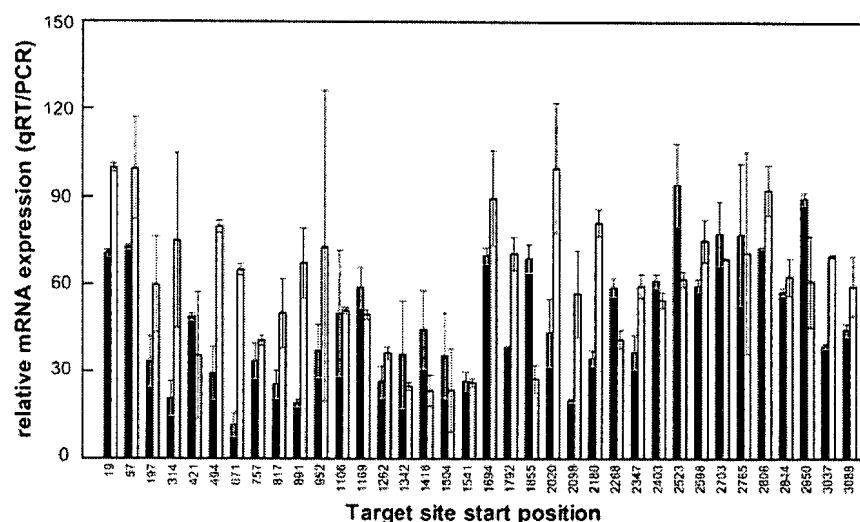
with a *p* value of 0.0032, and Spearman's correlation coefficient was 0.426 with a *p* value of 0.0039. The average difference between the two screens was 18.5% with a *p* value of 0.0056. These results indicate that a significant overlap exists between siRNA and RNase H oligonucleotide screens in terms of correlation coefficients and average difference. The association between siRNA and RNase H oligonucleotide activity was further analyzed using ROC analysis. The area under the ROC curve is a summary of the overall diagnostic accuracy of the test that measures the correspondence between potent siRNA and RNase H-dependent oligonucleotide sites. The area under the ROC curve is 0.75 for CD54, suggesting that a significant concordance exists between siRNA and RNase H-dependent oligonucleotide binding sites on target RNAs.

A second comparative analysis was performed using 36 2'-MOE chimeric oligonucleotides, 18 nucleotides in length, and a series of corresponding siRNAs (Table II) targeted to the human PTEN message. PTEN mRNA is constitutively expressed in T24 cells. Cells were treated with siRNAs or RNase H-dependent oligonucleotides as described under "Experimental Procedures." As defined by a target mRNA reduction of 50% or greater, 22 of the 36 RNase H-dependent oligonucleotides (*solid bars*) were identified as active (Fig. 4). In contrast, the siRNA screen (*open bars*) identified only 12 of 36 sites as active, defined by the same criteria. Of the 12 active siRNA oligonucleotide sites, 10 were shared as active with the RNase H-dependent oligonucleotide screen, with only 2 of the active siRNAs not identified in the RNase H-dependent oligonucleotide screen.

The RNase H/siRNA oligonucleotide screens for PTEN were repeated three separate times. A statistical analysis of the composite data from the three experiments was performed as detailed above. Pearson's correlation coefficient was determined to be 0.425 with a *p* value of 0.0049, and Spearman's correlation coefficient was 0.318 with a *p* value of 0.0299. The average difference between the two screens was 21.3% with *p* value of 0.0038. These results suggest that a significant association exists between siRNA- and RNase H-dependent oligonucleotide screens in terms of Pearson's correlation coefficient and average difference. ROC analysis of these data give a value of 0.588 for PTEN. Whereas the data for the PTEN screens are not as highly significant as those for CD54, they do demonstrate a reasonable, although not perfect, correlation between siRNA and RNase H-dependent oligonucleotide binding sites.

Effect of RNA Secondary Structure on Activity—We have previously demonstrated that the secondary structure of the mRNA target strongly influences activity of RNase H-dependent oligonucleotides in cell culture (26). A luciferase reporter system was developed in which the target site for ISIS 5132 was cloned into the 5'-UTR of the luciferase reporter plasmid pGL3-Control. Sequence immediately adjacent to the target sequence was altered to form various RNA secondary structures that included the 5132 target sequence. These structures ranged from one in which the entire target site was sequestered in a 20-base stem closed by a UUGC tetraloop (pGL3-5132-S20) to one that had little predicted secondary structure likely to inhibit hybridization of RNase H oligonucleotide to target (pGL3-5132-S0) (26).

FIG. 4. PTEN oligonucleotide screen. A series of 36 chimeric RNase H-dependent oligonucleotides and a series of corresponding siRNAs were administered to T24 cells in the presence of Lipofectin reagent. After 16 h, total RNA was harvested, and PTEN mRNA levels were accessed by quantitative RT-PCR as detailed under "Experimental Procedures." Results are the percentage of PTEN mRNA relative to untreated control. *Solid bars*, chimeric RNase H oligonucleotides; *open bars*, siRNA oligonucleotides.



The activities of ISIS 5132 and si5132 were compared using the pGL3-5132-S20 and pGL3-5132-S0 constructs. The reporter plasmids were transfected into COS-7 cells as detailed under "Experimental Procedures." Following the plasmid transfection, cells were seeded in 24-well plates and treated with ISIS 5132 or si5132 at doses ranging from 10 to 300 nM. Lysates from the treated cells were assayed for luciferase activity 16 h later. When directed against the message with no structure (pGL3-5132-S0), both ISIS 5132 (*open circles*) and si5132 (*open triangles*) effectively reduced luciferase expression in a dose-dependent manner with IC_{50} values between 30 and 100 nM (Fig. 5), which is consistent with the observed IC_{50} for endogenous message reduction. Conversely, neither the RNase H oligonucleotide (*solid circles*) nor siRNA (*solid triangles*) were found to inhibit luciferase expression when directed against the highly structured target (pGL3-5132-S20) at concentrations up to 300 nM. Therefore, the secondary structure of the target has an equally important effect on reduction of target RNA by both types of antisense oligonucleotides.

Sequence Specificity of RNase H-dependent Oligonucleotides and siRNA—The sequence fidelity of the RNA interference pathway has been evaluated to a limited extent in several hallmark systems, including *C. elegans* (35) and *Drosophila* cell extracts (28), and more recently in mammalian cell culture (9, 36). Several investigators have reported that incorporation of one or two mismatches into a siRNA construct, with respect to the target RNA, is sufficient to disable RNA interference activity against the target RNA. A common attribute of each of the mismatch constructs tested thus far, however, has been location of the mismatches in the center domain of the construct. To further define the fidelity of the RNA interference pathway for perfect Watson-Crick base pair matched sequences, we tested an additional type of construct, wherein a mismatch was incorporated in each of the 5'- and 3'-terminal domains of the siRNA targeting PTEN (si116847). The same mismatches were also incorporated into ISIS 116847, an RNase H oligonucleotide. When the mismatches were placed in the center of the sequence, a complete loss of activity was observed for both siRNAs and RNase H-dependent oligonucleotides at a concentration of 100 nM (Fig. 6). In contrast to the duplex with two mismatches positioned in the center of the siRNA (*gridded bars*), the siRNA with mismatches in the outside domains (*open bars*) demonstrated only a moderate loss of activity in comparison with the perfect match construct (*cross-hatched bars*). The results for the RNase H-dependent oligonucleotide were similar, although the RNase

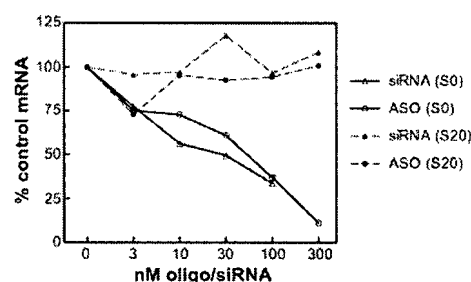


Fig. 5. Inhibition of alternate structure clones by ISIS 5132/si5132. Cells were transfected with luciferase reporter plasmids and then treated with chimeric RNase H-dependent oligonucleotide/siRNA at doses ranging from 3 to 300 nM. Luciferase expression was measured the following day. Results are the percentage of luciferase expression compared with the untreated control. *Open circles/triangles*, pGL3-5132-S0 target; *solid circles/triangles*, pGL3-5132-S20 target. *Circles*, ISIS 5132. *Triangles*, si5132.

H-dependent oligonucleotide containing mismatches on the ends demonstrated a greater loss of activity than was observed for the homologous siRNA (71% versus 52% control).

Comparison of Potency and Efficacy—Comparison of the relative potency of siRNAs directed to the same site on the target RNA as an optimized RNase H oligonucleotide revealed that the RNase H oligonucleotide exhibited similar or better potency as defined by IC_{50} values compared with the siRNA (Fig. 1). The siRNA and RNase H-dependent oligonucleotides also exhibited a similar level of efficacy as defined by the maximal level of target RNA reduction. Since the siRNA molecules used for these analysis were not selected as the optimal siRNA molecules for the respective target based upon screening numerous siRNA sequences, we compared the most effective siRNA molecule derived from the siRNA screen (Fig. 4) with an optimized second generation chimeric oligonucleotide to PTEN. The different antisense agents, tested at concentrations ranging from 10 to 200 nM in T24 cells, produced a similar dose-response curve with IC_{50} values near 10 nM (Fig. 7A). Additionally, both agents reduced PTEN mRNA levels by greater than 90%.

Similarly, the most effective siRNA from the CD54 screen was compared with its corresponding second generation RNase H oligonucleotide, which showed a similar degree of efficacy in the primary screen. T24 cells were treated with either the siRNA, si121747, or the oligonucleotide, ISIS 121747, at concentrations ranging from 10 to 200 nM. As with PTEN, the

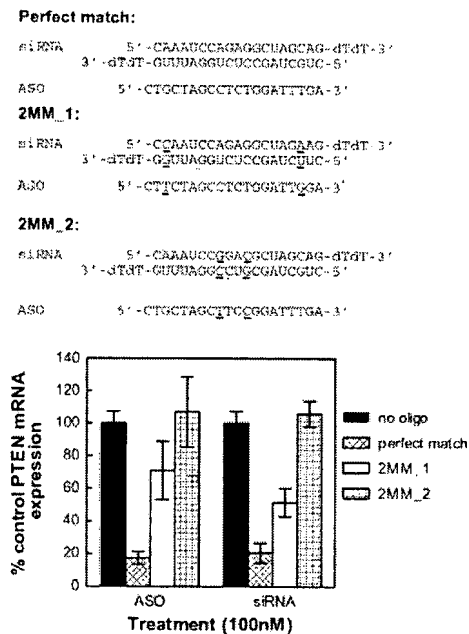


FIG. 6. **Sequence specificity of RNase H and siRNA oligonucleotides.** The effect of base mismatches on siRNA/RNase H-dependent oligonucleotides activity was evaluated. Two mismatches were incorporated in the center (MM2_2) or on the ends (MM2_1) of the ISIS 116847 or si116847 sequence as shown. Sequence changes in the MM2_1 and MM2_2 oligonucleotides are *underlined*. The day after RNase H-dependent oligonucleotide/siRNA treatment, total RNA was harvested, and PTEN mRNA reduction was assessed by quantitative RT-PCR. The results shown are percentage of untreated control expression. *Black bars*, mock-treated; *cross-hatched bars*, perfect match; *open bars*, 2MM_1; *gridded bars*, 2MM_2.

CD54 siRNA and chimeric oligonucleotide produced similar dose-response curves with IC₅₀ values of ~15 nM for the siRNA and 30 nM for the oligonucleotide for reduction of TNF- α -induced CD54 mRNA expression. The efficacy was almost identical with maximal reduction of ~85% for both antisense agents.

Duration of Action—We compared the duration of action of a second generation RNase H oligonucleotide and siRNA in T24 cells using human Bcl-X as a target (Fig. 8). Cells were seeded in six-well dishes so that they would be 80–90% confluent at the time of harvest. In T24 cells, inhibition of Bcl-X by siRNA (*open bars*) was found to be maximal at 24 h post-transfection and returned to normal levels by day 5. The results were similar for RNase H oligonucleotide treatment (*solid bars*) except that maximal activity was achieved at 8 h. In both cases, activity began to taper off between 48 and 96 h, and by 120 h, no significant inhibition of targeted message was seen with either the RNase H oligonucleotide or the siRNA.

Effects of Targeting Intron Sequences—To compare the site of activity of siRNA oligonucleotides and RNase H-dependent oligonucleotides and directly, siRNA duplexes were designed based upon several previously identified active RNase H oligonucleotide sites that target intron sequences (shown in Table III), with the assumption that RNA transcripts containing introns would only be found in the nucleus. The target sites for COREST and PAK1 are contained completely within the introns, whereas the target sites for caspase recruitment domain 4 and Notch homolog 2 overlap the indicated intron/exon boundary with 10 nucleotides on either side (Table III). T24 cells were treated with the RNase H oligonucleotide or the corresponding siRNA at a single dose of 200 nM as described above. The results are shown in Fig. 9. In all cases, the RNase H oligonucleotides effectively reduced the targeted message

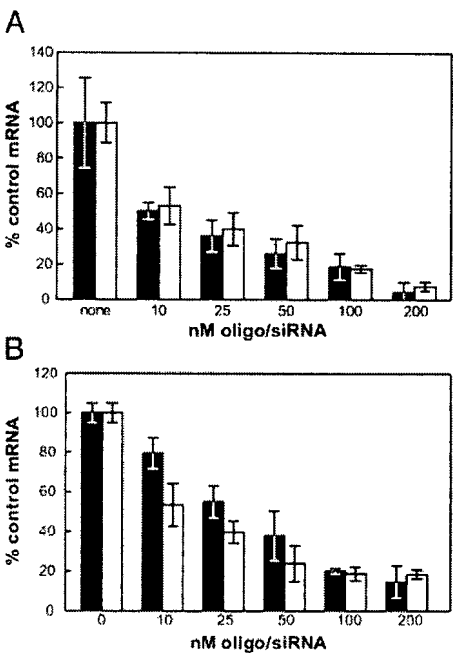


FIG. 7. **Comparative potency.** T24 cells were dosed at 10–200 nM with optimized RNase H oligonucleotides or siRNAs. Total RNA was harvested the following day, and mRNA expression was assessed by quantitative RT-PCR. Results shown are percentage of untreated control expression. All expression data are normalized to GAPDH mRNA expression. *A*, PTEN; *B*, CD54 (ICAM-1). *Solid bars*, RNase H-dependent oligonucleotides; *open bars*, siRNA.

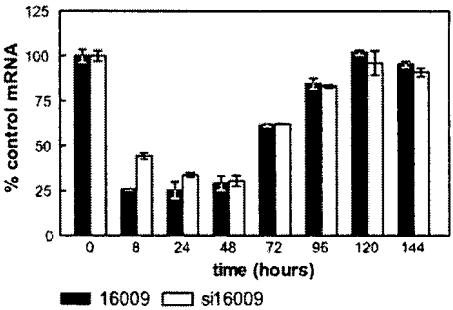


FIG. 8. **Duration of action.** Cells were seeded in six-well dishes so that they would be 80–90% confluent at the time of harvest. RNase H-dependent oligonucleotide/siRNA treatment was at 100 nM with ISIS 16009 or si16009 as detailed above. Total RNA was harvested 8, 24, 48, 72, 96, 120, and 144 h after the initiation of transfection. Bcl-x mRNA levels were accessed by quantitative RT-PCR and normalized to GAPDH expression in the same cells. *Solid bars*, RNase H-dependent oligonucleotide; *open bars*, siRNA.

(*striped bars*), whereas an RNase H oligonucleotide targeted to another gene, tumor necrosis factor receptor 2, had no effect on gene expression (*gray bars*). In contrast, the homologous siRNAs did not reduce mRNA levels for any of the four genes in which introns were targeted (*open bars*); nor was any nonspecific reduction observed using siRNAs targeted to tumor necrosis factor receptor 2 (*cross-hatched bars*). As a control, another gene, *c-ras*, was included, in which the target was in the exon. As previously demonstrated (Fig. 1A), the siRNA targeted to the *c-ras* exon did reduce message expression. These data support the hypothesis that siRNA activity is primarily cytoplasmic and therefore does not interact with pre-mRNA.

DISCUSSION

Multiple mechanisms exist by which synthetic oligonucleotides can be used to regulate gene expression in mammalian cells (12). To date, the most successful strategy has been to

TABLE III
Sequences of RNase H oligonucleotides targeting intron sequences

Gene name	ISIS no.	Sequence	Location
CoRest	165031	AATCCCAGCTACTCGGGAGG	Intron 2
Notch (<i>Drosophila</i>) homolog 2	226968	AAGCCCTTACTTGCATGTCT	Exon 25:intron 25
PAK1	232214	GCCTGAAGCACTGAACAGTA	Intron 5
Caspase recruitment domain 4	199213	CGAGCTATTACCACAGTATT	Exon 11:intron 11

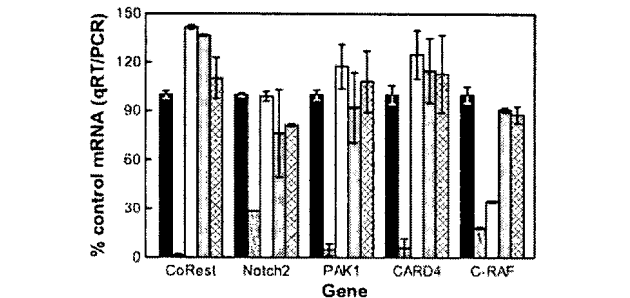


FIG. 9. Effect of intron targeting. siRNA duplexes were designed based upon several previously identified active RNase H-dependent oligonucleotide sites that target intron sequence (shown in Table III). Cells were treated with siRNAs and homologous RNase H-dependent oligonucleotides at 200 nM, and total RNA was harvested 20 h after the initiation of transfection. mRNA levels for each gene were accessed by quantitative RT-PCR normalized to GAPDH expression. Black bars, mock-transfected; striped bars, specific RNase H-dependent oligonucleotide; open bars, specific siRNA; gray bars, RNase H-dependent oligonucleotide control; cross-hatched bars, siRNA control.

design oligonucleotides that hybridize to a target RNA by Watson-Crick base-pairing rules (*i.e.* antisense oligonucleotides). Once bound, antisense oligonucleotides can disable target RNAs by two broadly defined processes: disruption of RNA function by occupancy of critical sites and degradation of targeted RNA. Within these two broadly defined processes, multiple mechanisms are possible. Examples of “occupancy only” mechanisms include inhibition of translation (37), modulation of pre-mRNA splicing (38), and modulation of polyadenylation (39). In each case, the antisense oligonucleotides were found to be potent and selective regulators of gene expression.

Several endogenous enzymes can be exploited to promote targeted cleavage of RNAs in cells. One of the most widely exploited mechanisms is RNase H-mediated cleavage of targeted RNA. RNase H represents a ubiquitously expressed family of cellular enzymes that hydrolyze the RNA strand of an RNA-DNA heteroduplex. There are additional RNases present in mammalian cells that can be exploited for antisense inhibition of gene expression. As an example, we have reported that a single-stranded phosphorothioate modified RNA molecule can promote selective loss of Ha-ras in human cells (40).

Small interfering RNAs have been gaining widespread acceptance as a valuable tool for inhibiting gene expression in mammalian cells. In mammalian cells, like RNase H-dependent oligonucleotides, siRNAs bind to targeted RNA by Watson-Crick base pairing and induce site-specific cleavage of the RNA by specific RNases. The RNase that recognizes the duplex formed by the siRNA molecule has not been identified to date; however, the substrate specificity suggest that it is a double-stranded specific RNase (28). Since siRNA is an antisense mechanism resulting in loss of target RNA, we sought to directly compare siRNA-mediated with RNase H-mediated degradation of target RNA (12).

It has recently been reported that siRNA efficacy is highly dependent upon target position (36). Since RNase H-dependent oligonucleotides are also known to be dependent upon target position (34, 41), siRNAs were designed to previously identified RNase H-dependent oligonucleotide binding sites to determine

whether active RNase H-dependent oligonucleotide binding sites would be predictive of active siRNA sites. In three of four cases (ISIS 5132, ISIS 116847, and ISIS 16009), active siRNAs that targeted a site previously shown to be a good target site for RNase H-dependent oligonucleotides showed activity comparable with that of the RNase H-dependent oligonucleotide. One hypothesis for the lack of activity observed against the ISIS 2302 target, CD54, was that the siRNA mechanism is not amenable to silencing of TNF- α -induced genes. This turned out not to be the case, since screening 40 siRNA molecules targeting different regions of the CD54 mRNA identified several active siRNAs.

Analysis of oligonucleotide screens against both CD54 and PTEN confirmed that target position is an important factor in determining siRNA activity. Our data suggest that there is an imperfect correlation between RNase H and siRNA oligonucleotide activity when they are designed to bind different regions of the target RNA. In general, sites on the target RNA that were not active with RNase H-dependent oligonucleotides were similarly not good sites for siRNA. Conversely, a significant degree of correlation between active RNase H oligonucleotides and siRNA was found, suggesting that if a site is available for hybridization to an RNase H oligonucleotide, then it is also available for hybridization and cleavage by the siRNA complex. However, some exceptions were noted, with sites identified that apparently were poor RNase H-dependent oligonucleotide targets but effective siRNA targets and *vice versa*. This dichotomy could be due to additional factors other than RNA accessibility, such as sequence preferences for the respective nucleases. Differences in activity between siRNAs and RNase H-dependent oligonucleotides may also result from structural differences between pre-mRNA and mRNA, which appear to be the targets for RNase H and siRNA oligonucleotides, respectively. Our data suggest that the secondary structure of the target RNA is an important determinant of activity for both siRNA and RNase H antisense oligonucleotides, and it can be assumed that the structure of a pre-mRNA containing intron sequences will be different from the structure of mature mRNA.

To determine whether siRNA molecules were more potent or effective inhibitors of gene expression in human cells, we compared an optimized siRNA molecule to an optimized 2'-MOE chimeric antisense molecule targeting either PTEN or CD54. In both cases, the oligonucleotides working by either antisense mechanism exhibited similar potencies in T24 cells. Additionally, both types of oligonucleotides inhibited the respective target genes by more than 90%. Some investigators have reported greater siRNA efficacy in cultured cells (9, 36). However, others have reported activity comparable with what we report in this paper (11, 42, 43). One possible explanation for this difference would be that the siRNA molecules used in our studies were not optimally designed. For example, it has been demonstrated that a 5'-phosphate group is required for optimal siRNA activity (44). Since the siRNA oligonucleotides used in these experiments were not synthesized with 5'-phosphates, it is possible that greater potency would have been observed had the siRNA oligonucleotides been phosphorylated. However, published experiments (45) have revealed that there are no

differences in efficiencies of 5'-phosphorylated and nonphosphorylated siRNAs in mammalian cells, since siRNA duplexes with free 5'-hydroxyls and 2-nt 3' overhangs are readily phosphorylated in the cell. Our own results confirm these observations (*i.e.* we did not see an increase in activity with siRNA molecules containing a 5'-phosphate) (data not shown). A more plausible explanation for the decreased siRNA potency in our study compared with others is the method chosen to quantify target reduction. Most other published studies have measured siRNA efficacy at the protein level. We chose instead to assay target reduction at the mRNA level using quantitative RT-PCR. Comparison of target protein and mRNA reduction demonstrates that several oligonucleotides appear to produce a more robust reduction of protein compared with the corresponding mRNA (Fig. 3, A and B). Additionally, the extreme sensitivity of quantitative RT-PCR compared with other assay methods may overrepresent RNA reduced to very low levels.

Both siRNA and the RNase H-dependent oligonucleotides gave similar duration of action in cultured cells, each showing a gradual recovery of mRNA expression over 4–6 days. These results are in agreement with those reported previously for siRNAs (36) and second generation RNase H oligonucleotides (22). First generation phosphorothioate-modified oligodeoxynucleotides exhibit a duration of action and tissue half-life ranging from 24 to 48 h (46, 47). In contrast, the second generation 2'-MOE-modified oligonucleotides used for the these studies exhibit a significant increase in nuclease resistance, resulting in a prolonged duration of action and tissue half-life from 5 to 10 days (22, 23). If one takes into account the biostability of phosphorothioate-modified 2'-MOE antisense (23, 48) and predicted stability of unmodified double-stranded RNA oligonucleotides, this result is somewhat surprising and suggests that the siRNA molecules may be protected from nucleases in cells. From this limited comparison, the onset of the RNase H-dependent activity appears to be slightly earlier than that of the siRNA. This may be a result of differences arising from the RNase H oligonucleotide acting in the nucleus on the pre-mRNA while siRNA acts cytoplasmically on the mature mRNA. Our data as well as other published data (49, 50) suggest that the siRNA mechanism of action is restricted to the cytoplasm. In contrast, our results as well as previous publications (51, 52) suggest that RNase H oligonucleotides are capable of binding to pre-mRNA in the cell nucleus. There may be specific applications in which it may be desired to utilize an RNase H oligonucleotides to inhibit all RNA variants derived from a single transcript or alternatively to selectively discriminate alternative spliced transcripts using siRNA in the cytoplasm.

The fidelity for perfect base pair matches for both types of oligonucleotides was investigated by designing oligonucleotides with internal or external 2-base mismatches. Activity was completely lost when 2-base mismatches were made in the central domain of either the RNase H-dependent oligonucleotide or siRNA. When mismatches were placed near the ends of the sequence, activity was reduced, but not completely. The loss of activity was greater for the RNase H-dependent oligonucleotide than the siRNA but not significantly so. Therefore, the two types of antisense oligonucleotides exhibit similar sequence selectivity.

In conclusion, we have compared RNase H-dependent antisense oligonucleotides with siRNA molecules targeting several human genes in cell-based assays. These studies have demonstrated that optimized siRNA and RNase H-dependent oligonucleotides behave similarly in terms of potency, maximal effects, specificity, and duration of action and efficiency. It remains to be determined whether siRNA molecules work

broadly for *in vivo* applications. In a preliminary report of a siRNA molecule delivered to mice, the authors administered the oligonucleotide by rapid tail vein injection of a large volume of fluid (high pressure delivery) (53). It is not clear whether administration of siRNA molecules by more clinically acceptable practices will result in effective delivery to target tissues. In contrast, delivery of RNase H oligonucleotides to a variety of target tissues by a parenteral and nonparenteral routes of administration with subsequent inhibition of gene expression has been well documented in rodents, non-human primates, and humans (17, 23, 30, 32, 48, 54–57). Both strategies, however, appear to be equally valid approaches for cell-based analysis of gene function *in vitro*.

Acknowledgment—We thank John Reed for critical review of the manuscript.

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Clarke, Modet & Cº

22

COLOMBIA

ANEXO 10

000131

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ABSTRACT

The ability to manipulate the RNA interference (RNAi) machinery to specifically silence the expression of target genes could be a powerful therapeutic strategy. Since the discovery that RNAi can be triggered in mammalian cells by short double-stranded RNAs (small interfering RNA, siRNA), there has been a tremendous push by researchers, from academia to big pharma, to move siRNAs into clinical application. The challenges facing siRNA therapeutics are significant. The inherent properties of siRNAs (polyanionic, vulnerable to nuclease cleavage) make clinical application difficult due to poor cellular uptake and rapid clearance. Side effects of siRNAs have also proven to be a further complication. Fortunately, numerous chemical modification strategies have been identified that allow many of these obstacles to be overcome. This unit will present an overview of (1) the chemical modifications available to the nucleic acid chemist for modifying siRNAs, (2) the application of chemical modifications to address specific therapeutic obstacles, and (3) the factors that must be considered when assessing the activity of modified siRNAs. *Curr. Protoc. Nucleic Acid Chem.* 39:16.3.1-16.3.22. © 2009 by John Wiley & Sons, Inc.

Keywords: chemical modification • siRNA • oligonucleotide • immunostimulatory effects • off-target effects

INTRODUCTION

The first report that long double-stranded RNA could elicit specific gene silencing was published in 1998 (Fire et al., 1998). This discovery prompted a tremendous research effort to understand and manipulate the gene-silencing pathway now referred to as RNA interference (RNAi). Three years later a synthetic trigger of the RNAi pathway in mammalian cells was identified (Elbashir et al., 2001a). These short RNAi triggers are 21-nt double-stranded RNAs aligned such that each strand has a 2-nt 3'-overhang, and are referred to as siRNAs. In recent years, siRNAs have generated tremendous interest in the therapeutic field, and rightly so. siRNAs can be used to silence the expression of virtually any gene. The potential applications of siRNAs in drug development are immediately obvious: siRNAs can be used in cell culture to inhibit specific genes as a means of target validation, and/or siRNAs can themselves be drugs used to silence therapeutic targets.

The transition of siRNAs from the laboratory to the clinic has met with some significant obstacles. The polyanionic phosphodiester backbone of siRNA hinders cellular uptake, and numerous cellular enzymes are capable of rapidly degrading RNA strands, making for poor pharmacokinetics. Further-

more, it is becoming increasingly clear that siRNAs can elicit undesirable side effects. siRNAs can act like microRNAs (miRNAs) and affect the expression of unintended genes through partial complementarity. More recently, it has been discovered that siRNAs can be potent stimulants of the innate immune system (Robbins et al., 2009). Mammalian cells have receptors that recognize dsRNA (often a hallmark of viral infection), which can be triggered by siRNAs to produce inflammatory cytokines. Recent findings suggest that these immune responses to siRNAs may confuse gene-silencing outcomes, and may produce unwanted and potentially dangerous side effects. All of these side effects are collectively termed "off-target" effects.

Fortunately, a variety of chemical modifications have been proposed to address these issues. This overview describes a wide range of chemical modifications that have been applied to siRNAs, and provides examples of how these modifications can improve potency and serum stability, and reduce off-target and immunostimulatory side effects. The goal of this review is to provide the nucleic acid researcher with an overview of the state of the art, and a comprehensive guide for the design of chemically modified siRNAs.

Current Protocols in Nucleic Acid Chemistry 16.3.1-16.3.22, December 2009

Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).

DOI: 10.1002/0471142700.nc1603s39

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RNA Silencing

16.3.1

Supplement 39

000132

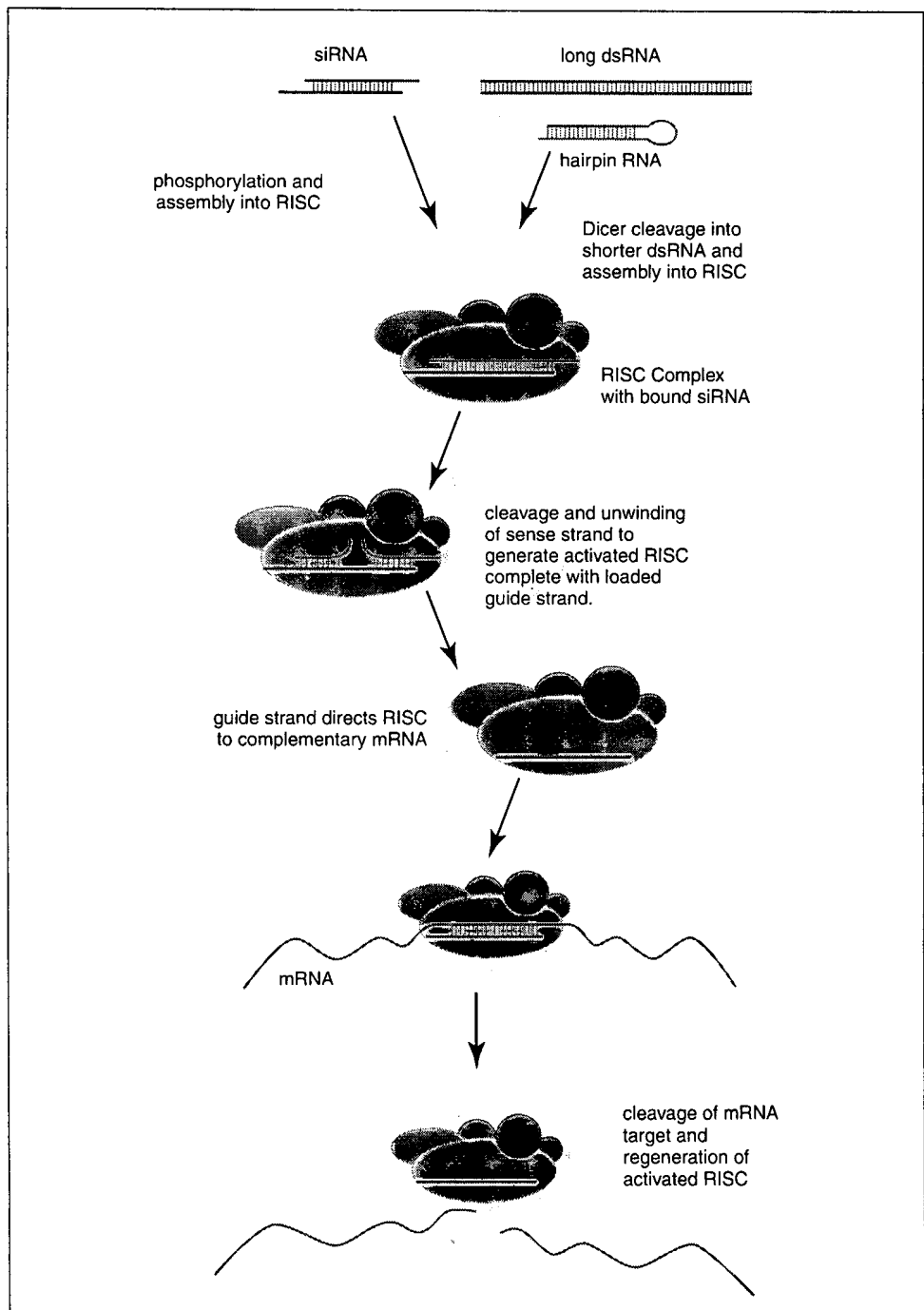


Figure 16.3.1 The mechanism of RNAi in human cells triggered by exogenously introduced dsRNAs. The largest of the ellipses represents AGO2, the catalytic engine of RISC. Adapted from Watts et al. (2008), with permission from Elsevier.

A detailed understanding of the mechanism of RNAi and the enzymes involved is invaluable when designing chemically modified siRNAs, an overview of which can be found in *UNIT 16.1*. For the purposes of this overview, a brief description of the RNAi process is included in Figure 16.3.1. Briefly, when an exogenous 19- to 21-bp siRNA is

introduced into a mammalian cell, the 5'-end is phosphorylated by Clp-1, a cellular kinase (Weitzer and Martinez, 2007). The siRNA is then loaded into the RNA-induced silencing complex (RISC). This multiprotein complex includes Argonaute2 (AGO2), Dicer, TRBP (HIV-1 TAR RNA-binding protein), and PACT (a dsRNA binding protein), as

well as other proteins, some of which remain unknown (Rana, 2007). The structural and mechanistic aspects of both Dicer and Argonaute2 have been recently reviewed (Jinek and Doudna, 2009).

siRNAs are double-stranded molecules, and the two strands play very different roles in RNAi. One strand, referred to as the guide or antisense strand, is loaded into the RISC complex and will function to direct RISC to complementary mRNAs for post-transcriptional gene silencing (PTGS). The other strand, referred to as the passenger or sense strand, is simply cleaved and/or unwound from the guide strand upon RISC loading. It appears that RISC differentiates between the guide and passenger strands based on the thermodynamics of the siRNA duplex. It has been reported that the strand with lower binding affinity at its 5'-end preferentially becomes the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). The other strand is designated as the passenger strand. It is cleaved and unwound, generating a single RNA guide strand associated with AGO2, which is an endonuclease at the heart of RISC and promotes hybridization and cleavage of mRNAs complementary to the guide strand (Jinek and Doudna, 2009). When modifying an siRNA duplex, it is important to recognize that different modification approaches are required for the sense and antisense strands, because of their very different roles (Parrish et al., 2000; Hamada et al., 2002).

It is typically assumed that the mechanism of siRNA-mediated gene silencing is unchanged in chemically modified siRNAs. Studies using modified siRNA have confirmed this by showing that the cleavage of complementary mRNA occurs between bases 10 and 11, counting from the 5'-end of the guide strand, which is the same result as for unmodified duplexes (Soutschek et al., 2004; Kraynack and Baker, 2006; Ui-Tei et al., 2008; Judge et al., 2009). In principle, this should be verified for each new pattern of modification.

METHODS FOR DESIGNING MODIFIED siRNA

Choosing the Right Sequence

Many factors must be considered in the design of an effective and specific siRNA. Firstly, the chosen siRNA sequence must be unique to the intended mRNA target. Even incomplete complementarity to unintended mRNAs can result in significant unwanted off-target effects (Jackson et al., 2006a). For exam-

ple, the silencing ability of siRNAs doubly mismatched to mRNA targets has been thoroughly analyzed. Results indicate that in some cases doubly mismatched siRNAs could still effect >50% gene silencing (Dahlgren et al., 2008). Because siRNAs can sometimes enter microRNA-type pathways, which silence gene expression based on complementarity to the miRNA "seed region" (the 6-7 nucleotide region at the 5' end of the guide strand), siRNAs should be designed to reduce seed-region complementarity to other genes as much as possible.

Additional considerations include the availability of the targeted region of the mRNA for RISC binding. Secondary structure can interfere with RISC-mediated mRNA cleavage, and so prediction of mRNA secondary structure can be useful (Brown et al., 2005; Gredell et al., 2008; Rudnick et al., 2008). Experiments can also be used to identify accessible mRNA regions (Sokol et al., 1998; Lee et al., 2002).

These factors can complicate siRNA design. Fortunately, computer programs have been developed that take these and other factors into account. Many of them are extremely user friendly, and can be invaluable when designing siRNAs. Some noteworthy programs include:

BIOPREDSi (<http://www.biopredsi.org>): This site was developed by the Novartis Institutes for BioMedical Research. It requires and provides only essential information. Input is a gene Accession number or gene sequence, and output is a user-defined number of optimized siRNA sequences (Huesken et al., 2005).

Whitehead siRNA Selection Web Server (<http://jura.wi.mit.edu/bioc/siRNAext>): This Web site was developed and is hosted by the Whitehead Institute. It is somewhat more complex, giving a large number of possible duplexes along with their properties, e.g., thermodynamic properties. An off-target search can be done for each duplex, within the site, but in a separate step (Yuan et al., 2004).

For an excellent guide to siRNA sequence selection, see Pei and Tuschl (2006). Other siRNA design algorithms are reviewed by Boese et al. (2005).

It is useful to test several duplexes targeting a gene, even after using a selection Web site. The most thorough way to identify highly active siRNA sequences is to test as many as 100 to 200 sequences against a given mRNA (Choong et al., 2006; Manoharan and Rajeev, 2007), looking for the duplexes with the best gene silencing activity and minimum off-target effects. Furthermore, individual sequences

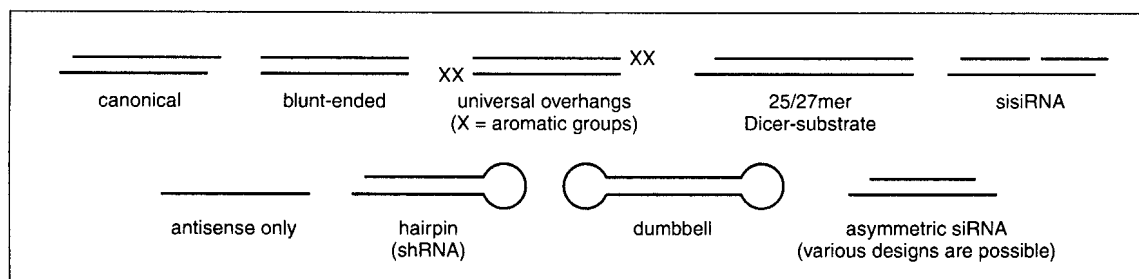


Figure 16.3.2 Functional siRNA architectures. The sense strand is always shown on top, in the 5' to 3' direction, and the antisense strand is on the bottom, in the 3' to 5' direction. Note that three of the structures (25/27mer, hairpin, and dumbbell) require the activity of Dicer before incorporation into RISC. Adapted from Watts et al. (2008), with permission from Elsevier.

respond differently to chemical modifications, and modifications beneficial for one siRNA sequence may be detrimental to another. Therefore, a variety of chemical modifications should be included in the sequence screen from fairly early in the process.

Choosing an siRNA Architecture

The canonical siRNA architecture is a 21-nt antiparallel, double-stranded RNA with 2-nt 3' overhangs (Elbashir et al., 2001a). The canonical siRNA design mimics the product of Dicer processing of long dsRNAs (Zamore et al., 2000; Bernstein et al., 2001). In the naturally occurring cellular pathway, these Dicer cleavage products are the effector molecules loaded into RISC to effect gene silencing. However, researchers have discovered that a much wider variety of duplexes can be incorporated into the RNAi pathway.

Indeed, we now understand that siRNA overhangs are particularly amenable to modifications. The use of 2'-deoxynucleotide units in the 3'-overhangs has been common since the earliest siRNA experiments; it reduces the cost of oligonucleotide synthesis and may increase resistance to 3'-exonucleases (Elbashir et al., 2001a). Of course, RNA units also work well (Holen et al., 2002). Perhaps more interestingly, most of the chemistries reviewed below are well tolerated in the overhangs as well (i.e., accepted by the RNAi machinery without loss of potency).

Blunt-ended siRNAs have also been used (Czauderna et al., 2003) because blunt-ended duplexes are more resistant to 3'-exonucleases. In addition, one study reported a greater tolerance to chemical modifications in combination with blunt-ended duplexes (Kraynack and Baker, 2006). However, blunt-ended siRNAs can be more immunogenic than duplexes with 3'-overhangs (Marques et al., 2006).

It has been shown that the 3' overhang is bound within a hydrophobic pocket of the

PAZ domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Ma et al., 2004). So, the 3' nucleotide overhangs can be replaced by aromatic groups (described as "universal overhangs" due to the lack of base pairing), improving nuclease stability without harming activity (Ueno et al., 2009).

Chemical phosphorylation of the 5'-end of the antisense strand can help ensure high potency, and may be necessary when the 5'-end of the strand is modified (Fisher et al., 2007; Watts et al., 2007; Zhang et al., 2009). Conversely, 5'-phosphorylation of the sense strand can be blocked without loss of activity (Nykanen et al., 2001; Czauderna et al., 2003). In fact, blocking sense strand phosphorylation can be beneficial, ensuring proper guide strand selection by RISC (Chen et al., 2008).

The siRNA architecture itself can be modified via chemical synthesis (Fig. 16.3.2). While most siRNA duplexes are made up of two strands, an siRNA made of three strands (an intact antisense strand and two 9- to 13-nt sense strands) can reduce off-target effects and increase potency; the resulting nicked duplex is termed small internally segmented interfering RNA (sisiRNA; Bramsen et al., 2007).

Functional siRNA can also be made from just one strand, in any of the following ways: Hairpin-type duplexes, made from a single strand, can be introduced exogenously (Siolas et al., 2005) or expressed within a cell (Brummelkamp et al., 2002; reviewed in Banan and Puri, 2004). Dumbbells or nanocircles, made by closing the open end of the hairpin, retain RNAi activity while providing complete protection from exonucleases (Abe et al., 2007). Finally, a single-stranded antisense RNA (not folded into a duplex at all) has been shown to enter the RNAi pathway, with potency occasionally approaching that of duplex siRNA (Martinez et al., 2002; Amarzguioui et al., 2003; Holen et al., 2003).

Recent findings have also demonstrated that asymmetrically designed siRNA duplexes can be potent gene silencing agents, comparable to canonical siRNAs. Indeed, siRNA duplexes can vary significantly in the lengths of either the sense or antisense strands. Asymmetrical siRNAs can contain ssRNA regions and comparatively short dsRNA regions without impairing activity in many cases. Active asymmetrical designs include 15 (sense, S) + 21 (antisense, AS) (Sun et al., 2008), 16 (S) + 21 (AS), 18 (S) + 18 (AS) (Chu and Rana, 2008), and 16 (S) + 19 (AS) (Chang et al., 2009). Asymmetrical siRNA designs are intriguing because several of these designs significantly decrease off-targeting, especially the sense strand mediated off-targeting that occurs when the wrong strand is loaded into RISC.

Asymmetry in siRNA overhangs can also be beneficial in directing the proper guide strand into RISC. Asymmetric siRNAs designed such that the guide strand has a 2-nt 3'-overhang and the passenger strand lacks a 3'-overhang (blunt ended) can improve siRNA potency (Sano et al., 2008). This effect is useful for siRNAs with unfavorable thermodynamics of loading.

The length of an siRNA duplex can be altered. Most synthetic duplexes are 19 to 21 bp in length, designed to mimic the natural products of the Dicer enzyme, which cleaves long dsRNAs into substrates for RISC. On the other hand, using a longer siRNA duplex can make it a *substrate* for Dicer, and has been found to increase potency of gene silencing and stability to degradation (Kim et al., 2005; Amarzguoui et al., 2006; Kubo et al., 2007). Enhanced potency may result from a physical transfer of newly dicer-produced siRNA to RISC. It is important to maintain a length of <30 nt, to avoid triggering the interferon response (Minks et al., 1979).

INCORPORATING MODIFIED NUCLEOSIDES AND BACKBONE LINKAGES

While careful sequence selection is essential and varying the architecture of an siRNA duplex can provide certain advantages, this review will focus on changing the chemical nature of the oligos themselves. The rationale for chemical modification is clear, and the right chemical modifications can address nearly all the shortcomings of siRNA therapeutics:

1. Nuclease stability can be significantly improved.

2. Cellular uptake can be increased.
3. Immunostimulation can be reduced (or increased, if desired).
4. Off-targeting can be reduced.

This section will describe a wide variety of chemical modifications compatible with siRNA.

Sugar Modifications

The ribose sugar moiety is perhaps the most attractive component for chemical modification. Changes in the sugar chemistry can strongly influence sugar conformation, and thus overall siRNA duplex structure. Sugar modifications can improve endonuclease resistance. siRNAs containing RNA-like nucleoside analogues can pass under the immune receptor radar screen without impairing gene silencing activity. A wide variety of sugar modifications can be used to modify siRNAs, although often with limitations on position and quantity. Various sugar modifications compatible with RNAi are described below (see Fig. 16.3.3), with reference to features and properties that are useful when designing modified siRNAs.

A number of successful siRNA modifications have focused on modification of the 2' position. One of the earliest studies on chemically modified siRNA showed that while A-form duplex structure is important, the 2'-OH is not required for active siRNA (Chiu and Rana, 2003). DNA is perhaps the simplest modification accepted within siRNA duplexes. DNA has been used in the 3'-overhangs of synthetic siRNAs since the beginning (Elbashir et al., 2001a), and can be tolerated in limited numbers within the duplex region as well (Parrish et al., 2000; Elbashir et al., 2001b; Ui-Tei et al., 2008). An antisense strand made entirely of DNA purines and 2'-F-RNA pyrimidines is functional (Chiu and Rana, 2003), since 2'-F-RNA strongly favors a northern (C3'-*endo*) sugar pucker and A-form helical structure, presumably directing the conformation of the more flexible 2'-deoxynucleotides towards northern, RNA-like conformations. dsDNA can be used in the 8-bp region at the 5'-end of the guide strand to produce active siRNAs with reduced off-target effects (Ui-Tei et al., 2008).

Binding affinity and nuclease stability are increased by 2'-O-methylation of RNA, and 2'-O-Me-RNA can be well-tolerated throughout the siRNA duplex. This makes it a popular and versatile siRNA modification. Many groups have found that large numbers

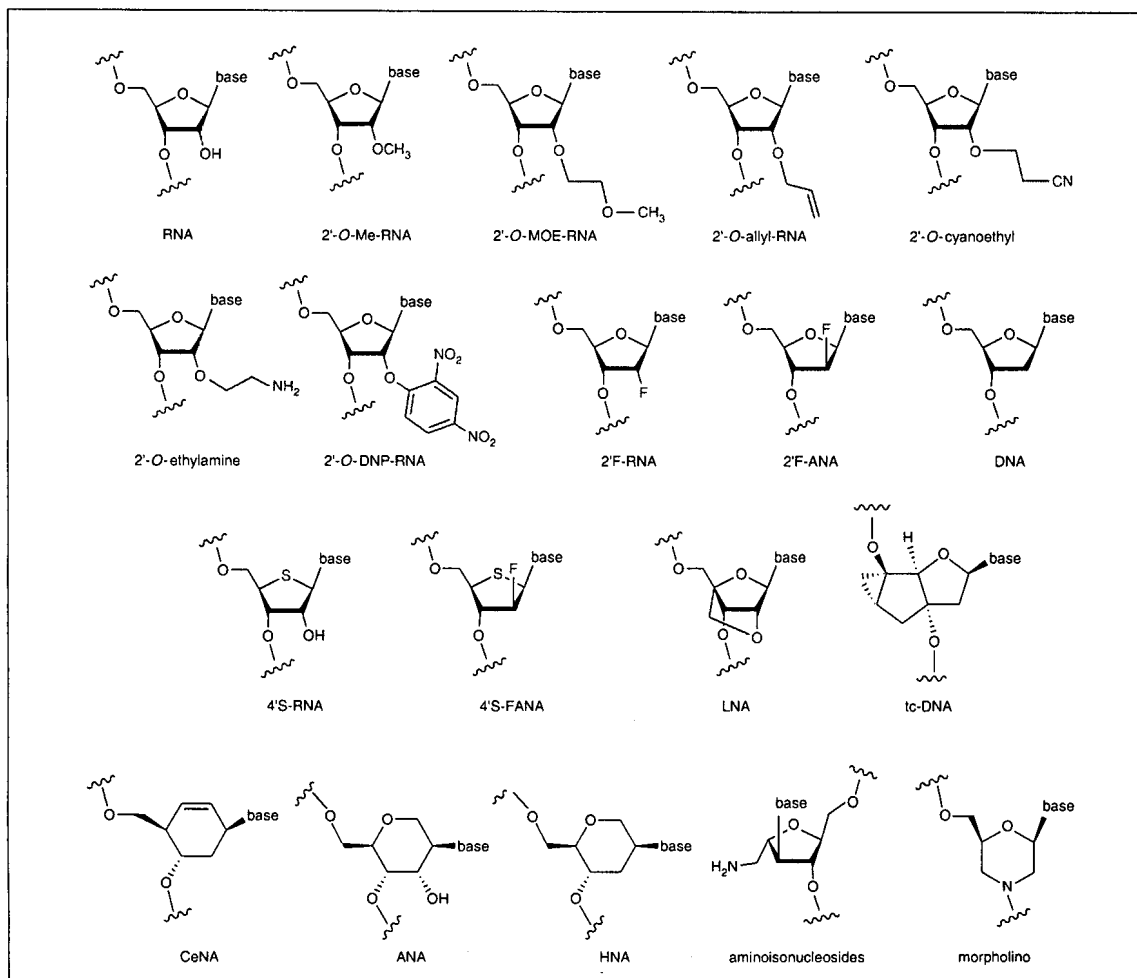


Figure 16.3.3 Sugar units that have been successfully used to modify siRNA duplexes. Top row, 2'-O-alkyl modifications (2'-O-Me, 2'-O-MOE, 2'-O-allyl, 2'-O-cyanoethyl); second row, other 2'-modifications (2'-O-ethylamine, 2'-O-DNP, 2'-F-RNA, 2'-F-ANA, and DNA); third row, 4'-modifications (4'S-RNA and 4'S-2'-F-ANA) and bicyclic/tricyclic modifications (LNA and tc-DNA, respectively); bottom row, six-membered ring modifications (CeNA, ANA, HNA) and dramatically redesigned sugar structures (aminoisucleosides, morpholino). Adapted from Watts et al. (2008), with permission from Elsevier.

of 2'-O-Me modifications (in either strand) decrease siRNA activity (Elbashir et al., 2001b; Braasch et al., 2003; Chiu and Rana, 2003; Czauderna et al., 2003), but others report that fully modified 2'-O-Me sense strands are functional (Choung et al., 2006; Kraynack and Baker, 2006). The retention of functionality may be due to the 2'-O-Me modifications being made in blunt-ended duplexes (Kraynack and Baker, 2006), but at least one other group reports that even in this context, activity is greatly reduced by heavy 2'-O-Me modification (Czauderna et al., 2003). The 2'-O-methoxyethyl (2'-O-MOE) modification has been used in the 3'-overhangs of siRNA targeting the pain-related cation-channel P2X₃ and has resulted in successful gene targeting in vivo (Dorn et al., 2004). Another group found that

2'-O-MOE modifications could be used in the sense strand, especially at the termini, but not in the antisense strand (Prakash et al., 2005). Similarly, 2'-O-allyl-modifications reduce activity at most positions in the duplex, but can be used (Odadzic et al., 2008). These modifications do not reduce activity in the 3'-overhangs (Amarzguoui et al., 2003).

A cyanoethyl group at the 2' position increases binding affinity and nuclease resistance, and may find applications in siRNA (Saneyoshi et al., 2005; Bramsen et al., 2009). 2'-O-acetalester-protecting groups are being developed for production of "proRNA," which remains protected until demasked by cellular esterases (Martin et al., 2009). These molecules may find application as "pro-siRNA" (Martin et al., 2009). In a similar fashion, 2'-esterified units (levulinates) may

be used to create protected siRNA molecules (Lackey et al., 2007).

2'-*O*-alkylamines also appear to be active in siRNA duplexes (Odadzic et al., 2008; Bramsen et al., 2009). Minimal modification with 2'-*O*-ethylamine units at the 3' end of the antisense strand appears to be well-tolerated (Odadzic et al., 2008). Further characterization will improve our understanding of their gene silencing potential.

In most cases, bulkier 2'-modifications are not well tolerated in siRNA duplexes, except for limited modifications on the termini. Interestingly, siRNAs with 70% of the 2'-OH groups converted at random into 2,4-dinitrophenyl ethers (2'-*O*-DNP) show a variety of improved properties, including higher binding affinity, nuclease resistance, and potency (Chen et al., 2004). Recently Bramsen et al. (2009) have reported a large-scale screen of siRNA activity elicited by several nucleoside analogues, showing that a variety of 2' modifications can be tolerated, particularly in the sense strand. In some cases, bulky 2'-modifications were tolerated, but with slightly reduced activity.

Functional siRNAs can also contain fluorine at the 2'-position. One of the best-known and most versatile siRNA modifications is 2'-F-RNA. Partial 2'-F-RNA modification is tolerated throughout sense and antisense strands (Braasch et al., 2003; Chiu and Rana, 2003; Harborth et al., 2003), and there are reports of active siRNAs that are fully modified 2'-F-RNA (Blidner et al., 2007). 2'-F-RNA-modified siRNA duplexes have significantly increased serum stability (Layzer et al., 2004) and increased binding affinity of the duplex (Kawasaki et al., 1993; Monia et al., 2003). The 2'-F-RNA modification has also allowed the synthesis of siRNAs containing 5'-amino-2',5'-dideoxy-2'-fluororibonucleic acid units that confer improved hydrolytic stability (Ueno et al., 2008).

Changing the stereochemistry of the fluorine of 2'-F-RNA results in 2'-deoxy-2'-fluoroarabinonucleic acids (2'-F-ANA). Considering that 2'-F-ANA was originally developed as a DNA mimic (Rosenberg et al., 1993; Berger et al., 1998; Damha et al., 1998; Ikeda et al., 1998; Wilds and Damha, 2000), it is somewhat surprising that 2'-F-ANA is also well-tolerated in siRNA duplexes, including fully modified sense strands and partially modified antisense strands (Dowler et al., 2006; Watts et al., 2007). Like 2'-F-RNA, 2'-F-ANA binds with high affinity and increases nucle-

ase stability. Both 2'-fluorinated analogues are highly resistant to acidic conditions (Watts et al., 2009).

In addition to the oxygen at the 2'-position, the ring oxygen can be modified for siRNA applications. One example is 4'S-RNA, a high-affinity modification that gives a significant advantage in nuclease stability. Incorporation of 4'S-RNA units does not reduce silencing when placed near the termini of siRNA duplexes (Hoshika et al., 2005, 2007; Dande et al., 2006). An siRNA architecture consisting of four 4'-thioribonucleotides on each end of the sense strand and four at the 3'-end of the antisense strand has consistently worked well for silencing two target genes in three cell lines (Hoshika et al., 2007). When the antisense strand was modified with 4'S-RNA, especially at the center, potency was reduced (Hoshika et al., 2005; Dande et al., 2006). Combinations of 4'S-RNA with 2'-*O*-Me and 2'-*O*-MOE modifications at the termini of both strands have also shown excellent potency and serum stability (Dande et al., 2006).

4'S-2'-F-ANA, featuring modifications at both the 2' and 4' positions, has a northern, RNA-like conformation (Watts et al., 2007), and is compatible with siRNA activity at various positions in both strands. 4'S-2'-F-ANA in the antisense strand shows synergy with 2'-F-ANA modifications in the sense strand (Watts et al., 2007). However, the low-binding affinity of 4'S-2'-F-ANA means that only a small number of modifications should be used.

Locked nucleic acid (LNA), a conformationally constrained nucleotide adopting a northern structure (Petersen and Wengel, 2003), has been extensively studied in siRNA (Braasch et al., 2003; Elmen et al., 2005; Hornung et al., 2005; Bramsen et al., 2007; Mook et al., 2007). Its conformational rigidity causes significant increases in binding affinity. Careful placement of LNA in siRNA duplexes can lead to functional duplexes of various types. Common terminal modification sites are the termini of the sense strand (Hornung et al., 2005) and the 3'-overhang of the antisense strand (Elmen et al., 2005; Mook et al., 2007). Minimal modification of most internal positions of the antisense strand is also tolerated (Braasch et al., 2003; Elmen et al., 2005), but heavier modification of the antisense strand is tolerated only in combination with a segmented sense strand (sisiRNA, described above; see Bramsen et al., 2007).

LNA is one example of a larger family of nucleoside analogues, the bicyclic nucleic

acids (BNAs). Another BNA, ethylene-bridge nucleic acid (ENA), has been tested in the siRNA overhang and been found to impair siRNA activity (Hamada et al., 2002). A rigid tricyclic modification (tc-DNA) has been successfully used at either end of the sense strand, and even improved activity when placed in the overhangs (Ittig et al., 2008). The development of a variety of BNAs has been reviewed by Obika (2004), although much remains to be understood regarding their compatibilities to elicit siRNA activity. Toward this end, Bramsen et al. (2009) have published a large-scale screen of siRNA activity elicited by several nucleoside analogues, including several members of the BNA family. For the siRNA sequence tested, several BNAs appear to be tolerated in siRNA, especially within the sense strand.

Interestingly, siRNA potency can be retained, and in some cases improved, when the 5-membered sugar moiety is replaced with a 6-membered ring. Cyclohexenyl nucleic acids (CeNA), altritol nucleic acids (ANAs), and hexitol nucleic acids (HNAs) have all been successfully incorporated into siRNAs. Incorporation of one or two CeNA units can produce siRNAs with similar or enhanced activity compared to unmodified siRNA (Nauwelaerts et al., 2007). ANAs support A-form structures, and can improve potency and duration of activity when incorporated at certain positions (Fisher et al., 2007), but can also slightly decrease potency in some cases (Fisher et al., 2009). HNA can improve siRNA potency, and is tolerated in the sense strand opposite the position on the antisense strand at which mRNA cleavage occurs (Fisher et al., 2009).

siRNAs can be minimally modified with aminoisonucleosides (see Fig. 16.3.3) without reducing activity (Li et al., 2007). Aminoisonucleosides were better tolerated by the RNAi machinery in the sense strand than in the antisense strand, and a single insert could be used at either the 5' or the 3' region of the sense strand without impairing gene silencing. However, modifications were less tolerated internally in the sense strand, reduced activity when placed in the antisense strand, and may be detrimental to overall nuclease stability. Aminoisonucleoside modification of siRNA is awaiting optimization.

Morpholino nucleoside analogues can also be used in siRNA. Morpholino analogues are tolerated in the sense strand and on the overhangs, but reduce activity in the antisense strand (Zhang et al., 2009).

Backbone Linkage Modifications

Chemical modifications to the oligonucleotide backbone are also tolerated by the RNAi machinery. Indeed, several variations on the phosphodiester linkage can be used for gene silencing (Fig. 16.3.4). Phosphorothioate (PS) linkages, a popular modification in antisense technology, can be used in siRNA, but they often reduce potency relative to native siRNA (Chiu and Rana, 2003; Hall et al., 2004). However, in some cases their potencies are comparable to native siRNAs (Amarzguoui et al., 2003; Harborth et al., 2003). Some observations indicate that PS linkages are not accepted at the center of the duplex, especially at the sense strand scissile phosphate (Leuschner et al., 2006). Some cytotoxicity has been observed with extensive PS modification (Amarzguoui et al., 2003). In marked contrast to PS-DNA antisense constructs (Geary et al., 2001), PS modifications do not appear to have a major effect on biodistribution of siRNA (Braasch et al., 2004).

siRNAs with boronophosphate linkages are functional. They show increased potency relative to PS-modified siRNAs, and often to native siRNAs as well. To maximize potency, the center of the antisense strand should not be modified (Hall et al., 2004). Boronophosphate linkages in siRNAs provide a significant increase in nuclease stability over native RNA (Hall et al., 2004).

Two nonionic amide linkages are tolerated in the 3' overhangs of siRNA: a simple amide linkage that maintains the ribose structure while increasing duplex thermodynamic stability and nuclease resistance (Iwase et al., 2007) and peptide nucleic acid, in which the entire sugar-phosphate backbone is replaced by an amide moiety (Potenza et al., 2008).

Phosphonoacetate linkages (Fig. 16.3.4) are recently described backbone modifications with exciting properties (Sheehan et al., 2003; Yamada et al., 2007). These linkages can be esterified, affording nucleic acids with neutral backbones, which can be taken up by cells without transfection reagent (Sheehan et al., 2003; Yamada et al., 2007). Cellular esterases are then believed to cleave the ester groups, producing phosphonoacetate backbones, which are negatively charged and thus retained inside cells (Yamada et al., 2007). The properties of phosphonoacetate backbones are beneficial for siRNA applications, and preliminary reports of siRNA activity elicited by these modifications are encouraging.

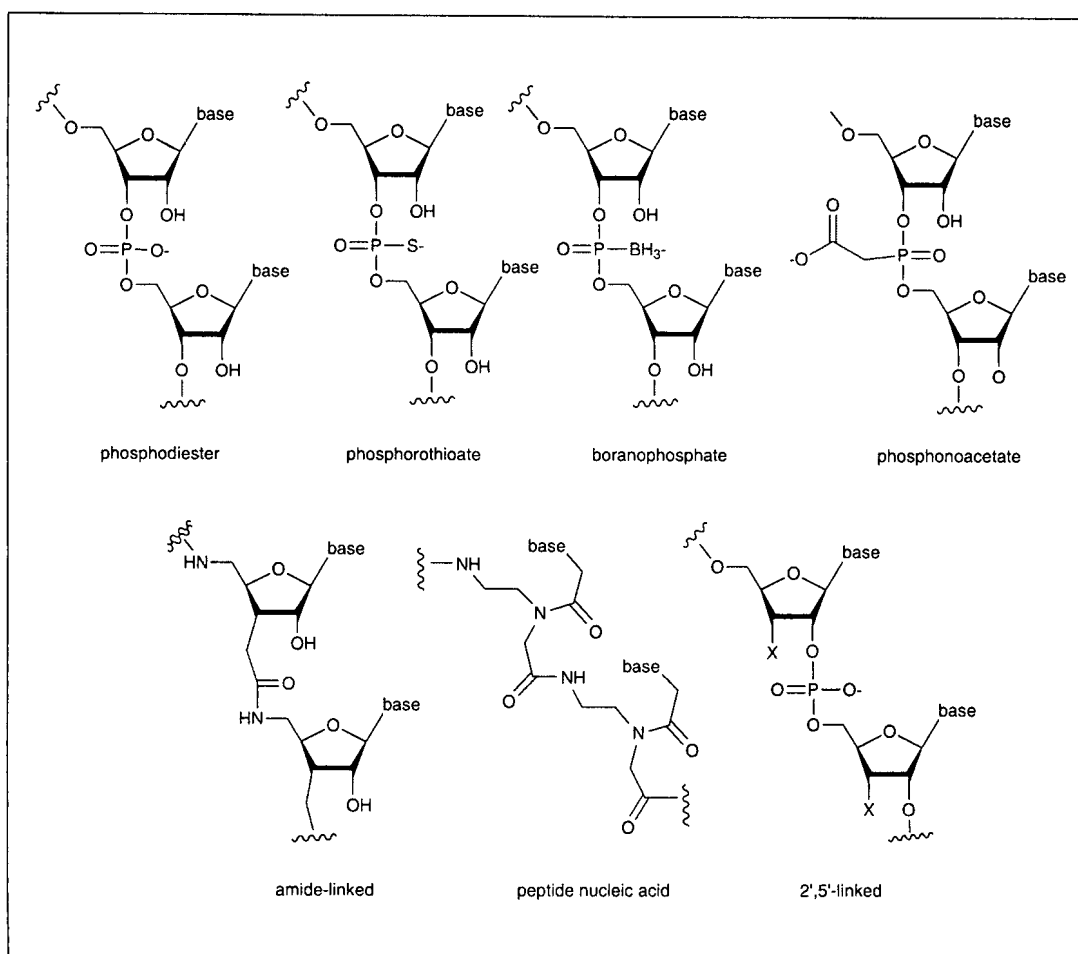


Figure 16.3.4 Internucleotide linkages used in siRNA. The phosphodiester linkage can be replaced by phosphorothioate, boranophosphate, or phosphonoacetate linkages, which retain the negative charge of a phosphate. Neutral amide-linked RNA or peptide nucleic acid (PNA) can be used at select positions. Either 2',5'-linked DNA (X=H) or 2',5'-linked RNA (X=OH) can be used in the sense strand. Adapted from Watts et al. (2008), with permission from Elsevier.

A 2',5'-linkage (either 2',5'-DNA or 2',5'-RNA) can substitute for the native 3',5' linkage, but only in the sense strand of the duplex, and with some reduction in potency (Prakash et al., 2006).

Base Modifications

The use of modified bases in siRNA has been more limited than other modifications, but there have been several examples (Fig. 16.3.5). Modified bases that stabilize A-U base pairs (5-Br-Ura and 5-I-Ura instead of uracil, and diaminopurine instead of adenine) are tolerated in siRNA duplexes, although activity is somewhat reduced (Chiu and Rana, 2003).

4-Thiouracil has also been successfully used (Parrish et al., 2000). 2-Thiouracil (Hornung et al., 2006; Sipa et al., 2007) and the C-linked base pseudouracil (Hornung et al., 2006; Sipa et al., 2007) increase binding affinity

and can be used to increase potency and specificity if placed appropriately within the duplex (see the sections *Increasing potency* and *Modulating immunostimulatory activity*). 5-Methylation of pyrimidines (i.e., T and 5-Me-C instead of U and C) is common in conjunction with sugar modifications such as DNA, 2'-F-ANA, and LNA. Indeed, small base modifications projecting into the RNA major groove (5-Me-U and 5-Me-C) can improve siRNA stability without impairing gene silencing, whereas bulkier groups (5-propynyl-U) are detrimental to activity (Terrazas and Kool, 2009). Bulky or cationic base modifications have not been well-tolerated (Parrish et al., 2000).

Some surprisingly atypical base structures have been used in siRNA. A difluorotoluoyl base, which has the same shape as thymine but cannot form classical hydrogen bonds, can replace uracil at single positions throughout

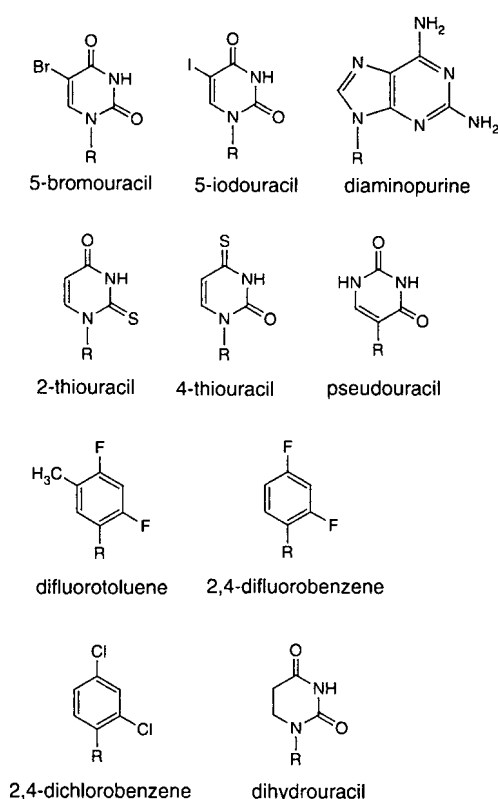


Figure 16.3.5 Base-modified nucleotides used in siRNA. Top two rows: 5-halouracils, 2-thiouracil, pseudouracil, and diaminopurine increase the strength of A-U base pairs; bottom two rows: highly atypical modified bases including bases that cannot form classical hydrogen bonds (difluorotoluene, 2,4-difluorobenzene, 2,4-dichlorobenzene) and a non-aromatic base (dihydrouracil). R represents the ribose phosphate backbone. Adapted from Watts et al. (2008), with permission from Elsevier.

an siRNA duplex, without significantly decreasing the potency or changing the mechanism of cleavage (Xia et al., 2006). Non-polar nucleobases (2,4-difluorobenzene and 2,4-dichlorobenzene; see Fig. 16.3.5) are also tolerated in certain siRNA positions, and can in some cases improve siRNA specificity (Somoza et al., 2008). These studies provide evidence that non-hydrogen-bonding nucleobases can be tolerated at positions 1, 3, 4, 7, and 19 of the guide strand (Somoza et al., 2008). A nonaromatic base, dihydrouracil, can also be used, but because it cannot contribute to base stacking, it lowers the binding affinity of the duplex and is best placed at the 5'-end of the duplex, as defined by the antisense strand (Sipa et al., 2007).

Terminal Conjugates

Various groups can be conjugated to the termini of siRNA duplexes, especially the termini of the sense strand. An inverted abasic end cap

(David et al., 2005; Morrissey et al., 2005) helps with exonuclease stability. Conjugation of fluorescent dyes (Harborth et al., 2003) or biotin (Chiu and Rana, 2002; Schwartz et al., 2008) has allowed important biophysical/biochemical studies. Conjugation of lipophilic groups, including steroids and lipids (Lorenz et al., 2004; Soutschek et al., 2004), has helped with delivery. Cholesterol conjugation has even been shown to allow siRNA-mediated protection from HSV-2 following intravaginal administration of the siRNAs (Wu et al., 2009).

A combinatorial library of lipid-like conjugates, termed "lipidoids" has also been described as potential delivery agents (Akinc et al., 2008). siRNA delivery is a rapidly developing research field; a recent study showed that including 5 to 8 dA and dT units on the 3'-ends of the strands can lead to reversible concatemerization via sticky ends, which in turn leads to higher efficiency delivery in complex

with linear polyethylenimine (PEI) (Bolcato-Bellemin et al., 2007).

The general consensus is that the 5'-end of the antisense strand is the most sensitive to modifications (Chiu and Rana, 2002; Martinez et al., 2002; Czauderna et al., 2003). However, one group observed that fluorescein could be conjugated to any of the termini except the 3'-end of the antisense strand (Harborth et al., 2003). As long as an antisense 5'-phosphate is present, attaching a group to it does not necessarily eliminate RNAi activity (Schwarz et al., 2002).

Combining Modifications

The various modifications described above can confer advantageous properties to siRNAs. However, it is also clear that the effectiveness of modifications has a strong sequence dependence. Many modifications are beneficial in some siRNA regions but detrimental in others, and enhance activity in some sequence contexts, but reduce potency in others. An interesting concept then becomes the combination of different, complementary modifications within the same siRNA. Most siRNA modification studies focus on individual modifications to minimize experimental variables. However, a few studies have demonstrated that combining modifications can produce siRNAs with impressive features. Indeed, many interesting modified siRNAs consist of a combination of multiple chemical modifications (Allerson et al., 2005; Morrissey et al., 2005; Dande et al., 2006; Koller et al., 2006; Bramsen et al., 2009).

Indeed, by combining various modifications, these researchers were able to design potent siRNAs completely free of the native ribose. We have been investigating the use of chimeric siRNAs composed entirely of 2'-F-ANA and 2'-F-RNA modifications, and have observed synergy between these two modifications when assessing gene silencing activity against a few different mRNA targets (G.F. Deleavey et al., unpub. observ.). Primarily, research on new chemically modified siRNA involves only one type of modification. However, if the field is to move toward widely applicable chemical modification strategies, there is an ongoing need to combine chemistries in creative ways.

APPLYING MODIFICATIONS TO ADDRESS siRNA SHORTCOMINGS

The following sections were adapted from Watts et al. (2008), with permission from Elsevier.

Serum Stability

RNA is rapidly degraded in cells. The double-stranded nature of siRNA improves stability relative to ssRNA, but these molecules are still not ideal for in vivo therapeutic applications. A nuclease called *eri-1* has been found to play a key role in degradation of siRNA (Kennedy et al., 2004), and expression levels of *eri-1* inversely correlate with duration of siRNA activity (Takabatake et al., 2007). This and other data suggest that increasing the nuclease resistance of siRNAs can prolong their activity. Chemical modification is the principal strategy used to improve the nuclease resistance of siRNAs.

Essentially all of the modifications discussed can be used to increase the serum half-life of siRNAs. In the therapeutic siRNA community, two approaches are being studied for protecting siRNAs against nucleases. The first strategy favors extensive or full chemical modification of siRNA. Sirna Therapeutics (<http://www.sirna.com>) has published work on heavily modified siRNA duplexes, demonstrating the advantages of this strategy. A fully modified siRNA with a sense strand made of 2'-F-RNA pyrimidines, DNA purines, and 5' and 3' inverted abasic end caps, and an antisense strand made of 2'-F-RNA pyrimidines, 2'-O-Me purines, and a single phosphorothioate linkage at the 3'-terminus, had significantly increased potency in an HBV mouse model (David et al., 2005). This fully modified duplex had a half-life in serum of 2 to 3 days, as compared with 3 to 5 min for the unmodified duplex (David et al., 2005). This improved stability translated into higher efficacy in vivo. Still higher potency was later obtained by including 1 to 3 RNA inserts at the 5'-end of the antisense strand, and this heavily modified siRNA still had a serum half-life nearly 30× longer than that of unmodified siRNA (Morrissey et al., 2005).

Several other examples of fully modified duplexes have been reported. An siRNA made entirely of alternating 2'-F-RNA and 2'-O-Me units had greatly increased stability in serum (Allerson et al., 2005). This architecture maintains or improves potency, as discussed in the next section. An siRNA made with DNA overhangs, a 2'-O-Me-RNA sense strand, and a PS-RNA antisense strand was nearly all intact after 48 hr in serum (Choung et al., 2006), and a functional fully 2'-F-RNA-modified siRNA has also shown excellent nuclease resistance (Blidner et al., 2007).

However, extensive chemical modification may not always be necessary. The second

paradigm for protecting siRNAs against nucleases involves minimal, selective modification. This strategy is favored by, among others, Alnylam Pharmaceuticals (<http://www.alnylam.com>). Endonucleolytic hydrolysis is a major mechanism of degradation of siRNAs (Czaderna et al., 2003), and so the endonuclease cleavage pattern of a given siRNA duplex is characterized. Degradation is often dominated by cleavage after a pyrimidine nucleotide, and can be readily characterized by mass spectrometry (Turner et al., 2007) or denaturing polyacrylamide gel electrophoresis (Xia et al., 2006). Vulnerable positions are selectively modified, usually with 2'-O-Me or 2'-F-RNA nucleotides, which considerably increase the stability of the siRNA with only minimal modification (Manoharan, 2006; de Fougères et al., 2007). Besides these experimentally determined vulnerable internal positions, key regions for modification include the strand termini (Choung et al., 2006; Hoerter and Walter, 2007).

Increasing Potency

siRNA is a potent gene silencing agent, although potency depends on cell type, mRNA target, and siRNA sequence. Increasing potency is usually not considered the primary objective of chemical modification: it is sufficient to maintain the potency of unmodified siRNA. It is more important to increase serum half-life and specificity. However, as our understanding of the requirements for effective RNAi develops, we can foresee an increase in the use of chemical modifications to optimize potency as well, through features such as target-binding affinity (by enhancing hybridization on-rates and off-rates), preorganization into appropriate conformations (to reduce entropic penalties of binding), flexibility (to allow for enzyme dynamics), and optimized binding to RISC (in various other ways).

Unfortunately, patterns of chemical modification that universally increase potency are rare. However, there are several known modifications that increase potency, sometimes very significantly, for particular sequences or systems. One of the most dramatic increases was observed for a fully modified siRNA made of a combination of 2'-O-Me and 2'-F-RNA, which was 500-fold more potent than unmodified siRNA (Allerson et al., 2005). Such impressive improvement was not observed for other sequences, but this architecture showed equal or greater potency and efficacy than unmodified siRNA for several other targets (Koller et al., 2006).

Part of the increase in potency observed in some studies may arise from the increased nuclease stability of the chemically modified siRNA. Beyond this, however, in most cases where increased potency is observed, it is not yet clear why. In addition, it is not yet clear why so many patterns of chemical modification work for one system/sequence and not for another. This reflects paucity in our understanding of the requirements of the RNAi pathway, and of the interplay between chemical modifications, nucleotide sequence, and duplex structure.

Rational chemical modification can, however, be used to improve potency through improving the selective loading of the antisense strand. Strand differentiation by RISC is determined by the thermodynamics of the siRNA duplex (Khvorova et al., 2003; Schwarz et al., 2003). The strand with lower binding at its 5'-end is preferentially selected as the guide strand by RISC. The sequence of an siRNA should therefore be chosen to be AU-rich at the 5'-end of the antisense strand. Chemical modifications can also be used to increase incorporation of the appropriate guide strand. By placing high-affinity modifications like LNA at the 5'-end of the sense strand (Elmen et al., 2005), duplex thermodynamics can be altered to affect strand loading. The activity of a moderately active siRNA duplex was significantly improved using the high-affinity 4'-S-RNA modification in both strands (Hoshika et al., 2007). However, a duplex that already had optimal strand thermodynamics was not further improved by 4'-S-RNA modification (Hoshika et al., 2007). Base modifications can be used for similar purposes. Of a series of modified siRNA duplexes, the most potent siRNAs contained a high-affinity 2-thiouracil base at the 3'-end of the antisense strand, and a low-affinity dihydrouracil base at the 3'-end of the sense strand (Sipa et al., 2007).

In addition to chemical modification, a recent report has demonstrated that a small molecule, enoxacin, can actually enhance siRNA-mediated mRNA degradation (Shan et al., 2008). Enoxacin is believed to enhance TRBP-binding to RNA (Shan et al., 2008), and enhanced interactions between TRBP and siRNA may facilitate RISC loading (Katoh and Suzuki, 2007). This interesting discovery suggests that RNAi potency can be improved via indirect methods as well as with direct chemical modification, and also suggests that it would be worth designing chemical modifications that optimize protein interactions directly.

Immunostimulation

Long dsRNA can be a hallmark of viral infection. It has long been known that dsRNAs longer than 30 bp are potent activators of immune responses (Minks et al., 1979), preventing the application of RNAi to mammalian cells in the same fashion originally described in *C. elegans* (Fire et al., 1998). It was eventually discovered that 21-nt siRNA duplexes were capable of effecting potent and specific gene knockdown in mammalian cells. These molecules were below the 30-bp threshold, and were expected to avoid the immunostimulation characteristic of longer dsRNAs (Elbashir et al., 2001a).

However, the immunogenic activity of short RNA duplexes is more significant than originally perceived. Reports of off-target effects resulting from immunostimulation caused by dsRNAs shorter than 30 bp are widespread (Kim et al., 2004; Hornung et al., 2005, 2006; Judge et al., 2005; Marques and Williams, 2005; Morrissey et al., 2005; Reynolds et al., 2006; Schlee et al., 2006). Nonspecific immune responses to siRNA therapeutics may trigger unwanted side-effects unless the immunogenic properties of siRNA can be addressed.

Immune response activation by short RNAs is a complex process, and has been reviewed elsewhere (Marques and Williams, 2005; Schlee et al., 2006). Briefly, RNAs are recognized by three main types of immunoreceptors: Toll-like receptors (TLRs) (specifically TLR3, TLR7 and TLR8), protein kinase R (PKR), and helicases like RIG-I and MDA5. Receptor recognition can lead to a number of cellular responses including release of cytokines and changes in gene expression (Schlee et al., 2006). The types of immunoreceptors involved and the level of immune activation depend on a number of factors, including the length, sequence, and cellular delivery method of the RNA, as well as the type of immune cell involved. These receptors exist on cell surfaces (TLR3) (Kleinman et al., 2008), in endosomes (TLR3/7/8; Takeda and Akira, 2005; Judge and Maclachlan, 2008; Zamanian-Daryoush et al., 2008), and in the cytoplasm (RIG-I, MDA5, PKR; Judge and Maclachlan, 2008).

Immune responses are affected by RNA sequence; however, the sequence dependence is not fully understood. Judge et al. have identified immunostimulatory motifs in mice and in vitro human blood, most notably 5'-UGUGU-3' regions (Judge, et al., 2005). Hornung et al.

demonstrated that 5'-GUCCUCAA-3' is also a potent immunostimulatory motif (Hornung et al., 2005). In fact, any U-rich RNA sequence may be sufficient for recognition by TLR7 (Diebold et al., 2006). The presence of a 5'-triphosphate or blunt-end can lead to RIG-I-mediated immunostimulation (Kim et al., 2004; Hornung et al., 2006; Marques et al., 2006). Duplexes between 23 and 30 bp may still induce the interferon response in a length-dependent manner, depending on cell type (Reynolds et al., 2006).

Fortunately, chemical modifications can be used to reduce the immunostimulatory properties of siRNAs (Eberle et al., 2008; Judge and Maclachlan, 2008). siRNA duplexes >90% modified with 2'-F-RNA, 2'-O-Me, and DNA residues were shown to cause no detectable effect on interferon levels or cytokines, while unmodified siRNA duplexes caused significant activation (Morrissey et al., 2005). Because of immunostimulation, mice treated with unmodified siRNA showed increased levels of serum transaminases and signs of systemic toxicity such as decreased body weight, transient lymphopenia and thrombocytopenia, and piloerection, but these adverse effects were not evident in those mice treated with modified siRNA (Morrissey et al., 2005). Although minimal 2'-O-Me modification is sufficient to reduce immunostimulatory activity, 2'-O-methylation of cytidine residues was, however, found ineffective in fulfilling this function (Judge et al., 2006). 2'-F-RNA and/or 2'-O-Me modification of uridine resulted in elimination of immune off-target effects (Hornung et al., 2006), including both TLR-dependent and TLR-independent immune effects (Cekaite et al., 2007). Notably, even the presence of 2'-O-Me modified RNAs on separate, non-complementary strands abrogates the TLR7-dependent immunostimulatory activity of unmodified siRNAs, indicating that 2'-O-Me-RNA itself is a potent antagonist of TLR7 (Robbins et al., 2007).

In addition to 2'-O-Me and 2'-F-RNA modifications, LNA has been used to modify the ends of an siRNA sense strand containing an immunostimulatory motif, abrogating the IFN- α immunostimulatory activity of the duplex without affecting the silencing activity (Hornung et al., 2005). Base modifications can also reduce immune activation: modification with pseudouracil or 2-thiouracil prevented the RIG-I-mediated immunostimulation due to a 5'-triphosphate (Hornung et al., 2006), and various base modifications abrogated immune

effects mediated by TLR3, TLR7, and TLR8 (Karikó et al., 2005).

Different types of chemical modifications can reduce the immune effects from different receptors. For example, of the base modifications useful for siRNA, one study found that only 2-thiouridine was able to reduce TLR3-mediated immunostimulation, but several others were able to reduce TLR7- or TLR8-mediated effects as well (Karikó et al., 2005). In another recent study, the cytokine induction profile was different upon treatment with unmodified, 2'-O-Me-modified, and 2'-F-RNA modified siRNAs (Zamanian-Daryoush et al., 2008).

Immunogenic side effects from siRNA administration have not always been observed. However, it is clear that when immune activation is a problem, many chemical modifications are available to minimize this predicament, and surely, many more modifications will be tested and found successful in the years to come. Thus, much work lies ahead before the full immunomodulatory potential of chemical modifications can be both realized and understood. Encouragingly, our understanding of dsRNA recognition by TLR-3 has been greatly improved by very recent reports of the structural basis of dsRNA recognition (Liu et al., 2008; Pirher et al., 2008). Studies such as these may allow the rational design of siRNAs that can avoid receptor recognition.

In closing this section, we note that the immunostimulatory activity of siRNA may actually prove useful in some cases. A recent paper showed that the therapeutic anti-angiogenesis activity of an unmodified siRNA currently in clinical trials might actually be due to TLR3-mediated immune effects (Kleinman et al., 2008). Furthermore, immunostimulatory siRNAs may find use as drugs capable of facing cancer and chronic viral infections on two fronts: eliciting both gene knockdown and activation of immune responses (Schlee et al., 2006; Poeck et al., 2008). Currently, however, immune activation is generally considered an unwanted and potentially dangerous off-target effect (Judge and Maclachlan, 2008).

Hybridization-Dependent Off-Target Effects

Off-target side effects of siRNA can arise from partial hybridization with unintended mRNAs (Jackson et al., 2003). This primarily occurs when siRNAs function as miRNAs via 6 to 8 nt complementarity at the 5'-end of the antisense strand (Birmingham et al., 2006; Jackson et al., 2006a), but can also occur with a

~15-nt region of significant complementarity in the center of the antisense strand (Jackson et al., 2003). If some of the sense strand is mistakenly incorporated into RISC instead of the antisense strand, it can cause even more off-target effects (Jackson et al., 2003).

Two major strategies are used to minimize off-target effects: (1) increasing the selective loading of the antisense strand (disfavoring incorporation of the sense strand) as the RISC guide, and (2) disfavoring miRNA-type activity.

The first of these strategies can have the benefit of increasing potency, as well as reducing off-target effects, and is typically accomplished by increasing the binding affinity of the 3'-end of the antisense strand and/or the 5'-end of the sense strand, as discussed in the section *Increasing potency*. To ensure that the sense strand makes absolutely no contribution to off-target effects, it can be further modified. An extreme example of this is small internally segmented interfering RNA (sisiRNA), in which the sense strand is nicked to prevent it from being incorporated into RISC (see Fig. 16.3.2, and *Choosing an siRNA Architecture*) (Bramsen et al., 2007). The high affinity of LNA modifications is essential for such strand architecture (Bramsen, et al., 2007), since 10- to 12-bp duplexes of unmodified RNA are too unstable to effect gene silencing.

Another method of reducing RISC loading of the sense strand is to block 5'-phosphorylation by methylation (Chen et al., 2008). Finally, incorporation of eight DNA nucleotides at the 3'-end of the sense strand significantly reduces sense-strand mediated off-target effects (Ui-Tei et al., 2008). However, even if the antisense strand is incorporated into RISC with perfect selectivity, the problem remains that siRNAs can function as miRNAs (Doench et al., 2003; Zeng et al., 2003) and downregulate many genes through partial complementarity (Lim et al., 2005; Jackson et al., 2006a), especially in the seed region. Furthermore, these off-target effects can cause toxicity (Fedorov et al., 2006). 2'-O-Me nucleotides at the 5'-end of the antisense strand can reduce miRNA-type off-target effects significantly, with little effect on siRNA gene silencing (Jackson et al., 2006b). If the seed region is made of DNA instead of RNA, miRNA-type off-target effects are also significantly reduced (Ui-Tei et al., 2008).

A third strategy for reducing off-target effects is adopted by Dharmacon RNA Technologies (<http://www.dharmacon.com>) who have championed the idea of delivering four

different siRNA duplexes at once against the target gene. In this way, the desired gene silencing is maintained, but hybridization-dependent off-target effects are diluted, since they are different for each of the siRNA duplexes. Thus, the overall off-target signature of a pool of siRNAs is usually less than that of any of the individual duplexes that make it up. This strategy mimics the natural siRNA pathway, in which long dsRNA is diced into a large number of siRNA duplexes, each targeting the same gene, but with a different off-target signature. This strategy can be combined with the chemical modification strategies described above.

To reduce or avoid both immune-mediated and hybridization-dependent off-target effects, careful sequence selection is an essential first step (see Choosing the Right Sequence above). Chemical modifications can then provide further protection.

CONSIDERATIONS WHEN ASSESSING THE ACTIVITY OF MODIFIED siRNAs

The wide range of nonspecific and off-target effects that can be triggered by siRNAs can complicate experimental results. Controlling for off-target and immunostimulation effects is an essential aspect of siRNA experimentation. At a minimum, siRNA experiments should include mismatch, scrambled, and nontargeting (e.g., luciferase) controls. These measures control for nonspecific effects. Several positive controls (independent siRNA sequences targeting the same mRNA) should also be used. Unfortunately, these simple controls do not provide the full picture of nonspecific effects caused by siRNA. The following section will look in more detail at ways to rule out nonspecific effects when using siRNAs.

Off-Target Effects

siRNA off-target effects can significantly affect the phenotypic outcome of gene silencing experiments. Off-target effects can arise from partial complementarity of unintended mRNA targets to either the sense or antisense strand of an siRNA. Several methods have been developed to determine the off-target effects of siRNAs.

Perhaps the most thorough method to observe off-target effects is the use of microarray technology to monitor changes in cellular gene expression following siRNA treatment. (Jackson et al., 2003)

Another technique for observing sense-strand mediated versus guide-strand mediated mRNA targeting involves the use of two psiCheck plasmids containing sequence complementarity to either the sense strand or the guide strand, which allows determination of the extent of sense-strand mediated off-targeting (Ui-Tei et al., 2008).

In order to verify that the observed phenotype resulting from RNAi-mediated gene silencing is a result of gene knockdown and not off-target effects, it is often useful to attempt to obtain the phenotype using different siRNA sequences targeting the same gene. This technique should control for off-target effects since different siRNA sequences have different off-target effects, but in each case, the intended gene should be silenced (Echeverri and Perrimon, 2006). Conversely, rescue experiments can be performed, whereby the target mRNA can be modified to remove complementarity to an siRNA without changing the coded protein (Echeverri and Perrimon, 2006). In the rescue condition, the phenotype should disappear since the mRNA is no longer degraded. If the phenotype remains, it could be attributed to siRNA-mediated off-targeting (Kittler et al., 2005).

Finally, chemically modified siRNAs with reduced off-targeting properties can be used to further verify that effects are arising from specific gene silencing.

Immunostimulation

siRNA knockdown experiments can be further confused by immunostimulation triggered by siRNA. Indeed, when siRNA is being used to target cancer and viral mRNAs, experimental outcomes must be carefully interpreted due to the high sensitivity many of these diseases have towards innate immune responses (Robbins et al., 2008). When comparisons are made with scrambled controls (duplexes that have been rearranged to prevent base pairing to the mRNA), caution should also be observed due to the possibility that the scrambled controls may possess significantly different immunostimulatory properties in comparison to the targeting siRNAs under study (Robbins et al., 2008). Use of a control siRNA that is less immunostimulatory than the experimental siRNA can lead to false positives or overestimated efficacies, if a phenotype mediated by the innate immune response is attributed to the RNAi pathway (Judge and MacLachlan, 2008). Use of chemically modified analogues of siRNAs can also help to

rule out immune effects, as discussed above. Two recent studies that demonstrated the potential of siRNAs to induce immune effects also rigorously demonstrated that chemically modified siRNA analogues operated through the RNAi mechanism *in vivo* (Kleinman et al., 2008; Judge et al., 2009).

Metabolites

Depending on endo and exonuclease cleavage patterns of siRNAs, several degradation products can be created when siRNAs are metabolized *in vivo*, all of which should be tested for safety. This is equally true for chemically modified siRNAs. Mass spectroscopy analysis following serum incubation is a method for determining metabolic products. Mass spectroscopy is a common tool for oligonucleotide characterization, and effective protocols have been developed for siRNA analysis (Shah and Friedman, 2008; Yan et al., 2008). The ultimate test of safety in humans, however, is clinical trials. Several siRNA clinical trials are underway and include both modified and unmodified duplexes.

CONCLUSIONS

The chemical modification of siRNA is a rapidly developing field of study. New and exciting advancements are reported on a regular basis. The discovery of siRNA-mediated gene silencing by short double-stranded RNAs generated tremendous interest in the therapeutics field. Nevertheless, the progression of siRNAs from discovery to clinical application has been hampered by a number of obstacles, including stability issues, cellular uptake problems, and undesirable side effects arising from off-targeting and immunostimulation. Encouragingly, in the 8 years since the discovery of siRNA, a vast array of chemical modifications has become available to the nucleic acid chemist. Indeed, rapid development in this field, owing in no small part to the successes of research on previous and ongoing antisense technologies (Corey, 2007), has provided the understanding and tools with which siRNA therapeutics may begin to realize their full potential.

While rapid progress has been made in this field of study, several frontiers remain to be explored with chemically modified siRNAs. Most of the currently used strategies have significant sequence dependence; thus, a remaining challenge is to find universal chemical modification strategies, or to elucidate rules

to allow reliable prediction of which modifications and architectures will be effective for a given sequence. Significant advances in siRNA delivery technologies have also been achieved, and in combination with chemical modification strategies, the prospects for siRNA therapeutics are looking up.

ACKNOWLEDGEMENTS

We acknowledge financial support from NSERC Canada (fellowship to GFD), FQNRT (fellowship to JKW), and CIHR (grant to MJD). Sections of this work originated from a review previously published in *Drug Discovery Today* (Watts et al., 2008), and have been revised and updated for inclusion in this overview, with permission from Elsevier.

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ANEXO 11

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RNA major groove modifications improve siRNA stability and biological activity

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ANEXO 11

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Received October 20, 2008; Revised November 10, 2008; Accepted November 11, 2008

ABSTRACT

RNA 5-methyl and 5-propynyl pyrimidine analogs were substituted into short interfering RNAs (siRNAs) to probe major groove steric effects in the active RNA-induced silencing complex (RISC). Synthetic RNA guide strands containing varied combinations of propynyl and methyl substitution revealed that all C-5 substitutions increased the thermal stability of siRNA duplexes containing them. Cellular gene suppression experiments using luciferase targets in HeLa cells showed that the bulky 5-propynyl modification was detrimental to RNA interference activity, despite its stabilization of the helix. Detrimental effects of this substitution were greatest at the 5'-half of the guide strand, suggesting close steric approach of proteins in the RISC complex with that end of the siRNA/mRNA duplex. However, substitutions with the smaller 5-methyl group resulted in gene silencing activities comparable to or better than that of wild-type siRNA. The major groove modifications also increased the serum stability of siRNAs.

INTRODUCTION

RNA interference (RNAi) represents a powerful molecular strategy for inhibition of gene expression (1–3). This process can be induced by 21–23 nt double-stranded RNAs (dsRNAs), known as short interfering RNAs (siRNAs) (4–6). These oligoribonucleotides (formed by a sense and an antisense strand) are recognized by the RNA-induced silencing complex (RISC) (7,8), a protein complex located in the cytoplasm. After siRNA binding, RISC is activated by ATP and carries out unwinding and strand separation of the siRNA duplex, loading one RNA (the antisense 'guide' strand) into the activated RISC complex. The RISC complex uses the guide strand as a template to find the complementary target mRNA (9) and induces endonucleolytic cleavage of the mRNA (10–12),

preventing its translation into protein. Despite the significance of RNAi both as a biological tool and as a potential therapeutic strategy, some important challenges remain, including development of strategies for effective siRNA delivery, improving stability against serum nuclease degradation (13) and addressing low sequence specificity that results in undesired 'off-target' activities (14,15). To address these limitations and to further understand the mechanism of silencing, several research groups have been actively studying siRNAs with various chemical modifications (16–19). However, most of the focus to date has been on modifying the RNA backbone, and few laboratories have modified the bases of siRNAs, despite their central involvement in target recognition.

In recent studies, we and others (20–22) have begun to investigate the physicochemical factors contributing to mRNA sequence recognition by the RISC complex using siRNAs containing modified nucleobases. In one study, we used nonpolar base replacements to probe steric effects of altering nucleobase shapes on the sequence specificity of mRNA recognition; we found that base shape had a large effect on the preferred target sequence, and that with the unnatural bases, sequence specificity could be increased over that of natural RNA (21). In a different study, we incorporated a nonhydrogen bonding uridine isostere into 11 different positions in place of natural uridine along an RNA guide strand (20). This experiment allowed us to perturb the stability of the duplex in a systematic way. We found that at 9 of 11 positions there was a very close correlation of RNA duplex stability with gene suppression activity, such that more stable siRNA duplexes showed better activity in suppressing the target mRNA expression in HeLa cells. In those experiments, the nonpolar analog decreased stability, resulting in lower activity. However, if such a correlation were general, then in principle, increases in RNA duplex stability (as a result of chemical modifications) might increase RNAi activity over that of natural RNA. Such duplex stabilization might add favorable free energy that could compensate for the unwinding of the mRNA target from its native structure. In general, the experiments to date have revealed that both

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steric effects and thermodynamic stability of siRNAs play substantial roles in their biological activity.

A survey of the literature shows that few nucleobase modifications known to affect stability or sterics have been tested for their effect on siRNA activity. In this context, Rana and co-workers incorporated 5-bromouridine, 5-iodouridine and 2,6-diaminopurine (23) into siRNAs and tested the cellular activity of the modified oligonucleotides (24). The former two substitutions add a small amount of steric size in the RNA major groove, and all three may increase duplex stability to a small extent. It was found that RNAs with these modifications could induce RNAi-directed gene suppression, although to a level significantly lower than the one observed for the wild-type siRNA. On the other hand, several sugar modifications known to stabilize double helices, such as 2'-fluoro and 2'-*O*-Me substitutions (25), have been studied in siRNAs. In particular, siRNAs having 2'-fluoro substitutions at internal positions along the guide strand have been shown to display near wild-type activity (24).

Another class of major groove modification that is known to increase RNA duplex affinity is the substitution of alkyl and alkynyl functionality at the C-5 position of pyrimidine nucleobases (26–30). The methyl group adds $\sim 23 \text{ \AA}^3$ of steric bulk in the major groove, while the propynyl group adds $\sim 53 \text{ \AA}^3$. Both types of substitution not only add steric size, but also stabilize duplexes. For example, in one context, each methyl in a 12-mer RNA increased the favorable free energy of duplex formation by 0.3 kcal/mol, yielding several kilocalories per moles of total stabilization in short duplexes with multiple substitutions (26). Interestingly, the larger, unsaturated propyne group is expected to exert even greater effects on duplex stabilization (28). This substitution in DNA was shown to increase the melting temperature (T_m) of a DNA:RNA hybrid by 0.9–2.6°C per modification (28,31). Both the methyl and propynyl stabilization effects are likely due to enhanced base stacking propensity (25,31,32,33). The application of propynyl-substituted oligonucleotides as antisense agents in cell cultures has been well documented (34–38). However, to the best of our knowledge, there are no published studies of the effects of the 5-propynyl substitution in siRNAs. As for methyl substitution, we know of no previous reports of methyl effects on ribonucleotides in siRNA. However, Eberle *et al.* (39) recently described siRNAs containing deoxythymidine substitutions, in which methyl groups and deoxy sugars were incorporated together. Interestingly, they found that four substitutions led to a decrease in off-target effects in plasmacytoid dendritic cells without affecting gene silencing activity. However, they did not test for positional effects. It is well-known that the 5'-half of the siRNA duplex (as defined by the antisense strand) is functionally distinct from the 3'-half, due to the asymmetric nature of siRNA recognition for initiation of unwinding (24,40), and in addition, interactions of the RISC protein(s) with the guide and mRNA are expected to be different at the two ends as well. Thus, a detailed study comparing the positional effects of 5-substitutions of different size on RNAi activity would be valuable in probing major groove steric effects in the active RISC complex.

Here we explore the effects of methyl and propynyl substitution on siRNA duplex stability and on cellular RNAi activity. The goals of these studies are (i) to probe major groove steric effects in the active RISC complex and (ii) to evaluate whether enhanced duplex stability has a positive effect on RNAi activity. Furthermore, we explore the effects of these nucleobase modifications on the sensitivity to siRNA degradation in human serum, since the unnatural C-5 substitution might alter biostability, thus contributing in a different way to the activity.

MATERIALS AND METHODS

Nucleoside phosphoramidite synthesis

The 5-propynylU ribonucleoside was prepared as described (41). For automated RNA synthesis, the 5-dimethoxytrityl, 2'-*O*-TOM (TOM = [(triisopropylsilyl) oxy]methyl), 3'-*O*-phosphoramidite derivative was prepared. Details are given in the Supplementary Data.

RNA preparation

Modified and unmodified 21-nt RNAs (passenger strand and guide strands 1–9) were synthesized on the 1 μ mol scale on an applied Biosystems 394 synthesizer using 2'-TOM-phosphoramidites (42,43). Acetonitrile (synthesis grade), the 2'-TOM protected phosphoramidite monomers of A, C, G, U and 5-methylC and the 2'-OTBDMS protected phosphoramidite monomer of 5-methylU were from commercial suppliers. 5-Ethylthio-1*H*-tetrazole was used as activator. The standard coupling time of 6 min was used for the four standard phosphoramidites and an increased coupling time of 15 min was used for the modified 5-propynylU, 5-methylC and 5-methylU phosphoramidites. All oligonucleotides were synthesized in DMT-off mode. After the solid-phase synthesis, the solid support was transferred to a screw-cap vial and incubated at room temperature for 16 h with 1.5 ml methylamine solution (prepared by mixing one volume of 40% aqueous methylamine with one volume of 33% methylamine in ethanol). The vial was then cooled on ice and the supernatant was transferred into a 2 ml eppendorf tube and the support was rinsed with 50% ethanol (2 $\times$ 0.25 ml). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue that was obtained was dissolved in 1 ml of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) and rocked at 37°C for 12 h. Then, 1 ml of 1 M Tris-HCl (pH 7.4) was added and the oligonucleotide was desalted on a NAP-25 column using water as the eluant and evaporated to dryness. After purification by 20% denaturing polyacrylamide gel, the oligonucleotides were isolated by the crush and soak method, quantified by absorption at 260 nm and confirmed by MALDI mass spectrometry (Supplementary Data). Sequence-scrambled control siRNAs were synthesized by the protein and nucleic acid (PAN) facility at Stanford University.

UV-monitored thermal denaturation

Absorbance versus temperature curves of duplexes were measured at 1 μ M strand concentration in 15 mM

HEPES-KOH (pH 7.4), 1 mM MgCl₂ and 50 mM KOAc buffer and in low salt buffer (2 mM sodium phosphate, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA)). Experiments were performed on a Varian Cary UV-vis spectrophotometer equipped with thermoprogrammer. The samples were heated to 95°C, allowed to slowly cool to 25°C, and then warmed during the denaturation experiments at a rate of 1°C/min to 95°C, monitoring absorbance at 260 nm. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computer-fit of the first derivative of absorbance with respect to $1/T$.

RNAi methods

HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four hours before transfection at 50–80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1\text{--}3 \times 10^5$ cells/ml) and transferred to 24-well plates (500 µl per well). Two luciferase plasmids, *Renilla* luciferase (pRL-CMV) and firefly luciferase (pGL3) from Promega, were used as reporter and control, respectively. Cotransfection of plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 µg pGL3-Control, 0.1 µg pRL-CMV and 0.03–26 nM siRNA duplex formulated into liposomes, were applied; the final volume was 600 µl per well. The cells were harvested 22 h after transfection, and lysed using passive lysis buffer, 100 µl per well, according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a Fluoroskan Ascent FL Luminometer (Thermo Electron Corporation, USA) with a delay time of 2 s and an integrate time of 10 s. The following volumes were used: 20 µl of sample and 30 µl of each reagent (Luciferase Assay Reagent II and Stop and Glo Reagent). The inhibitory effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter (*Renilla*) luciferase gene and the control (firefly) luciferase gene.

Stability assays

The dsRNAs (14 µM) were incubated in 90% human serum, and the mixture was incubated at 37°C for various lengths of time (0 min, 15 min, 30 min, 120 min and 240 min). At the time points indicated, 1% sodium dodecyl sulfate was added and mixtures were heat-denatured for 5 min at 100°C. siRNAs were isolated by hot phenol extraction in the presence of sodium dodecyl sulfate followed by ethanol precipitation. Re-suspended RNA samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide. RNA bands were visualized with the 'Stains All' reagent (Sigma-Aldrich) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Design and synthesis of modified guide-RNA strands containing 5-methyl and 5-propynyl substitutions

The 5-propynylU (^pU), 5-methylU (^mU) and/or 5-methylC (^mC) monomers (Figure 1) were incorporated into different positions in place of natural rU and rC along a 21-mer RNA guide strand that targets an A-rich site 501–519 of *Renilla* luciferase mRNA (20,21). The 5-methylU and 5-methylC monomers were obtained from commercial sources. The 5-propynylU monomer was synthesized using known Sonogashira chemistry according to the literature (41,44) (see the Supplementary Data for details). We prepared RNAs containing no substitutions (wt, sequence 1), 5-methyl groups at internal positions [methylC in place of C (2)], propynyl groups at spaced-out positions along the guide strand (3), fully substituted with 11 propynyl groups (4) and substituted with propynyl and methyl groups [methylC and propynylU (5)]. Moreover, we focused attention on predominantly 5'-modified and predominantly 3'-modified siRNA duplexes, in order to localize any possible steric effects. To this end, 5-propynylU or 5-methylU were incorporated within the antisense (guide) strand predominantly on the 5'-half (RNAs 6 and 7) or predominantly on the 3'-half (RNAs 8 and 9).

Thermal denaturation studies

First we measured the effects of these substitutions on thermal stability of the siRNA duplex formed by these sequences paired with the passenger strand (Figure 2). Compared to the unmodified RNA sequence, all substituted oligoribonucleotides had greater duplex stability (as evaluated by higher T_m values) when hybridized to their RNA complement (see data in Figure 2 and in Supplementary Table S2). As expected, the incorporation of the propyne group at the position 5 gave rise to a substantially larger stabilization of the RNA duplex compared with the 5-methyl modification. The incorporation of 5-propynylU residues resulted in an increase of 1.3–1.8°C per modification depending on the position of substitution. The contribution of the 5-methylC was smaller (1.0°C per methyl group), and 5-methylU substitution yielded the smallest stabilization, at 0.2–0.7°C per substitution. The rank order of duplex T_m was: eleven 5-propynylU (4) > five 5-propynylU and five methylC (5) > six 5-propynylU on the 3'-half (8) > six 5-propynylU on the 5'-half (6) = five propynylU at spaced-out positions along the guide strand (3) > five

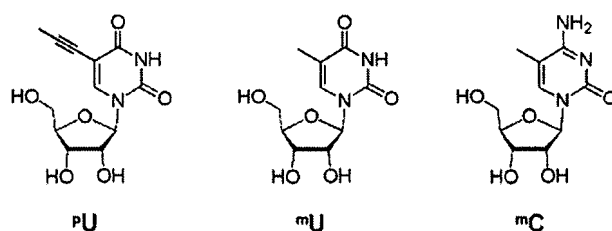


Figure 1. The 5-substituted nucleosides employed in this study.

5-methylC at internal positions (2) = six 5-methylU on the 3'-half (9) > six 5-methylU on the 5'-half (7).

Effect of 5-methyl and 5-propynyl substitutions on RNAi activity

We then carried out separate RNAi studies in HeLa cells with siRNA duplexes containing each of the eight modified guide-RNA strands containing 5-methylU, 5-propynylU and/or 5-methylC substitutions (2-9), as well as with the unmodified (wt) RNA (1). Experiments were carried out in triplicate. The cells were first transfected with dual reporter plasmids that express *Renilla* luciferase (the target) and nontargeted firefly luciferase as an internal nontargeted control. The effects of the different RNAs on luciferase expression were evaluated after dosing with 0.21-210 ng (0.03-26 nM) of dsRNA in the cell media, and measuring luminescence responses after 22 h. The results, showing *Renilla* luciferase activity

normalized to firefly luciferase, are represented in Figure 3. Surprisingly, 5-propynylU siRNAs disrupted *Renilla* luciferase-specific RNAi activity despite the strong stabilization that this substitution causes. Incorporation of 11 5-propynylU units within the guide strand (RNA 4) caused loss of most of the interference activity even at the highest siRNA concentration (26 nM). Similar results were observed for the guide RNA containing six 5-propynylU substitutions on the 5'-half (6). Interestingly, and in contrast, incorporation of six 5-propynylU groups on the 3'-half (8) did not disrupt RNAi activity, although gene silencing was not as efficient as for the wild-type RNA. At siRNA concentration of 0.3 nM, *Renilla* luciferase expression was ~59% for sequence 6, ~24% for sequence 8 and ~12% for the wild-type siRNA (1). These contrasting results support the idea that the major groove of the siRNA/mRNA active substrate is more sterically sensitive in the 5'-half of the antisense strand of the duplex than the 3'-half. This is also consistent with previous observations that mismatches near the 5'-half of a siRNA caused negative effects on RNAi activity, whereas siRNAs mismatched at the 3' retained a significant level of gene silencing (24). We surmise that close fitting of protein around the guide/mRNA complex may result in more costly disruptions at the 5'-end.

In contrast, the 5-methyl substitution did not disrupt RNAi activity. When 5-methylC was incorporated into positions 6, 8, 9, 12 and 15 in place of natural C along the guide RNA strand containing five 5-propynylU substitutions (3), gene silencing was more efficient than that observed for 3, which contained the propynyl groups alone (81% knockdown for 5 versus 73% for 3). Moreover, guide RNAs having five 5-methylC substitutions at internal positions (2), six 5-methylU residues on the 5'-half or six 5-methylU's on the 3'-half (7 and 9, respectively) showed activity comparable to or better than that of the wild-type siRNA (Figure 4). For example, at a siRNA concentration of 0.3 nM, *Renilla* luciferase gene silencing was 86.0±0.8% for sequence 2,

passenger	TTAAAAAGAGGAAGAAGUCUA	5'			T _m (°C)
guide	5'	UUUUUCUCCUUCUUCAGAU	1	70.2	
		UUUUU ¹ CU ² C ³ CUU ⁴ CUU ⁵ CAGAU	2	74.0	
		U ¹ UU ² UUCUCCU ³ UCU ⁴ UCAGA ⁵ UTT	3	78.0	
		¹ U ² U ³ U ⁴ U ⁵ U ⁶ U ⁷ U ⁸ U ⁹ U ¹⁰ U ¹¹ U ¹² U ¹³ U ¹⁴ U ¹⁵ U ¹⁶ U ¹⁷ U ¹⁸ U ¹⁹ U ²⁰ U ²¹	4	87.0	
		U ¹ UU ² U ³ CU ⁴ C ⁵ CU ⁶ U ⁷ CU ⁸ U ⁹ CAGA ¹⁰ UTT	5	82.0	
		¹ U ² U ³ U ⁴ U ⁵ U ⁶ U ⁷ U ⁸ U ⁹ U ¹⁰ U ¹¹ U ¹² U ¹³ U ¹⁴ U ¹⁵ U ¹⁶ U ¹⁷ U ¹⁸ U ¹⁹ U ²⁰ U ²¹	6	77.8	
		¹ U ² U ³ U ⁴ U ⁵ U ⁶ U ⁷ U ⁸ U ⁹ U ¹⁰ U ¹¹ U ¹² U ¹³ U ¹⁴ U ¹⁵ U ¹⁶ U ¹⁷ U ¹⁸ U ¹⁹ U ²⁰ U ²¹	7	71.2	
		UUUUUCUCC ¹ U ² U ³ U ⁴ UCAGA ⁵ U ⁶ U ⁷ U ⁸ U ⁹ U ¹⁰ U ¹¹ U ¹² U ¹³ U ¹⁴ U ¹⁵ U ¹⁶ U ¹⁷ U ¹⁸ U ¹⁹ U ²⁰ U ²¹	8	81.0	
		UUUUUCUCC ¹ U ² U ³ U ⁴ U ⁵ U ⁶ U ⁷ U ⁸ U ⁹ U ¹⁰ U ¹¹ U ¹² U ¹³ U ¹⁴ U ¹⁵ U ¹⁶ U ¹⁷ U ¹⁸ U ¹⁹ U ²⁰ U ²¹	9	74.0	

Figure 2. Sequences of modified guide-RNAs (1-9) and of passenger RNA used in the RNAi experiments and T_m data. Sites of propynylU (¹P), methylU (¹M) and methylC (¹C) substitutions are indicated in bold. T_ms were measured in 15 mM HEPES-KOH (pH 7.4), 1 mM MgCl₂ and 50 mM KOAc. T_m data from lower ionic strength solution are included in the Supplementary Table S2.

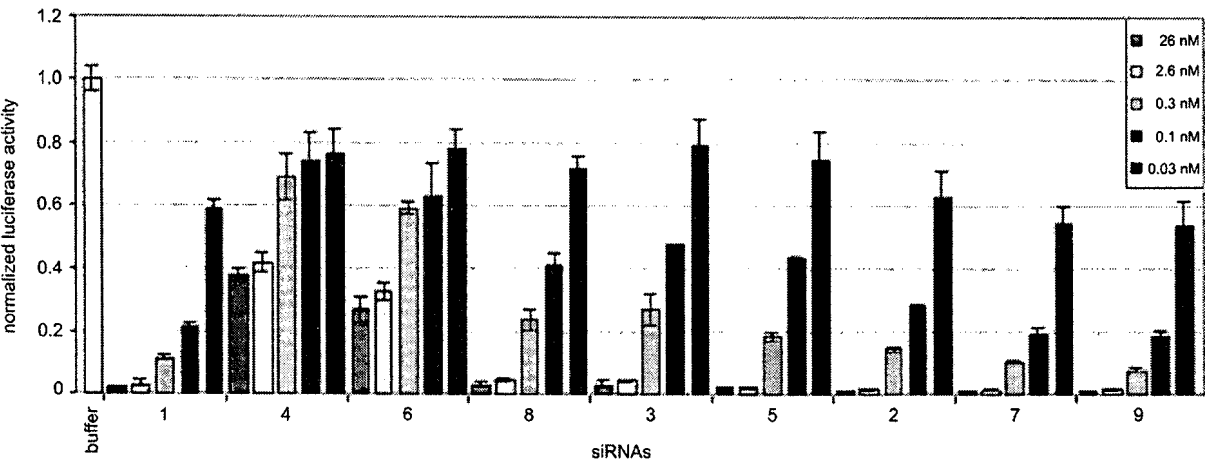


Figure 3. Plot of gene-specific RNAi activity for 5-propynyl and 5-methyl substituted siRNAs at different sites in the *Renilla* luciferase mRNA expressed in HeLa cells. Varied amounts of RNA were added as shown.

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89.0 ± 0.3% for 7, 92.1 ± 0.3% for 9 and 88 ± 0.4% for the wild-type siRNA (1). At all siRNA concentrations from 0.1 nM to 26 nM, the methylated RNAs 7 and 9 showed small but statistically significant increases in gene suppression activity.

To ensure the specificity of the observed effects, sequence-scrambled siRNAs (as negative controls) were used. Cells cotransfected with scrambled versions of the unmodified siRNA 1 and scrambled versions of two of the more active chemically modified siRNAs (2 and 9) (see sequences in Supplementary Figure 1) showed levels of *Renilla* luciferase activity similar to those of cells transfected with plasmids alone (see data in Supplementary Figure 2). The results indicate that the siRNAs used in this study do indeed specifically induce inhibition of expression of the target gene.

The above results showed that the significant increase in thermal stability caused by the 5-propynyl substitution did not have positive effects on activity. As a result, there is no general correlation of T_m with gene suppression ability (Figure 5). However, in contrast to this, the less stabilizing 5-methyl substitution resulted in siRNAs displaying activity at least comparable to that of the unmodified siRNA (Figure 4). Thus the methyl substitution, possibly as a result of its positive influence on stability, favorably affects biological activity, while the larger propynyl group does not.

We hypothesize that this difference between methyl and propynyl substitution may result from steric effects in the RNA duplex. Rana and co-workers reported that RNAi

absolutely requires A-form helix formation between target mRNA and its guiding antisense strand (40). Structurally, methyl and propynyl functionalities located at the position 5 of pyrimidine nucleosides would lie in the major groove of the dsRNA. Our results show that both of these groups stabilize the RNA duplex, and thus likely do not affect major groove structure strongly. However, the RNAi activities suggest that the propynyl substituent, as a bulky group, may sterically hinder protein contacts in the major groove around the 5'-half of the active RISC complex. In contrast, the methyl group is substantially smaller (by ~2.6 Å in length) (45,46), and appears to be well tolerated at all regions of the active siRNA. A recent crystal structure of the PIWI domain of the Ago 2 protein (a key component of RISC) in complex with a 16-nt siRNA-like duplex (47) reveals potential steric clashes between bulky substituents at the C-5 position of U1 and U2 residues and the protein. This structure contains a highly conserved metal-binding site that recognizes the 5'-end of the guide strand. Methyl or propynyl groups introduced at the C-5 position of U1 and U2 residues would point toward the Ile142 and the Tyr124 residues of the PIWI domain, respectively (Figure 6). The distances between the C-5 atom of U1 and the C γ atom of Ile142 (3.99 Å) and between the C-5 atom of U2 and the hydroxyl group of Tyr124 (4.07 Å) suggest that a 5-propynyl substitution might cause a serious steric conflict, whereas a 5-methyl group might be well tolerated. Based on these observations we speculate that such steric clashes might lead to loss of important interactions between the 5'-end of the guide strand and the protein residues involved in its recognition (47,48). On the other hand, 3'-nucleobases of the guide strand are not likely involved in direct contacts with the protein. A crystal structure of the PAZ domain of Ago 2 in complex with a 9-mer siRNA-like duplex, which mimics the 3'-overhanging end of a guide strand bound to a target mRNA (pdb code 1S12) (49), reveals interactions predominantly between the phosphodiester backbone of the 3'-end of the guide strand and the protein. Thus, based on the above described hypotheses one could expect

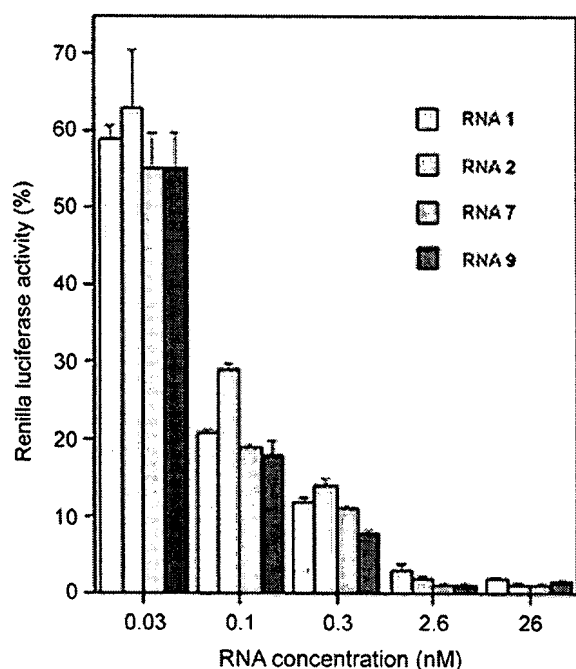


Figure 4. Plot comparing RNAi activity of methylated siRNAs (2, 7, 9) to unmodified siRNA (1). Data are from Figure 3, plotted on a scale that allows for ready comparison of highest activity RNAs. Note the consistently greater gene suppression by 7 and 9 relative to 1.

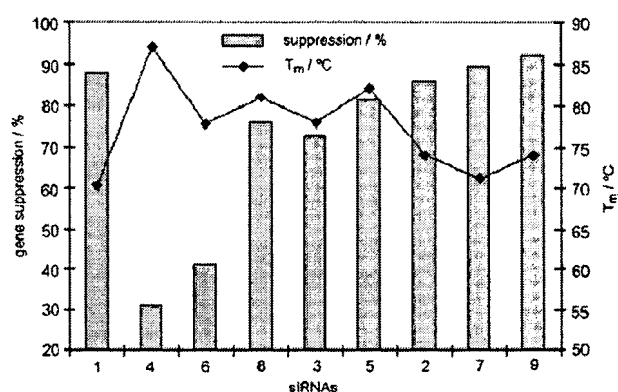


Figure 5. Dual plot comparing helix stability (T_m) and RNA gene knockdown activity (percent suppression) with 5-propynyl and 5-methyl substitutions along the guide strand of siRNA duplexes at different positions. Suppression data are for siRNA concentrations of 0.3 nM.

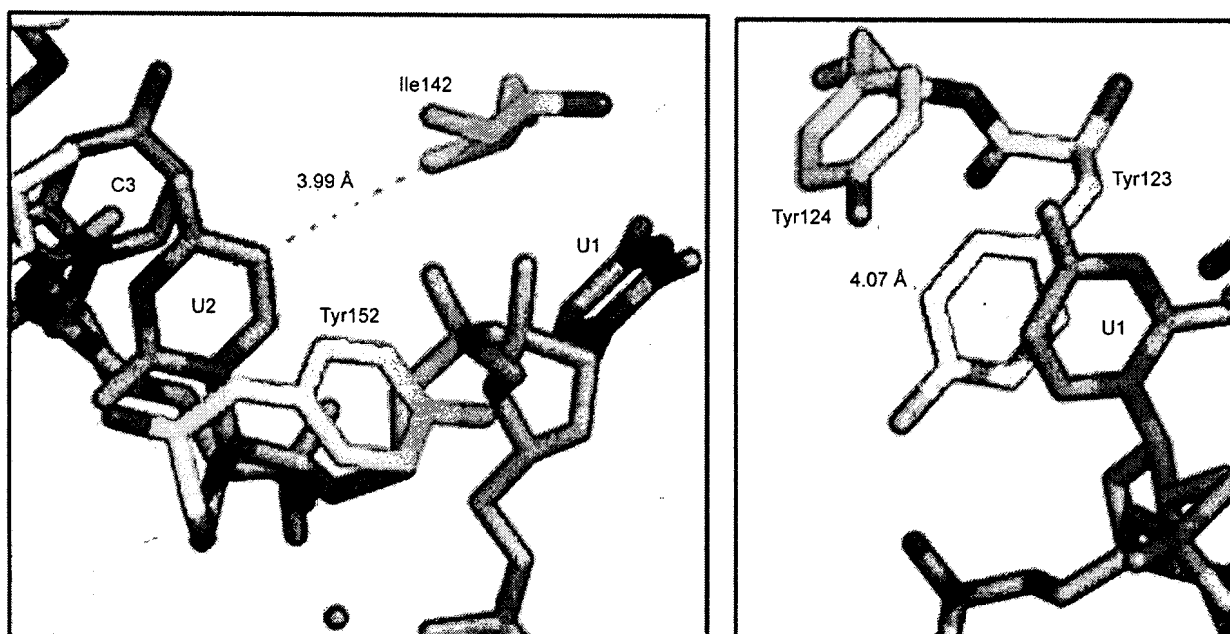


Figure 6. Models of interactions between the PIWI domain and the 5'-nucleobases of a siRNA-like guide strand. Adapted from the crystal structure of a Piwi protein from *Archaeoglobus fulgidus* (AIPiwi) in complex with a siRNA-like duplex (pdb code 2bgg). The figures were prepared with PyMOL (<http://pymol.sourceforge.net/>).

negative effects on RNAi activity for siRNAs having five bulky substituents at positions 1 and 2 of the guide strand. This hypothesis needs further exploration but may provide a satisfactory explanation to the observation that RNAi activity was significantly low for guide-siRNAs having 5-propynyl substitutions at positions 1 and 2 (4 and 6), markedly higher for siRNA 3, which has a 5-propynyl substitution at position 2 and a natural U nucleobase at position 1, and much higher activity for predominantly 3'-propynyl-modified siRNA 8.

Stability of modified siRNAs in human serum

Since the above experiments showed that siRNAs modified with 5-substituted nucleobases could effectively mediate RNAi activity to levels comparable to wild-type RNA, it was of interest to study the serum stability of these oligonucleotides. To carry out such studies, unmodified or modified dsRNAs were incubated in 90% human serum. At various time points, siRNAs were extracted, analyzed on a 20% polyacrylamide gel under denaturing conditions and visualized by staining (Supplementary Data). Unmodified double-stranded siRNA (wt, 1) showed low stability with ~50% and 0% of the original siRNA remaining intact through 30 min and 2 h in serum, respectively. Double-stranded siRNAs with six 5-methylU's on the 5'-half and six 5-methylU on the 3'-half (7 and 9, respectively) demonstrated an increase in stability over the course of the experiment, with half-lives twice that of unmodified guide strand (Figure 7). Similar results were observed for strand 2, which contained five 2-methylC residues at internal positions. Interestingly, double-stranded siRNA having five 5-methylC

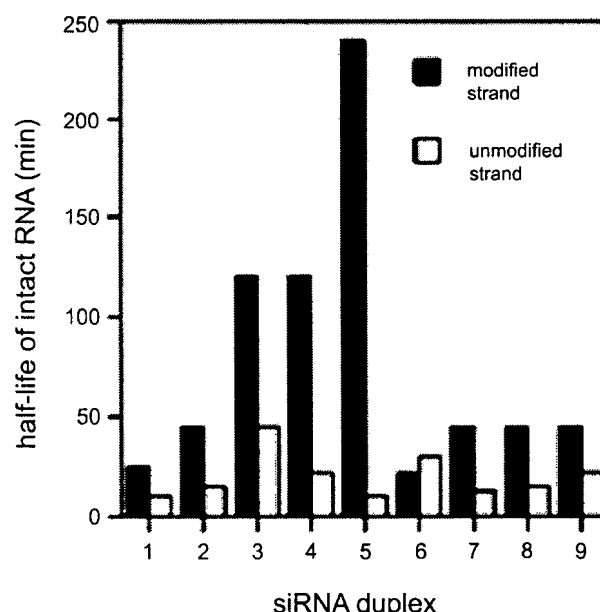


Figure 7. Oligoribonucleotide [modified (guide) and unmodified (passenger) strands] half lives in 90% human serum, from stained gel data. Half-life is defined as the time at which full-length RNA staining density is half that of the density at time zero. Error is estimated at $\pm 30\%$.

and 5-propynylU residues along the antisense strand (5) displayed strongly enhanced stability, with the original siRNA population remaining intact for the duration of the experiment (4 h). In order to determine the effect

of the 5-methyl substitutions on serum stability, the 5-methylC residues of strand 5 were replaced by natural cytidine. Although the resulting modified strand (3) was significantly more stable than the wild-type siRNA (1), it showed strongly decreased stability as compared with the siRNA containing five 5-methylC and five 5-propynylU residues (5), showing the positive effect of propynyl groups. Finally, the results for strand 11, which was fully substituted with 11 propynyl groups, were similar to those observed for 3. Thus, we can conclude that, in general, incorporation of 5-methyl and 5-propynyl substitutions along the guide strand cause an increase in serum stability. The above results further suggest that the half-life of the resulting modified siRNAs likely depends also on the position of the substitution along the guide strand.

We and others (39) have noted that incorporation of 5-methyl substitutions into guide-siRNAs did not disrupt RNAi activity, while in contrast, previous studies with 5-bromo- and 5-iodo-substitutions revealed lowered activity of the siRNAs containing them (24). The origin of this difference is unclear at present, and may be due either to chemical differences associated with methyl versus halogen substitution, or to differences in the RNA targets in the two studies. Although a number of base substitutions have been studied previously in RNAi, only the methyl substitution has shown to yield gene suppression activity comparable to that of substituted RNA.

CONCLUSIONS

In summary, we have evaluated steric and stability effects on RNAi activity by use of siRNAs modified with propynyl and methyl functionalities at the C-5 position of pyrimidine nucleobases. Our results suggest that at the 5'-half of the guide RNA, large increases in the size of the functionality at the position 5 of pyrimidine nucleobases can be detrimental to RNAi activity. Despite the strong stabilizing effects of the 5-propynyl modification, this bulky substitution caused negative effects in RNAi activity, probably due to disruption of interactions in the major groove of the active RISC complex. However, the smaller 5-methyl substitution did not adversely affect gene silencing activity. Furthermore, this modification contributed positively to stability of siRNAs in human serum. Thus, our work offers an avenue for the design of future RNA agents for biomedical applications of siRNAs, and adds support to the notion that modified nucleobases can play an important role in biological activity and sequence selectivity of siRNAs (16–22,24).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank the National Institutes of Health (GM072705) for support. M.T. acknowledges support from Beatriu de Pinós Fellowship (AGAUR, Generalitat de Catalunya, Spain).

FUNDING

National Institutes of Health (GM072705). Funding for open access charge: National Institutes of Health (GM072705).

Conflict of interest statement. None declared.

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ANEXO 12

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Volume 389, 2004, Pages 244–265

Regulators of G-Protein Signaling, Part A



Modular Architecture and Novel Protein
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Given recent efforts to determine the sequence information on thousands of genes in the human genome, the current challenge is to identify the functions of these genes, including those encoding the regulator of G-protein signaling protein gene superfamily, and to establish their roles in particular signaling pathways in a native system. Increasingly, reverse genetic approaches are being used to address these questions. This article compares two powerful approaches [ribozyme and "short interfering" RNA (siRNA) techniques] under identical conditions for the first report on the suppression of endogenous RGS domain-containing RhoGEFs. The siRNA technique was found to be much more potent than ribozyme targeting at the same mRNA site of RGS-RhoGEFs. Also, the three siRNAs targeting LARG, PDZ-RhoGEF, and p115-RhoGEF are able to discriminate the closely related sequences within this RGS-RhoGEF gene family.

Figures and tables from this article:



Fig. 1. Targeting sites for human RGS-RhoGEF siRNA and ribozymes. Different targeting sites for each siRNA (lettered A through E) and ribozymes (RZ) are shown. "inv" indicates mutant siRNA obtained by inverting the sequence of siRNA. Inhibition of SRC-1 promoter/luciferase (Luc) activity was performed as described in the text for screening siRNA function at different targeting sites. The bottom schematic map indicates the relative location of each siRNA/ribozyme on each RGS-RhoGEF gene structure.

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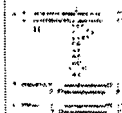


Fig. 2. Structure of human PDZ-RhoGEF hammerhead ribozyme and siRNA. (A) Hammerhead structure of PDZ-RhoGEF ribozyme. The targeted PDZ-RhoGEF mRNA transcript is shown in the 5' to 3' direction (*upper sequence*), and the constructed DNA-RNA chimeric ribozyme is shown in the 3' to 5' direction (*lower sequence*). The targeted GUC trinucleotide in the mRNA is underlined, and the cleavage site is indicated by the arrow. Ribonucleotides required for ribozyme activity in the core region are shown in lowercase letters, and complementary flanking regions of deoxyribonucleotides are shown in capital letters. The two phosphorothioate linkages at the 3' end of ribozyme to enhance the stability are indicated by asterisks. (B) Structure of the PDZ-RhoGEF double-stranded siRNA "PDZ-B." A 18 nucleotide sequence from PDZ-RhoGEF cDNA is selected and synthesized chemically with 3' "TT" overhangs and annealed as double-stranded siRNA. (C) Mutant PDZ-RhoGEF siRNA (PDZnv). The mutant siRNA is designed by inverting the siRNA sequence from the 3' to 5' direction and then annealing to form the double-stranded RNA duplex as a negative control.

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ANEXO 13

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Comparison of the Suppressive Effects of Antisense Oligonucleotides and siRNAs Directed Against the Same Targets in Mammalian Cells

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ANEXO 13

ABSTRACT

RNA interference appears to be a potentially powerful tool for studies of genes of unknown function. However, differences in efficacy at different target sites remain problematic when small interfering RNA (siRNA) is used as an effector. Similar problems are associated with attempts at gene inactivation using antisense oligonucleotides (ODNs) and ribozymes. We performed a comparative analysis of the suppressive effects of three knockdown methods, namely, methods based on RNA interference (RNAi), antisense ODNs, and ribozymes, using a luciferase reporter system. Dose-response experiments revealed that the IC₅₀ value for the siRNA was about 100-fold lower than that of the antisense ODN. Our results provide useful information about the positional effects in RNAi, which might help to improve the design of effective siRNAs.

INTRODUCTION

THE DRAFT SEQUENCE OF THE HUMAN GENOME (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001) includes many genes of unknown function, and identification of these genes is of great importance. Methods for disrupting the expression of specific genes are critical for accurate analysis of the functions of unknown genes, and at present, three major tools, namely, antisense oligonucleotides (ODNs), ribozymes, and RNA interference (RNAi), are available for suppression of the expression of individual specific genes.

Antisense ODNs are short pieces of synthetic, chemically modified DNA that are designed to bind to their target mRNA via Watson-Crick base pairing, thereby specifically inhibiting the expression of the target mRNA (Dove, 2002). As opposed to the apparently simple theory, the mechanism of inhibition by antisense ODNs

seems to involve several complicated steps, including inhibition of translation, splicing, or transport of the target mRNA or degradation of the DNA/RNA hybrid by RNase H.

Ribozymes are RNA molecules with enzymatic activities that bind selectively to and cleave specific target RNAs (Kuwabara et al., 2000a,b; Takagi et al., 2001). It was expected initially that ribozymes might be useful as therapeutic agents and as tools for the functional analysis of genes of interest. However, high-level activities or ribozymes *in vivo* depend on the stability and localization of the transcript, the cleavage activity, and the accessibility of the target RNA to the ribozyme. Nonetheless, a chimeric tRNA^{Val} ribozyme, transcribed at a high level from a pol III promoter, with considerable stability in cells and the capacity for translocation to the cytoplasm, has yielded many successful results in cultured cells and animals (Kato et al., 2001; Koseki et al., 1999; Tanabe et al., 2000).

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RNAi is a recently discovered knockdown phenomenon that is induced by double-stranded RNA (dsRNA) and that has been demonstrated in plants, nematodes, *Drosophila*, protozoa, and mammalian cells (Fire et al., 1998; Fire, 1999; Hammond et al., 2001b; Sharp, 2001; Zamore, 2001; Caplen et al., 2001; Elbashir et al., 2001a). The mechanism of RNAi is not fully understood, but recent genetic and biochemical studies have revealed some details at the molecular level. Inside the cell, dsRNAs are digested into fragments of 21–23 nucleotides (nt), with a 2-nt 3'-overhang, by an RNase III-related enzyme, designated Dicer (Bernstein et al., 2001). Subsequently, these small fragments, known as small interfering RNAs (siRNAs), are incorporated into the RNA-induced silencing complex (RISC) (Hammond et al., 2001a; Nykanen et al., 2001). The active complexes containing the guide RNA recognize and cleave the target RNA.

The activity of siRNA is high even at low concentrations, without apparent toxicity. However, recent reports suggest that the efficacy of siRNA exhibits serious dependence on the target site, as is the case for antisense ODNs and ribozymes, which might significantly limit the conve-

nient use of siRNA (Holen et al., 2002; Miyagishi and Taira, 2002). In the present study, we compared the efficacy of suppression and positional dependency of these knockdown methods, using antisense ODNs and RNAi.

MATERIALS AND METHODS

Cell culture, transfections, and assays of expression of reporter genes

HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed with Lipofectamine™ 2000 reagent (Life Technologies, Rockville, MD) using 1 nM siRNA or 100 nM ribozymes, as described by the manufacturer. For experiments with antisense ODNs, cells were transfected with 30 ng of an RSV-Renilla luciferase plasmid (pRL-RSV) (Miyagishi et al., 2000), 30 ng of a firefly luciferase expression vector pGL3 (Promega, Madison, WI) using FuGENE 6 (Roche Applied Science, Mannheim, Germany), and 200 nM antisense ODNs using Oligofectamine™

Site 1	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-CUUACGCUGAGUACUUCGAUU-3' 5'-UCGAAGUACUCAGCGUAAGUU-3' 5'-CATTTCTGAAGTACTCAGCGT 5'-GGUUGCAACCUGAUGAGGCCGA AAGGCCGAAACUCAGCGUAAG-3'
Site 2	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UUCGUCACAUCUCAUCUACUU-3' 5'-GUAGAUGAGAUGAGACGAAUU-3' 5'-GGAGGTAGATGAGATGTGAC 5'-GGGGUAGAUCUGAUGAGGCCGA AAGGCCGAAAGAUGUGA-3'
Site 3	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UGCCAGAGUCCUUCGAUAGGG-3' 5'-CUAUCGAAGGACUCUGGCACA-3' 5'-CCTATCGAAGGACTCTGGCA-3' 5'-GGATCGAAGCTGATGAGGCCGAA AGGCCGAAAACCTCTGGC-3'
Site 4	siRNA(sense) siRNA(antisense) Antisense ODN	5'-GUGCGCUGCUGGUGCCAACUU-3' 5'-GUUGGCACCAGCAGCGCACUU-3' 5'-TTGGCACCAGCAGCGCACUU-3'
Site 5	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-UAAGGAAGUCGGGGAAGCGGU-3' 5'-CGCUUCCCCGACUCCUUAAG-3' 5'-CCGCTTCCCCGACTTCCTTA-3' 5'-GGCUUCCCCCUGAUGAGGCCGA AAGCCGAAACUCCUU-3'
Site 6	siRNA(sense) siRNA(antisense) Antisense ODN Ribozyme	5'-CAGGUGGCUCCCGCUGAAUUG-3' 5'-AUUCAGCGGGAGCCACCUGAU-3' 5'-AATTCAGCGGGAGCCAGGTG-3' 5'-GGUCAGCGGCUGAUGAGGCCGA AAGGCCGAAAGCCACCU-3'

FIG. 1. Design of siRNAs and antisense ODNs targeted to six sites in the firefly gene for luciferase.

(Life Technologies) in Optimem medium (Life Technologies). Three hours after transfections, the medium was replaced by serum-containing medium. Luciferase activities were analyzed after 24 hours with the dual-luciferase system (Promega).

RNA and antisense ODNs

RNA ODNs were synthesized by an automated synthesizer (model 394) (Applied Biosystems, Foster City, CA). The synthetic RNAs were deprotected and purified by electrophoresis on a denaturing polyacrylamide gel. After elution from the gel, the RNAs were desalted by passage through a NAP-10 column (Amersham Pharmacia Biotech, Piscataway, NJ) in RNase-free water. Fractions were reduced to dryness under a vacuum, and residues were resuspended in annealing buffer (phosphate-buffered saline [PBS], pH 6.8, 2 mM MgCl₂). RNA oligonucleotides (10 μM) were annealed by incubation at 95°C for 1 minute, cooled to 70°C, and subse-

quent slowly cooled to 4°C for 2 hours. The sequences of the sense and antisense strands of negative control siRNA were follows: sense, 5'-CUU UCA GCU UCG AUG UAG GTT-3', antisense, 5'-CCU ACA UCG AAG CUG AAA GTT-3'. HPLC-purified phosphorothioate ODNs were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The sequence of the negative control antisense ODN was 5'-CCA ATG TCA AGC ACT TCC GTT-3'. Ribozymes were synthesized from annealed synthetic ODNs by transcription from a T7 promoter using MEGAShortscript™ T7 kit (Ambion, Austin, TX) and purified by electrophoresis on a urea-polyacrylamide gel (7 M urea, 20% polyacrylamide).

RESULTS

Selection of target sites in firefly gene for luciferase

We used a dual-luciferase assay system to evaluate the suppressive effects of the three knockdown methods, us-

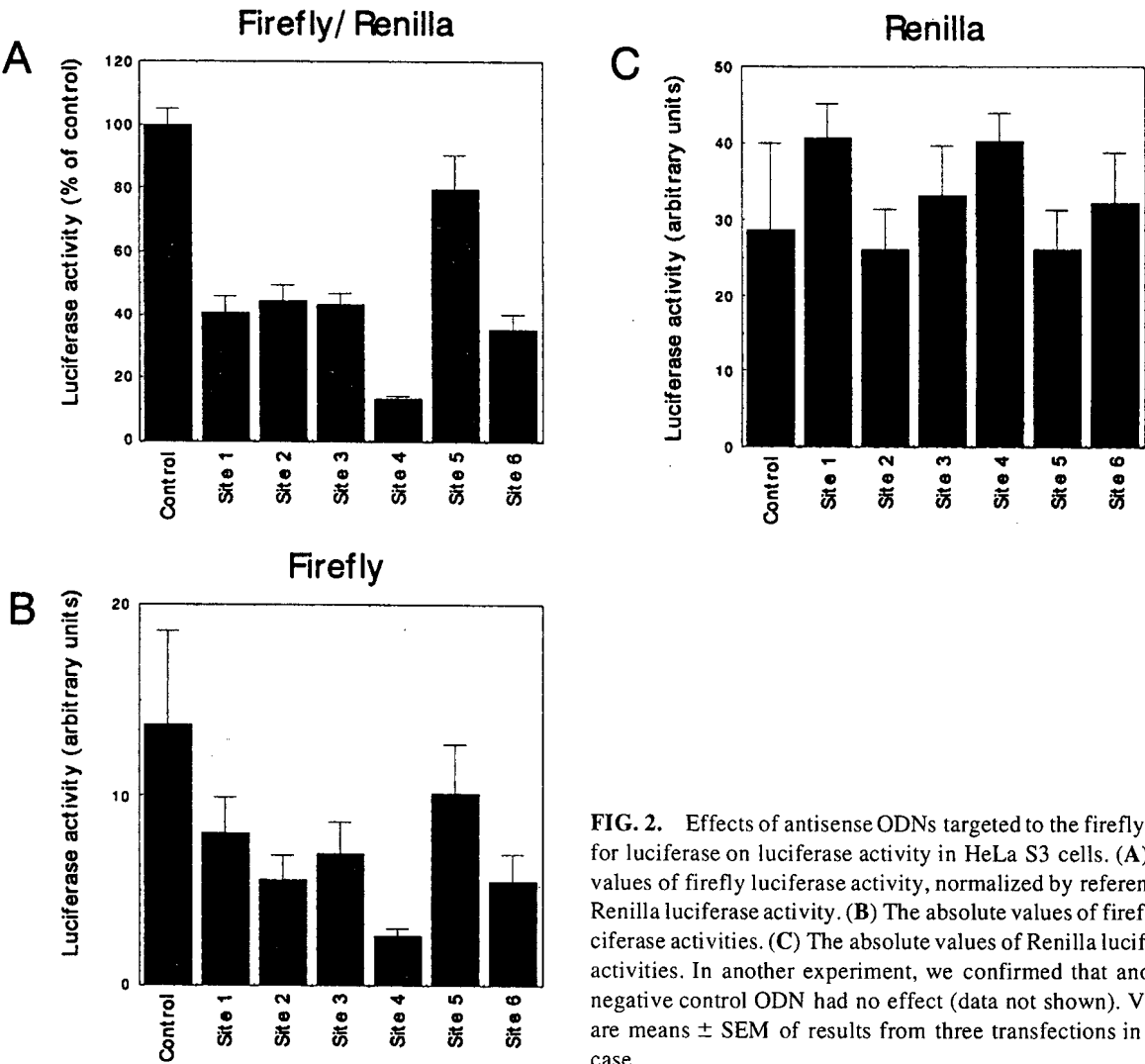


FIG. 2. Effects of antisense ODNs targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. (A) The values of firefly luciferase activity, normalized by reference to Renilla luciferase activity. (B) The absolute values of firefly luciferase activities. (C) The absolute values of Renilla luciferase activities. In another experiment, we confirmed that another, negative control ODN had no effect (data not shown). Values are means ± SEM of results from three transfections in each case.

ing synthetic phosphorothioated antisense ODNs, synthetic siRNAs, and synthetic ribozymes. In this system, we measured firefly and Renilla luciferase activities in the same sample, and suppressive effects on the expression of firefly luciferase were standardized by reference to the expression of Renilla luciferase, which allowed accurate evaluation of the suppressive effects. We selected six target sites within the coding region of the firefly gene for luciferase. Each target sequence included the NUX triplet motif at the center of the sequence, as this is the motif at which cleavage by the hammerhead ribozyme occurs. N can be any residue, and X can be C, U, or A. The length of each arm of the ribozyme was set at 7–9 nt.

In the case of RNAi, the cleavage site for a target RNA has been identified as being 11 nt downstream of the target position that is complementary to the 3'-most nucleotide of the antisense strand of the siRNA (Elbashir et al., 2001a,b). We selected six sites for siRNAs that overlapped the cleavage site as the ribozyme (the cleavage sites were not necessarily identical, but the annealing regions overlapped each other). A length of 21 nt and a 2-nt 3'-overhang were chosen for the siRNA because this form of siRNA was shown to be most effective with *Drosophila* lysate *in vitro* (Elbashir et al., 2001b).

In our antisense experiments, we used 20-nt phosphorothioate-modified oligonucleotides. However, as we used ribozymes as controls for the stability of siRNAs, we did not chemically modify the ribozymes. We used the naked RNAs. Details of the various sense and antisense siRNAs and antisense ODNs are shown in Figure 1.

Suppressive effects of antisense ODNs

In the first experiment, we examined the effects of antisense ODNs against the gene for firefly luciferase. We cotransfected HeLa S3 cells with a series of antisense ODNs, the firefly luciferase expression vector, and the Renilla luciferase expression vector, using various cationic transfection lipids, including Lipofectin™, Lipofectamine, Lipofectamine 2000, FuGENE 6, and Effectene™. The luciferase assays gave poor results because the activities of Renilla luciferase, used as an internal control, were too low or were unstable when we used antisense ODNs at 200 nM or higher concentrations for transfections. High doses of ODNs might have an inhibitory effect on the efficiency of transfection of plasmids, or they might act nonspecifically to block protein synthesis.

Oligofectamine appeared to be a much better reagent for transfection of antisense ODNs. It is a recently developed low-toxicity reagent that was optimized for delivery of ODNs. We continued to use FuGENE 6 for the transfection of plasmids. We obtained greatly improved luciferase activities, with stable values and low toxicity. Us-

ing this procedure, we evaluated the effects of antisense ODNs targeted to six sites in the firefly gene for luciferase. As shown in Figure 2, cotransfections with antisense ODNs that corresponded to site 1, site 2, site 3, site 5, and site 6 of the firefly gene for luciferase plus the firefly luciferase expression vector and the Renilla luciferase expression vector caused decreases in firefly luciferase activity of 59%, 55%, 57%, 23%, and 65% respectively. The antisense ODN targeted to site 4 had stronger suppressive activity (87%) without any negative effects on Renilla luciferase activity, compared with the negative control (Fig. 2C).

Effects of RNAi on expression of firefly gene for luciferase

We transfected cells with siRNAs using the Lipofectamine 2000 reagent. This lipid allows cotransfection of HeLa S3 cells with siRNAs and plasmids at high efficiency (Elbashir et al., 2001a; Miyagishi and Taira, 2002). We examined the suppressive activities of siRNAs that corresponded to the six sites in the firefly gene for luciferase and found that the siRNAs directed against site 1 and site 4 strongly suppressed the luciferase activity (92% and 95%), whereas the corresponding val-

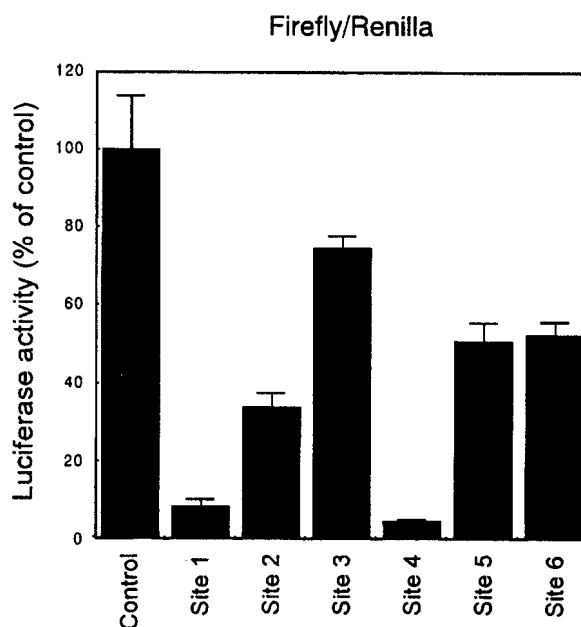


FIG. 3. Effects of siRNAs targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and 1 nM siRNA directed against individual target sites in the firefly gene for luciferase or with control siRNA. Values were normalized by reference to Renilla luciferase activity. Values are means $\pm$ SEM of results from three transfections in each case.

ues for site 2, site 3, site 5, and site 6 were much lower (66%, 25%, 49%, and 48%, respectively) (Fig. 3). The results demonstrated the positional dependence of RNAi, as was also the case for the antisense ODNs. Although the correlation coefficient between the results for antisense ODNs and siRNAs was low (0.42), it is of interest that the highest efficacy was observed at the same posi-

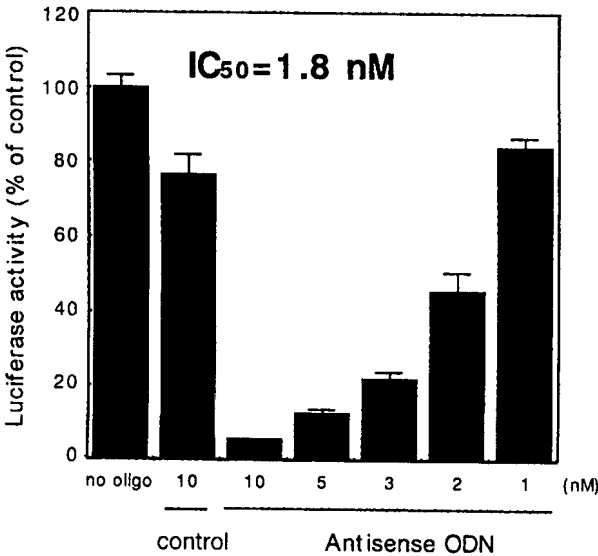
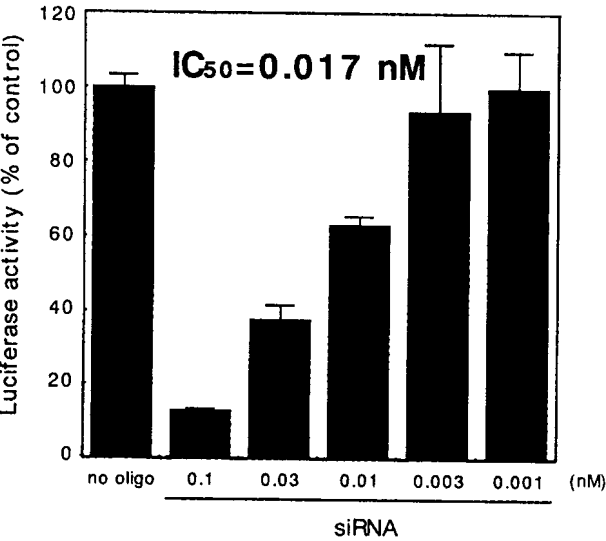


FIG. 4. Dose dependence of siRNAs and antisense ODNs on luciferase activity. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and the indicated amounts of siRNA or antisense ODN directed against site 4 in the firefly gene for luciferase or with control siRNA or control antisense ODN. Values are means $\pm$ SEM of results from three transfections in each case.

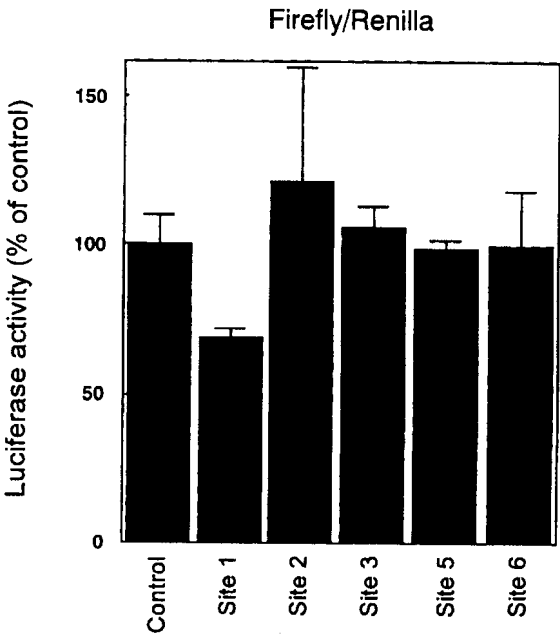


FIG. 5. Effects of ribozymes targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. Values were normalized by reference to Renilla luciferase activity. Values are means $\pm$ SEM of results from three transfections in each case.

tion (site 4), even though the two systems are quite different. The low correlation between the antisense ODN and siRNA may originate from the small difference of target sites for siRNA and ODN at single nucleotide level (especially at site 1 and site 2).

To compare the suppression efficacy of two methods, we performed dose-response experiments of the siRNA and the ODN on the suppression effect of luciferase gene expression at site 4. Under this experimental condition, the inhibitory concentration of 50% reduction for luciferase activity (IC_{50}) for the siRNA and the ODN is 0.017 nM and 1.8 nM, respectively (Fig. 4). It is important to note that the IC_{50} value of the siRNA was about 100-fold lower than that of the antisense ODN.

Suppressive effects of ribozymes

We examined the effects of unmodified ribozymes directed against the six sites in the firefly gene for luciferase. We demonstrated previously that ribozymes function effectively in cells when transcribed as tRNA constructs under the control of a pol III promoter (Kato et al., 2001; Koseki et al., 1999; Tanabe et al., 2000). However, in the present study, in order to compare the effects of concentration as an index of stability in cells between siRNAs and ribozymes, we introduced naked RNA ribozymes directly into cells, in the same way as we had introduced antisense ODNs and siRNAs, in order to evaluate directly the activities of siRNAs and ribozyme. Ribozymes

were synthesized by T7 polymerase, purified by electrophoresis, and introduced into HeLa S3 cells with the firefly luciferase expression vector and the Renilla luciferase expression vector under the same conditions as those used for siRNA, with the exception of significantly higher final concentrations (100 nM; note that the concentration of siRNA was 1 nM; with Lipofectamine 2000 reagent) (Fig. 5). Even at these higher concentrations, we observe no significant reduction in the expression of firefly luciferase (data not shown).

DISCUSSION

The dual-luciferase system allows the accurate evaluation of gene-suppressive effects and has been used widely in analyses of the mechanism of RNAi (Elbashir et al., 2001a,b; Miyagishi and Taira, 2002). Nonetheless, there are only a few reports of application of the system to analysis of the effects of antisense ODNs (Faria et al., 2001). In present study, we established a system that allowed us to evaluate antisense effects accurately by use of different cationic lipids for antisense ODNs and reporter plasmids. Our protocol allowed us to compare site-dependent effects between antisense ODNs and siRNAs.

Analysis of the effects of the siRNAs targeted to six sites in the firefly gene for luciferase revealed that positional effects were not well correlated with positional effects on the efficacy of antisense ODNs, but both an siRNA and an antisense ODN were most effective when directed against site 4. This result implies that a major factor in site dependence of the effect of siRNA might be accessibility to the target mRNA, as is also the case for antisense ODNs and ribozymes (Kawasaki et al., 2002; Kawasaki and Taira, 2002; Krupp and Gaur, 2000; Warashina et al., 2001). It is possible that the effects of siRNA and ODN might share some common features. However, we could not find any significant correlation between structures of mRNA, as predicted by Mfold (Zuker and Jacobson, 1998), and the suppressive effects of siRNAs. However, more experimental data about the accessibility of antisense ODN to target RNA *in vitro* and *in vivo* are needed for more precise estimations of such a relationship.

In our experiment with naked ribozymes, we failed to detect suppressive activity even at a concentration of ribozyme of 100 nM during transfection, despite the apparent repressive activity of siRNA under similar conditions at a concentration of 1 nM. This result was most likely due to the poor resistance to nuclease of naked single-stranded ribozymes in cells. By contrast, siRNAs, which are 21–23-nt RNA duplexes with a 2-nt or 3-nt 3'-overhang, are considered to be much more resistant than ribozymes to nucleases. Indeed, we showed previously that an siRNA duplex was significantly more stable in

cells than the cognate single-stranded sense or antisense RNA, with transcription under the control of the identical promoter in each case (Miyagishi and Taira, 2002). Double-stranded siRNAs appear to be protected by RISC from attacks by RNases, whereas ssRNA and ribozymes are not protected by such proteins in cells. Thus, constitutive expression of ribozymes from vectors is required for the effective exploitation of ribozymes.

In our comparative analysis of three major methods for suppression of gene expression, we found a low but significant correlation between the effects of siRNA and antisense ODN. Our results led to the hypothesis that one of the major factors responsible for the dependence of the activity of siRNA on its target site is accessibility to the target mRNA. This working hypothesis should help us focus on such effects and help us to clarify the mechanisms of positional effects in the suppression of gene expression.

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ANEXO 14

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REVIEW

ANEX 024

Hiroaki Kawasaki · Renu Wadhwa · Kazunari Taira

World of small RNAs: from ribozymes to siRNA and miRNA

Received November 7, 2003; accepted December 11, 2003

Abstract RNAs, besides bridging genetic information to proteins, the major determinants of bio-structures and functions, serve as active regulators of gene expression. Initiated nearly 20 years ago with ribozymes (the small RNAs with catalytic activity providing fine tuning of gene expression and function, used as molecular scissors and tools for gene discovery), an era of more complex and coordinated gene regulation by small RNAs, siRNA, and miRNA has recently started. Simple nucleotide complementarity results in highly ordered and regulated events, such as assembly of RNA and proteins, resulting in gene silencing either by mRNA degradation or suppression of translation. This article reviews our contributions to the understanding of structure, the function of small RNAs, their use in biotechnology, and the understanding of phenotypes such as apoptosis, metastasis, and differentiation.

Key words ribozymes · siRNA · miRNA · regulation of gene expression · neuronal differentiation

The world of small RNA inaugurated by the ribozyme: from gene silencing to gene discovery

RNA was previously viewed as a mere bridge between DNA (reservoir of all genetic information) and protein

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(determinants of biological structure, function, and integrity). Its perception as a simple vehicle for deciphering the genetic code to a translatable readout changed about 20 years ago when RNA with catalytic activity, ribozyme—RNA molecule with enzymatic properties—were discovered. Hammerhead ribozymes (because of the resemblance of their two-dimensional structure to a hammerhead) are the smallest ribozymes that are used as molecular scissors in molecular biology and biotechnology to elucidate and eliminate gene functions (Haseloff and Gerlach, 1988; Rossi, 1999). The RNA is induced to fold into its active conformation by the binding of metal ions. It forms two domains: the scaffold (domain 2), on which the ribozyme is built, and the active center (catalytic domain called domain 1) of the ribozyme. Over the last two decades, mechanisms of action of hammerhead ribozymes describing the requirement of divalent metal ions, definition of catalytic domains, and sequence specificity (usually referred to as target site specificity) have been largely demonstrated (Minoshima et al., 2003).

Ribozyme activity *in vivo* critically depends on its effective level of expression, specificity, intracellular stability, target co-localization, and accessibility to the target site. These technical issues impose a major difficulty in the use of ribozymes *in vivo*. Various modifications of plasmids to express ribozymes in cells have thus come into play. It was shown that the ribozymes expressed under the control of a modified RNA polymerase III promoter (tRNA^{Val} promoter) were efficiently expressed, highly stable, and exported to the cytoplasm (Koseki et al., 1999; Kato et al., 2001; Kuwabara et al., 2001a, 2001b). Such expression improved ribozyme activity *in vivo* by many fold. However, efficiently expressed and highly stable ribozymes may still lack activity due to their inaccessibility to the target sites that in turn impose a major hurdle because of unforeseeable secondary and tertiary RNA structures. The rate-limiting step for the cleavage of phosphodiester bonds *in vivo* is the association and annealing of the ribozyme with its target site (Kato et al., 2001; Warashina et al., 2001; Kawasaki et al.,

2002). In a long RNA molecule, with its secondary and tertiary folded structure, a significant number of target sites are inaccessible to the ribozyme. This phenomenon is often a serious problem in attempts to exploit ribozyme activity, in particular *in vivo*. To overcome the problems of accessibility, computer-generated predictions of secondary structure can be typically used to identify target sites that are most likely to be in an open and accessible conformation. However, such predictions are not always accurate because of unpredictable interactions between RNAs and proteins that influence the intracellular structures of RNAs.

A significant recent modification of ribozymes that overcomes their target inaccessibility has been demonstrated. In this modification, a helicase-binding motif that recruits helicase protein and its unwinding activity *in vivo* was linked to the 3' end of the ribozyme sequence in the RNA polymerase III promoter (tRNA^{Val})-driven expression plasmid (Warashina et al., 2001; Kawasaki and Taira, 2002a; Fig. 1). Such RNA helicase-coupled ribozymes were indeed shown to have substrate-unwinding as well as strong cleavage activity in *in vitro* and *in vivo* assays (Warashina et al., 2001; Kawasaki and Taira, 2002a; Kawasaki et al., 2002) and were effective where conventional ribozymes did not cause any change in gene expression (Wadhwa et al., 2003). Subsequent to this significant modification of ribozymes, it became possible to prepare effective libraries of hybrid ribozymes with randomized binding arms and to examine their effects after being introduced into cells (Kawasaki and Taira, 2002b; Kawasaki et al., 2002; Suyama et al., 2003a, 2003b). This led us to develop a novel approach for cloning functional genes. Briefly, libraries of hybrid ribozymes in which the target arm sequence was randomized were generated. These were introduced into cells by either plasmid or retroviral vectors and were combined with the selection of a loss or gain of a cellular phenotype. Isolation of ribozymes from selected cells, sequence analysis, and identification of genes in databases could lead to the identification of genes involved in these phenotypes (Fig. 2). The application of the ribozyme-based gene discovery system to apoptosis, metastasis, Alzheimer's disease, and muscle differentiation led to the isolation of several genes that are implicated in these phenotypes, confirming the validity and usefulness of this technology (Kruger et al., 2000; Li et al., 2000; Welch et al., 2000; Begger et al., 2001; Kawasaki and Taira, 2002a, 2002b; Kawasaki et al., 2002; Nelson et al., 2003; Suyama et al., 2003a, 2003b; Onuki et al., 2004; R.W. et al., unpublished data). Besides, many novel genes have been isolated, supporting it as an effective tool to isolate new genes and elucidate novel functional pathways. This system has also led to the isolation of small RNAs involved in regulation of neuronal differentiation (T. Kuwabara et al., personal communication).

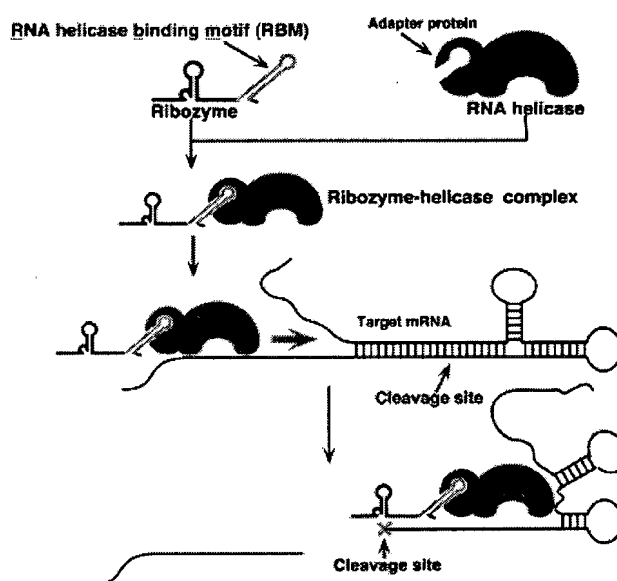


Fig. 1 Schematic representation of cleavage of a normally inaccessible target site by a sliding hybrid ribozyme. The ribozyme is linked to an RNA helicase-binding motif (RBM or connector RNA) that is capable of recruiting a helicase(s) to the target site where it unwinds the substrate structure to expose the cleavage site. The helicase might even be able to slide the ribozyme along the transcript. The coupling of helicase activity to the ribozyme allows suppression of the expression of genes whose transcripts were found previously resistant to cleavage by the conventional ribozyme.

Another significant modification of ribozymes was the construction of allosterically controllable ribozymes that bind to two different target sites (Kuwabara et al., 1999). Such ribozymes were effectively used for suppression of genetic disorders that arise from gene

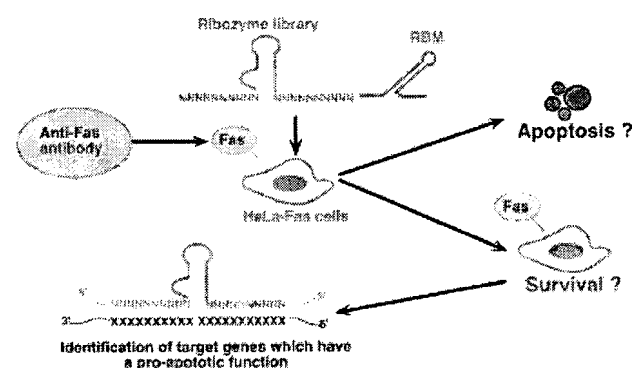


Fig. 2 Schematic presentation of a gene discovery system that can identify functional genes in the Fas-mediated pathway to apoptosis. Randomized RBM-connected ribozyme libraries were introduced into HeLa-Fas cells using the retrovirus expression system. After infection with the randomized RBM-Rz library expressing retrovirus, HeLa-Fas cells that expressed the randomized library were treated with Fas-specific antibody (α -Fas). Surviving clones were picked up and genomic DNA was purified from each clone. Sequences of RBM-connected ribozyme were determined by direct sequencing and the target genes of ribozymes were identified in databases by a BLAST search.

rearrangements. For example, chronic myelogenous leukemia is a hematopoietic malignant disease associated with the expression of a chimeric *BCR-ABL* gene. A ribozyme that can disrupt chimeric *BCR-ABL* mRNA exclusively and is neutral to normal *ABL* mRNA was designed and shown to specifically cleave *BCR-ABL* mRNA (Kuwabara et al., 1998; Tanabe et al., 2000).

The world of small RNA extended to siRNA and miRNA

In the post-genome era, when curiosity in the field of biology centered on "how gene expression and function are regulated," an amazing discovery in biological regulation flashed in science news and made its way to the top scientific journals. The articles, "brave new world of RNA," "a small fortune," "tiny regulators with great potential," "small RNAs: the genome's guiding hand," "something new under the sun," "breakthrough of the year," "small RNAs make big splash," and "big things from little RNA" talked about regulation of gene expression by small RNAs (Ambros, 2001; Couzin, 2002; Moss and Poethig, 2002; Pasquini, 2002; Moberg and Hariharan, 2003). These tiny regulators come from the large proportion of non-coding genome referred to as junk DNA and regulate the fate of cells, not by coding for any proteins with functional motifs, but by causing gene silencing by a highly coordinated and sequence-specific mechanism known as RNA interference (RNAi) and/or microRNA-mediated regulation. Small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs), have emerged as the key components of an evolutionarily conserved system of RNA-based gene regulation in eukaryotes. They are involved in molecular interactions, including defense against viruses and regulation of gene expression during development (Cullen, 2002; Carrington and Ambros, 2003). How these 21- to 23-nucleotide (nt) small non-coding RNAs can act as small interfering RNAs (siRNAs) or miRNAs is just beginning to be understood. The two kinds of small RNAs, that is, siRNAs and miRNAs, are produced by the cleavage of double-stranded RNA precursors by Dicer, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein et al., 2001; Provost et al., 2002; Zhang et al., 2002). siRNAs are 21-nt double-stranded RNAs that contain 19 base pairs, with 2-nt, 3' overhanging ends, that act as functional intermediates in RNAi. These are formed in cells when transposons, viruses, or endogenous genes express long dsRNA or when dsRNA is introduced experimentally. In contrast, single-stranded miRNAs are the products of endogenous, non-coding genes whose precursor RNA transcripts can form small stem loops from which

mature miRNAs are produced by Dicer (Ambros et al., 2003; Lim et al., 2003a, 2003b). miRNAs are encoded in genes distinct from the mRNAs whose expression they control. In contrast to siRNA, which can direct RNA destruction mediated by RISC (RNA-induced silencing complex; functions as an siRNA-directed endonuclease), miRNAs suppress protein expression by inhibition of mRNA translation (Fig. 3). Because both siRNAs and miRNAs are found in similar, if not identical, complexes, it is suggested that RISC might be a bi-functional complex that mediates both cleavage and translational control (Mourelatos et al., 2002). Furthermore, recent evidence suggests that siRNAs and miRNAs are functionally interchangeable, in which the target mRNA can be either cleaved or translationally repressed as determined by the degree of complementarity between the small RNA and its target (Hutvagner and Zamore, 2002; Doench et al., 2003; Zeng et al., 2003).

MicroRNAs (*miR* genes) are a large family of highly conserved non-coding genes transcribed as short hairpin precursors (~70 nt) that are processed into active 21- to 22-nt RNAs by a ribonuclease, Dicer, which recognizes target mRNAs via base-pairing interactions. The miRNA-miRNP (ribonucleoprotein) complex represses mRNA translation by partial base pairing to the 3' UTR of target mRNAs. Small RNA of 18–25 bases called microRNA (miRNA) was reported for the first time by the group of Victor Ambros (Dartmouth College) in 1993. They found that miRNA that interacts with the 3' UTR of *lin-4* mRNA regulates nematode

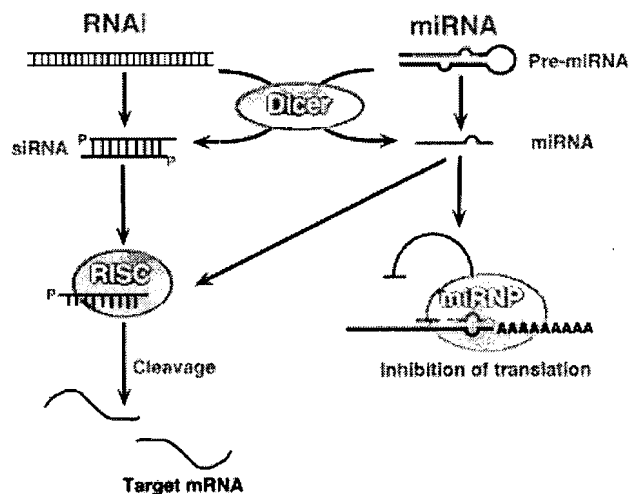


Fig. 3 Diagram to show siRNA and miRNA action. siRNAs are produced from long dsRNAs by ribonuclease Dicer. Then siRNAs are incorporated in RISC and the RISC cleaves the target RNAs. MicroRNAs are expressed as short hairpin RNAs (pre-miRNAs) and are transported to the cytoplasm. Pre-miRNAs are processed to mature miRNAs by Dicer. The miRNAs are incorporated in miRNPs and then miRNPs inhibit translation of target mRNAs by forming partial duplexes with target mRNAs. In addition, miRNA can function as siRNAs in the case of complete matching with target mRNAs.

development. Similarly, miRNA to *let-7* mRNA was identified. Both *lin-4* and *let-7* act as repressors of their respective target genes, such as *lin-14*, *lin-28*, and *lin-41*. Both *lin-4* and *let-7* small temporal RNAs were shown to play a central role in controlling the timing of *Caenorhabditis elegans* cell fate decisions. *let-7* has been conserved through evolution, and its expression correlates with adult development in animals, including *Drosophila* (Bashirullah et al., 2003). A best match for *lin-4* in *Drosophila*, miR-125, is also expressed during pupal and adult stages of *Drosophila* development (Sempere et al., 2003). To date, over 300 miRNAs from diverse eukaryotic organisms have been identified. Annotation of new miRNAs, so that miRNAs can be reliably distinguished from siRNAs, has been proposed along with the establishment of the Rfam database of RNA families (Ambros, 2001, 2003; Ambros et al., 2003; Grad et al., 2003). How these tiny RNAs work is only beginning to be understood; the major hurdle is to identify their target mRNAs. The fact that makes it even more arduous is that miRNA require only a partial homology (in some cases, about 70%). Some of the *Drosophila* miRNAs that align to the K box motif were shown to mediate a negative post-transcriptional regulation of the Hairy *HES* gene family in *Drosophila* (Lai and Posakony, 1997; Lai et al., 1998; Lai, 2002). The human miR-23 containing the antisense sequence to the K box motif was also identified, of which the target gene is not known.

We initiated a study to investigate whether human Hairy *HES* gene is the target of human miR-23 (Kawasaki and Taira, 2003). *HES1* is a basic helix-loop-helix (bHLH) transcriptional repressor that is expressed in undifferentiated cells but not in differentiated cells (Kageyama et al., 2000). It participates in the Notch signaling pathway in mammals and acts as an anti-differentiation factor. miR-23 aligns to a coding region of human *HES1* mRNA near the termination codon and to mouse *Hes1* mRNA (nearly the same position as human *HES1* including the stop codon; Fig. 4a). A duplex of *HES1* and miR-23 was also observed using a prediction program of mRNA secondary structure (Mulfold), suggesting that miR-23 may be conserved phylogenetically as a regulator of human and mouse *Hes1*.

We used NT2 cells as a model for neuronal differentiation. When subjected to retinoic acid (RA)-induced differentiation, NT2 cells showed suppression of *Hes1* expression that was independent of the level of mRNA, as detected by Northern analysis. miR-23, on the other hand, was easily detected in differentiated NT2 cells in contrast to undifferentiated cells. Introduction of the synthetic wild-type miR-23, but not the mutant miR-23, into undifferentiated NT2 cells resulted in the reduction of *Hes1* protein. Of note, the level of *HES1* mRNA did not change in response to the synthetic miR-23, suggesting that synthetic miR-23

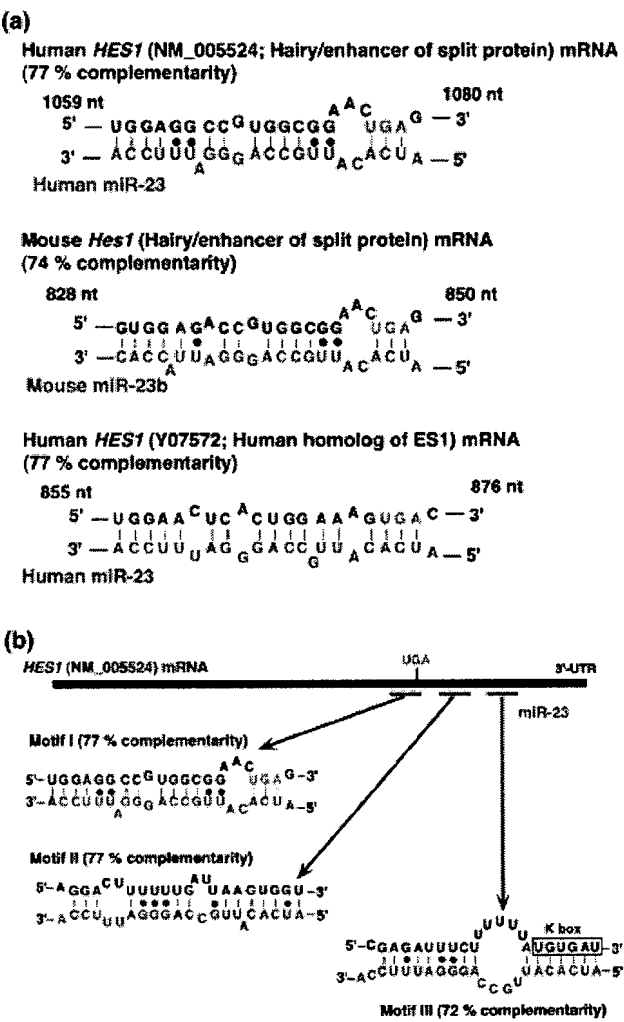


Fig. 4 *HES1* is a target of miR-23. (a) The prediction of secondary structures between miR-23 and its target RNAs. The region nearly complementary to human and mouse miR-23 (blue) is located in the coding region, near the termination codon (red) of human Hairy *HES1* (NM_005524), mouse *Hes1*, and human homolog *HES1* (Y07572) mRNAs (top). (b) Human Hairy *HES1* (NM_005524) mRNA has three target regions (motifs I, II, and III) of miR-23 (bottom). Motif III has a K box sequence (black box) that is known, at least in the case of *Drosophila*, to be involved in post-transcriptional negative regulation.

causes gene silencing at the translational level. As expected, targeting of endogenous miR-23 by synthetic siRNA (siRNA-miR-23) indeed resulted in an elevation of *HES1* protein, but the mutant siRNA-miR-23 had no effect. These data strongly support that *HES1* is a target for miR-23. NT2 cells did not undergo RA-induced neuronal differentiation (as confirmed by cell morphology and differentiation markers such as MAP2) in the presence of siRNA-miR-23, suggesting that miR-23 plays a critical role during RA-induced neuronal differentiation of NT2 cells. The functional regulation of *HES1* by an miRNA mechanism was further confirmed by using two more miRNA motifs (Fig. 4b)

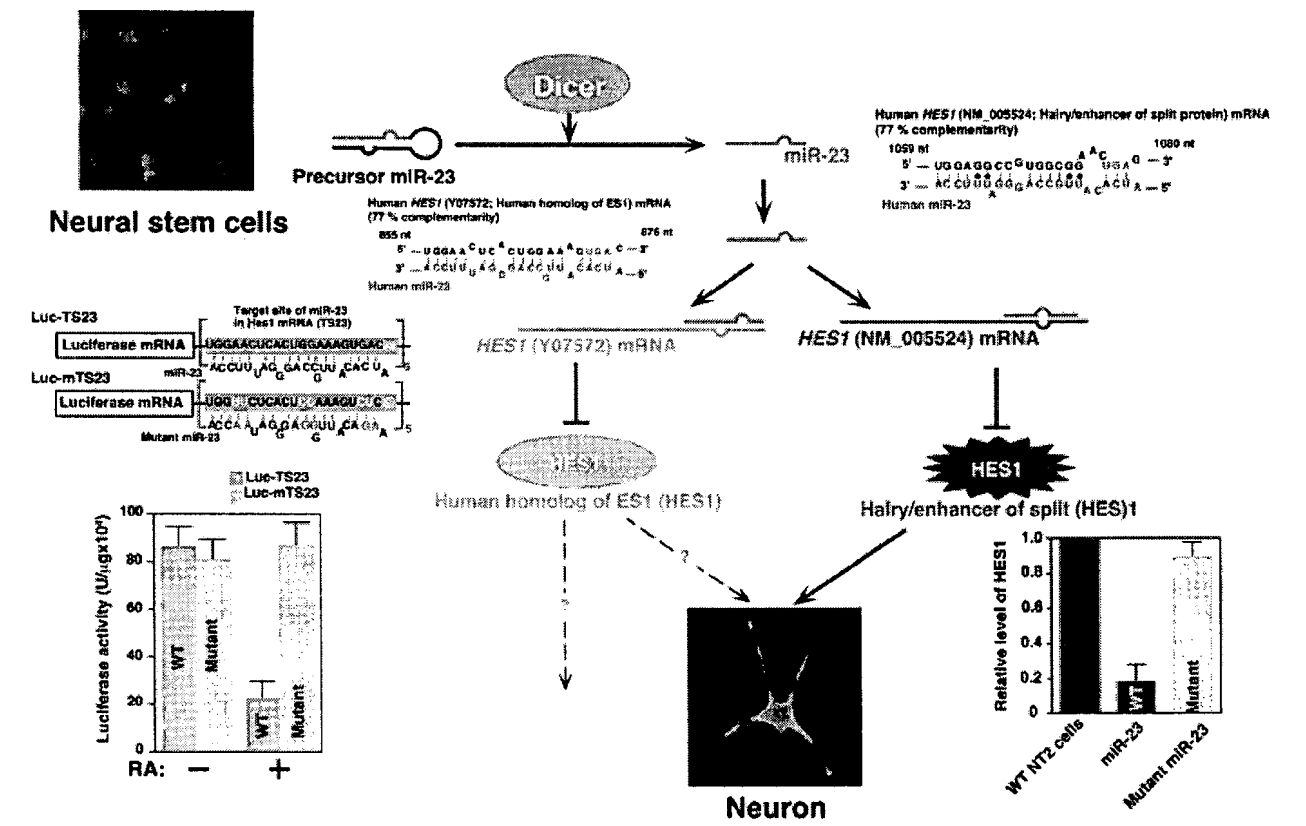


Fig.5 The model of the function of miR-23 in RA-induced differentiation. When cells were treated with RA, pre-miR-23 is expressed. Then the pre-miR-23 is processed to mature miR-23 by ribonuclease Dicer. The mature miR-23 negatively regulates expression of Hairy *HES1* gene strongly and homolog *HES1* gene weakly. Then these events promote neuronal differentiation of NT2

cells. Left lower panel: sequences of genes for Luc-TS23 and mutant Luc-TS23 (Luc-mTS23). The activity of luciferase, due to the reporter genes, in NT2 cells in the presence and absence of RA. Right lower panel: the level of HES1 mRNA in undifferentiated NT2 cells that had been treated with synthetic miR-23 or synthetic mutant miR-23.

and their various combinations along with the original miR-23 (Figs. 4b,5), which happened to match another cDNA also called *HES1* (human homolog of *Escherichia coli* and zebrafish; GenBank accession number Y07572; Scott et al., 1997; Kawasaki and Taira, 2003; unpublished data). We were informed of this ambiguity by Debbie Marks (Columbia University). Hairy/enhancer of split protein, also called *HES1* gene (accession number NM_005524; Feder et al., 1994), which actually encodes the transcriptional repressor HES1, was the subject of our study and is referred to as Hairy *HES1* (Kawasaki and Taira, 2003). Of note, in our study, the HES1 protein was detected by anti-HES1 antibody and the miR-23 was seen to suppress HES1 protein levels (Kawasaki and Taira, 2003). Thus, although the database sequence homologies raise doubts on the validity of the data demonstrating hairy HES1 as a target for miR-23, protein analysis stands by it.

To further clarify this issue, we investigated the effect of two additional independent miRNA target motifs (Fig. 4b) that were found in the 3' UTR of Hairy *HES1* mRNA and did not match to homolog *HES1* (Fig. 5). Analysis of the activity of the luciferase reporter that

was linked to the 5' end of the miR-23 target site or the sequence of the other two unique potential target motifs in Hairy *HES1* mRNA revealed that the three independent target motifs (Fig. 4b) act as targets of miR-23 in Hairy *HES1* mRNA; each of these sites have only about 72–77% homology to the target. Thus, although detoured by the long route of confirmation and validation, the consequence of the study remains unchanged in describing that HES1 is regulated by miRNA such as miR-23.

A model for the function of miR-23 in regulation of RA-induced neuronal differentiation of NT2 cells is proposed (Fig. 5). When cells were treated with RA, pre-miR-23 is expressed. The pre-miR-23 is then processed to mature miR-23 by the ribonuclease Dicer. The mature miR-23 negatively regulates expression of Hairy *HES1* gene strongly and homolog *HES1* gene weakly. These events then promote neuronal differentiation of NT2 cells. However, the mechanism of expression of pre-miR-23 and inhibition of translation of both *HES1* mRNAs by miR-23 remains to be addressed in future studies.

In an independent study, we were compelled to recall what we referred to as pseudo-positives in our screens to

search for novel genes using a ribozyme-based discovery system. Many of the target sequences were ignored and forgotten because they did not belong to the coding genome. Those of us who could not ignore the pseudopositives won this time. T. Kuwabara et al. (2004) used a randomized ribozyme library to isolate genes involved in neuronal development and isolated small RNAs that determine the fate of cells. It is becoming clear that nature has structured sequence information to embed in it not only structural and functional aspects of proteins but also non-coding regulatory elements such as siRNA and miRNA. An era of an unexplored functional RNA world has begun, and is predicted to go a long way in providing information for the understanding of the regulation of biological phenotypes.

Note added in proof: Kawasaki, H. and Taira, K. (2003) Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. *Nature* 423, 838-842. In this Article, the mRNA that is identified to be a target of microRNA-23 (miR-23) is from the gene termed human 'homolog of ES1' (HES1), accessionnumber Y07572, and not from the gene encoding the transcriptional repressor Hairy enhancer of split HES1 (accession number NM_00524) as stated in this paper. We incorrectly identified the gene because of the confusing nomenclature. Our experiments in NT2 cells had revealed that the protein levels of the repressor Hes1 were diminished by miR-23. Although we have unpublished data that suggest that miR-23 also interacts with Hes1 repressor mRNA specially in NT2 cells, given the incorrect use of the probe for the detection of the target mRNA resulting from our error, we respectfully retracted this paper [Kawasaki, H. and Taira, K. (2003). retraction: Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. *Nature* 426, 100].

Acknowledgments This study was partly supported by grants from the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

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ANEXO 15

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Evidence that siRNAs Function as Guides, Not Primers, in the *Drosophila* and Human RNAi Pathways

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Summary

In *Drosophila*, two features of small interfering RNA (siRNA) structure—5' phosphates and 3' hydroxyls—are reported to be essential for RNA interference (RNAi). Here, we show that as in *Drosophila*, a 5' phosphate is required for siRNA function in human HeLa cells. In contrast, we find no evidence in flies or humans for a role in RNAi for the siRNA 3' hydroxyl group. Our in vitro data suggest that in both flies and mammals, each siRNA guides endonucleolytic cleavage of the target RNA at a single site. We conclude that the underlying mechanism of RNAi is conserved between flies and mammals and that RNA-dependent RNA polymerases are not required for RNAi in these organisms.

Introduction

In diverse eukaryotes, double-stranded RNA (dsRNA) triggers the destruction of mRNA sharing sequence with the double strand (Hutvagner and Zamore, 2002b; Hanon, 2002). In animals and basal eukaryotes, this process is called RNA interference (RNAi) (Fire et al., 1998). There is now wide agreement that RNAi is initiated by the conversion of dsRNA into 21–23 nt fragments by the multidomain RNase III enzyme, Dicer (Billy et al., 2001; Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Martens et al., 2002). These short RNAs are known as small interfering RNAs (siRNAs), and they direct the degradation of target RNAs complementary to the siRNA sequence (Zamore et al., 2000; Elbashir et al., 2001c, 2001b, 2001a; Nykänen et al., 2001; Elbashir et al., 2002). In addition to its role in initiating RNAi, Dicer also cleaves ~70 nt precursor RNA stem-loop structures into single-stranded 21–23 nt RNAs known as microRNAs (miRNAs; Hutvagner et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Reinhart et al., 2002). Like siRNAs, miRNAs bear 5' monophosphate and 3' hydroxyl groups, the signatures of RNase III cleavage products (Hutvagner et al., 2001; Elbashir et al., 2001b). miRNAs are hypothesized to function in animals as translational repressors (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996; Moss et al., 1997; Olsen and Ambros, 1999; Reinhart et al., 2000; Zeng et al., 2002; Seggerson et al., 2002). The conversion of dsRNA into siRNAs requires additional protein cofactors that

may recruit the dsRNA to Dicer or stabilize the siRNA products (Tabara et al., 1999; Hammond et al., 2001; Grishok et al., 2001; Tabara et al., 2002). How siRNAs direct target cleavage and whether a single mechanism explains the function of siRNAs in posttranscriptional gene silencing in plants, quelling in fungi, and RNAi in animals remain unknown. Furthermore, how siRNAs are permitted to enter the RNAi pathway while other 21–23 nt RNAs seem to be excluded cannot yet be fully explained.

Three models have been proposed for RNAi in *Drosophila*. Each model seeks to explain the mechanism by which siRNAs direct target RNA destruction. In one model (Figure 1), target destruction requires an RNA-dependent RNA polymerase (RdRP) to convert the target mRNA into dsRNA (Lipardi et al., 2001). The RdRP is hypothesized to use single-stranded siRNAs as primers for the target RNA-templated synthesis of complementary RNA (cRNA). The resulting cRNA/target RNA hybrid is proposed to then be cleaved by Dicer, destroying the mRNA and generating new siRNAs in the process. Key features of this model are that the ATP-dependent, dsRNA-specific endonuclease Dicer acts twice in the RNAi pathway, that target destruction should require nucleotide triphosphates to support the production of cRNA, and that a 3' hydroxyl group is essential for siRNA function, since siRNAs are proposed to serve as primers for new RNA synthesis.

A second model proposes that single-stranded siRNAs do not act as primers for an RdRP, but instead assemble along the length of the target RNA and are then ligated together by an RNA ligase to generate cRNA (Lipardi et al., 2001; Nishikura, 2001). The cRNA/target RNA hybrid would then be destroyed by Dicer. This model predicts that target recognition and destruction should require ATP (or perhaps an NAD-derived high-energy cofactor) to catalyze ligation, as well as to support Dicer cleavage. Like the first model, the ligation hypothesis predicts that an siRNA 3' hydroxyl group should be required for RNAi. Furthermore, a 5' phosphate should be required for siRNA ligation, but ribonucleotide triphosphates other than ATP should not be required for target destruction.

A third model (Figure 1) hypothesizes that two distinct enzymes or enzyme complexes act in the RNAi pathway (Hammond et al., 2000; Zamore et al., 2000; Nykänen et al., 2001). As in the first model, Dicer is proposed to generate siRNAs from dsRNA. These siRNAs are then incorporated into a second enzyme complex, the RNA-induced silencing complex (RISC), in an ATP-dependent step or series of steps during which the siRNA duplex is unwound into single strands. The resulting single-stranded siRNA is proposed to guide the RISC to recognize and cleave the target RNA in a step or series of steps requiring no nucleotide cofactors whatsoever. The absence of a nucleotide triphosphate requirement for target recognition and cleavage is a key feature of this model.

We have previously demonstrated by two different experimental protocols that both recognition and endonucleolytic cleavage of a target RNA proceeds efficiently in the presence of less than 50 nM ATP, a concentration

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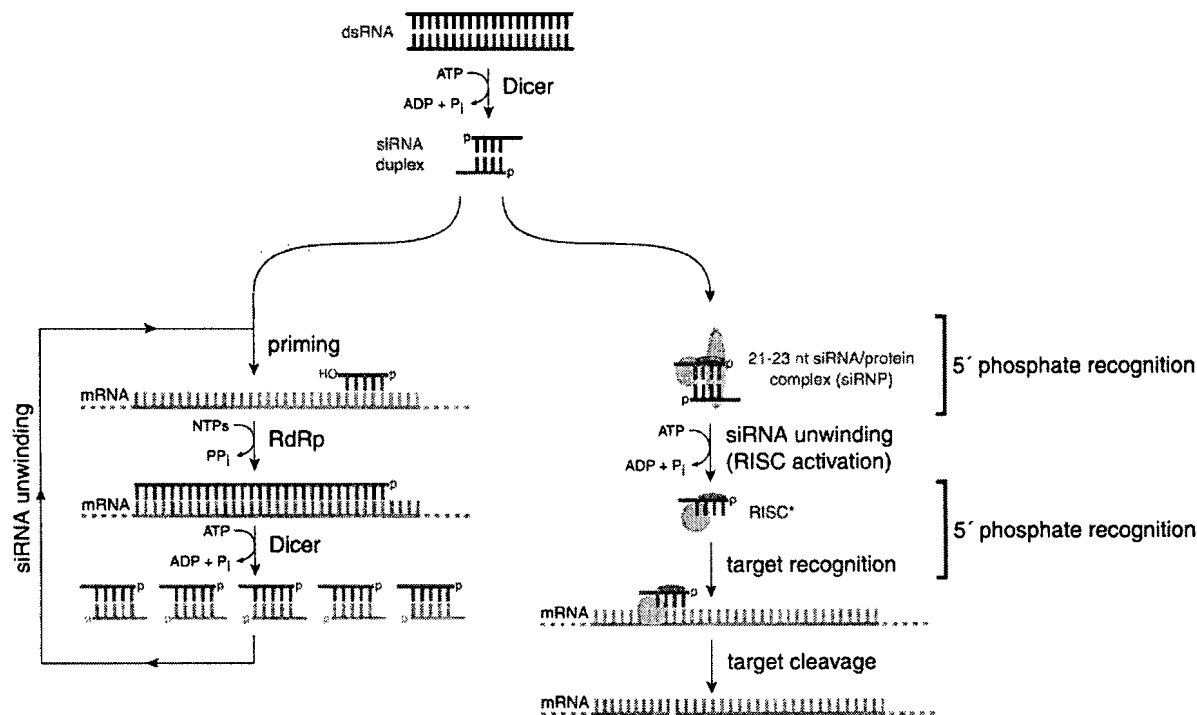


Figure 1. Two Models Proposed for the RNAi Pathway in *Drosophila*

Both models postulate that dsRNA is converted to siRNA by the ATP-dependent endoribonuclease Dicer, but the models differ as to the subsequent function of siRNAs. In the "random degradative PCR" model (at left), siRNAs are postulated to function as primers for the target RNA-templated synthesis of cRNA by an RdRP. The resulting dsRNA is then proposed to be cleaved by Dicer into a new crop of siRNAs, which can prime the conversion of additional target RNAs into dsRNA. In the endonucleolytic cleavage model for RNAi (at right), siRNAs are proposed to be incorporated into an endonuclease complex distinct from Dicer, the RISC. Assembly of the RISC is proposed to be ATP dependent, whereas endonucleolytic cleavage of the target RNA is postulated to require no high-energy cofactors.

likely to be insufficient to support either the synthesis of new RNA or the ligation of multiple siRNAs into cRNA (Nykänen et al., 2001). However, our data also revealed an absolute requirement for a 5' phosphate for siRNAs to direct target RNA cleavage in *Drosophila* embryo lysates, a finding we interpreted as reflecting an authentication step in the assembly of the RNAi-enzyme complex, the RISC. We envisioned that the 5' phosphate was involved in obligatory noncovalent interactions with one or more protein components of the RNAi pathway. Nonetheless, the 5' phosphate requirement might formally reflect a requirement for the phosphate group in covalent interactions, such as the ligation of multiple siRNAs to generate cRNA (Nishikura, 2001).

Here, we more fully define the mechanism of RNAi in flies and mammals by examining the requirement for a 5' phosphate and a 3' hydroxyl group on the antisense strand of the siRNA duplex. First, we analyze the role of these functional groups in siRNA function in vitro, using both *Drosophila* and human cell-free systems that recapitulate siRNA-directed target RNA destruction. Then, we validate our findings in vivo in human HeLa cells. Our data support a model for the RNAi pathway in which siRNAs function as guides for an endonuclease complex that mediates target RNA destruction. We find that the requirement for a 5' phosphate is conserved between *Drosophila* and human cells and that an siRNA 3' hydroxyl is dispensable in both systems. Our data

argue against an obligatory role for an RdRP in *Drosophila* or human RNAi, despite the clear requirement for such enzymes in PTGS in plants, quelling in *Neurospora crassa*, and RNAi in *C. elegans* and *Dictyostelium discoideum* (Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000; Sijen et al., 2001; Martens et al., 2002). In this respect, the mechanism of RNAi in flies and mammals appears to be distinct from that of PTGS, quelling, and RNAi in worms and *Dictyostelium*, suggesting that the pathway in flies and mammals may be more restricted in the range of triggers that can elicit an RNAi response.

Results and Discussion

Requirement for the siRNA 5' Phosphate in Human RNAi

Synthetic siRNAs bearing a 5' hydroxyl can efficiently mediate RNAi both in vitro in *Drosophila* embryo lysates and in vivo in cultured human cells (Elbashir et al., 2001b, 2001a; Nykänen et al., 2001). However, in the *Drosophila* in vitro system, an endogenous kinase rapidly converts the 5' hydroxyl group to a phosphate (Nykänen et al., 2001). Blocking siRNA phosphorylation by substituting the 5' hydroxyl with a methoxy moiety completely blocks RNAi in *Drosophila* embryo lysates (Nykänen et al., 2001). Furthermore, 5' phosphorylated siRNAs more efficiently trigger RNAi in vivo in *Drosophila* embryos than

do 5' hydroxyl-containing siRNAs (Boutla et al., 2001). 5' hydroxyl-containing, synthetic siRNAs that trigger RNAi in cultured mammalian cells (Elbashir et al., 2001a, 2002), in mice (McCaffrey et al., 2002; Lewis et al., 2002), and perhaps even in plants (Klahre et al., 2002) may likewise be phosphorylated by a cellular kinase prior to entering the RNAi pathway.

To determine if a 5' phosphate is required for RNAi in mammals, we first analyzed mammalian RNAi in vitro, using HeLa cell S100 extract. These reactions accurately recapitulate the known features of siRNA-directed RNAi in mammalian cell culture: exquisite sequence specificity (Elbashir et al., 2001a) and target RNA cleavage (Holen et al., 2002). RNAi reactions were performed in HeLa S100 extracts using siRNA duplexes in which the antisense strand, which we refer to as the guide strand, contained either a 5' hydroxyl or a 5' methoxy group (Figure 2A) and a chimeric target RNA in which nucleotides 62 to 81 were complementary to the siRNA (Figure 2B). When the guide strand of the siRNA duplex contained a 5' hydroxyl group, and could, therefore, be phosphorylated, it directed cleavage of the target RNA within the sequence complementary to the siRNA (Figure 3). Target cleavage directed by this siRNA occurred at the same site in the HeLa S100 as in *Drosophila* embryo lysate. These data suggest that endonucleolytic cleavage of the target RNA is a common feature of RNAi in flies and mammals. siRNAs with a 5' methoxy group cannot be phosphorylated by nucleic acid kinases and cannot direct RNAi in lysates of *Drosophila* embryos (Nykänen et al., 2001). Such siRNAs were likewise unable to direct cleavage of the target RNA in the HeLa S100 reaction (Figure 3A). Although the exogenous, methoxy-blocked siRNA did not trigger sequence-specific target cleavage, an endogenous HeLa RISC complex that contains the miRNA *let-7* (Hutvagner and Zamore, 2002a) cleaved the chimeric target RNA within the *let-7* complementary sequence near its 3' end (Figure 2B) in all of the human in vitro RNAi reactions. This diagnostic 5' cleavage product (indicated by an asterisk) serves as an internal control for these and subsequent in vitro HeLa S100 reactions. Our data suggest that mammalian RNAi, like RNAi in *Drosophila* (Nykänen et al., 2001; Boutla et al., 2001), requires the siRNA 5' phosphate for target cleavage and suggest that 5' hydroxyl-containing siRNA duplexes must be phosphorylated by a cellular kinase before they become competent to mediate RNAi in human cells. Consistent with this idea, 5' hydroxyl-containing siRNAs are rapidly 5' phosphorylated after only 5 min incubation in the HeLa S100 (Figure 3B). Thus, like *Drosophila*, human cells contain a nucleic acid kinase that can add a 5' phosphate to a synthetic siRNA.

Role of the siRNA 3' Hydroxyl Group in Flies and Mammals

Both siRNAs produced by enzymatic cleavage of dsRNA and those prepared by chemical synthesis contain 3' hydroxyl termini (Elbashir et al., 2001b). Experiments using nuclease-treated siRNAs suggested that a 3' phosphate blocks RNAi in *Drosophila* embryo lysates (Lipardi et al., 2001), a finding consistent with authentication of siRNA 3' structure by the RNAi machinery, with siRNAs acting as primers for cRNA synthesis, or with

RNA-templated ligation of multiple siRNAs into cRNA. To determine if the siRNA 3' hydroxyl group plays an essential role in RNAi, we synthesized two siRNAs in which the 3' hydroxyl group of the guide strand was blocked (Figure 2). In one siRNA, the 3' hydroxyl was replaced by a 2',3' dideoxy terminus. In the other, the 3' position contained 3-amino-propyl phosphoester (3' "amino modifier"). Each of the blocked siRNA guide strands was analyzed by electrospray mass spectrometry to confirm its identity and purity. The two modified siRNA guide strands, as well as a 3' hydroxyl-containing control strand, were annealed to a standard 21 nt siRNA sense strand. The three resulting siRNA duplexes were tested for their ability to direct cleavage of a complementary target RNA in an in vitro RNAi reaction containing *Drosophila* embryo lysate. Figure 4A shows that the two 3'-blocked siRNAs produced the same degree of target cleavage as the 3' hydroxyl-containing siRNA control.

Next, we repeated the experiment in HeLa S100 extract to determine if an siRNA 3' hydroxyl group is required for RNAi in mammalian cells. 3' modification of an siRNA has been reported to be permitted for RNAi in mammalian cells (Holen et al., 2002), but it was not shown in those experiments that all of the siRNA was 3' modified. In contrast to the 5' methoxy modification, which completely blocked target RNA cleavage in the HeLa S100 reaction, 3' modification had no effect on the efficiency or specificity of RNAi (Figure 4B). The identity and purity of these siRNAs was confirmed by electrospray mass spectrometry. However, we could envision that a fraction of the siRNA guide strand was cleaved within the single-stranded, two nucleotide, 3' overhang by a nuclease in the HeLa S100, regenerating the 3' hydroxyl. If this occurred, the cleaved siRNAs could then act as primers. To exclude this possibility, we performed RNAi reactions using progressively shorter guide siRNAs blocked at the 3' end by either a 2',3' dideoxy or a 3' amino modifier group. The 20 or 19 nt guide strands were annealed to the same 21 nt sense siRNA strand. Figure 4B shows that target RNA cleavage occurred in all cases, although the efficiency of cleavage decreased as the siRNA guide strand was shortened, even when it contained a 3' hydroxyl terminus. If the 3' blocked 21 nt siRNA was active because it had been shortened to a 20-mer, it could not have attained the activity of the 3' hydroxyl 21 nt siRNA. Similarly, if nucleolytic removal of the 3' block accounted for the activity of the 20 nt guide siRNA, it should have only been as active as the 19 nt, 3' hydroxyl-containing siRNA. These results suggest that the 3' hydroxyl group of the siRNA guide strand does not play an obligatory role in siRNA-directed RNAi in flies or mammals.

Single-Stranded siRNAs

All current models for RNAi—including those that propose siRNA to function as guides for an endonuclease and models that propose siRNAs to act as primers for target-RNA templated RNA synthesis—predict that siRNAs ultimately function as single strands. In fact, in *Drosophila* embryos, single-stranded antisense siRNAs corresponding to the *Notch* mRNA elicited *Notch* phenotypes in 12% of injected embryos, although the expres-

A

guide strand length (nt)	guide strand 5' end	guide strand 3' end	siRNA sequence
21	OH	OH	5'-UCGAAGUAAUCCCGGUACGUG-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
21	CH ₃ O	OH	5'-CH ₃ O-δTCGAAGUAAUCCCGGUACGUG-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
21	OH	2',3' ddC	5'-UCGAAGUAAUCCCGGUACGddC-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
21	OH	AM	5'-UCGAAGUAAUCCCGGUACGUG-AM-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
20	OH	OH	5'-UCGAAGUAAUCCCGGUACGU-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
20	OH	2',3' ddC	5'-UCGAAGUAAUCCCGGUACGddC-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
20	OH	AM	5'-UCGAAGUAAUCCCGGUACGU-AM-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
20	OH	OH	5'-UCGAAGUAAUCCCGGUACGC-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
19	OH	OH	5'-UCGAAGUAAUCCCGGUACG-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
19	OH	AM	5'-UCGAAGUAAUCCCGGUACG-AM-3' 3'-UUAGCUUCAUAAGGCGCAUGC-5'
21	OH	OH	5'-UGAGGUAGUAGGUUGUAUAGU-3' 3'-UUACUCCAUCACCAACAUAU-5'
21	OH, 2'dT	AM	5'-dTGAGGUAGUAGGUUGUAUAGU-AM-3' 3'-UUACUCCAUCACCAACAUAU-5'

B



Figure 2. RNAs Used in This Study
(A) *Photinus pyralis* (firefly) luciferase (blue) and *let-7* (red) siRNAs used in this study. The guide strand (antisense strand) is shown 5'-to-3' as the upper strand of each siRNA. Single-stranded siRNAs used in Figures 4, 5, and 6 correspond to the indicated guide strands. ddC, dideoxy Cytosine; AM, amino modifier. siRNAs corresponding to firefly luciferase sequence are blue; those corresponding to *let-7* sequence are red.
(B) A schematic representation of the chimeric target RNA, indicating the relative positions of firefly luciferase sequences and sequences complementary to the *let-7* miRNA found naturally in HeLa cells.

sivity was quite low (Boutla et al., 2001). Furthermore, single-stranded RNAs of various lengths trigger RNAi in *C. elegans*, but only when they contain a 3' hydroxyl group, suggesting that single-stranded siRNA functions in that organism as a primer for an RdRP (Tijsterman et al., 2002). Consistent with single-stranded siRNAs acting in nematodes as primers that direct the production of new dsRNA, they fail to trigger RNAi in the absence of Dicer (Dcr-1) (Tijsterman et al., 2002).
We examined if the guide siRNA strand alone could trigger target cleavage in an in vitro RNAi reaction containing either *Drosophila* embryo lysate or human HeLa

cell S100. We first examined if single-stranded siRNA could direct target RNA cleavage in *Drosophila* embryo lysates (Figure 5A). For this experiment, we used siRNA with the sequence of the miRNA *let-7* (Figure 2A). Cleavage of the target RNA (Figure 2B) by a *let-7*-containing siRNA duplex produces a diagnostic 522 nt 5' product (Hutvagner and Zamore, 2002a). When the synthetic siRNA was used as a single strand, the target RNA was not cleaved (Figure 5A). Similarly, a single-stranded siRNA of the same sequence but bearing a 2' deoxy thymidine (dT) instead of uracil as its first nucleotide, was also a poor trigger of target cleavage. However,

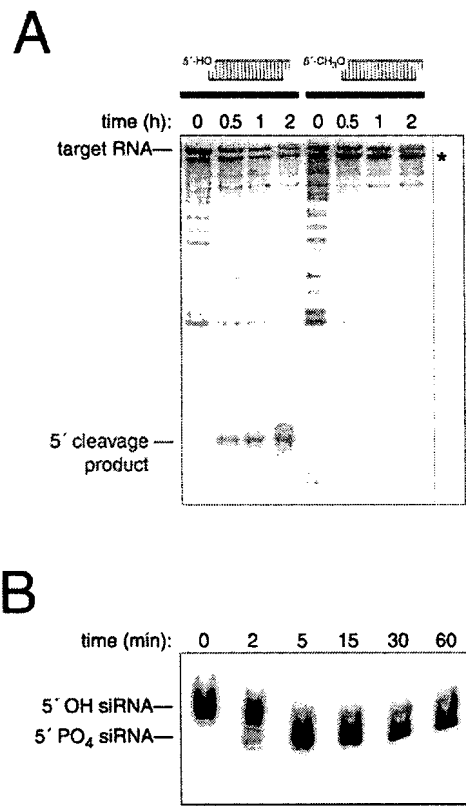


Figure 3. The siRNA 5' Phosphate Group Is Required for siRNA-Directed Target Cleavage in HeLa S100 Extracts

(A) RNAi in vitro in human HeLa cell S100 extract. At left, a time course of in vitro RNAi for a standard siRNA; at right, for an siRNA duplex bearing a 5' methoxy guide strand. The asterisk indicates the position of a 5' cleavage product catalyzed by an endogenous, human let-7-programmed RISC complex, which cleaves this target RNA within a let-7 complementary sequence located near the 3' end of the RNA (Hutvagner and Zamore, 2002a). This cleavage product serves as an internal control.

(B) Phosphorylation status of the guide strand of an siRNA duplex upon incubation in HeLa S100. An siRNA duplex containing a guide strand 3'-end-labeled with α -³²P cordycepin (3' deoxyadenosine triphosphate) was incubated in a standard HeLa S100 RNAi reaction, then analyzed on a 15% sequencing gel. Phosphorylation accelerates the gel mobility of the labeled siRNA strand, because it adds two additional negative charges. The radiolabeled RNA is 3' deoxy; therefore, we infer that the added phosphate is on the 5' end.

both these siRNAs contain a 5' hydroxyl, and a 5' phosphate is required for siRNA duplexes to trigger target RNA cleavage in *Drosophila* embryo lysates (Nykänen et al., 2001). Therefore, we considered that the defect with the single-stranded siRNAs might be that they lacked a 5' phosphate and cannot obtain one because they are not substrates for the *Drosophila* kinase. In support of this hypothesis, when the single-stranded siRNA starting with dT was prephosphorylated with polynucleotide kinase, it directed target cleavage.

To confirm these findings, we examined the activity of a second single-stranded siRNA, complementary to the luciferase portion of the target RNA. When prephosphorylated, this single-stranded siRNA again directed target cleavage in *Drosophila* embryo lysate, albeit less efficiently than the same molar concentration of an

siRNA duplex (Figure 5B). Cleavage occurred at precisely the same site in the target RNA for both single-stranded and double-stranded siRNAs, suggesting that the single-stranded siRNA entered the RNAi pathway, rather than triggered RNA destruction by a different route. The same single-stranded siRNA sequence bearing a 5' methoxy group did not direct target RNA cleavage (Figure 5B). Together, the experiments in Figure 5 demonstrate that single-stranded siRNAs—like the guide strands of siRNA duplexes—do not function in the RNAi pathway unless they bear a 5' phosphate.

To determine if single-stranded siRNAs trigger target destruction in *Drosophila* embryo lysates by acting as primers, we modified the 3' end of the siRNA to 2',3' dideoxy. As with double-stranded siRNAs, blocking the 3' end of the single-stranded siRNA had no effect on the efficiency or specificity with which the target was cleaved (Figure 5B). We note that the efficiency of target cleavage by single-stranded siRNAs is significantly less than that of siRNA duplexes. The lower efficiency might simply reflect the remarkably short lifespan of single-stranded siRNA in the *Drosophila* embryo lysate: the vast majority is destroyed within the first 2 min of incubation (Figure 5C). One explanation for the requirement for a 5' phosphate might be that, without it, the single-stranded siRNA is destroyed even faster. This explanation is unlikely, because the rate of single-stranded RNA destruction is only 1.4-fold faster for 5' hydroxy siRNAs (Figure 5C). More likely is that the 5' phosphate of the single-stranded siRNA is required for its entry into the RISC and that because a small fraction of 5' phosphorylated, single-stranded siRNA enters the RISC, it is protected from degradation, enhancing its stability in the lysate.

Next, we examined if single-stranded siRNAs could function to trigger RNAi in HeLa S100 extracts. Again, single-stranded siRNAs directed target cleavage at the same site as the corresponding siRNA duplex (Figure 6A). Prephosphorylation of single-stranded siRNA was not required for it to function in target cleavage in HeLa S100, but blocking the 5' end with a methoxy group completely eliminated RNAi (Figure 6B). These results suggest that a 5' phosphate is required for mammalian RNAi, but that the nucleic acid kinase(s) responsible for phosphorylating siRNAs in HeLa S100 acts on single-stranded siRNA, unlike its *Drosophila* counterpart. Blocking the 3' end of the single-stranded siRNA had no effect on the ability of the single-stranded siRNA to cleave the target RNA in HeLa S100 (Figure 6A). Thus, the structural requirements for single-stranded siRNA function in target cleavage are conserved between flies and mammals: a 5' phosphate is required, but a 3' hydroxyl is not.

Together, these data support the view that siRNAs do not direct target RNA destruction by priming the synthesis of new RNA, nor are siRNAs ligated together to generate cRNA. Both processes should require a 3' hydroxyl group, which is dispensable for target cleavage in either *Drosophila* or human cell extracts. Instead, our data suggest that siRNAs act as guides to direct a protein endoribonuclease to cleave the target RNA. The finding that single-stranded siRNAs can function as guides in the RNAi pathway suggests that each individual RISC contains only one siRNA strand. Consistent

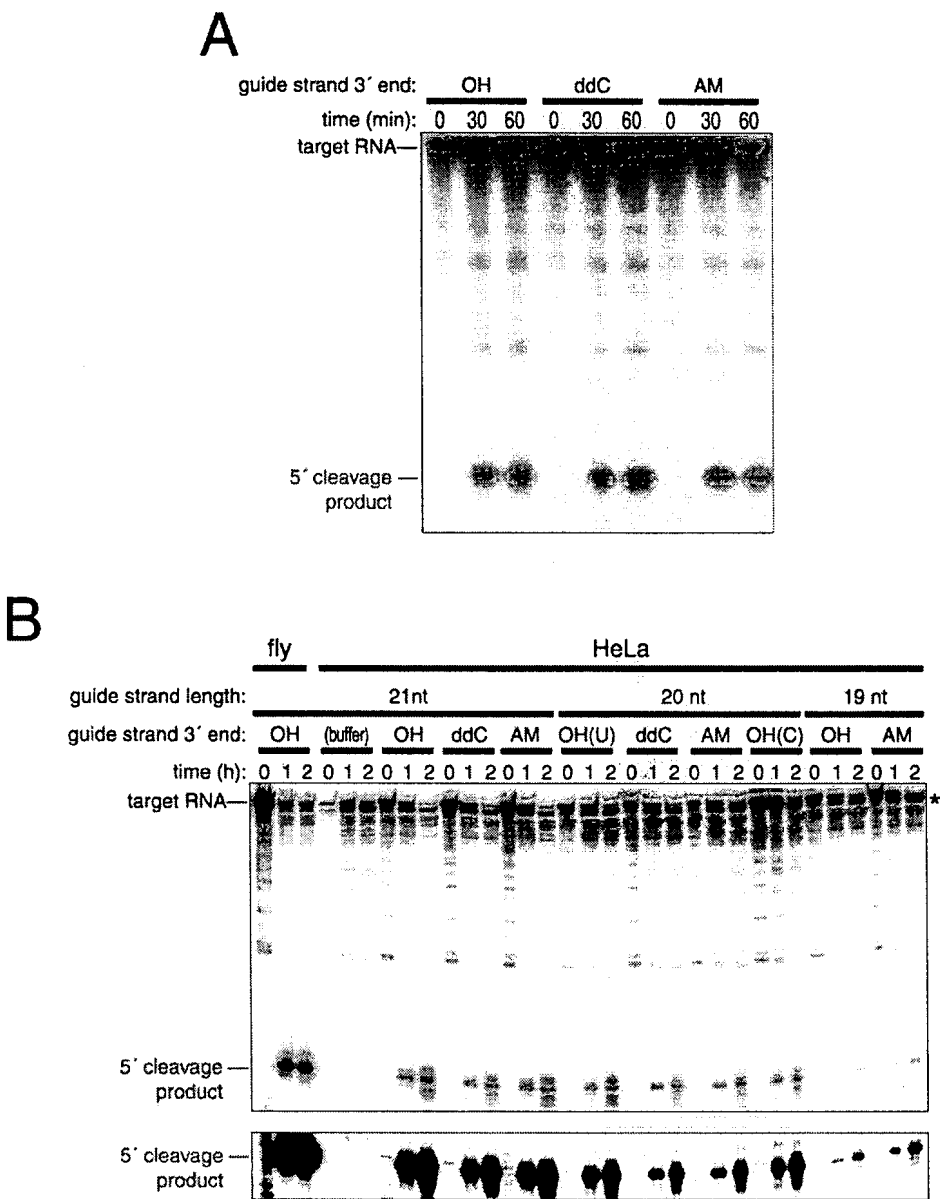


Figure 4. The siRNA 3' Hydroxyl Is Dispensable for siRNA-Directed Target Cleavage in *Drosophila* and Human Cell Extracts
(A) 3'-blocked siRNAs trigger RNAi in *Drosophila* embryo lysates with the same efficiency as 3'-hydroxyl-containing siRNAs. ddC, 2',3' dideoxy C; AM, amino modifier.
(B) 3'-blocked siRNAs trigger RNAi in HeLa S100 extracts with the same efficiency as standard, 3'-hydroxyl-containing siRNAs. An overexposure of the region of the gel containing the 5' cleavage product is shown in the lower panel. The asterisk marks the internal control 5' cleavage product described in Figure 3.

with this view, in HeLa cell S100 extracts, the single stranded miRNA, *let-7*, is in an endogenous RISC that catalyzes multiple rounds of cleavage of a perfectly complementary target RNA (Hutvagner and Zamore, 2002a). Previously, it was proposed that the siRNA 5' phosphate was recognized twice during the assembly of the siRNA-containing endoribonuclease complex (Nykänen et al., 2001) (Figure 1). That study placed one 5' phosphate recognition event before siRNA duplex unwinding but could not distinguish whether the 5' phosphate is

required subsequently at the unwinding step itself or after unwinding is complete. The absence of target cleavage by single-stranded siRNAs lacking a 5' phosphate suggests that the second phosphate recognition step occurs after the siRNA duplex is unwound. In both *Drosophila* embryo lysates and human HeLa S100, cleavage directed by single-stranded siRNA was less efficient than RNAi triggered by siRNA duplexes. This inefficiency correlated with the general instability of short RNA in the in vitro extracts, as determined by

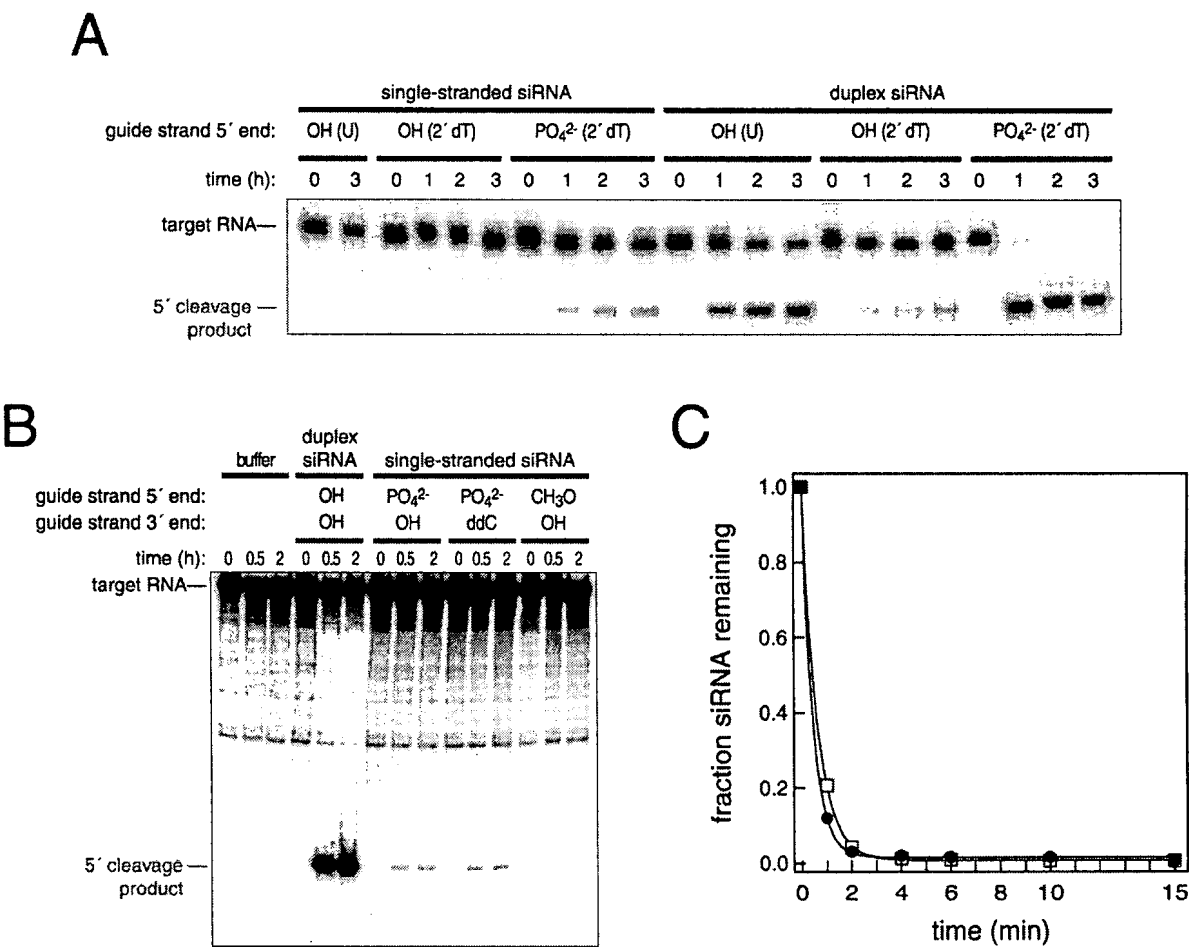


Figure 5. Single-Stranded siRNA Guides Target Cleavage in *Drosophila* Embryo Lysates
(A) Single-stranded siRNAs with the sequence of the miRNA *let-7* triggered target cleavage in *Drosophila* embryo lysate, but only if the 5' end was prephosphorylated.
(B) Single-stranded siRNAs complementary to firefly luciferase sequence triggered target cleavage in *Drosophila* embryo lysate, even if the 3' end was blocked (2',3'ddC). No target cleavage was observed using an siRNA with a 5' methoxy group.
(C) Rate of degradation of single-stranded siRNA in the *Drosophila* embryo lysate. siRNA single strands were 3' end-labeled with α -³²P cordycepin and their stability measured with (filled circles) or without (open squares) a 5' phosphate. The curves represent the best-fit to a single exponential, consistent with pseudo first-order kinetics for single-stranded siRNA decay. The difference in rates is 1.4-fold (with versus without a 5' phosphate).

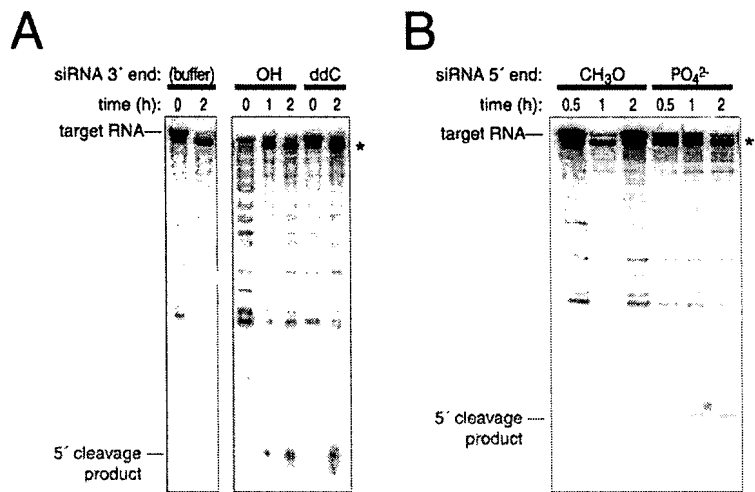


Figure 6. A 5' Phosphate but Not a 3' Hydroxyl Is Required for Single-Stranded Antisense siRNAs to Trigger RNAi in HeLa S100 Extract
(A) Single-stranded siRNA triggered target cleavage in HeLa S100, even if the 3' end of the siRNA was blocked (2',3' dideoxy).
(B) Blocking the 5' end of the siRNA with a methoxy group eliminated the ability of the single-stranded RNA to trigger RNAi. The asterisk marks the control 5' cleavage product described in Figure 3.

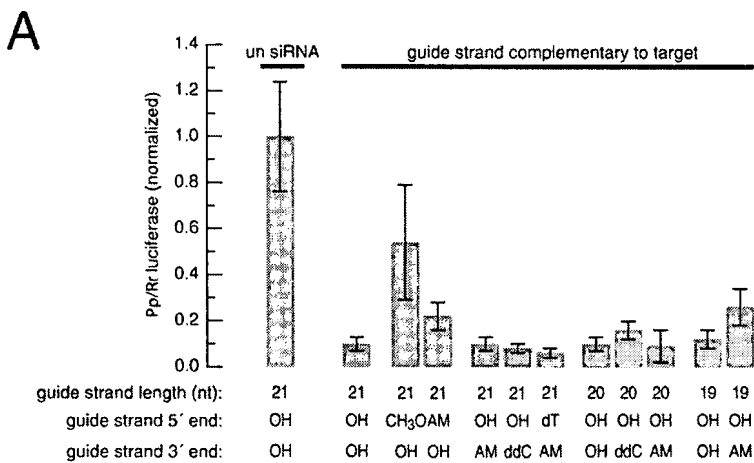
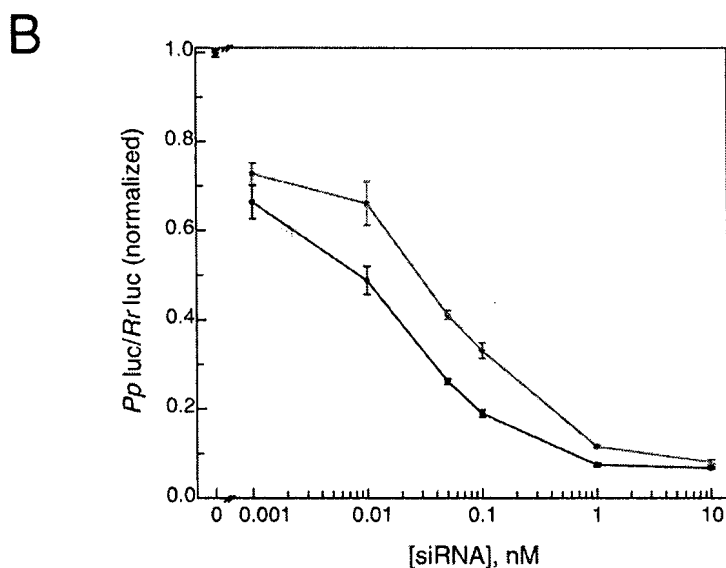


Figure 7. A 5' Phosphate but Not a 3' Hydroxyl Is Required for siRNA Duplexes to Trigger RNAi In Vivo in Cultured Human HeLa Cells

(A) siRNA duplexes were examined for their ability to silence the *Photinus pyralis* (Pp; firefly) luciferase target reporter, relative to the *Renilla reniformis* (Rr) luciferase control reporter. ddC, 2',3' dideoxy C; AM, amino modifier.

(B) Relative efficacy at limiting siRNA concentrations for siRNA duplexes with guide strands bearing either hydroxy (black symbols) or ddC (red symbols) 3' termini. Data are the average $\pm$ standard deviation for three trials.



measuring single-stranded siRNA half-life using 3' radiolabeled siRNAs (Figure 5C) and by Northern hybridization (data not shown).

siRNAs Need Not Function as Primers to Trigger RNAi in HeLa Cells

To assess if our in vitro results accurately predict the RNAi mechanism in vivo, cultured human cells were used to assess the structural requirements for siRNA function. Synthetic siRNAs were cotransfected into HeLa cells with plasmids expressing target (*Photinus pyralis*, Pp) and control (*Renilla reniformis*, Rr) luciferase mRNAs. Luciferase expression was measured, and target (firefly) luciferase levels were normalized to the *Renilla* control. The results of these experiments are shown in Figure 7.

First, the requirement for a 5' phosphate observed in *Drosophila* and HeLa extracts was conserved in vivo (Figure 7A). A 5' hydroxyl-containing siRNA duplex triggered efficient gene silencing in vivo, reducing expression of the target luciferase >90%. In contrast, a 5' methoxy-modified siRNA reduced firefly luciferase lev-

els by only 2-fold. This small reduction may reflect inhibition of translation, perhaps by an antisense mechanism. Alternatively, some of the methoxy-blocked siRNA may inefficiently enter the RNAi pathway in vivo. An siRNA in which the guide strand contained a 5' amino modifier group—6-amino-hexyl phosphoester—was significantly more effective in suppressing target mRNA expression than the siRNA with the 5' methoxy group (Figure 7A). This finding is consistent with the idea that a 5' phosphate group is required for siRNA function, but that the 5' phosphate participates in noncovalent interactions only, since the modified 5' phosphate should be less able to act as an electron acceptor. The in vivo studies agree with the in vitro results: a 5' phosphate is essential for efficient siRNA function in flies and mammals. However, in flies only duplex siRNAs can be 5' phosphorylated by cellular kinases, whereas in mammals, both single-stranded and double-stranded siRNAs are phosphorylated.

Consistent with the view that the core function of siRNA in human cells is as guides, not primers, blocking the 3' end of the siRNA guide strand had no effect on

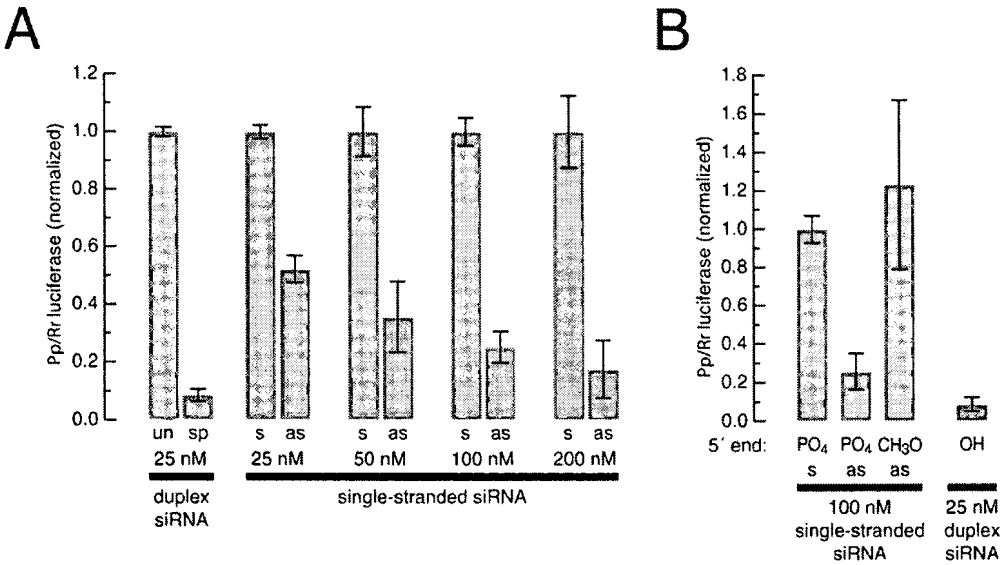


Figure 8. Single-Stranded siRNA Triggers Gene Silencing in HeLa Cells
(A) Single-stranded siRNA silencing as a function of siRNA concentration.
(B) Blocking the 5' end of single-stranded siRNAs prevented their triggering target gene silencing. Gray bars indicate the average $\pm$ standard deviation for three trials. Un, siRNA unrelated in sequence to the target RNA; sp, specific siRNA corresponding to the target RNA; s, sense strand; as, antisense strand.

RNAi in vivo. siRNA duplexes in which the guide strand contained a 3' hydroxyl, a 2',3' dideoxy, or a 3' amino modifier were all equally effective in triggering RNAi in vivo (Figure 7A). The silencing activity in vivo of a 21 nt, 3'-blocked siRNA guide strand was greater than that of a 20 nt, 3' hydroxy siRNA guide strand, indicating that the 3' block was not removed in vivo. We believe that these data exclude an obligatory role for the siRNA 3' hydroxyl group in RNAi in mammalian cells and argue that siRNAs do not normally trigger target destruction in human cells by functioning as primers.

These experiments were conducted at siRNA concentrations where the siRNA is not limiting for RNA silencing. An siRNA function in priming the synthesis of dsRNA might be used when siRNAs are limiting. We tested the relative ability of siRNA duplexes in which the guide strand either contained a 3' hydroxyl or a 2',3' dideoxy group at low siRNA concentrations (Figure 7B). We find that the efficacy of the two types of siRNAs did differ when siRNA was rate limiting for target mRNA silencing, but never by more than 1.8-fold. The observed difference in efficacy between the two types of siRNAs does not seem sufficiently great to support the view that the 3' hydroxyl group of the siRNA is used to prime the synthesis of dsRNA from the target mRNA. If the siRNA were used to prime dsRNA synthesis, the production of new dsRNA by an RdRP using the siRNA as a primer should have amplified the silencing activity of the 3' hydroxy but not the 2',3' dideoxy siRNA at limiting concentrations. For example, if the 3' hydroxy guide strand had primed synthesis of one molecule of dsRNA (~130 bp long based on the site of siRNA/target complementarity) for each target mRNA molecule, and this new dsRNA was then Diced into just two of the possible six new siRNAs, at least a 2-fold difference between the two

siRNAs should have been observed. This analysis fails to take into account the new crop of siRNAs acting in a subsequent cycle of priming, which would further amplify the difference between 3' deoxy and 3' hydroxy siRNA at limiting concentrations. The simplest interpretation of our finding that 3' hydroxy siRNAs trigger no significant amplification of RNA silencing relative to 3' blocked siRNAs is that an siRNA-primed, RdRP-dependent cycle of siRNA amplification plays no productive role in RNAi in cultured HeLa cells, even at low siRNA concentrations. The small difference in efficacy between 3' OH and 2',3' dideoxy siRNAs likely indicates that the blocked siRNAs have a subtle defect such as a lower affinity for components of the RNAi machinery, slightly reduced intracellular half-life, or a minor reduction in phosphorylation rate. This defect may result from the 2' deoxy modification of the terminal nucleotide, rather than the 3' block, since siRNAs with 2' deoxythymidine tails have been reported to be less efficient than those containing uracil in HeLa cells (Hohjoh, 2002).

Our in vitro studies suggest that single-stranded siRNAs can enter the RNAi pathway, albeit inefficiently. To test if single-stranded siRNAs could trigger mRNA silencing in vivo, we substituted various concentrations of single-stranded, sense or antisense siRNA for siRNA duplexes in our HeLa cell cotransfections (Figure 8A). As the concentration of antisense single strand was increased, the expression of the firefly luciferase decreased relative to the Renilla internal control. Note that single-stranded siRNAs are less efficient than siRNA duplexes: it takes nearly eight times more single-stranded siRNA to approach the potency of the corresponding duplex. This inefficiency may simply reflect rapid degradation of the majority of the transfected single-stranded siRNA before it can enter the RISC com-

plex. Cells may possess a mechanism that stabilizes siRNA duplexes and shuttles them to the RISC as single strands without exposing them to degradatory enzymes. Thus, if endogenous siRNAs are double-stranded in vivo, they may be double-stranded so as to facilitate their entry into the RNAi pathway and to exclude them from a competing pathway that degrades small, single-stranded RNA. Alternatively, single-stranded siRNAs may bypass a key step in RISC assembly, making them less efficient than duplexes in triggering RNAi. The dramatic instability of single-stranded siRNAs in vitro may simply reflect their inefficiency in assembling into a RISC, which could protect them from degradation.

Gene silencing by single-stranded siRNA was sequence specific, and single-stranded sense siRNA did not alter the expression of the target RNA (Figure 8B). Thus, it is unlikely that siRNAs themselves are copied by an RdRP in mammalian cells, since copying the sense siRNA should generate the antisense siRNA strand. However, copying sense siRNA into a duplex would not generate the characteristic 3' overhanging ends of siRNAs. Such 3' overhangs might be required for siRNA unwinding and/or efficient RISC assembly. Prephosphorylation of single-stranded siRNA did not enhance its potency in HeLa cells, consistent with our observations in HeLa S100 extracts, but blocking phosphorylation with a 5' methoxy group abolished silencing, pointing to the importance of 5' phosphorylation for single-stranded siRNA function in vivo (Figure 8B). Our findings are not entirely unexpected, since endogenous, single-stranded miRNAs enter the RNAi pathway in HeLa cells (Hutvagner and Zamore, 2002a). Superficially, the finding that single-stranded siRNAs can elicit RNA silencing blurs the distinction between RNAi and antisense effects. We have presented here evidence that single-stranded siRNAs trigger the same pathway as siRNA duplexes: both guide endonucleolytic cleavage of target RNAs at the same site, and both require 5' phosphates but not 3' hydroxyl groups to function.

Our in vitro experiments with *Drosophila* embryo lysates and HeLa S100 extracts and our in vivo experiments in HeLa cells argue against siRNAs functioning as primers in the RNAi pathway. These findings are consistent with the absence of any genes encoding canonical RdRPs in the currently available release of either the *Drosophila* or human genome. A hallmark of the involvement of RdRPs in posttranscriptional silencing is the spread of silencing beyond the confines of an initial trigger dsRNA or siRNA into regions of the target RNA 5' to the silencing trigger. In *C. elegans*, this spreading ("transitive RNAi") is manifest in the production of new siRNAs corresponding to target sequences not contained in the exogenous trigger dsRNA (Sijen et al., 2001). Furthermore, small RNAs as long as 40 nt can initiate silencing in worms, but only if they contain 3' hydroxyls, suggesting that they act as primers for the synthesis of cRNA (Tijsterman et al., 2002). In contrast, 5' spreading is not detected in *Drosophila*, either in vitro (Zamore et al., 2000), in cultured *Drosophila* S2 cells (Celotto and Graveley, 2002), or in vivo in flies (J.-Y. Roignant and C. Antoniewski, personal communication). Our data support the view that, in both flies and mammals, siRNAs trigger target RNA destruction not by acting as primers but rather by guiding a protein endoribonuclease

to a site on the target RNA that is complementary to one strand of the siRNA. The observation that the target cleavage site is across from the center of the complementary siRNA (Elbashir et al., 2001c, 2001b) is consistent with an enzyme other than Dicer acting in target RNA destruction and not with models that propose that Dicer destroys target RNAs. Furthermore, mammalian extracts depleted of Dicer still catalyze siRNA-directed target cleavage (Martinez et al., 2002).

Will it be possible to design siRNAs to degrade just one of several mRNA isoforms that differ at only a single nucleotide? If siRNAs do not act as RdRP primers in flies and mammals, then there is no fear that the silencing signal will spread 5' to a region of sequence common to the entire family of mRNAs. Despite earlier concerns that such siRNAs would not be possible (Nishikura, 2001), our data suggest that isoform- and polymorphism-specific siRNAs will be used in mammals in the future to dissect the function of individual gene isoforms and perhaps even to treat inherited autosomal dominant human diseases.

Experimental Procedures

General Methods

Drosophila embryo lysate preparation, in vitro RNAi reactions, and cap-labeling of target RNAs using Guanylyl transferase were carried out as previously described (Zamore et al., 2000). Human S100 extracts were prepared as described (Dignam et al., 1983). HeLa S100 was substituted for *Drosophila* embryo lysate in an otherwise standard RNAi reaction, except that incubation was at 37° instead of 25°C. Cleavage products of RNAi reactions were analyzed by electrophoresis on 8% denaturing acrylamide gels. 3' end labeling with α -³²P cordycepin and determination of 5' phosphorylation status were according to Nykänen et al. (2001). Gels were dried and exposed to image plates (Fuji), which were scanned with a Fuji FLA-5000 phosphorimager. Images were analyzed using Image Reader FLA-5000 version 1.0 (Fuji) and Image Gauge version 3.45 (Fuji).

siRNA Preparation

Synthetic RNAs (Dharmacon) were deprotected according to the manufacturer's protocol and processed as previously described (Nykänen et al., 2001). siRNA strands were annealed (Elbashir et al., 2001a) and used at 100 nM final concentration unless otherwise noted. siRNA single strands were phosphorylated with polynucleotide kinase (New England Biolabs) and 1 mM ATP according to the manufacturer's directions.

Tissue Culture

siRNA transfections were as described (Elbashir et al., 2001a). In brief, cultured HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies). Cells were trypsinized and seeded at 1×10^5 cells/ml in 24-well plates (5×10^4 cells/well) in DMEM supplemented with 10% FBS. Twenty-four hours after seeding, 1 μ g pGL2 control firefly luciferase (*Pp-luc* GL2; Promega) and 0.1 μ g pRL-TK Renilla luciferase (*Rr-luc*; Promega) plasmids and the luciferase siRNA (25 nM) were cotransfected with LipofectAMINE 2000 reagent (Invitrogen) in DMEM (Life Technologies) lacking serum and antibiotics according to manufacturer's instructions. Media was replaced 4 hr after transfection with DMEM containing 10% fetal bovine serum (Life Technologies); 1 day after transfection, the cells were lysed in 1x Passive Lysis Buffer (Promega) according to the manufacturer's instructions. Luciferase expression was determined by the Dual luciferase assay kit (Promega) using a Mediators PhL luminometer. Data analysis was performed using Excel (Microsoft) and IgorPro 5.0 (Wavemetrics). Experiments were performed in triplicate, and error was propagated through all calculations.

Acknowledgments

We thank David Bartel, Andrew Fire, Craig Mello, Tariq Rana, and Stephen Scaringe for discussions; Jean-Yves Roignant and Tom Tuschl for sharing data prior to publication; Melissa Moore and members of the Moore lab for a generous gift of HeLa S100 extract and for assistance in the preparation of HeLa S100 extracts; and members of the Zamore lab for helpful discussions and comments on the manuscript. G.H. is a Charles A. King Trust fellow of the Medical Foundation. P.D.Z. is a Pew Scholar in the Biomedical Sciences and a W.M. Keck Foundation Young Scholar in Medical Research. Supported in part by a grant to P.D.Z. from the National Institutes of Health (GM62862-01).

Received: July 16, 2002
Revised: August 30, 2002

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ANEXO 16

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ANEX 016

RNAi as Random Degradative PCR: siRNA Primers Convert mRNA into dsRNAs that Are Degraded to Generate New siRNAs

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Summary

In posttranscriptional gene silencing (PTGS), “quelling,” and RNA interference (RNAi), 21–25 nucleotide RNA fragments are produced from the initiating dsRNA. These short interfering RNAs (siRNAs) mediate RNAi by an unknown mechanism. Here, we show that GFP and *Pp*-Luc siRNAs, isolated from a protein complex in *Drosophila* embryo extract, target mRNA degradation in vitro. Most importantly, these siRNAs, as well as a synthetic 21-nucleotide duplex GFP siRNA, serve as primers to transform the target mRNA into dsRNA. The nascent dsRNA is degraded to eliminate the incorporated target mRNA while generating new siRNAs in a cycle of dsRNA synthesis and degradation. Evidence is presented that mRNA-dependent siRNA incorporation to form dsRNA is carried out by an RNA-dependent RNA polymerase activity (RdRP).

Introduction

Fire and associates first reported on RNA interference (RNAi) when they demonstrated potent and specific genetic interference upon the injection of dsRNA into *C. elegans* (Fire et al., 1998). However, the underlying phenomenon, known as posttranscriptional gene silencing (PTGS), was initially described in studies on transgenic plants (Napoli et al., 1990). PTGS was observed when the introduction of all or a portion of a transgene occasionally resulted in the loss of expression from the corresponding endogenous gene with no disruption in gene transcription. Mature mRNA transcripts simply did not accumulate in the cytoplasm of the affected plants (Kooter et al., 1999; Plasterk and Ketting, 2000). The viral stocks used to make the transgene constructs were also known to generate dsRNA during the replication cycle and the phenomenon of PTGS was linked to replication-competent virus (Baulcombe, 1999a; Burton et al., 2000; Ruiz et al., 1998). Similar findings were also described in *Neurospora* where the phenomenon was termed “quelling” (Cogoni et al., 1996).

A remarkable aspect of this process is that the local induction of gene silencing can spread throughout the organism and is often heritable to the next generation. This is particularly true in plants (Palauqui and Vaucheret, 1998; Sonoda and Nishiguchi, 2000; Voinnet et al., 1998) but it has also been demonstrated in *C. elegans* (Grishok et al., 2000). Furthermore, only a few molecules of dsRNA per cell or embryo completely silence the corresponding gene, a result that led to the speculation

the process might be catalytic in nature (Fire et al., 1998; Kennerdell and Carthew, 1998). Genetic studies in *Neurospora*, *Arabidopsis*, and *C. elegans* identified several genes involved in quelling, PTGS, and RNAi, and include members of the helicase family (Cogoni and Macino, 1999b; Dalmay et al., 2001), RNase III-related nucleases (Ketting et al., 1999), members of the Argonaute family (Catalanotto et al., 2000; Fagard et al., 2000; Hammond et al., 2001a; Tabara et al., 1999), and RNA-dependent RNA polymerases (RdRP) (Cogoni and Macino, 1999a; Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000).

Molecular studies in plants (Hamilton and Baulcombe, 1999), *C. elegans* (Parrish et al., 2000), and *Drosophila* (Zamore et al., 2000) have also shown that a common feature shared among these various targeted RNA degradation processes is the production of short 21–25 nucleotide RNA fragments of both sense and antisense orientation derived from the input dsRNA. Recent studies using Schneider cell extracts prepared from dsRNA treated cells have identified Dicer, an ATP-dependent ribonuclease related to the RNase III family of nucleases that specifically cleaves double stranded RNA into 21–25 nucleotide pieces (Bernstein et al., 2001). The resulting small dsRNA fragments have been proposed to act as “guide RNAs” that target an associated nuclease complex, called RISC (RNA-induced silencing complex), (Hammond et al., 2000) to the corresponding mRNA through strand complementarity (Bass, 2000). However, this has not been demonstrated experimentally and the exact mechanism of action involving the 21–25 nucleotide RNAs remains to be elucidated.

With the recent development of *Drosophila* embryo (Tuschl et al., 1999) and Schneider cell extracts (Hammond et al., 2000) capable of carrying out the dsRNA-dependent targeted degradation of mRNA in vitro, we sought to further clarify the role of the 21–25 nucleotide RNAs in mRNA silencing. Tuschl and coworkers have shown that synthetic short dsRNAs, called siRNAs for short interfering RNAs, can mediate RNAi in *Drosophila* extracts by directing a cleavage event in the target RNA (Elbashir et al., 2001b). Here, we show that the siRNAs produced upon the addition of GFP and *Pp*-Luc dsRNA to *Drosophila* embryo extract can be enriched in a micrococcal nuclease-resistant fraction. Once these short RNAs are treated with calf-intestinal phosphatase, they mediate efficient RNAi in vitro. These siRNAs, as well as a synthetic 21-nucleotide GFP duplex siRNA, are shown to act as mRNA-specific primers that are incorporated during the subsequent conversion of the target mRNA into dsRNA. Nascent dsRNA is then cleaved by RNase III-related enzymes to degrade the mRNA while generating new siRNAs in the process. RdRP activity is shown to mediate the incorporation of a synthetic siRNA into nascent dsRNA. We propose that repeated cycles of dsRNA synthesis and concomitant siRNA/primer production result in targeted mRNA degradation, and that this process can account for the underlying mechanism responsible for PTGS, quelling, and RNAi.

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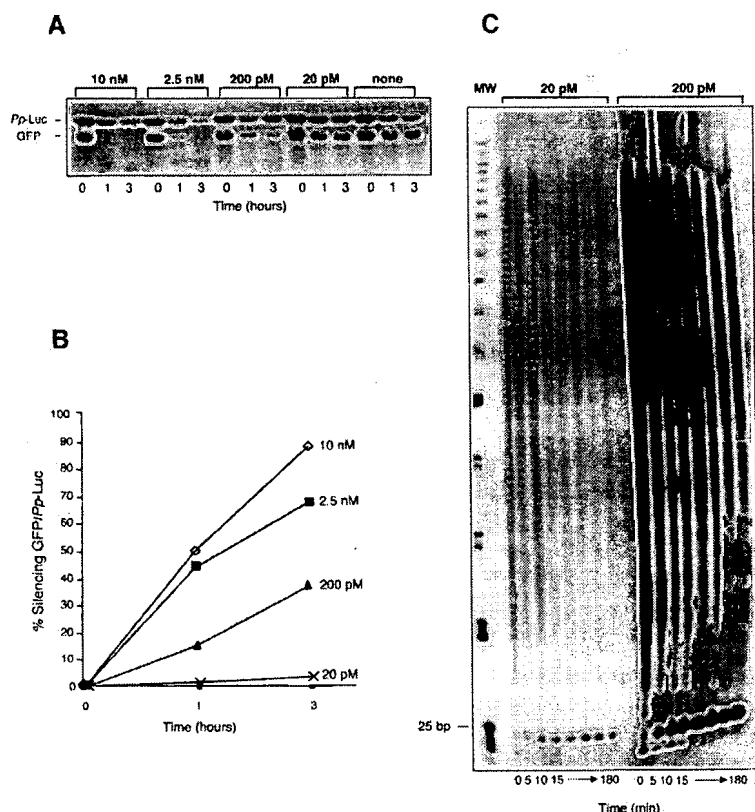


Figure 1. siRNAs Are Produced in the Absence of RNAi

(A) The rate of GFP mRNA (746 bp) degradation is proportional to the input concentration of GFP dsRNA (716 bp). Degradation does not occur below a threshold concentration of 20 pM dsRNA. Pp-Luc mRNA (1682 bp) is the nontargeted control mRNA. GFP and Pp-Luc indicate the positions of the full-length target and control RNAs, respectively.

(B) Data from (A) have been analyzed on the Fuji Phosphorimager and normalized to Pp-Luc mRNA over the concentration range of GFP dsRNA indicated.

(C) GFP siRNAs reach maximal levels in the processing reaction within 15 min in response to dsRNA concentrations that do (200 pM) or do not (20 pM) trigger RNAi. The specific activity of ^{32}P -UTP-labeled dsRNA added to the processing extract was approximately 1×10^5 cpm/ μg . MW: 25 bp DNA ladder with the 25 nucleotide position indicated.

Results

Production of siRNAs Can Occur in the Absence of RNAi

Drosophila embryo extracts proficient in RNAi were prepared (Tuschl et al., 1999) to study the dsRNA-dependent degradation of mRNA. ^{32}P -labeled GFP mRNA (746 bp, capped and adenylated with 30 A residues) was selected as the target with ^{32}P -labeled *Photinus pyralis* luciferase (Pp-Luc) mRNA (1682 bp, capped and adenylated with 30 A residues) serving as the nontargeted control. Full-length unlabeled GFP dsRNA (716 bp) was used to activate targeted GFP mRNA degradation. Similar to the findings reported for dsRNA injected into *C. elegans* (Fire et al., 1998), the rate of GFP mRNA degradation in *Drosophila* embryo extracts was dependent upon the concentration of GFP dsRNA added to the reaction. Using the same conditions described previously (Tuschl et al., 1999) with 10–50 pM mRNAs and varying concentrations of dsRNA from 20 pM to 10 nM, maximal degradation rates for GFP mRNA were observed with GFP dsRNA concentrations above 10 nM, whereas no targeting was detected at or below 20 pM GFP dsRNA (Figures 1A and 1B). ^{32}P -labeled full-length GFP dsRNA was also used to look at the concentration-dependent appearance of the siRNAs produced under silencing and nonsilencing conditions. Although 20 pM GFP dsRNA did not support detectable targeted GFP mRNA degradation, the time course of appearance for the siRNAs was the same for both 20 pM and 200 pM GFP dsRNA and reached a plateau value within 15 min of incubation (Figure 1C). A similar time course for the

appearance of siRNAs was also noted previously for *Drosophila* embryos injected with ^{32}P -labeled dsRNA (Yang et al., 2000). The amount of GFP siRNAs produced depended upon the input concentration of GFP dsRNA and this, in turn, was correlated with the level of RNAi in the reaction (Figure 1). Below a threshold concentration for the siRNAs, targeted mRNA degradation did not occur.

siRNAs Are Double Stranded and Protected in a Micrococcal Nuclease-Resistant Protein Complex

Previous studies have suggested that the processed short RNAs are double stranded and contained in a ribonucleoprotein complex (Elbashir et al., 2001b). We used micrococcal nuclease (Pelham and Jackson, 1976) to test whether or not the small RNAs could be isolated in a nuclease-resistant protein complex. In the first instance, extract was treated with micrococcal nuclease and blocked with EGTA prior to the addition of any RNAs. In agreement with previous observations (Hammond et al., 2000) micrococcal nuclease-treated extract was inactive in RNAi (Figure 2A, lane 2). This was not due to a nonspecific interaction between RNA and the nuclease (Wang and Gegenheimer, 1990) since the simultaneous addition of micrococcal nuclease, EGTA, and calcium to extracts was previously shown not to inhibit or affect RNAi (Hammond et al., 2000). The production of siRNAs was analyzed in the same reaction at 30 and 60 min time points using 10 nM full-length ^{32}P -labeled GFP dsRNA as a cleavage substrate. In micrococcal nuclease-treated extract, the rate of appearance

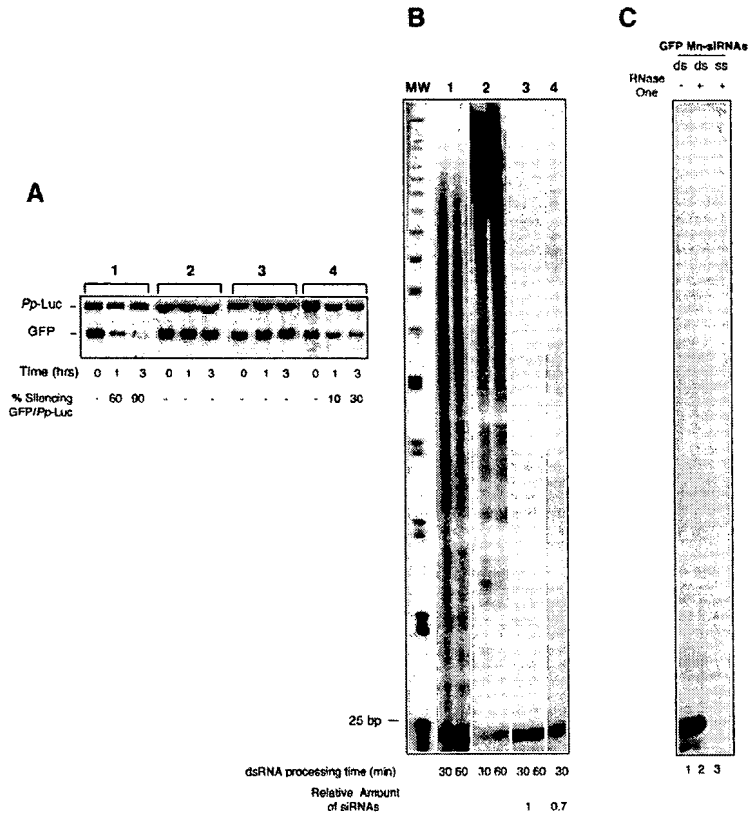


Figure 2. siRNAs Are Double Stranded and Associate in a Dynamic Ribonucleoprotein Complex Protected from Micrococcal Nuclease Digestion

(A) Treatment of *Drosophila* embryo extract with micrococcal nuclease before or after the addition of GFP dsRNA inhibits RNAi: Lane 1, the standard reaction; Lane 2, addition of micrococcal nuclease prior to dsRNA; Lane 3, addition of dsRNA prior to micrococcal nuclease; and Lane 4, addition of dsRNA to the reaction in Lane 3 restores modest levels of RNAi. Percent silencing shown as GFP/Pp-Luc ratios is indicated below each lane.

(B) Addition of ^{32}P -UTP-labeled GFP dsRNA (200 pM, $>10^9$ cpm/ μg) to extract prior to micrococcal nuclease treatment produces inactive siRNAs. Lane 1, control; Lane 2, addition of micrococcal nuclease prior to dsRNA; Lane 3, addition of dsRNA prior to micrococcal nuclease; and Lane 4, addition of 10 nM unlabeled GFP dsRNA to the reaction in Lane 3 chases the level of siRNAs. The initial input of labeled dsRNA was the same in all lanes shown, 2.5×10^5 cpm.

(C) The GFP siRNAs after micrococcal nuclease treatment, called Mn-siRNAs (Figure 2B, lane 3), are double stranded and resistant to Ribonuclease One digestion. Lane 1, siRNAs with no nuclease; Lane 2, siRNAs with RNase One; and Lane 3, denatured siRNAs with RNase One.

as well as the plateau value for the small RNAs was greatly reduced (Figure 2B, lane 2).

Extract incubated with unlabeled GFP dsRNA prior to micrococcal nuclease treatment also was unable to direct the degradation of GFP mRNA added subsequently to the reaction (Figure 2A, lane 3). However, an examination of the GFP siRNAs produced under these conditions revealed that the small RNAs remained resistant to nuclease digestion and were produced in amounts comparable to that seen in control reactions (Figure 2B, compare lanes 1 and 3). Furthermore, with the exception of the GFP siRNAs, most of the labeled GFP dsRNA was degraded during nuclease treatment (Figure 2B, lane 3).

When additional unlabeled GFP dsRNA was added to the reaction shown in Figure 2A, lane 3, modest RNAi was observed—about 30% of control values (Figure 2A, lane 4). Similarly, unlabeled GFP dsRNA added to extract preincubated with ^{32}P -labeled GFP dsRNA also resulted in roughly a 30% decrease in the amount of ^{32}P -labeled GFP siRNAs (Figure 2B, lane 4). Taken together, these results indicate that the production of the siRNAs is a dynamic process involving cleavage and displacement by the dsRNA substrate.

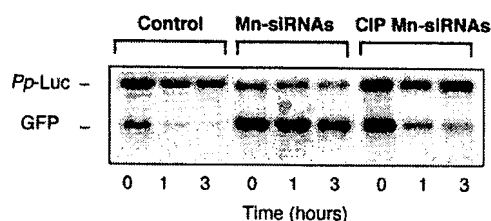
The resistance of the siRNAs to micrococcal nuclease digestion suggested the small RNAs were in a protein complex. This was confirmed upon treatment of the protected siRNAs with proteinase K, as described by Tuschl (Tuschl et al., 1999), which now rendered the siRNAs digestible by micrococcal nuclease (data not shown, but similar to Figure 2C). The same short RNAs were also resistant (Figure 2C) to digestion with Ribo-

nuclease One (RNase One), a single-strand-specific ribonuclease that can cut gaps and single base-pair mismatches (Meador et al., 1990).

siRNAs Require a 3'-Hydroxyl Group to Direct RNAi In Vitro

Using micrococcal nuclease and 500 ng of unlabeled GFP dsRNA, we prepared an RNA fraction enriched for the GFP-specific siRNAs for further testing in RNAi reactions. Addition of this RNA fraction to a silencing reaction did not trigger the degradation of GFP mRNA (Figures 3A and 3B, control versus Mn-siRNAs). The products of RNA digestion with micrococcal nuclease are mononucleotides and oligonucleotides with terminal 3'-phosphate groups (Krupp and Gross, 1979). Tuschl and coworkers have recently shown that the siRNAs derived from dsRNA incubated in *Drosophila* extracts have 5'-monophosphate and 3'-hydroxyl groups (Elbashir et al., 2001b). We speculated that a phosphate group on the 3'-hydroxyl position might inhibit the function of the GFP siRNAs in some aspect of the silencing process. In order to check this, the enriched GFP Mn-siRNAs were treated with calf-intestinal alkaline phosphatase (CIP) to remove any phosphate groups prior to retesting for RNAi (Mossner et al., 1980). Phosphatase treatment restored mRNA target degradation to a level comparable to that observed with 500 ng of full-sized GFP dsRNA (Figures 3A and 3B, control versus CIP-Mn-siRNAs). The slight trimming of the siRNAs during nuclease treatment did not impair their silencing activity and they remained within the 21–25 nucleotide size range, as noted in Figures 2B and 2C. This was a direct

A



B

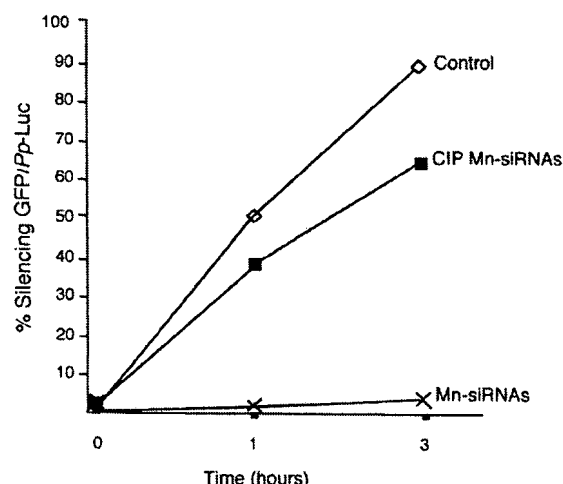


Figure 3. siRNAs Require a 3'-Hydroxyl Group to Function in RNAi (A) GFP siRNAs produced from 500 ng of full-length GFP dsRNA (716 bp) and treated with micrococcal nuclease trigger efficient RNAi if they are treated with calf-intestinal alkaline phosphatase (CIP). Control, 500 ng GFP dsRNA under standard reaction conditions; Mn-siRNAs, siRNAs derived from 500 ng of GFP dsRNA treated with micrococcal nuclease; CIP Mn-siRNAs, Mn-siRNAs treated with CIP. GFP (746 bp) and *Pp-Luc* (1682 bp) mRNA positions are indicated. (B) Quantification of the GFP/*Pp-Luc* band intensity ratios shown in (A) were measured with the Fuji Phosphoimager.

demonstration that the GFP siRNAs produced in a silencing reaction efficiently target degradation of the corresponding mRNA and that this activity required a 3' hydroxyl group on the siRNAs.

siRNAs Behave as Primers and Are Incorporated into dsRNA

The requirement for a 3'-hydroxyl group on the GFP siRNAs to degrade the target mRNA indicated that they might serve as primers for an RdRP activity in RNAi. If this was correct, labeled siRNAs would be incorporated into RNA product dependent specifically upon the use

of the complementary template RNA. ^{32}P -labeled siRNAs (2.5×10^5 cpm), corresponding to 250 pg of dsRNA, were prepared from full-size GFP and *Pp-Luc* ^{32}P -labeled dsRNAs using the micrococcal nuclease digestion procedure and assayed for template RNA-dependent incorporation into larger RNAs. Full-size GFP (716 bp) and *Pp-Luc* (1652 bp) single strand, double strand, and capped/adenylated RNAs were used as templates. The reaction products were analyzed on denaturing 1.5% agarose formaldehyde gels. Consistent with their role as primers for RdRP activity, the labeled GFP siRNAs were incorporated specifically into full-size RNA for all the templates (Figure 4A). Heterologous RNA/siRNA combinations did not result in any detectable siRNA incorporation (Figure 4A) and neither single- nor double-stranded DNA substrates served as templates for siRNA uptake (data not shown).

siRNAs Are Incorporated into dsRNA that Is Subsequently Degraded

We wanted to determine if the full-length RNAs labeled with siRNAs were double stranded. The reaction products were digested with RNase one and found to be resistant to nuclease digestion under conditions that completely degraded single-stranded GFP RNA transcribed in vitro with T7 RNA polymerase (Figure 4B). The slight sensitivity of the dsRNAs derived from the different RNA templates suggested that some of them might have contained gaps or mismatches susceptible to RNase one digestion. RNase T1, which does not degrade dsRNA but cleaves single stranded RNA 3' to G residues, also did not degrade the siRNA-labeled RNAs, as shown for GFP mRNA derived dsRNA (Figure 4B). Therefore, the siRNA-labeled RNAs are double stranded.

The siRNA-labeled RNAs could also serve as substrate for cleavage by RNase III-related enzymes. To test this, the full-length GFP dsRNA (shown in Figure 4A) was gel purified and incubated in extract. As shown in Figure 4C, lane 3, the siRNA-labeled RNA was processed to produce new GFP siRNAs.

Given this result, we speculated that longer incubation of the siRNA incorporation reaction would show degradation of the dsRNA and accumulation of the corresponding siRNAs. This was observed when ^{32}P -labeled GFP and *Pp-Luc* siRNAs and the cognate RNA templates were used to produce dsRNA (Figures 5A and 5B). The products were analyzed over a 3 hr period. The full-length dsRNAs appeared rapidly within 2–5 min for both the *Pp-Luc* and GFP templates (Figure 5A, panels 1 and 3) and reached a maximum level within 5–10 min of incubation before the onset of degradation was observed. A longer exposure revealed that the siRNAs were incorporated into shorter products that were degraded during the time course of the reaction (Figure 5A, panels 2 and 4). By the end of the incubation period, more than 90% of the siRNA-labeled dsRNA was degraded while nascent siRNAs continually increased over the time course of the reaction (Figures 5A and 5B). siRNA levels did not plateau within 15 min, as observed with the addition of labeled dsRNA to the extract, but continued to accumulate (Figure 5B). This result confirmed that the siRNA-labeled RNA was double stranded since single-stranded RNA is not a substrate for RNase III-related

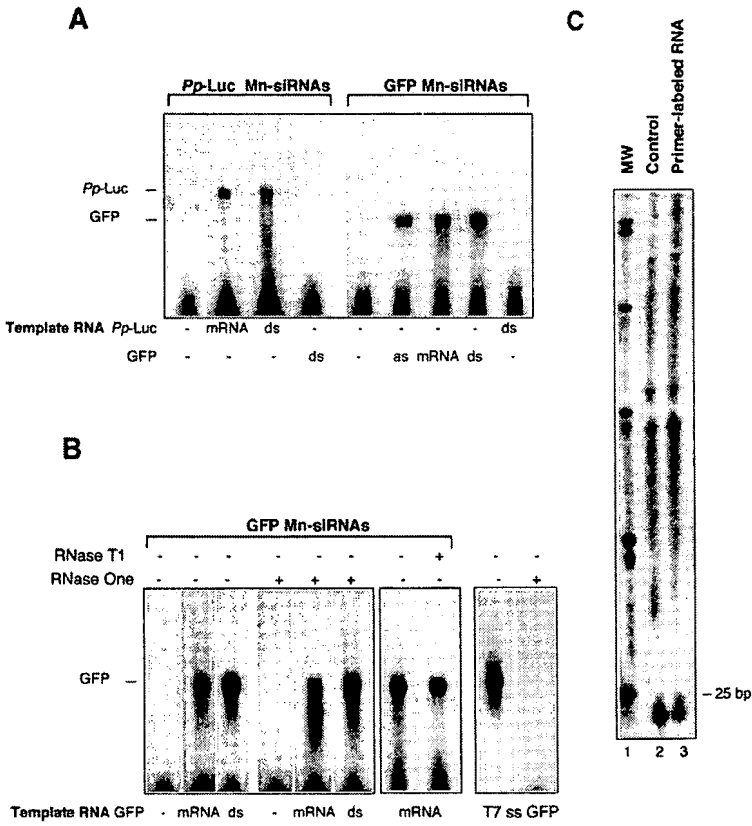


Figure 4. siRNAs Are mRNA-Specific Primers for dsRNA Synthesis

(A) 32 P-UTP labeled GFP or *Pp-Luc* Mn-siRNAs, prepared from the full-length dsRNAs by the micrococcal nuclease/CIP method (see Experimental Procedures), were incubated in extract with the cognate and heterologous RNA templates and assayed for incorporation into larger RNAs on 1.5% agarose formaldehyde gels. *Pp-Luc* and GFP siRNAs were incorporated up to the corresponding full-size RNAs only with the homologous single- or double-stranded RNA templates. Incubation of siRNAs in the absence of template or with the heterologous template resulted in no product. Single-stranded antisense RNA (as), double-stranded RNA (ds), and capped-polyA RNA (mRNA) were all templates for primer incorporation. GFP (746 bp) and *Pp-Luc* (1682 bp) mark the positions of the corresponding full-length RNAs.

(B) siRNAs are incorporated into RNA that is double-stranded and resistant to digestion by Ribonuclease One and Ribonuclease T1. siRNAs-labeled GFP RNA produced from either single- or double-stranded templates is resistant to two different nucleases that do not digest dsRNA. T7 ss GFP, single strand control RNA transcribed in vitro with T7 RNA polymerase. The same notation as in (A) is used here.

(C) Full-length siRNA-labeled GFP dsRNA is processed into new siRNAs. Full-length GFP dsRNA, as shown in (A), was gel purified as described in Experimental Procedures and incubated in extract for processing. Lane 1, 25 bp ladder; Lane 2, marked control, processed GFP dsRNA (90,000 cpm input); Lane 3, new GFP siRNAs produced from the full-length GFP RNA labeled by siRNA incorporation (90,000 cpm input).

enzymes and does not produce siRNAs. When GFP dsRNA was used as template for siRNA incorporation, the production of new siRNAs was significantly slower and reached a lower level compared to that seen with mRNA template (Figure 5B, compare dsGFP to ssGFP). This can be explained by the fact that cold dsRNA template competes with the newly labeled dsRNA for the cleavage reaction, as shown in the previous results (Figure 2B, compare lanes 3 and 4). SiRNAs incubated in the absence of template remained at the same position and intensity and did not shift into new products at all time points (Figure 5B, no template).

Therefore, siRNAs are used to convert the target mRNA into dsRNA, which is then cleaved by RNase III activity in the extract to eliminate the target RNA while producing a new set of siRNAs to repeat the process.

A Synthetic 21 nt GFP Duplex siRNA Is Incorporated into Full-Length GFP dsRNA

It has been recently shown that synthetic 21 nt duplex siRNAs can mediate RNAi in both *Drosophila* embryo extracts and in Schneider cells (Elbashir et al., 2001b). We prepared a 32 P-labeled 21 nt duplex siRNA corresponding to nucleotides 26–44 in the GFP coding region with two additional uridine residues on the 3' end of each

strand (Figure 6A). This siRNA was tested for template-specific incorporation into full-length GFP dsRNA using sense and antisense GFP and *Pp-Luc* RNAs. Specific incorporation would provide evidence for RdRP activity in the extract. This synthetic duplex siRNA primed the synthesis of essentially full-length GFP dsRNA only when the antisense RNA strand was used as template (Figure 6B). The sense strand template primed the synthesis of the predicted 44 nucleotide fragment corresponding to the number of base pairs from the 3' end of the siRNA to the GFP AUG start codon at the 5' end of the template (Figure 6B). *Pp-Luc* templates did not produce any incorporation of the GFP siRNA (Figure 6B). Therefore, both strands of the siRNA were used as primers on the appropriate template strand and were incorporated into dsRNA as a single primer event.

The dsRNA produced with the synthetic GFP duplex siRNA was also synthesized and degraded with kinetics similar to the GFP dsRNA produced with micrococcal nuclease-generated GFP siRNAs (Figure 6D). This result suggests that a single primer in the natural siRNA population would be capable of priming the entire GFP dsRNA. Using the synthetic GFP siRNA, we never saw the intermediate products noted with the natural siRNAs (Figure 6C, panel 2 versus Figure 5A, panels 2 and 4). These intermediate products may represent numerous

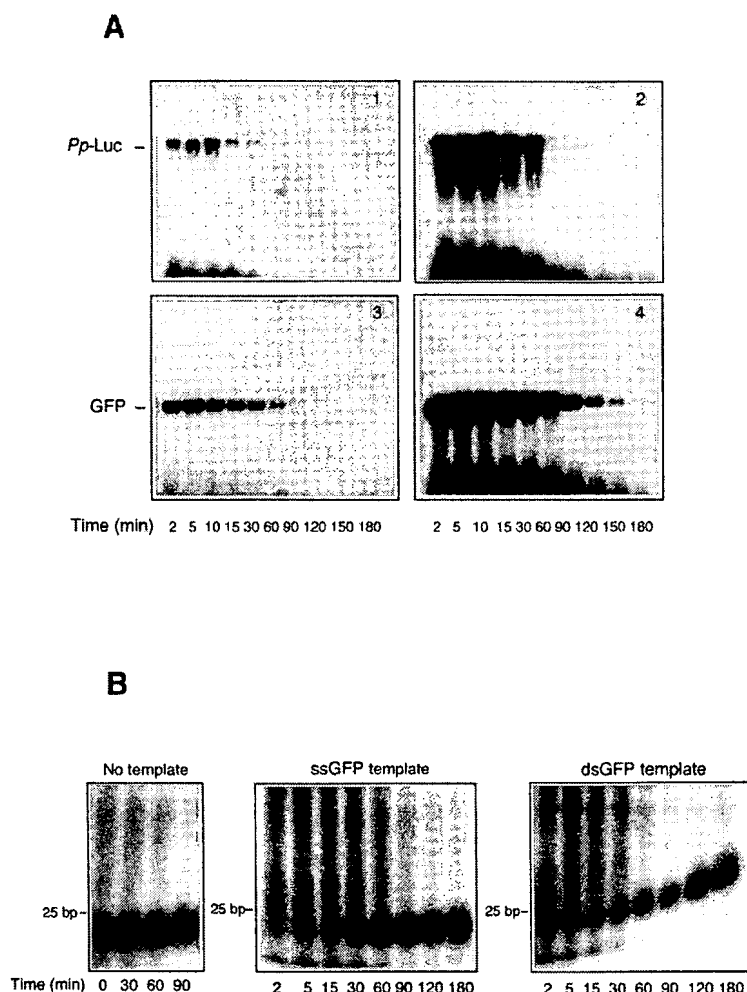


Figure 5. siRNAs Are Incorporated into dsRNA Prior to Degradation and the Formation of New siRNAs

(A) Nascent full-length GFP and *Pp-Luc* dsRNAs labeled by siRNA incorporation are synthesized then degraded upon prolonged incubation in extract. Labeled siRNAs are rapidly incorporated into dsRNAs up to the full-length of the template RNAs. Panels 1 and 3, short exposure (20 min) of *Pp-Luc* and GFP siRNA incorporation into dsRNA showing a maximal labeling rate at 5 to 10 min. Panels 2 and 4, long exposure (2 hr) of panels 1 and 3 to reveal shorter labeled products. The full-length products are essentially absent by 180 min of incubation.

(B) New siRNAs are produced during the degradation of the full-length GFP and *Pp-Luc* dsRNAs labeled by siRNA incorporation. No template, the siRNAs are not incorporated (see Figure 4A, minus template) and remain at a constant level at each time point; ssGFP template, using the reaction shown above in (A) panel 3, new siRNAs are derived from the labeled GFP dsRNA and continue to accumulate during the incubation period; dsGFP, GFP dsRNA was used as a template for siRNA incorporation. New siRNAs accumulate more slowly during the reaction period due to the competition for cleavage by cold GFP dsRNA. Images are from the Fuji Phosphorimager.

single primer extensions from various primer positions along the target RNA.

Efforts to use unlabeled siRNAs and ^{32}P -UTP to follow dsRNA synthesis were unsuccessful due to the high levels of nonspecific UTP incorporation in the extract that were unaffected by α -amanitin and actinomycin D. Nevertheless, the incorporation of the synthetic 21 nucleotide GFP duplex siRNA into dsRNA is consistent with the presence of RdRP in the extract and its role in the siRNA-dependent generation of dsRNA from the mRNA target.

Discussion

Model for RNAi

Our findings complement and clarify the previously proposed models concerning the mechanism of PTGS, quelling, and RNAi (Bass, 2000; Baulcombe, 1999b, 2000; Carthew, 2001; Cogoni and Macino, 2000; Gura, 2000; Hammond et al., 2001b; Hunter, 2000; Kooter et al., 1999; Maine, 2000; Marx, 2000; Meins, 2000; Plasterk and Ketting, 2000; Sharp, 2001; Sijen et al., 2001). Most importantly, we demonstrate the template-specific incorporation of the 21–25 nucleotide RNAs, or siRNAs, to generate dsRNA that is subsequently cleaved by RNase

III activity into new siRNAs. In this way, mRNA is degraded through a cycle of “degradative-PCR” (Figure 7). We present substantial evidence for RdRP activity in *Drosophila* extracts and suggest siRNA incorporation into dsRNA involves RdRP, the crucial step in the amplification of the target RNA for rapid degradation by RNase III-type activity. Although we cannot exclude that siRNAs may be incorporated into dsRNA by a direct “guide” mechanism not involving RdRP, such a process would not give the sufficient amplification of the double-stranded RNA target. This would be needed to trigger efficient RNAi with substoichiometric levels of the initiating double-stranded trigger RNA. Consistent with the genetic screens in other lower eukaryotes, our results suggest a role for RdRP in *Drosophila* RNAi as well.

Double-Stranded RNA as the Target for Degradation and the Source for New siRNAs

The requirement for a dsRNA trigger as the effector for silencing can be partially explained by the nature of the dsRNA cleavage step required for siRNA production. As previously noted by Fire and associates (Parrish et al., 2000) and reiterated by Sharp (Sharp, 2001), any factor that significantly alters the double-stranded nature of the dsRNA trigger, such as sequence divergence or

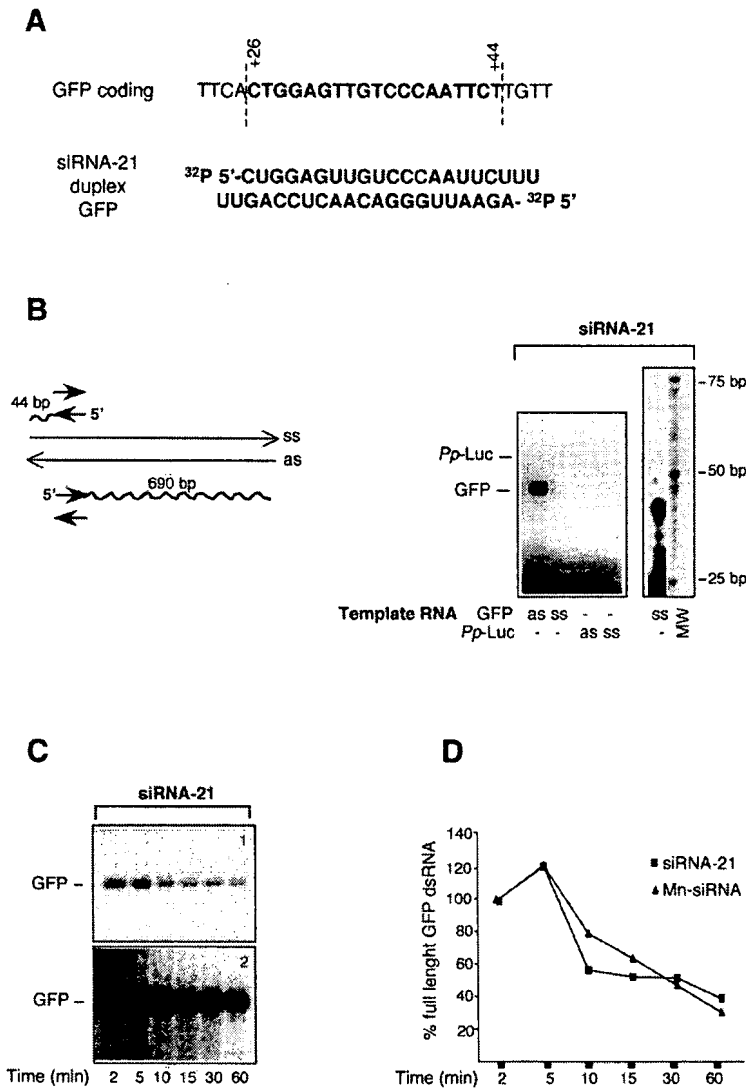


Figure 6. Incorporation of a Synthetic 21 Nucleotide Duplex GFP siRNA into dsRNA

(A) The GFP coding region and the sequence of the synthetic 21 nucleotide duplex GFP siRNA are indicated. The siRNA corresponds to nucleotides 26–44 in the GFP coding region with two additional uridine residues on the 3' end. The 5' end of each strand of the siRNA was labeled with [γ -³²P]ATP (see Experimental Procedures).

(B) Left, the expected product lengths for incorporation of each strand of the synthetic 21 nucleotide GFP siRNA using full-length GFP sense and antisense RNA templates are shown: 690 bp with the antisense strand template and 44 bp with the sense strand template. Right, the ³²P-labeled siRNA incorporation into full-length GFP dsRNA occurs only with the GFP antisense RNA template and gives the expected 44 bp product with the sense strand template. Pp-Luc templates give no product. GFP and Pp-Luc mark the positions of the full-length RNAs and MW denotes the 25 bp ladder.

(C) GFP dsRNA produced with the synthetic 21 nucleotide duplex GFP siRNA is rapidly synthesized and then degraded upon prolonged incubation in the reaction (Panel 1). Note with longer exposure the absence of intermediate products for the early time points for GFP dsRNA formation (Panel 2). GFP marks the position of the full-length RNA (690bp). Panels 1 and 2 were exposed for 30 min and 2 hr, respectively.

(D) GFP dsRNA produced either with micrococcal nuclease generated GFP siRNAs (Mn-siRNA, data from Figure 5A, GFP) or the synthetic 21 nucleotide duplex GFP siRNA (siRNA-21) is synthesized and degraded with the same kinetics in the incorporation reaction. Note that maximal levels of GFP dsRNA occur at 5 min for both synthetic and natural siRNAs. Data were quantified on the Fuji Phosphorimager.

chemical modification, affects silencing substantially. In the model proposed here, any changes in strand complementarity could presumably reduce the susceptibility of the triggering dsRNA to RNase III-type cleavage. The nature of the sense and antisense strands in the triggering dsRNA would also play a role in the efficacy of silencing since the RdRP amplification step would depend upon the production and functionality of the siRNAs. We show that both strands of a synthetic 21 nucleotide GFP duplex siRNA function as primers to give the expected RNA products when the appropriate GFP template strand is used (Figure 6).

The length of the siRNAs may be an important aspect of their function. Previous reports indicated that 29–36 nucleotide dsRNAs transcribed in vitro do not direct RNAi efficiently in *Drosophila* extracts (Elbashir et al., 2001b), and that a 26 nucleotide dsRNA, also transcribed in vitro, when injected into worms, triggered lower than expected levels of RNA interference at 25°C and none at 16°C (Parish et al., 2000). However, chemically synthesized 21 and 22 nucleotide siRNAs can mediate targeted RNA cleavage in *Drosophila* embryo ex-

tracts and in Schneider cells (Elbashir et al., 2001a, 2001b). The siRNAs produced in *Drosophila* embryo extract by micrococcal nuclease and CIP treatment are essentially as efficient on a weight basis in RNAi as the full-length dsRNA from which they were derived, suggesting there is some optimal length for siRNAs in RNAi. The conservation in the size range for the small RNAs associated with silencing in all the species examined proposes that it may be closely correlated with primer function. This could be due to some unique property of primer activity in a protein complex that has yet to be identified.

We demonstrate that siRNAs require a 3' hydroxyl group for function in RNAi. The authentic siRNAs, produced in *Drosophila* extracts by RNase III-related enzymes such as Dicer (Bernstein et al., 2001), have been chemically characterized and shown to have a 5' phosphate and a 3' hydroxyl group (Elbashir et al., 2001b). The micrococcal nuclease generated siRNAs described here are functional in RNAi and can be incorporated into dsRNA after phosphatase treatment (Figure 3) to remove the 3' phosphate group produced by the nuclease diges-

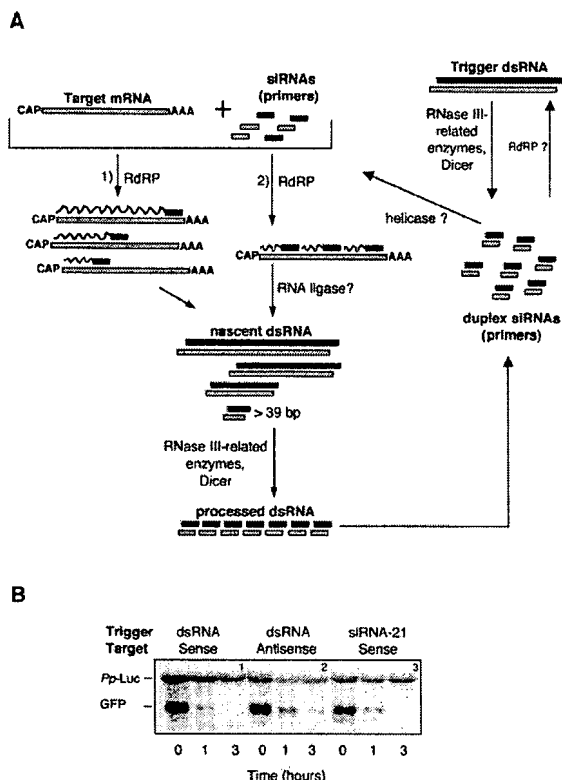


Figure 7. A Model for RNAi

(A) RNAi is initiated by a threshold concentration of the trigger dsRNA, which is immediately cleaved by RNase III-related enzymes, such as Dicer, to generate duplex siRNAs. The siRNAs are proposed to interact with the target mRNA as primers to form dsRNA in the following ways: (1) siRNA primers align randomly along the template strand, one per template, and are extended by RdRP to form dsRNA; and (2) multiple siRNA primers align along the template strand and are extended by RdRP then ligated by a putative RNA ligase. If the siRNAs were "guide" RNAs, they would only be ligated along the template strand by RNA ligase to form larger dsRNA. However, a "guide" mechanism for primer incorporation could not amplify the target dsRNA sufficiently when substoichiometric levels of dsRNA are used to initiate RNAi, so this mechanism is not considered likely. Importantly, unlike RdRP, there is no genetic evidence for the involvement of RNA ligase in RNAi. Even though dsRNA can direct siRNA incorporation in vitro, this may play a minor role in the overall RNAi process and the potential amplification of the trigger dsRNA since dsRNA is rapidly degraded. Genetic screens also suggest a role for helicase, which may be involved in unwinding the siRNAs for dsRNA synthesis. Ultimately, the nascent dsRNA greater than 39 bp in length is then cleaved by RNase III-related enzymes and this step generates new siRNAs to amplify mRNA degradation. It is not clear what fraction of the dsRNA generates siRNAs. This cycle is repeated with the new siRNAs until the target mRNA is degraded. (B) The model predicts that the GFP dsRNA trigger RNA should target both sense and antisense GFP transcripts, and that the GFP siRNA primer used in Figure 6 should direct GFP mRNA degradation since it generates dsRNA greater than 39 bp. This is what is observed using either 1 μ g of GFP dsRNA or 1 μ g GFP siRNA-21 to target GFP sense or antisense mRNA; Panel 1, GFP dsRNA with GFP mRNA; Panel 2, GFP dsRNA with GFP antisense mRNA; and Panel 3, GFP siRNA-21 with GFP mRNA.

tion. The 21 nucleotide synthetic siRNA primer (Figure 6) also has a 3' hydroxyl group that would be required for incorporation into dsRNA by RdRP activity. Whether or not the 3' hydroxyl group is also used in a primer ligation step remains to be determined.

In agreement with the results reported using the synthetic siRNAs hybridized to RNA (Elbashir et al., 2001b), we show that cleavage of the GFP target RNA occurs after the synthetic GFP siRNA is incorporated into dsRNA. If cleavage occurred in the template RNA immediately upon binding to the synthetic GFP siRNA, no full-length GFP dsRNA would have been observed. Therefore, cleavage occurs in the nascent dsRNA in regions inside and outside the zone represented by the initial siRNA since the primers are extended to make dsRNA. The fact that the synthetic GFP siRNA is extended to the 5' end of the sense strand template (Figure 6B) would also restrict cleavage, in this instance, to the region upstream of the 3' terminus of the siRNA. Any region of the target RNA converted into duplex by a given siRNA would be subject to digestion by RNase III activity.

A Role for RdRP in *Drosophila* RNAi

It has been proposed previously that there is no amplification of the trigger dsRNA in RNAi in *C. elegans* (Sharp, 2001), based upon the effects of asymmetric strand substitutions in the input dsRNA (Parrish et al., 2000). We provide evidence that both single- and double-stranded RNAs can serve as templates for siRNA incorporation into dsRNA in *Drosophila* extract. However, the rapid degradation of dsRNA suggests that amplification of the trigger dsRNA is of limited value. In the substitution experiments described by Fire et al. (Parrish et al., 2000), modifications on the sense strand had no effect, but the same substitutions on the antisense strand rendered the modified dsRNA inactive in RNAi when injected into worms. Since any amplification of the modified dsRNA would dilute out the substitution effects, it was concluded that the target dsRNA was not amplified (Sharp, 2001). Our results also indicate that the antisense siRNA strand would be the most important for the synthesis of new dsRNA from the mRNA template (Figure 6).

Genetic studies have identified several mutants in *C. elegans*, *Neurospora crassa*, and *Arabidopsis thaliana* that resist RNA interference. These include mutants that affect the initiation of silencing activity (*rde-1* and *rde-4* in *C. elegans* [Tabara et al., 1999], *qde-2* in *Neurospora* [Catalanotto et al., 2000], and *AGO-1* in *Arabidopsis* [Fagard et al., 2000]), mutants in the effectors of silencing (*rde-2* and *mut-7* in *C. elegans* [Ketting et al., 1999], the later related to RNase D), mutants in helicase (*qde-3* in *Neurospora* [Cogoni and Macino, 1999b], and *SDE-3* in *Arabidopsis* [Dalmay et al., 2001]), and mutants in RNA-dependent RNA polymerase (*ego-1* in *C. elegans* [Smardon et al., 2000], *qde-1* in *Neurospora* [Cogoni and Macino, 1999a], and *SGS-2/SDE-1* in *Arabidopsis* [Dalmay et al., 2000; Mourrain et al., 2000]). Although by sequence comparison an RdRP homolog in *Drosophila* has not yet been identified, our results suggest the presence of an RdRP gene.

RdRP-dependent as well as -independent mechanisms may be involved in the generation of dsRNA up to the full-length of the target RNA, according to one of the following schemes: (1) a single siRNA primer would be extended from various positions along individual template strands by RdRP to generate dsRNAs; and (2) different siRNAs would associate along a single template RNA and be extended by RdRP to the adjacent

siRNA primer. The extension products would be ligated to generate dsRNAs. This model would require RdRP activity as well as an RNA ligase step; and (3) dsRNAs would be formed by a primer "guide" mechanism where they would align along the template for subsequent ligation. All these mechanisms could generate dsRNA of sufficient length to be cleaved by RNase III-type activity since this requires a minimum of 39 base pairs (Elbashir et al., 2001b). Our results favor the first and second models since RdRP activity would be required to amplify the target dsRNA sufficiently when substoichiometric amounts of the trigger dsRNA are involved in initiating RNAi (Figure 7). A primer "guide" mechanism would require the involvement of an RNA ligase in order to generate larger dsRNAs, and genetic screens have not identified related genes as candidates essential for RNAi. As previously noted, RdRP genes have been shown to be involved in posttranscriptional gene silencing in three different lower eukaryotes. In addition, the "guide" primer ligation model is not supported by observations using synthetic siRNAs. siRNAs generated from dsRNAs greater than 111 nucleotides in length are not well defined and are derived from several overlapping regions of different lengths (18–24 nucleotides) to make the siRNA population heterogeneous in composition (Elbashir et al., 2001b). It is unlikely that a "guide" mechanism could sort out the precise siRNAs for ligation along the target RNA to rapidly generate the full-length dsRNA. The most convincing evidence for the involvement of RdRP activity in *Drosophila* RNAi comes from our results using the synthetic 21 nucleotide GFP duplex siRNA where full-length GFP dsRNA was produced from a single primer (Figure 6) with the same time course of synthesis and degradation as dsRNA produced using the micrococcal-nuclease-generated GFP siRNAs (Figure 6D). The extension of both strands of the synthetic siRNA in a template-dependent manner to yield the expected dsRNA products (Figure 6B) would specifically require an RdRP. The role for helicase activity in RNAi, as shown for *qde-3* in *Neurospora* (Cogoni and Macino, 1999b) and *SDE-3* in *Arabidopsis* (Dalmay et al., 2001) in the genetic screens, may be to unwind the primers or the dsRNA trigger, but this remains to be demonstrated.

Predictions from the Degradative PCR Model

Double-stranded RNA is processed into siRNA primers that convert the target mRNA into dsRNA for subsequent degradation and the formation of new siRNAs. Since the siRNA primers are double stranded, they should direct the degradation of either sense or antisense cognate target RNAs. This is exactly what is observed when either sense or antisense GFP mRNA is incubated in extract with GFP dsRNA (Figure 7B). Therefore, dsRNA representing transcripts derived from opposite strands of a complementary template would be targeted simultaneously to effect silencing of more than a single gene in some instances. The siRNA primer model also suggests a single siRNA should target transcript degradation as long as the primer extended product is of sufficient length to be cleaved by RNase III activity, roughly 39 nucleotides. When the siRNA-21 shown in Figure 6 is used in a silencing assay, GFP mRNA is selectively degraded, as predicted, since the extended primer produces dsRNA 44 bp long (Figure 7B). Therefore, a single

primer is sufficient to target mRNA silencing. This result also predicts that a primer producing the longest dsRNA product would be the most efficient since the second generation of siRNAs would represent more of the target RNA.

Experimental Procedures

Drosophila Embryo Extracts and RNAi Assay

Drosophila embryo extracts were prepared and subfractionated using the procedures previously described by Tuschl et al. (1999) and Hammond et al. (2000). Aliquots of all extract preparations were stored at -80°C . In vitro RNAi reactions were performed as described by Tuschl with 10–50 pM (final concentration) ^{32}P -UTP-labeled capped and adenylated target and control mRNAs and 10 nM unlabeled dsRNA as the trigger RNA unless otherwise noted. Reaction products were analyzed on 1.5% formaldehyde gels and processed as previously described (Tuschl et al., 1999).

RNA Synthesis

Unlabeled and labeled RNAs were prepared using one of the following Ambion kits, as recommended by the company. After synthesis, the reactions were phenol extracted and precipitated with isopropanol as recommended. For unlabeled dsRNA, the MegaScript T3 and T7 kits were used. For labeled dsRNA, the T3 and T7 MaxiScript kits were used with ^{32}P UTP (800 Ci/mmol) as the only source of UTP. For capped and adenylated labeled mRNA, the SP6 Message Machine Kit was used with 40 μCi UTP (Redivue 3000 Ci/mmol) and the appropriate GFP (716 bp) and *Pp-Luc* (1652 bp) plasmids. Where indicated, the RNAs were annealed, as previously described by (Misquitta and Paterson (1999) and Tuschl et al. (1999) and digested with RNase one to eliminate single strand contamination.

Analysis of dsRNA Processing

High specific activity GFP dsRNA ($>1 \times 10^8$ cpm/ μg) was incubated for various times, up to 3 hr, at the indicated concentrations in *Drosophila* extract, as described for RNAi assays. After treatment, samples were prepared for analysis on 6% sequencing gels, as previously noted (Zamore et al., 2000). A 25 bp DNA ladder was used as a molecular weight marker and was labeled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ according to the manufacturer (Life Sciences). In order to process siRNA-labeled GFP dsRNA, the RNA was fractionated on a 4% mini DNA-sequencing gel, the band of full-length dsRNA was excised, and the material electro-eluted. The eluted material was phenol extracted and precipitated with 20 μg of glycogen carrier, dissolved in TEN (10 mM Tris [pH 7.5], 0.1 mM EDTA, and 20 mM NaCl), annealed, and processed for siRNA production in a 30 min incubation as described above.

Preparation of siRNAs

For preparative amounts of siRNAs, 500 ng to 10 μg of unlabeled dsRNA was added to 100 μl of S100 extract, prepared from embryo lysate according to Hammond et al. (2000), and adjusted to a final concentration of 100 mM potassium acetate, 30 mM HEPES (pH 7.4), 2 mM Mg acetate, 5 mM DDT, 10 mM creatine phosphate, 1 $\mu\text{g}/\text{ml}$ creatine kinase, 500 μM ATP, and 500 μM α -amanitin and incubated 30 min at 25°C . After processing, micrococcal nuclease (Roche) was added to a final concentration of 60 U/50 μl of reaction in the presence of 2 mM CaCl_2 and incubated for 30 min at 37°C . This step was repeated and the reaction was stopped by the addition of EGTA to a final concentration of 10 mM. The siRNA preparation was digested with 100 $\mu\text{g}/\text{ml}$ proteinase K for 15 min at 65°C in 100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, and 1% SDS, phenol extracted and precipitated with ethanol using 40 μg of glycogen as carrier (Tuschl et al., 1999). The precipitated material was dissolved in 50 μl of water. Ten microliters of the siRNAs were treated with 20 U of calf-intestinal phosphatase in New England Biolabs buffer 3 in a final volume of 50 μl for 1 hr at 37°C . The sample was phenol extracted and precipitated with ethanol. The material was dissolved in 10 μl of water for further use. For the preparation of very high specific activity siRNAs, RNA was transcribed in vitro (see below) using ^{32}P UTP (800 Ci/mmol) as the only source of UTP to give dsRNAs with specific activities $>10^8$

cpm/ μ g. Typically, 1 μ g of labeled dsRNA was processed as above to generate highly labeled siRNAs.

Two synthetic 21 nucleotide RNAs were synthesized by the Yale Keck Labs (trityl-off) representing nucleotides 26–44 in the GFP coding region and containing two additional U residues on the 3' end: RNA-1, CUGGAGUUGUCCAAUUCUUU and RNA-2, UUGACCUCAACAGGGUUAAGA. To label the 21 nucleotide RNAs, 0.1 μ g of each RNA was labeled in a 10 μ l reaction using T4 polynucleotide kinase (NEB) with 3 μ l of [γ - 32 P]ATP (crude, ICN 6000 Ci/mmol) and passed over a G-25 spin column in 50 μ l final volume (preadjusted with TE) to yield a specific activity of $>10^8$ cpm/ μ g for each 21 nucleotide RNA. Kinase was inactivated by incubation at 70°C for 10 min after the addition of 1 μ l of 0.5 M EDTA, as recommended by the manufacturer. Twenty microliters of each labeled synthetic RNA was annealed in a 50 μ l reaction to form the siRNA as described (Elbashir et al., 2001b). Unlabeled siRNAs were prepared by annealing 7.5 μ g of each RNA in 50 μ l as previously noted.

SiRNA Incorporation Assay

S100 fraction, prepared from embryo extract, was used instead of crude extract under standard RNAi reaction conditions with 500 μ M α -amanitin, 2.5×10^5 cpm of 32 P-UTP labeled siRNAs, and 3 μ g of RNA template (either double- or single-stranded RNA), incubated at 25°C for 60 min or the indicated times for the time course reactions. The primer incorporation reaction was stopped with proteinase K/SDS buffer and extracted as before (Tuschl et al., 1999). Labeled RNA products were analyzed on 1.5% agarose formaldehyde gels or 6% DNA sequencing gels. For incorporation reactions using the 21 nucleotide synthetic duplex siRNA, 1 μ g of unlabeled siRNA was mixed with 3×10^6 cpm of labeled siRNA along with 3 μ g of template and incubated in extract as described above.

Ribonuclease One and Ribonuclease T1 Digestions

Digestions of single- and double-stranded RNAs as well as the products of the siRNA incorporation reactions were carried out using Ribonuclease One (Promega, 10 μ U/3 μ g of cold template) for up to 5 min at 37°C in buffer supplied by the manufacturer. Ribonuclease One digests RNA to nucleotide monophosphates so gel purification of dsRNA was not required. Reactions were then phenol extracted as before and analyzed on 1.5% agarose formaldehyde gels. For Ribonuclease T1 (Roche) digestion, identical RNA samples were dissolved in 50 μ l of TE (10 mM Tris [pH 7.5] and 0.1 mM EDTA) and digested with 100–500 units of enzyme for 30 min at 37°C. The reactions were phenol extracted and precipitated with 20 μ g of glycogen carrier prior to gel analysis.

Image Analysis

Radioactivity was detected by exposing the dried agarose gel (dried under vacuum) onto an Immobilon-NY+ membrane or sequence gel directly to Kodak AR-2 film at –80°C with a Dupont screen or a Fuji Image plate, the latter quantified with a Fujix Bas 2000 using Image Gauge 3.0 software.

Acknowledgments

We would like to acknowledge Claude Klee for help and suggestions. Also we would like to thank the anonymous referee for describing our results as “Nature’s own PCR machine” and for inspiration on the title. We thank Carl Wu for the use of his *Drosophila* cages.

Received June 25, 2001; revised September 17, 2001.

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