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Señor

**DIRECTOR DIVISIÓN DE NUEVAS CREACIONES  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

E.

S.

D.

REF: Expediente No. **11-64192-** solicitud de patente de invención a favor de **MAGNACHEM INTERNATIONAL LABORATORIES, INC.**  
Título **MÉTODO PARA CLASIFICAR COMPUESTOS QUE INTERACTÚAN SELECTIVAMENTE CON RAD9**

**RESPUESTA A EXAMEN DE FONDO**

**JUAN PABLO CADENA SARMIENTO**, portador de la tarjeta profesional No. 57.492 del C.S.J., obrando como apoderado de la sociedad solicitante me permito dar respuesta al oficio número 9644 relacionado con la patentabilidad de la presente invención y notificado por fijación en lista el 4 de Junio de 2013.

Con el ánimo de subsanar las objeciones planteadas por el señor Examinador, nos permitimos presentar un nuevo pliego reivindicatorio compuesto por seis (6) reivindicaciones, en donde no se amplía en ningún momento la materia inicialmente divulgada, y se encuentra enteramente sustentado en la descripción.

**Claridad**

Nos permitimos mostrar nuestro desacuerdo frente a la objeción del señor Examinador relacionada con que la descripción no divulga la invención de manera suficiente completa, ya que no se divulga la producción de un ser vivo con una mutación en el complejo de proteínas incluyendo Rad9.

Al respecto, nos permitimos aclarar a su Despacho que el objeto técnico de la presente invención **no** corresponde a la generación de mutaciones sobre moléculas proteicas, sino al desarrollo de un método que permite el cribado de compuestos de prueba en términos de su actividad anticancerígena. En este sentido, la divulgación de la producción de un ser vivo con una mutación en el gen Rad9 no corresponde a un aspecto esencial de la presente invención, toda vez que el normalmente versado en la materia se encuentra en plena capacidad (a partir de los conocimientos básicos del arte previo) de obtener las mutaciones de interés, más aun al tener en cuenta que la descripción explica en términos generales las líneas celulares adecuadas y los modelos animales que se encuentra disponibles comercialmente o que pueden ser preparados mediante prácticas de rutina.

Así las cosas, la descripción establece lo siguiente:

***"Sistemas de Ensayo***

*Los estudios de la eficacia del Securolide en inducir la muerte celular selectiva en las líneas de células de levadura de mutante Rad9 y/o p53 demuestra la viabilidad de usar las líneas de células para clasificar otros compuestos que inducen selectivamente la muerte celular en las líneas de mutantes Rad9 y/o p53 como es comparado con las líneas de tipo silvestre o modelos animales. Las líneas de células adecuadas y los modelos animales son comercialmente disponibles o se pueden preparar con esfuerzo de rutina. Por ejemplo, the American Type Culture Collection, Manassas, VA 20108, lista varias cepas de mutante Rad9 de levadura (números de catálogo 90731; 90730; 74154, 4003576, 4023576, y 4033576. Un pez cebra que contiene un RAD9 clonado homólogo es disponible de ATCC como número de catálogo 10169289. También existen numerosas publicaciones científicas en las cepas de levadura que tienen varios defectos que implican no solo RAD9 y/o p53 sino otros aspectos del complejo que implica RAD9 y/o p53, los cuales también se pueden usar para clarificar compuestos que inducen la muerte celular como es comparada con las células normales. Las células normales, especialmente células de mamífero, también se pueden diseñar para inducir mutaciones en el hRAD9 y/o gen p53, para el uso en*

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ensayos. El homólogo humano se lista en la base de datos NCBI, junto con los genes para ratones y ratas. (LocusID 5883).

*Las mutaciones se introducen de manera preferible mediante mutagénesis dirigida al sitio. La mutagénesis dirigida al sitio es una técnica de biología molecular en la cual se crea una mutación en un sitio definido en una molécula de DNA. En general, la mutagénesis dirigida al sitio requiere que la secuencia de genes de tipo silvestre sea conocida. Un oligonucleótido con su secuencia que contiene una mutación deseada se sintetiza químicamente. El oligonucleótido se une por el hidrógeno de par base que se enlaza a la secuencia de genes de tipo silvestre complementarios. El oligonucleótido sintético se usa como un cebador para la síntesis in vitro de una nueva hebra de DNA que es complementaria a la hebra original (plantilla). La síntesis de DNA se realiza al agregar la enzima DNA polimerasa a la plantilla de DNA. La hebra recientemente sintetizada de DNA tiene el cebador y la mutación deseada incorporada en el. Al usar un par de cebadores en la reacción en cadena de la polimerasa es posible amplificar la molécula de DNA recientemente creada y producir suficientes copias para hacer la manipulación adicional del nuevo DNA posible. Típicamente, el DNA mutado luego se inserta en un vector de expresión por medio de enzimas de restricción y la DNA ligasa. El vector de expresión luego se inserta típicamente en una célula donde se puede usar como una plantilla genética para la síntesis de una proteína mutada. La actividad biológica de la proteína mutada luego se puede comparar a aquella de la proteína de tipo silvestre."*

Por tanto, es claro que el versado en la materia puede generar mutaciones a partir de las secuencias *in vivo*, lo cual se encuentra considerablemente desarrollado en un amplio número de referencias del estado del arte que describen métodos de mutagénesis sitio dirigida *in vivo* en células de levadura y en células bacteriales. En este sentido, adjuntamos las referencias **Storici et al.** (Methods Enzymol., 2006, 409:329-345) y **Valledor et al.** (IUBMB Life, 2012, 64:684-689) para la contextualización de su Despacho.

Así las cosas, la descripción sustenta debidamente la materia reivindicada, y por tanto, damos por superada la objeción emitida.

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Ahora bien, el señor Examinador objetó el pliego reivindicatorio a la luz de la descripción, por cuanto asegura que las únicas estructuras probadas corresponden a las de fórmula Ia a Ie. Al respecto, nos permitimos recordar a su Despacho que los ejemplos contenidos en el capítulo descriptivo se constituyen únicamente como una ilustración de la invención, teniendo en cuenta que la misma no puede verse limitada por dichas divulgaciones.

No obstante lo anterior, y con el ánimo de facilitar la aceptación de la presente solicitud, el pliego reivindicatorio ha sido modificado con el fin de especificar que el método objeto de la invención involucra los compuestos de formula Ia-Ie.

Por tanto, consideramos que los aspectos relacionados con la descripción han sido completamente esclarecidos.

Partiendo de lo anteriormente expuesto, no existe ninguna necesidad de especificar (i) el desarrollo de las mutaciones sobre el gen Rad 9, ni (ii) las líneas celulares mencionadas a lo largo del pliego reivindicatorio, toda vez que dichas líneas celulares se encuentran comercialmente disponible y por tanto el versado en la materia se encuentra en plena capacidad de desarrollar y aislar el material biológico de interés sin tener que recurrir a experimentación excesiva.

En este mismo sentido, reiteramos como ejemplo la existencia de seis (6) depósitos de cepas mutantes Rad9 de *Saccharomyces cerevisiae* bajo los números ATCC: 90731, 90730, 74154, 4003576, 4023576 y 4033576, los cuales se anexan al presente memorial, y del depósito de un pez cebra (*Danio rerio*) que contiene un Rad9 clonado homólogo bajo el deposito ATCC 10169289.

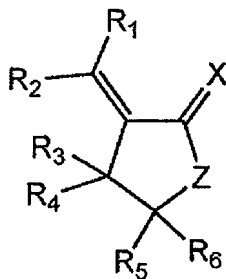
Por lo tanto, insistimos en que existen numerosas publicaciones científicas relacionadas con cepas de levadura que contienen varios defectos relacionados no solo con Rad9 o p53 sino con otros aspectos de interés del complejo que involucra Rad9 y p53, las cuales pueden ser usadas para el cribado de compuestos que inducen la muerte celular en comparación con células normales.

Sumado a lo anterior, y tal como lo establece la descripción, las células normales, particularmente las correspondientes a células de mamífero, también pueden ser diseñadas con el fin de inducirles mutaciones sobre los genes hRad9 y/o p53, con el fin de hacerlas aptas para los ensayos de interés.

Así las cosas, las cepas celulares de levadura empleadas a lo largo de los ejemplos contenidos en la descripción corresponden a cepas públicamente disponibles previa presentación de la presente solicitud, tal como se demuestra en los documentos anexos al presente memorial. (Ver documento anexo **Winzeler EA, et al.** Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901-906, 1999)

En consecuencia, la nueva reivindicación 1 es completamente clara y concisa, al establecer que las características esenciales del método reivindicado corresponden a:

1. *Un método para la evaluación de compuestos que tienen actividad anti cancerígena, que comprende:*
  - (a) *proporcionar células que tienen una o más mutaciones en el complejo de proteínas de control del ciclo celular que incluye Rad9, en donde las células tienen una mutación en el gen Rad9, y en donde las células son defectuosas en la proteína Rad9 mediada por la detención del ciclo celular.*
  - (b) *adicionar un compuesto a ser evaluado a las células, en donde el compuesto tiene la estructura de Formula Ia:*



Fórmula Ia

en donde

*R<sub>1</sub>-R<sub>6</sub> se seleccionan independientemente de un átomo de hidrógeno, un átomo de halógeno, un grupo hidroxilo, alquilo, alquilo sustituido, alquenilo, alquenilo sustituido, alquinilo, alquinilo sustituido, fenilo, fenilo sustituido, arilo, arilo sustituido, heteroarilo, heteroarilo sustituido, halo, hidroxilo, alcoxi, fenoxi, fenoxi sustituido, aroxi, aroxi sustituido, alquiltio, feniltio, feniltio sustituido, ariltio, ariltio sustituido, ciano, isociano, isociano sustituido, carbonilo, carboxilo, carboxilo sustituido, amino, amino sustituido, amido, amido sustituido, sulfonylo, ácido sulfónico, fosforilo, fosfonylo, poliarilo, poliarilo sustituido, C<sub>3</sub>-C<sub>20</sub> cíclico, C<sub>3</sub>-C<sub>20</sub> cíclico sustituido, heterocíclico, heterocíclico sustituido, un aminoácido, un péptido, o grupo polipéptido; o R<sub>3</sub>-R<sub>6</sub> tomados en conjunto forman un anillo sustituido o no sustituido que opcionalmente incluye uno o más heteroátomos seleccionado del grupo que consiste de oxígeno, azufre y nitrógeno.*

*Z es un heteroátomo seleccionado de oxígeno, azufre o nitrógeno; y*

*X es un heteroátomo seleccionado de oxígeno, azufre, o nitrógeno.*

- (c) *determinar si el compuesto genera muerte o induce apoptosis en algunas o en la totalidad de las células;*
- (d) *comparar la determinación resultante de la etapa (c) con la determinación resultante inducida por el compuesto en células normales que no tienen la una o más mutaciones en (a) para determinar si el compuesto tiene actividad anti cancerígena.*

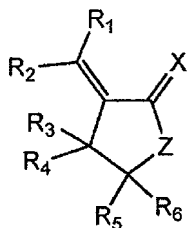
toda vez que el método reivindicado se encuentra debidamente caracterizado en término de las etapas y condiciones técnicas específicas que permiten dar solución al problema técnico propuesto.

En consecuencia solicitamos respetuosamente aceptar el nuevo pliego reivindicatorio como claro y conciso.

**Novedad y nivel inventivo**

Teniendo en cuenta que el señor Examinador reconoció la novedad de la inclusión de los compuestos de formula Ia como parte del método reivindicado damos por superada la objeción sobre la nueva reivindicación 1, toda vez que la misma establece en la etapa b) lo siguiente:

*(b) adicionar un compuesto a ser evaluado a las células, en donde el compuesto tiene la estructura de Formula Ia:*



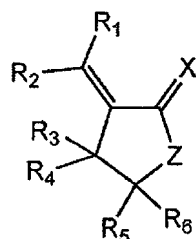
Fórmula Ia

Así las cosas, la presente invención debe ser reconocida como novedosa a la luz de la referencia D1.

Ahora bien, es fundamental poner en conocimiento de su Despacho que la presente invención surge de la necesidad de identificar compuestos altamente selectivos, en términos de su capacidad citotóxica, los cuales permitan inducir la muerte celular de células tumorales y preserven la integridad de las células normales. Así las cosas, la presente invención se constituye como una respuesta a dicha necesidad al proporcionar un método de cribado de la actividad anti-tumoral selectiva de compuestos específicos mediante la aplicación de cuatro etapas esenciales que emplean células que presentan una o más mutaciones en el complejo de proteínas del punto de control del ciclo celular incluyendo Rad9, o un gen que codifica una proteína en el complejo. Así las cosas, las etapas del método comprenden:

- (a) proporcionar células que tienen una o más mutaciones en el complejo de proteínas de control del ciclo celular que incluye Rad9, en donde las células tienen una mutación en el gen Rad9, y en donde las células son defectuosas en la proteína Rad9 mediada por la detención del ciclo celular.
- (b) adicionar un compuesto a ser evaluado a las células, en donde el compuesto tiene

la estructura de Formula Ia:



Fórmula Ia

- (c) determinar si el compuesto genera muerte o induce apoptosis en algunas o en la totalidad de las células; y
- (d) comparar la determinación resultante de la etapa (c) con la determinación resultante inducida por el compuesto en células normales que no tienen la una o más mutaciones en (a) para determinar si el compuesto tiene actividad anti cancerígena.

En este sentido, notamos que el señor Examinador objetó el nivel inventivo del método reivindicado a la luz de las divulgaciones de las referencias D1 y D2 al considerar que el versado en la materia habría combinado las enseñanzas de las mismas para llegar al objeto de la presente invención. Al respecto, nos permitimos incluir los argumentos que defienden el nivel inventivo de la presente invención, y que por tanto, desvirtúan las conclusiones de su Despacho.

Como primera medida notamos que a pesar de que la referencia **D1 (Methods in Molecular Biology, 223, 555-576 (2004))** se encuentra relacionada con ensayos realizados sobre líneas celulares de *Saccharomyces cerevisiae* en lo concerniente a la reparación de ADN de agentes anticancerígenos citotóxicos, dicha referencia es completamente silenciosa frente a un método para el cribado de compuestos farmacéuticamente activos a partir de compuestos de formula Ia, el cual se lleve a cabo mediante el empleo de células que presentan una o más mutaciones en el complejo de proteínas de control del ciclo celular incluyendo Rad9, o un gen que codifica una proteína en el complejo.

En este sentido, es claro que lo anteriormente no solo refuerza la novedad de la presente invención a la luz de D1 sino que nos permite concluir que el versado en la

materia enfrentado a la necesidad de métodos alternativos para la identificación de compuestos con actividad citotóxica selectiva no encontraría motivación alguna en las divulgaciones de la referencia en mención.

No obstante lo anterior, notamos que el señor Examinador centró la base de la argumentación que afecta la patentabilidad de la presente invención en un estudio citado en la referencia D1 que hace mención de mutantes Rad9, el cual se encuentra relacionado con la sensibilidad de la reparación del ADN y los mutantes del punto de control de *Saccharomyces cerevisiae* de agentes anticancerígenos aprobados por parte de la FDA. En este sentido, la mención acerca de la mutación en cuestión corresponde a que una de las cepas ensayadas presentaba un defecto en la proteína Rad9 del punto de control de ciclo celular.

Teniendo en cuenta lo anterior, consideramos pertinente hacer claridad en que a partir de las divulgaciones de D1 el versado en la materia es consciente del hecho de que el estado de la proteína Rad9 como proteína control del ciclo celular no implica que la misma sea de utilidad para ser aplicada en un método de cribado de agentes anticancerígenos. Lo anterior se encuentra plenamente expuesto en la referencia científica (*Simon, et al.*, Cancer research, 60:328-333 (2000)), la cual se encuentra anexa a la presente respuesta y se desarrolla a continuación.

De esta manera, *Simon, et al.* corresponde a un estudio de investigación de la sensibilidad de la reparación de ADN y mutantes de punto de control de *Saccharomyces cerevisiae* dirigido a agentes anticancerígenos aprobados por la FDA, en donde, tal como es el caso de D1, se parte de cepas de levadura que presentan una deficiencia en la reparación de ADN o en la función del punto de control del ciclo celular. Así las cosas, a partir de dicha referencia, es de alta relevancia tener en cuenta que agentes ampliamente conocidos como agentes anticancerígenos, tal como lo son el metotrexato, el fluorouracilo y la doxorubicina, evidenciaron una diferencia insignificante en su toxicidad frente a células de levadura de tipo silvestre y varias cepas de levadura mutante ensayadas (Ver *Simon, et al.* Página 331).

Por tanto, a partir de *Simon, et al.* es posible concluir que la sensibilidad de un agente anticancerígeno conocido hacia células que presentan mutaciones en varias proteínas

del punto de control del ciclo celular no influye necesariamente para concluir que dicho agente cuenta con la habilidad de funcionar como un agente anticancerígeno toda vez que:

- (i) la totalidad de agentes ensayados son conocidos como agentes que poseen actividad anticancerígena, y
- (ii) algunos agentes que nunca evidenciaron actividad anticancerígena, generaron por igual la muerte de cepas celulares mutantes y de tipo silvestre, sin evidenciar selectividad.

De manera similar, el artículo en mención establece que catalogar una proteína como una proteína de punto de control del ciclo celular no es suficiente para concluir que las células que presenten una mutación en dicha proteína tendrían utilidad alguna en un ensayo destinado al cribado de la actividad anticancerígena, al llevarse a cabo una comparación con las células de tipo silvestre.

Por tanto, es fundamental hacer énfasis en que al tomar como base las divulgaciones de la referencia *Simon, et al.* se podría emitir una conclusión errónea, en cuanto a que ninguno de los agentes comprendidos en el grupo de metotrexato, fluorouracilo y doxorubicina presenta actividad anticancerígena cuando se parte de la comparación entre los datos obtenidos con células de tipo silvestre y células mutantes (Ver: página 330 de *Simon, et al.* en donde se establece que las mutantes rad1 y rad14 no fueron sensibles a lomustina o carmustina).

En consecuencia, frente a la ausencia de una indicación clara el normalmente versado en la materia no procedería a la selección de células mutantes de las proteínas de punto de control del ciclo celular en espera de obtener éxito en la identificación de la actividad terapéutica de un compuesto, aun si dicho compuesto presenta actividad anticancerígena conocida.

Además, es relevante tener en cuenta que en lo que se refiere a ensayos de cribado, la referencia D1 hace mención de un método para la identificación de agentes anticancerígenos que exhiben selectividad hacia células cancerígenas, mediante el uso

de un panel de mutantes de levadura, pero dichas divulgaciones son completamente ajenas al empleo de mutantes de levadura que correspondan a mutantes Rad9.

Por tanto, a la luz de la única mención relacionada con mutantes rad9 y de las enseñanzas de **Simon, et al.** el normalmente versado en la materia no se vería motivado de manera alguna a la selección de células que presentan una o más mutaciones en el complejo de proteínas del punto de control del ciclo celular incluyendo Rad9, o un gen que codifique o proteína en el complejo, con una expectativa real de éxito de lograr la identificación de compuestos que caigan en el alcance de la formula Ia como agentes con actividad farmacéutica.

En otras palabras, la referencia D1 no se constituye como una referencia que pueda ser tomada como punto de partida para llegar a las enseñanzas de la presente invención, debido a que las enseñanzas de la misma y del arte previo, alejan al versado en la materia de la selección de las condiciones requeridas para llevar a cabo el método reivindicado. Por tanto, las conclusiones del señor Examinador según las cuales:

*"La diferencia entre la invención, definida en la reivindicación 1 y la invención divulgada en D1 consiste en los compuestos específicos con actividad anti-cancerígena, que de acuerdo a lo divulgado en la descripción corresponden a compuestos de fórmula Ia."*

*"Por tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el método de clasificación quimioterapéutico procedimiento conocido en D1 para la clasificación de compuestos farmacéuticamente activos selectivos para células tumorales"*

Se alejan completamente de la argumentación previamente expuesta, y por tanto, carecen de sentido técnico en el contexto de la presente invención. Además, la segunda conclusión presenta una redacción deficiente que no permite la plena comprensión del objeto a tratar por el señor Examinador.

Ahora bien, notamos que posteriormente el señor Examinador estableció que la referencia **D2 (W02004041217)** se encuentra relacionada con las propiedades anticancerígenas de los compuestos de fórmula Ia, y que a pesar de que la misma se aleje completamente de un método para el cribado de compuestos farmacéuticamente activos, “la persona del oficio normalmente versada en la materia incluiría compuestos farmacéuticamente activos de fórmula Ia del documento D1 en el procedimiento divulgado en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud”.

Frente a dicha apreciación, consideramos que el señor examinador sobre simplificó al extremo el desarrollo del método acá reivindicado e ignoró por completo la totalidad de aspectos técnicos relacionados en el arte previo (expuestos a partir de la referencia *Simon, et al.*).

Por tanto, para proceder al desarrollo exitoso de un método de cribado de compuestos anticancerígenos como el acá reivindicado no basta con (i) el conocimiento de la actividad anticancerígena de ciertos agentes químicos ni de (ii) catalogar a una proteína como una proteína de punto de control del ciclo celular, puesto que dicha situación no es suficiente para concluir que las células que presentan una mutación en dicha proteína tendrían utilidad alguna en un ensayo destinado al cribado de la actividad anticancerígena.

Además, tal como se concluyó previamente frente a la ausencia de una indicación clara, el normalmente versado en la materia no procedería a la selección de células mutantes de las proteínas de punto de control del ciclo celular en espera de obtener éxito en la identificación de la actividad terapéutica de un compuesto, aun si dicho compuesto presenta actividad anticancerígena conocida.

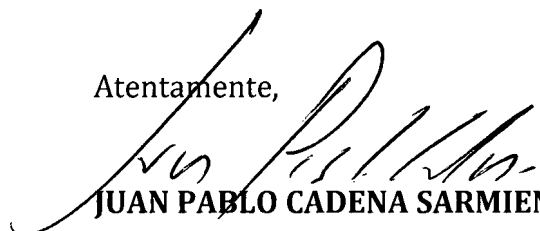
Es decir, el hecho de que D2 haga mención de la actividad biológica de los compuestos de fórmula Ia no suple de manera alguna las deficiencias identificadas en D1, y por tanto, a la luz de los lineamientos del Instructivo de Patentabilidad (2.13.5.1 Método “problema-solución”), la combinación de las referencias D1 y D2 no hace obvio el concepto inventivo que caracteriza a la presente invención.

En conclusión, las referencias D1 y D2, ni solas ni en combinación, afectan el nivel inventivo del método reivindicado.

Así las cosas, solicitamos de manera respetuosa al señor Examinador analizar a profundidad las divulgaciones de la referencia D1, a la luz de *Simon, et al.*, con el fin de evidenciar que la presente invención cumple a cabalidad con el requisito de nivel inventivo.

En vista de lo anteriormente expuesto, respetuosamente solicitamos el retiro de las objeciones de la presente solicitud y la concesión del privilegio solicitado, ya que en opinión del solicitante cumple con todos los requisitos establecidos en la decisión 486.

Atentamente,

  
**JUAN PABLO CADENA SARMIENTO**

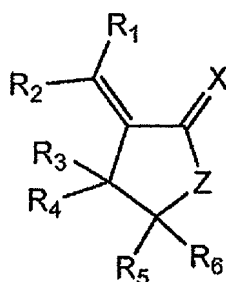
T.P. No. 57.492 del C.SJ.

C.C. 80.410.337 de Usaquén

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## REIVINDICACIONES

1. Un método para el cribado de compuestos que tienen actividad anti cancerígena, que comprende:
  - (a) proporcionar células que tienen una o más mutaciones en el complejo de proteínas de control del ciclo celular que incluye Rad9, en donde las células tienen una mutación en el gen Rad9, y en donde las células son defectuosas en la proteína Rad9 mediada por la detención del ciclo celular.
  - (b) adicionar un compuesto a ser evaluado a las células, en donde el compuesto tiene la estructura de Formula Ia:



Fórmula Ia

en donde

R<sub>1</sub>-R<sub>6</sub> se seleccionan independientemente de un átomo de hidrógeno, un átomo de halógeno, un grupo hidroxilo, alquilo, alquilo sustituido, alquenilo, alquenilo sustituido, alquinilo, alquinilo sustituido, fenilo, fenilo sustituido, arilo, arilo sustituido, heteroarilo, heteroarilo sustituido, halo, hidroxilo, alcoxi, fenoxi, fenoxi sustituido, aroxi, aroxi sustituido, alquiltio, feniltio, feniltio sustituido, ariltio, ariltio sustituido, ciano, isociano, isociano sustituido, carbonilo, carboxilo, carboxilo sustituido, amino, amino sustituido, amido, amido sustituido, sulfonilo, ácido sulfónico, fosforilo, fosfonilo, poliarilo, poliarilo sustituido, C<sub>3</sub>-C<sub>20</sub> cíclico, C<sub>3</sub>-C<sub>20</sub> cíclico sustituido, heterocíclico, heterocíclico sustituido, un aminoácido, un péptido, o grupo polipéptido; o R<sub>3</sub>-R<sub>6</sub> tomados en conjunto forman un anillo sustituido o no sustituido que opcionalmente incluye uno o más heteroátomos seleccionado del grupo que consiste de oxígeno, azufre y nitrógeno.

Z es un heteroátomo seleccionado de oxígeno, azufre o nitrógeno; y

X es un heteroátomo seleccionado de oxígeno, azufre, o nitrógeno.

- (c) determinar si el compuesto genera muerte o induce apoptosis en algunas o en la totalidad de las células;
  - (d) comparar la determinación resultante de la etapa (c) con la determinación resultante inducida por el compuesto en células normales que no tienen la una o más mutaciones en (a) para determinar si el compuesto tiene actividad anti cancerígena.
2. El método de conformidad con la reivindicación 1, en donde las células son células de levadura.
  3. El método de conformidad con la reivindicación 1, en donde las células son células de mamífero.
  4. El método de conformidad con la reivindicación 1, en donde las células son células tumorales.
  5. El método de conformidad con la reivindicación 1, en donde las células tienen una proteína Rad9 inactiva o menos activa.
  6. El método de conformidad con la reivindicación 3, en donde el método es un ensayo *in vitro*.

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## [19] The *Delitto Perfetto* Approach to *In Vivo* Site-Directed Mutagenesis and Chromosome Rearrangements with Synthetic Oligonucleotides in Yeast

By FRANCESCA STORICI and MICHAEL A. RESNICK

### Abstract

*In vivo* genome manipulation through site-directed mutagenesis and chromosome rearrangements has been hindered by the difficulty in achieving high frequencies of targeting and the intensive labor required to create altered genomes that do not contain any heterologous sequence. Here we describe our approach, referred to as *delitto perfetto*, that combines the versatility of synthetic oligonucleotides for targeting with the practicality of a general selection system. It provides for an enormously wide variety of genome modifications via homologous recombination. Exceptional high frequencies of mutations are reached when a site-specific double-strand break (DSB) is induced within the locus targeted by the synthetic oligonucleotides. Presented in this chapter is an in-depth description of a series of applications of the *delitto perfetto* strategy for mutagenesis and chromosome modification both with and without the induction of a DSB, along with the procedures and materials.

### Overview

Site-directed mutagenesis systems in which specific DNA sequences are targeted for alteration *in vitro* have been instrumental in dissecting genetic pathways and gene regulation and in understanding structure-function

A

relationships in proteins. Often there is a need for direct *in vivo* modification. However, the modification of genomic DNA within cells, without retention of heterologous sequences, is generally an elaborate and inefficient process that includes multiple cloning steps and extensive DNA sequencing of each mutant allele that is created (Erdeniz *et al.*, 1997; Langle-Rouault and Jacobs, 1995; Scherer and Davis, 1979).

There has been a longstanding interest in developing systems for the effective *in vivo* modification of chromosome structure and chromosomal sequences. Oligonucleotides have been proposed for creating localized mutational changes and were initially shown to target homologous chromosome sequences in yeast by Moerschell *et al.* (1988). The approach was restricted to the generation of mutants with a selectable phenotype. Other oligonucleotide-based strategies for genome modification include triple-helix-forming oligonucleotides (TFOs) (Barre *et al.*, 2000; Vasquez *et al.*, 2000) and chimeric RNA/DNA oligonucleotides folded into a double hairpin conformation (Kmiec *et al.*, 2000; Liu *et al.*, 2001). However, since these oligonucleotide approaches are limited to the generation of point mutations and there is no genetic selection system, extensive screening is required. The *delitto perfetto* approach developed in the budding yeast *Saccharomyces cerevisiae* (Storici and Resnick, 2003; Storici *et al.*, 2001) is the only oligonucleotide-based genome targeting system that provides direct selection for almost any chromosomal modification. Presented in this chapter is the rationale for the original *delitto perfetto* approach followed by a detailed description of the break-mediated oligonucleotide targeting system (Storici *et al.*, 2003).

The *delitto perfetto* approach that was developed in yeast is a simple and rapid method for *in vivo* genome mutagenesis using synthetic oligonucleotides. It eliminates many of the steps required to produce multiple changes in the genome. The name ascribed to this versatile and accurate system for *in vivo* targeted mutagenesis derives from the idiomatic Italian term for perfect murder since there is complete elimination (i.e., perfect deletion) of the marker sequences that are used for selection. Mutagenesis is accomplished in two steps (Fig. 1), each of which requires the homologous recombination function of Rad52 (Storici *et al.*, 2003). The first step involves integration of a *C*ounterselectable *RE*porter (*CORE*) cassette that is integrated into a desired genomic locus using standard targeting procedures. The second step is accomplished by transformation with the appropriate targeting oligonucleotides such that the *CORE* cassette is lost and the desired change is generated. Since no heterologous sequence remains, mutagenesis is accomplished via a self-cloning process. Once the *CORE* cassette is inserted, the resulting strain provides many opportunities, as

1. Insert core  
(COUNTERselectable  
REporter)  
Select  $URA3^+/G418^R$

2. Replace core  
with oligos  
Select on 5-FOA  
against  $URA3^+$   
confirm by  $G418^S$

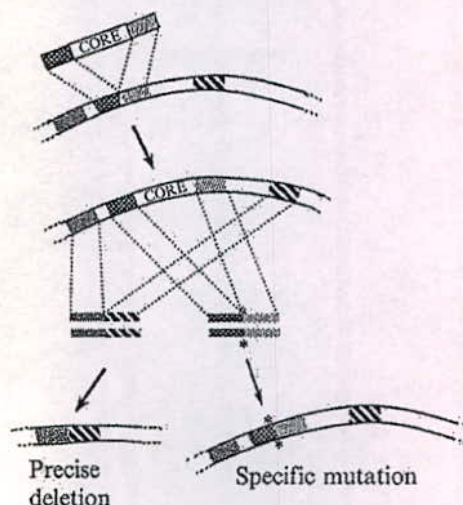


FIG. 1. Schematic drawing of the *delitto perfetto* system that uses Integrative Recombinant Oligonucleotides (IROs). This illustrates the deletion of a sequence and the creation of a specific point mutation. In Step 1, a CORE (COUNTERselectable REporter) cassette with *URA3* (counterselectable) and *kanMX4* (reporter) is inserted by standard DNA targeting procedures at a DNA sequence. The insertion site is anywhere in the sequence that has been chosen to be deleted or is close to a site where a specific mutation is to be created. In Step 2, cells containing the CORE cassette are transformed with IROs. This leads to loss of the CORE cassette and deletion of the desired region or introduction of the desired mutation (\*). Generic DNA sequences are indicated as stippled or striped boxes. In this example the IROs have a short overlap. (This model is printed with permission from Storici *et al.*, 2001.)

described later, for mutation over a large distance from the CORE. This is particularly relevant to structure-function studies, since many different mutations can be targeted to sequences coding for key protein motifs.

Following the initial development of the *delitto perfetto* system, a subsequent approach, known as break-mediated *delitto perfetto*, took advantage of efficient recombinational repair of double-strand breaks in yeast (Storici *et al.*, 2003). The CORE cassette was modified to include both a galactose regulatable *I-SceI* endonuclease and an *I-SceI* cut site (Colleaux *et al.*, 1986; Plessis *et al.*, 1992). Generation of a DSB prior to oligonucleotide transformation (Fig. 2) increased oligonucleotide targeting more than 1000-fold, reaching efficiencies as high as 20% of all cells. This level is over 2 orders of magnitude higher than any reported DNA integration frequency in yeast. Surprisingly, we found that the DSB can also strongly stimulate recombination with single-strand DNA, suggesting new twists on present models of DSB repair (Storici *et al.*, 2003, submitted). The extremely high transformation frequencies and versatility of the break-mediated *delitto perfetto* system has

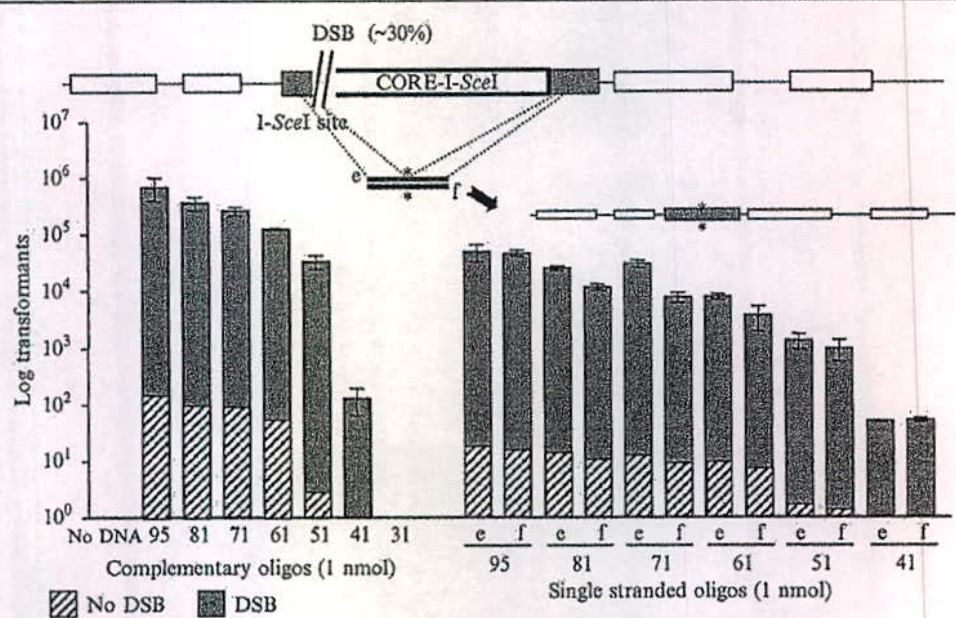


FIG. 2. Factors affecting IRO targeting efficiency: complementary pairs, single IRO molecules, size, and presence of a DSB. Presented are the frequencies of targeting by various IROs to a CORE-I-SceI cassette inserted in the *TRP5* gene. The diagram above the graph shows the position of the DSB and the result of the oligonucleotide-targeting event. The IROs examined are referred to as "e" and "f", fully complementary with equal lengths of homology to both sides of DSB and from 31 to 95 nucleotides in length. The types of IROs analyzed are indicated below the bars. The amount of total IRO DNA used in each transformation is 1 nmole (~10–30  $\mu$ g), unless otherwise indicated. The frequency of Trp<sup>+</sup> transformants are presented as the Log<sub>10</sub> Transformants, where the transformants correspond to the total transformants per 10<sup>7</sup> viable cells plated from the transformation mix. The vertical bars represent the average values from three to six determinations and include standard deviations. Dark-grey bars represent values from pairs of fully complementary IROs or single-strand IROs, and striped bars represent values from no-DSB controls (corresponding to cells not incubated in galactose media). The control value (no DNA) corresponds to no Trp<sup>+</sup> colonies.

resulted in powerful new tools for rapid genome modification as well as dissection of DNA repair mechanisms.

Presented later are protocols for the development and use of *delitto perfetto* systems that provide for targeted oligonucleotide recombination events. The first part describes the development of a CORE and transformation procedures. In the second part, emphasis is placed on the break-mediated *delitto perfetto* approach. Presented in Fig. 2 (striped bars) are frequencies of targeting with different sizes of complementary and single IROs using the CORE without a DSB generating system. The frequencies are much higher if targeting is mediated by a DSB (dark-grey bars in Fig. 2).

## Creation and Utility of *Delitto Perfetto* Systems

### *The Delitto Perfetto System Without a DSB*

**CORE Cassettes.** There are five CORE cassettes available (Fig. 3A). The original CORE cassette (Storici *et al.*, 2001), which contains the counterselectable *URA3* gene from *Kluyveromyces lactis* (*KIURA3*) and the reporter geneticin (G418) resistance gene *kanMX4*, is amplified as a 3.2 kb DNA fragment from pCORE using chimeric 70-mers consisting of 50 nucleotides homologous to the appropriate flanking region of the genomic target locus plus, as shown here, 20 nucleotides that allow for the amplification of the CORE-cassette:

P.1: 5'-...GAGCTCGTTTTTCGACACTGG-3' for the *kanMX4* side

P.2: 5'-...TCCTTACCATTAAGTTGATC-3' for the *KIURA3* side.

The genomic target site can be anywhere in a sequence that has been chosen to be deleted or within 100 bp of a site at which a specific mutation is to be created. Four additional CORE cassettes have been constructed in order to make the *delitto perfetto* approach applicable to a much wider set of yeast strains, including those that are *URA*<sup>+</sup> and G418 resistant. Two heterologous markers have been utilized: a reporter that provides resistance to hygromycin (*hyg*) (Goldstein and McCusker, 1999) and a new, counterselectable marker coding for the p53 mutant V122A (Storici and Resnick, 2003). High expression of this mutant p53 results in growth inhibition and toxicity (Inga and Resnick, 2001). These markers along with the previous markers (*KIURA3* and *kanMX4*) have been used in the construction of the following cassettes (see Fig. 3A): CORE-UK (*KIURA3* and *kanMX4*), CORE-UH (*KIURA3* and *hyg*), CORE-Kp53 (*kanMX4* and *GALI/10-p53*), and CORE-Hp53 (*hyg* and *GALI/10-p53*) (Storici and Resnick, 2003). The CORE-UK, -UH, -Kp53, and -Hp53 cassettes are amplified as 3.2, 3.5, 3.7, and 4.0 kb DNA fragments, respectively, from the corresponding vectors using chimeric 70-mers consisting of 50 nucleotides that are homologous to the flanking region of the genomic target locus plus the following 20 common nucleotide sequences that allow for the amplification of all four CORE cassettes (Note: these primers do not amplify the original CORE cassette from the pCORE plasmid):

P.I: 5'-...TTCGTACGCTGCAGGTCGAC-3' for the *KIURA3* side in pCORE-UK and pCORE-UH, and for the *kanMX4* or *hyg* side in pCORE-Kp53 and pCORE-Hp53

P.II: 5'-...CCGCGCGTTGGCCGATTCAT-3' for the *kanMX4* or *hyg* side in pCORE-UK and pCORE-UH, and for the *GALI/10-p53* side in pCORE-Kp53 and pCORE-Hp53.

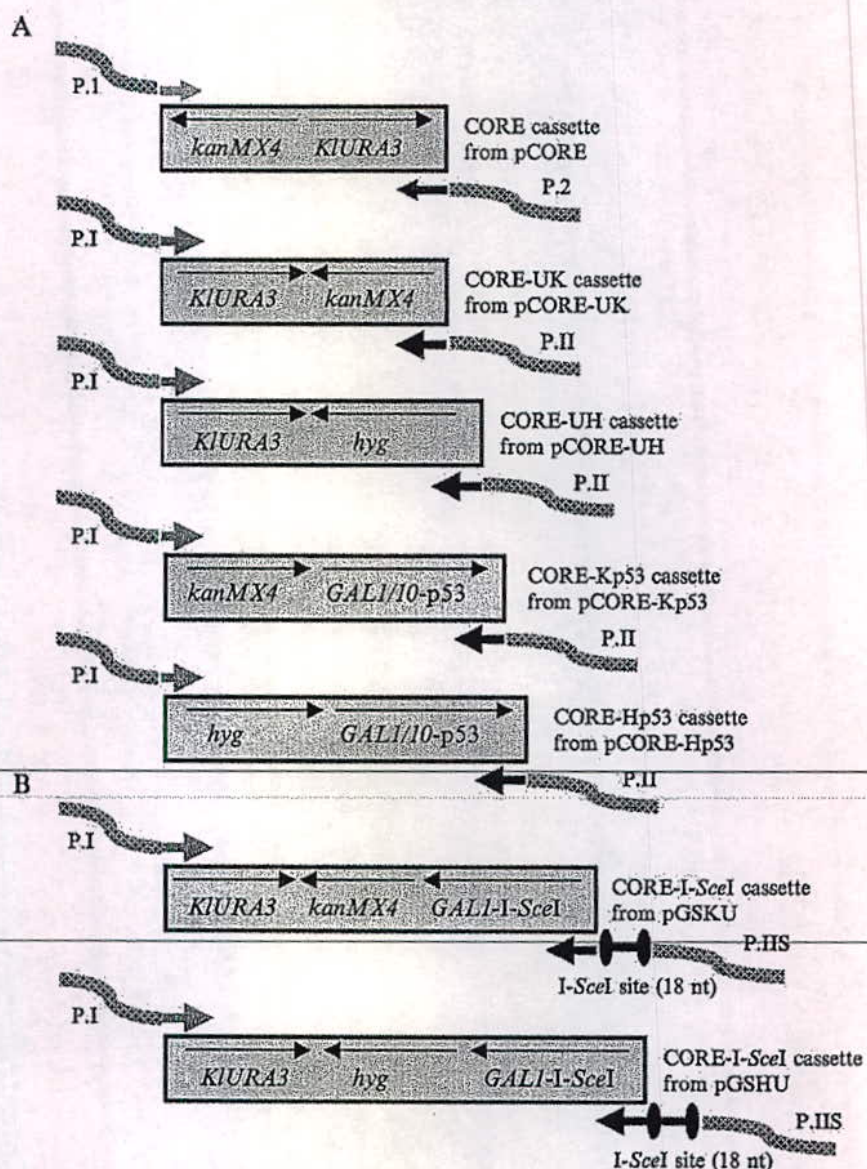


FIG. 3. CORE cassettes. (A) CORE cassette with *KIURA3*, or *GAL1/10-p53* as a counterselectable marker and with *kanMX4*, or *hyg*, as reporter markers. (B) CORE-I-SceI cassettes with *KIURA3* as the counterselectable marker, with *kanMX4*, or *hyg*, as reporter markers and with *GAL1-I-SceI* for the induction of the DSB. Orientation of each gene in the cassettes is indicated by an arrow. Cassettes with *KIURA3* derived from CORE-UK, CORE-UH, and CORE-I-SceI should have *KIURA3* oriented opposite to the transcription direction of the target gene (see text in the section CORE Cassettes). Primers used to amplify each cassette are also shown.

It should be noted that when inserting the CORE-UK, CORE-UH, and CORE-I-SceI (see later) cassettes into a gene, *KIURA3* should be oriented opposite to the transcription direction of the gene. If *KIURA3* is in the same orientation as the targeted gene, transcription from the promoter of the targeted gene may interfere with the transcription of *KIURA3*. This may delay the growth on Ura<sup>-</sup> media and may increase the background during selection of oligonucleotide transformants on 5-FOA. In such cases, to reduce the background during selection of oligonucleotide targeting events, we suggest replica plating the cells growing on 5-FOA on to a new 5-FOA plate once every day for a few days until the Ura<sup>-</sup> (CORE-loss) clones are clearly distinguishable from the background.

**PCR Reaction Conditions.** PCR amplification of CORE cassettes from circular plasmids (about 0.1  $\mu$ g) is performed with high yield in a final volume of 40  $\mu$ l using Takara Ex Taq DNA polymerase (Takara Bio Inc., Temecula, CA), with 2 min at 94°, 32 cycles of 30 s at 94°, 30 s at 57°, and 4 min at 72°. For all PCRs of CORE cassette amplification a higher yield is obtained with Takara DNA polymerase than Taq DNA polymerase. The product of three PCR reactions is collected and precipitated with ethanol and resuspended in 20  $\mu$ l of water. Ten  $\mu$ l are used for each transformation.

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#### *Steps in the Development of the Delitto Perfetto System*

##### *Integration of CORE Cassette*

**MEDIA AND COMPONENTS.** Standard yeast media and growth conditions are described in Sherman *et al.* (1986). Transformation is done using the lithium acetate protocol described by Wach *et al.* (1994) with some modifications. Stock solutions are as follows: LiAc 1 M, filter sterilized; TE 10X (Tris 100 mM pH 7.5, EDTA 10 mM) pH 7.5, filter sterilized; PEG 4000 50%, sterilized by autoclave. Working solutions are Solution 1 (LiAc 0.1 M, TE 1X pH 7.5) and Solution 2 (LiAc 0.1 M, TE 1X pH 7.5 in PEG 4000 50%). Salmon sperm carrier DNA, in slight excess volume relative to the amount needed is denatured at 100° for 5 min and then put on ice.

**TRANSFORMATION PROTOCOL.** Below are the steps involved in transforming cells with the oligonucleotides.

1. Inoculate 5 ml of YPDA (standard rich media supplemented with adenine) medium with the chosen strain and shake at 30° over night (O/N).
2. Inoculate 50 ml of YPDA medium (volume of flask) with 1.5 ml of the O/N culture and shake vigorously at 30° for 3 h.

3. Transfer culture to a 50-ml tube and spin at 3000 rpm for 2 min.
4. Wash cells with 50 ml of sterile water and spin as stated previously.
5. Resuspend cells in 5 ml of Solution 1 and spin as stated previously.
6. Resuspend cells in 250  $\mu$ l of Solution 1. This amount of cells is sufficient for about 7–8 transformations.
7. Aliquot 50  $\mu$ l of the cell suspension in Eppendorf tubes and add 10  $\mu$ l of concentrated CORE PCR product, 5  $\mu$ l of denatured carrier DNA, and 300  $\mu$ l of Solution 2 for each transformation reaction. Mix briefly by vortexing.
8. Incubate transformation reactions at 30° for 30 min with shaking.
9. Heat shock at 42° for 15 min.
10. Collect cells by centrifugation at 5000 rpm for 4 min.
11. Remove supernatant and resuspend cells in 100  $\mu$ l of water.
12. Plate all cells from each transformation tube on one plate of synthetic media lacking uracil (SD-Ura<sup>-</sup> plate) and incubate at 30° for 3 days or plate on one YPDA plate and incubate at 30° O/N and then replica-plate on YPDA media containing 200  $\mu$ g/ml of G418 (Gibco BRL, Grand Island, NY) or 300  $\mu$ g/ml of hygromycin B (Invitrogen, Carlsbad, CA) (Goldstein and McCusker, 1999), depending on the CORE cassette used.
13. Once transformant colonies appear (typically 5 to 30), streak a few colonies on YPDA to obtain single colony isolates.
14. Patch the single colony isolates (3 to 6) on YPDA and test for the presence of the other marker of the CORE, if applicable, and for various other phenotypes (according to the strain used) and on YP glycerol (YPG) to eliminate petite mutants.

**COLONY PCR REACTION CONDITIONS TO IDENTIFY DESIRED ISOLATES.** To identify clones with the correct CORE-cassette integration, colony PCR is performed using primers designed for annealing upstream and downstream of the integration locus and within the cassette and designed to give a band from 500 bp to 1500 bp, according to Wach *et al.* (1994) with modifications. Specifically, approximately 1 mm<sup>3</sup> of cells from the single-colony derived patch is resuspended in 50  $\mu$ l of water containing 1 unit of Lyticase (Sigma Chemical Co., St. Louis, MO) in an Eppendorf tube and incubated at room temperature (RT) for 10 min. Cells are then collected by centrifugation at 5000 rpm for 2 min. Supernatant is discarded and tubes with cell pellets are put in a 100° heat block for 5 min. Cell debris are finally resuspended in 40  $\mu$ l of water. 10  $\mu$ l of cell debris are used for each PCR reaction containing 5  $\mu$ l of Buffer 10 $\times$  with magnesium (Roche, Indianapolis, IN), 1  $\mu$ l of dNTPs (Roche), 1  $\mu$ l of each primer (from a 50  $\mu$ M primer solution), 0.2  $\mu$ l of Taq (Roche), and water to 50  $\mu$ l. PCR is

performed as follows: 2 min at 94°, 30 cycles of 30 s at 94°, 30 s at 55°, and 1 min at 72°.

*Oligonucleotide Targeting and CORE Removal*

**OLIGONUCLEOTIDE DESIGN.** The Integrative Recombinant Oligonucleotides (IROs) (Storici *et al.*, 2001) are desalted custom primers synthesized at the 50 nM scale (Invitrogen, Carlsbad, CA). They can be used in transformation experiments either singly or as pairs. Pairs of fully complementary oligonucleotides generally transform 5- to 10-fold more efficiently than corresponding single strand molecules (Fig. 2). Longer oligonucleotides also transform better than shorter (Fig. 2). Fully overlapping 80 to 100-mers are suggested for the introduction of point mutations, substitutions, or small insertions. Since the external 30 to 40 bases are used for efficient targeting, only the central sequence of the oligonucleotide pair can be designed for mutagenesis. The longer the oligonucleotide, the larger is the window available for modifications (Fig. 4). Therefore, if only a single point mutation is desired in a specific region, the CORE should be inserted at that site, and fully complementary IROs or single IROs can be used to introduce the mutation.

The window for mutagenesis can be expanded using IROs that share a short overlap of 20 nucleotides (nt) (Fig. 4). Using a pair of 100-mers that have a 20 nt complementary overlap at the 3' end, the window available for mutagenesis can be increased to 100 nt (Fig. 4). To increase the efficiency of targeting of IRO pair sharing only a short overlap of 20 nt, we suggest annealing and extension of the IROs *in vitro* (this is not required when using the break-mediated *delitto perfetto* system, see later). In order to extend the sequence of 3'-overlapping IROs *in vitro*, 0.5 nmoles (15–20 µg) of each IRO is added to a 50-µl reaction mix containing 4 units Platinum Pfx (Gibco BRL), 5 µl 10× Buffer, 1 µl 50 mM MgSO<sub>4</sub>, and 2 µl 10 mM dNTPs (Roche). Extension is performed as follows: 1 min at 94° and 30 s to 3 min at 68°. Samples are ethanol precipitated and resuspended in 10–20 µl of water. For more details about oligonucleotide design, see Storici and Resnick (2003).

**PROTOCOL FOR TRANSFORMATION WITH OLIGONUCLEOTIDE.** Before adding IRO DNA to the cells, the oligonucleotides are denatured at 100° for 2 min and placed immediately on ice in order to eliminate possible secondary structures. Strains containing the CORE cassette are transformed with chosen oligonucleotides. Other steps in transformation are the same as previously except for the following differences in steps 7 and 12.

Change in Step 7. To 50 µl of the cell suspension in Eppendorf tubes add 20 µl of 50 µM solution (i.e., 1 nmole) of oligonucleotides (1 nmole of a single oligonucleotide or for a pair of complementary

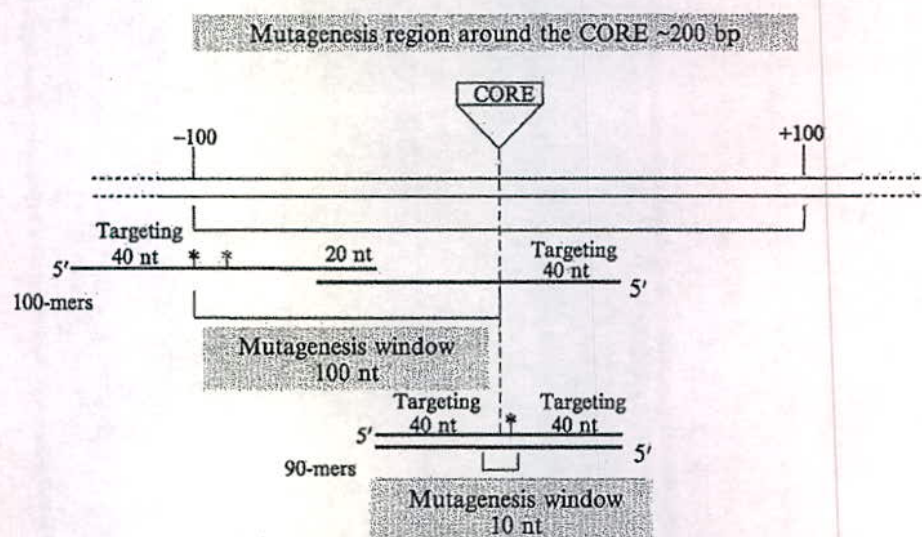


FIG. 4. Use of IROs for rapid production of a variety of mutations. Site-specific mutations can be accomplished at positions as far as 100 bp upstream or downstream of the CORE cassette integration point with different IRO pairs without changing position of CORE cassette. A pair of 100-mers, overlapping for 20 nt, is filled-in with Pfx and has a 100-nt region that can be modified. Within the 100-nt region, 40 nt on each side of the overlap can also be used for random mutagenesis. 40 nt at each end of the pair are designed to have identity upstream and downstream of the CORE cassette integration for efficient homologous integration. Complementary 90-mers have a central region of 10 nt, which can be used for site-directed mutations. 40 nt at each end must remain unchanged for efficient homologous integration. The figure shows examples of oligonucleotides designed to introduce different mutations (\*) from close to the CORE site, up to 100 bp upstream of the CORE-cassette integration point. The efficiency of mutagenesis may decrease as the mutation position in the IRO sequence is placed closer to the 5' end since the IRO recombination event leading to excision of the CORE could occur without inclusion of the mutation in the IRO (Storici *et al.*, 2001).

IROs use 0.5 nmole of each) and 300  $\mu$ l of Solution 2 for each transformation reaction. Vortex briefly. At this concentration of oligonucleotides, carrier DNA is not needed.

Change in Step 12. Plate all cells from each transformation tube on one YPDA plate and incubate O/N at 30°. The day after, if the counterselectable marker is *KIURA3*, replica-plate on media (synthetic complete) containing 60 mg/liter of uracil and 1 g/liter of 5-fluoroorotic acid (5-FOA) (Toronto Research Chemicals Inc., North York, ON, Canada). Three days later replica-plate colonies from 5-FOA to YPDA and YPDA containing G418 or hygromycin. If the counterselectable marker is *GALI10-p53*, replica-plate cells on synthetic

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complete media containing 2% galactose. Replica-plate every day for two days on a new plate of synthetic complete with 2% galactose. On the third day, replica-plate colonies from synthetic complete with 2% galactose to YPDA and YPDA containing G418 or hygromycin. In both cases, isolate colonies that are G418 or hygromycin sensitive.

**IDENTIFICATION OF RELEVANT SINGLE COLONY ISOLATES.** The procedure is similar to that described previously. Colony PCR products are sequenced after phenol-chloroform extraction and purification using the QIAquick PCR Purification Kit (Qiagen USA, Valencia, CA).

#### *The Break-Mediated Delitto Perfetto System*

**Integration of CORE-I-SceI Cassette.** The break-mediated *delitto perfetto* system utilizes a CORE that contains the gene for the double strand endonuclease I-SceI and the DSB target site. The components are diagrammed in Fig. 3B. The CORE-I-SceI cassette, starting from the 5' end, contains *KIURA3*, *kanMX4* (or *hyg*), and *GAL1-I-SceI*. The cassette is amplified as a 4.6 kb or 4.8 kb DNA fragment from pGSKU, or pGSHU, respectively, using Takara Ex Taq DNA polymerase, with 2 min at 94°, 32 cycles of 30 s at 94°, 30 s at 57° and 5 min at 72°. For integration of the cassette into chromosomal loci, chimeric primers are used, consisting of 50 nucleotides homologous to the genomic target and an additional 20 nucleotides for the amplification of the CORE-I-SceI cassette:

P.I for the *KIURA3* side primer: 5'-TTCGTACGCTGCAGGTC-GAC-3'

P.IIS for the *GAL1-I-SceI* side that also contains 18-nt of the I-SceI target double-strand break site (italics): 5'-. . .TAGGGA-TAA-CAGGGTAAT-CCGCGCGTTGGCCGATTCAT-3'

Transformation of CORE-I-SceI cassettes and identification of clones with the correct cassette integration are performed as described previously.

**Protocol for Targeting Oligonucleotides to a Region Containing a DSB.** The procedures described previously (transformation protocol) are used with the following modifications:

Change in Step 2. Inoculate 50 ml of synthetic complete media containing 2% galactose with 1.5 ml of the O/N culture in YPDA and shake at 30° for 4 h to express *GAL1-I-SceI*, which will induce a DSB at the cloned target site. In experiments with strains having the CG379 background, about 30% of cells were cut by I-SceI at the *TRP5* locus on chromosome VII after 4-h induction of *GAL1-I-SceI* (Storici *et al.*, submitted).

Change in Step 7. Aliquot 50  $\mu$ l of the cell suspension in Eppendorf tubes and add 20  $\mu$ l of 50  $\mu$ M solution (i.e., 1 nmole) of oligonucleotides (1 nmole of a single oligonucleotide or 0.5 nmoles of each strand of a pair of complementary oligonucleotides) and 300  $\mu$ l of Solution 2 for each transformation reaction. Mix briefly by vortexing. At this concentration of oligonucleotides, carrier DNA is not needed.

Change in Step 12. Plate a dilution of the cells (over the range 10- to 10,000-fold, depending on oligonucleotide chosen) onto YPDA plates and incubate at 30° O/N. Replica-plate to media (synthetic complete) containing 60 mg/liter of uracil and 1 g/liter of 5-fluoroorotic acid (5-FOA). Three days later replica-plate cells from 5-FOA to YPDA and YPDA containing G418 or hygromycin depending on selective markers used. Isolate colonies that are G418 or hygromycin sensitive.

*Frequencies of Targeting and Identification of Relevant Single Colony Isolates.* The frequencies of targeting with different sizes of complementary and single IROs are shown in Fig. 2 (dark gray bars). In all transformation experiments 10<sup>5</sup>-fold dilutions are plated directly to YPDA in order to determine viability of the cell population. Survival is normally ~25% after transformation with or without IROs, in glucose or in galactose (not shown). To directly measure events when there is a very high frequency of targeting, the 10<sup>-5</sup>-fold diluted suspensions are plated to YPDA directly. Subsequently, the plates (containing 100–1000 colonies) are replica-plated to 5-FOA and to YPDA containing G418 or hygromycin.

The procedures to identify and isolate single colony isolates of transformant clones are the same as described previously. Colony PCR products are sequenced after phenol-chloroform extraction and purification using the QIAquick PCR Purification Kit.

#### *Break-Mediated Delitto Perfetto with Oligonucleotides in Diploid Cells*

The ability to apply the *delitto perfetto* approach to diploid cells has many utilities for gene and genome modification. Recent results have demonstrated that a pair of 95-mers (1 nmole) can be targeted to a single DSB in diploid yeast, in spite of the competition by an unbroken homologous chromosome (Storici *et al.*, 2003). Loss of the CORE-I-*SceI* cassette was primarily due to recombination with the homologous chromosome; however, approximately 4% of the loss events were due to direct oligonucleotide targeting. These results suggest that clones with the desired IRO generated mutation can be isolated by screening a pool of clones selected

for CORE loss. For productive targeting in diploid cells, IROs used for the generation of point mutations are designed to also create a restriction site. That way the desired clones can be identified within a pool of samples by PCR analysis followed by restriction digestion. Mutations that generate several base deletions or insertions or rearrangements can be identified simply by PCR analysis of pooled samples.

*High-Throughput Mutagenesis and Rapid Gene Diversification with the Delitto Perfetto Systems*

Use of the CORE-I-SceI cassette provides the opportunity for direct, highly efficient generation of many mutations within a gene. The cassette is placed in the middle of the desired region (<200 bp) of a gene. This provides a window for mutagenesis that is 100 bases upstream and downstream from the CORE insertion site. If the domain of interest is longer than 200 bases, or covers the entire gene, two or more CORE-I-SceI cassettes are placed at appropriate positions in different strains at 150 nucleotide intervals across the region of interest. This leads to a panel of isogenic yeast strains, each containing one CORE-I-SceI cassette, as described in Fig. 5. Placing the cassettes at intervals of 150 bases ensures some overlap between contiguous windows of mutagenesis. The chosen mutations are generated simply by replacement of the CORE cassette with IROs. The oligonucleotides can be specific for a single desired mutation, or the IROs could be degenerate in order to produce random mutations in the region of interest (Figs. 4 and 5).

*Generation of Chromosomal Rearrangements*

IROs can be targeted to sites of homology distant from a DSB, in fact, the distance can be quite large based on recent observations (Storici *et al.*, 2003). A pair of complementary 83-mers that were designed to delete 16.6 kb around a CORE-I-SceI inserted at the *TRP5* locus on chromosome VII resulted in more than 1% deletion efficiency. They had 40-nt of homology at a position 4.7 kb on one side of the DSB and 11.9 kb on the other. Single-strand oligonucleotides also could be targeted to the sites distant from the DSB, although with 10-fold less efficiency. The maximum distance for targeting is currently under investigation.

The oligonucleotide-mediated process enables the production of a variety of rearrangements including circularization, interchromosomal fusion, and reciprocal translocations. Briefly, a CORE-I-SceI cassette is integrated near the border of the proposed rearrangement. Oligonucleotides are designed that will repair the break, eliminate the cassette, and generate the desired genomic modifications.

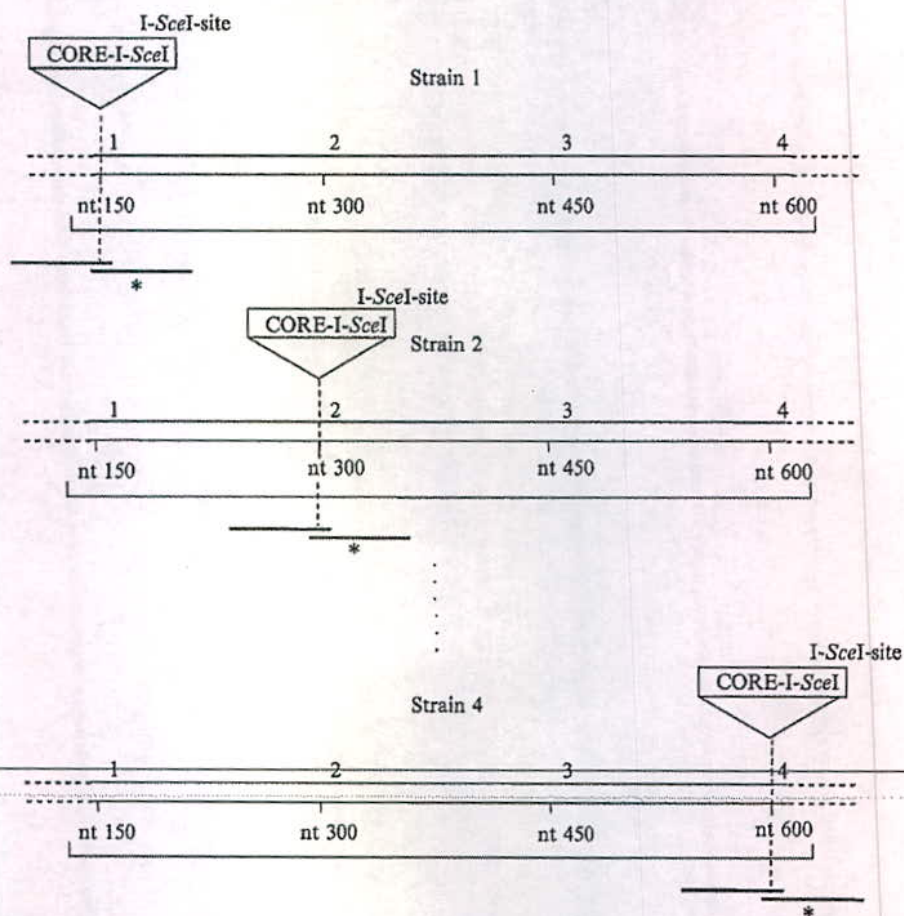


FIG. 5. Strategy for *in vivo*, site-directed mutagenesis in a large DNA region. In this example, four isogenic yeast strains are created each containing a CORE-I-SceI cassette integrated at a different position (every 150 nucleotides) along the gene sequence. Mutations can be created simply by designing IROs that contain the desired change. Transformation with oligonucleotides, which are homologous to a region surrounding the CORE cassette, results in the elimination of the CORE cassette and creation of a site-specific change. A mutation can be designed 100 bases upstream and downstream of each CORE cassette insertion point (see Fig. 4). The specific scheme shows the introduction of a mutation upstream from a CORE cassette that was inserted at nucleotide positions 150, 300, and 600. (To decrease the likelihood that a distant mutation is not included during the targeting of the IRO, part of the sequence surrounding the site where the CORE is to be targeted can be deleted during the step of CORE insertion and reintroduced with IRO transformation.)

An example of redesigning chromosomes is presented in Fig. 6. To generate a reciprocal translocation between chromosome V and VII, a CORE-I-SceI cassette was integrated on chromosome VII at the site of translocation. A DSB was induced and the break was repaired using two

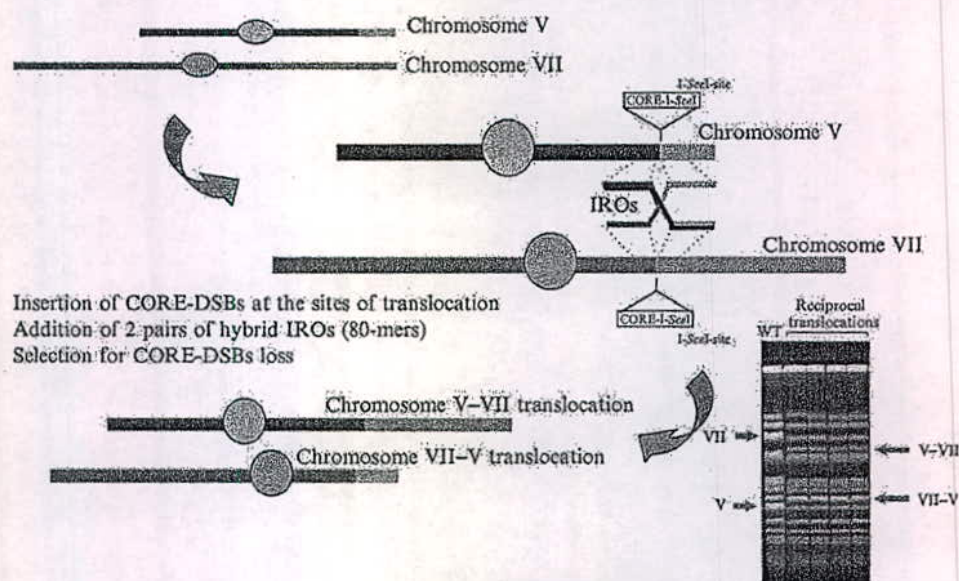


FIG. 6. Example of genome reconstruction: reciprocal translocation between the arms of chromosome V and VII. The first step involves the insertion of one CORE-I-SceI cassette at one site of translocation. (Insertion of a CORE-I-SceI cassette at each site of translocation on both chromosomes [using the cassette with *kanMX4* and the one with *hyg*, respectively] is predicted to enhance translocation frequency by IROs.) The second step involves transformation with two pairs of IROs, each containing sequence from both chromosomes adjacent to the translocation point. Selection of translocation events is accomplished by isolating 5-FOA resistant clones, which also have lost the reporter marker (or both reporter markers if using two cassettes). The picture on the right shows the karyotype display using pulse-field gel electrophoresis. Bands corresponding to chromosome V-VII and VII-V translocations are indicated.

pairs of complementary 80-mers. One pair had half of its sequence identical to a region upstream of the translocation point on chromosome V and half of the sequence identical to a region downstream of the translocation point on chromosome VII. The other pair had the opposite configuration. Cells were selected for loss of the markers in the CORE and screened by colony PCR of small pools. Using this system, the frequency of events was approximately  $10^{-6}$  (unpublished). The frequency of targeting might be higher if two CORE-I-SceI cassettes are used, one with *KIURA3* and *kanMX4* markers (from pGSKU) and the other with *KIURA3* and *hyg* markers (from pGSHU) at the two translocation sites, since two DSBs would be generated. Transformant colonies would be selected for loss of *KIURA3* (loss of both cassettes) on media containing 5-FOA and checked for the loss of both *kanMX4* and *hyg* markers. This approach would be expected to eliminate most false positive clones (spontaneous *KIURA3* mutants; see Fig. 6). A similar strategy could be used to circularize or fuse chromosomes.

### Conclusions

The protocols and examples described previously provide the essence of the *delitto perfetto* strategy and describe examples for the *in vivo* modification of genes and chromosomes in yeast. The efficiency of genetic modification is greatly enhanced when oligonucleotide targeting is stimulated by a DSB. Targeting frequencies as high as 20% of all cells can be obtained. More remarkable is the fact that the IROs can generate almost any kind of genetic modification *in vivo*, from a single-base mutation to a large deletion or a reciprocal chromosomal translocation. While developed in the yeast *S. cerevisiae*, the approach could be applied to other organisms where homologous recombination is efficient.

The *delitto perfetto* system has proven useful not only for the engineering of genetic material; studies on the mechanisms of DSB repair by single-strand oligonucleotides are revealing new features of DNA recombination and repair (Storici *et al.*, submitted). The information about repair and recombination mechanisms in yeast is expected to provide insights into gene/chromosome targeting and modification in other systems, including mammalian cells. We also anticipate that the oligonucleotide targeting procedure can be expanded to develop genome-wide analysis of spontaneous and induced DNA damage. Given the almost unlimited number of oligonucleotide variants that can be designed, there are vast numbers of genomic targets, substrates, and genomic modifications that can be investigated with the *delitto perfetto* approach using oligonucleotides.

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## [20] Assays for Transcriptional Mutagenesis in Active Genes

By DAMIEN BRÉGEON and PAUL W. DOETSCH

### Abstract

Cells exposed to DNA-damaging agents in their natural environment do not undergo continuous cycles of replication but are more frequently engaged in gene transcription. Despite the relatively high efficiency of the different DNA repair pathways, some lesions remain in DNA. During transcription, RNA polymerase can bypass DNA damage on the transcribed strand of an active gene. This bypass can be at the origin of the production of "mutated" mRNA because of the transcriptional miscoding

## Research Communication

### Fluorescent Protein Engineering by *In Vivo* Site-directed Mutagenesis

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#### Summary

*In vivo* site-directed mutagenesis by single-stranded deoxyribonucleic acid recombineering is a facile method to change the color of fluorescent proteins (FPs) without cloning. Two different starting alleles of GFP were targeted for mutagenesis: *gfpmut3\** residing in the *Escherichia coli* genome and *egfp* carried by a bacterial/mammalian dual expression lentiviral plasmid vector. Fluorescent protein spectra were shifted by subtle modification of the chromophore region and residues interacting with the chromophore of the FP. Eight different FPs (Violeta, Azure, Aqua, Mar, Celeste, Amarillo, Mostaza, and Bronze) were isolated and shown to be useful in multicolor imaging and flow cytometry of bacteria and transgenic human stem cells. To make *in vivo* site-directed mutagenesis more efficient, the recombineering method was optimized using the fluorescence change as a sensitive quantitative assay for recombination. A set of rules to simplify mutant isolation by recombineering is provided. © 2012 IUBMB

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**Keywords** recombineering; fluorescent proteins; gene targeting; mutagenesis; recombination; oligonucleotides.

**Abbreviations** CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; *E. coli*, *Escherichia coli*; FBS, fetal bovine serum; FP, fluorescent protein; LB, lysogeny broth; MIAMI, marrow-isolated adult multilineage inducible; MMR, DNA mismatch repair; oligo, oligonucleotide.

#### INTRODUCTION

The ability to easily change the spectra of fluorescent proteins (FPs) expands the palette for routine molecular cell biology.

Additional Supporting Information may be found in the online version of this article.

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Although *in vitro* mutagenesis has been used to modify the spectra of green FP and many spectral variants are commercially available, we have developed a simple method for producing spectrally distinct variants on demand by making modest changes to FP genes without subcloning. Based on previous studies (1–3) and our analysis of sequences encoding green, cyan, blue, and yellow FP variants (Supporting Information Fig. 1), amino acids at positions 64 (FIL), 65 (GIT), 66 (YIWIH), and 203 (SITY) appear to be primarily responsible for the “color” of each protein, whereas other residues are less likely to be important for spectral shifts. We surmised that it might be possible to produce several spectrally distinct FPs by oligonucleotide (oligo)-directed mutagenesis *in vivo* by recombineering.

Recombineering uses viral recombination proteins [e.g., Beta protein expressed from a defective lambda prophage (4, 5)] to anneal a mutagenic oligo to its homologous target sequence inside the cell (6). Unlike *in vitro* site-directed mutagenesis, recombineering can directly modify any size of DNA molecule, including chromosomes and large bacterial artificial chromosomes (7–9). As oligo-mediated recombineering is most efficient in bacteria, we manipulated *gfp* gene variants stably integrated into the *Escherichia coli* genome (10) or in lentiviral plasmid vectors (11) in *E. coli*.

#### EXPERIMENTAL PROCEDURES

##### Cell Culture

293T cells (obtained from Dr. Priya Rai) were grown in Dulbecco's modified Eagle's medium (DMEM)-high glucose medium with 10% fetal bovine serum (FBS) and antibiotics-antimycotics (50 U/mL Penicillin G, 0.25 µg/mL Amphotericin B, and 10 µg/mL Streptomycin) at 37 °C in 5% atmospheric CO<sub>2</sub>. Marrow-isolated adult multilineage inducible (MIAMI) 3515 adult human stem cells (12) (isolated from whole bone marrow from a 20-year-old living male donor) were grown in DMEM-low glucose, 3% FBS, 20 mM ascorbic acid, 12.9 nM arachidonic acid,

1.12  $\mu$ M cholesterol, 290 nM DL- $\alpha$ -tocopherol acetate, 85.9 nM myristic acid, 69.4 nM oleic acid, 76.5 nM palmitic acid, 77.1 nM palmitoleic acid, 68.9 nM stearic acid, 100 U/mL penicillin, and 0.1 mg/mL streptomycin under low oxygen conditions (3% O<sub>2</sub>, 5% CO<sub>2</sub>, and 92% N<sub>2</sub>) at 37 °C. Media were changed every 2–3 days and the cells were detached using trypsin upon reaching ~ 60% confluency and pelleted. Pelleted cells were resuspended in medium and plated in 10 ng/mL fibronectin-treated flasks at 100 cells per square centimeter.

### Plasmids

pDual-eGFP was created by ligating a PCR product [produced using 5' phosphorylated primers (oligos 61 and 62) to amplify the T7 promoter region from pET28a] to SmaI digested pNL-eGFP/CEF (11) and screening transformed Rosetta-gami<sup>TM</sup>2(DE3) colonies for gain of green fluorescence. The inserted sequence contains the T7 promoter, the LacI-binding site (Lac operator), a Shine-Dalgarno sequence, a His<sub>6</sub> tag, and a T7 tag. The resulting His<sub>6</sub>-T7 tag-eGFP fusion protein is strongly expressed from pDual-eGFP in Rosetta-gami<sup>TM</sup>2(DE3), and the construct was verified by sequencing using oligo 44 as a primer. pDual-eGFP(W<sub>66</sub>) and pDual-eGFP(Stop<sub>66</sub>) were created from pDual-eGFP by recombineering with oligos 59 and 60, respectively, in strain RIK473. pDual-eGFP(H<sub>66</sub>) and pDual-eGFP(Y<sub>66</sub>) were created from pDual-eGFP using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with oligos 68–71. Mutagenesis was confirmed by allele-specific PCR (Y<sub>66</sub> with oligos 49 and 45 or oligos 72 and 46, W<sub>66</sub> with oligos 50 and 45, Stop<sub>66</sub> with oligos 53 and 45, H<sub>66</sub> with oligos 73 and 46, T<sub>203</sub> with oligos 45 and 74, and Y<sub>203</sub> with oligos 45 and 75) and sequencing using oligos 45 and 46. pCMV-VSV-G and pCMV-dR8.2dvpr used to create lentivirus-transducing particles were gifts from Dr. Priya Rai. All oligo sequences are listed in Supporting Information Table 2.

### Recombineering

Recombineering was performed essentially as in ref. 4. Oligos were designed with the desired change centrally located with  $\geq 35$  nt of targeting homology at each side. Oligos were obtained from Sigma Genosys (St. Louis, MO, USA), polyacrylamide gel electrophoresis purified, resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.55) to 100 ng/ $\mu$ L, and stored at -20 °C until used. All oligo sequences are listed in Supporting Information Table 2. Recombineering host strains were grown overnight in LB medium at 32 °C, diluted 1/100 into fresh LB, and grown at 32 °C to mid-log phase. Recombinase expression was induced at 42 °C for 15 min. Cells were chilled on ice for 10 min to stop induction and to prepare cells for electroporation. Cells were then washed in ice-cold water and concentrated 200-fold. Forty microliters of induced competent cells was mixed with 1  $\mu$ L of oligo (100 ng); for plasmid recombineering, 10 ng of the plasmid containing the target gene

(13) was also added. Cells were electroporated in chilled 1-mm gap electroporation cuvettes using a Bio-Rad Gene Pulser (Hercules, CA, USA) at 1.75 kV, 200  $\Omega$ , and 25  $\mu$ F. Cells were allowed to recover in 1 mL LB for 30 min at 32 °C before plating dilutions on LB agar medium and incubation at 32 °C. For FP engineering in pDual-eGFP by recombineering, cultures were grown overnight in LB broth and then plasmid DNA was isolated and used to transform the *E. coli* Rosetta-gami<sup>TM</sup>2 strain to permit expression of FP genes from the T7 promoter. In all cases, fluorescent recombinant colonies were scored using a plate imager (DR46B Dark Reader transilluminator, Clare Chemical Research, Dolores, CO, USA). Occasionally, recombinants were screened by allele-specific PCR. Recombination efficiency was calculated as the recombinant titer divided by the total titer. Transformation efficiency was determined by parallel electroporation of the recombineering strain with pUC19. Mutagenesis was validated by allele-specific PCR followed by sequencing. The detailed recombineering protocol is available in the Supporting Information Methods.

### Confocal Microscopy

*E. coli* grown to OD<sub>600</sub> = 0.4 in LB at 32 °C were pelleted and resuspended in 1.05% K<sub>2</sub>HPO<sub>4</sub>, 0.45% KH<sub>2</sub>PO<sub>4</sub>, 0.005% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05% sodium citrate, and 0.2% glucose to 10% of the initial volume. One microliter of the concentrated cells was added to slides with mounting medium (P7481 Prolong Antifade, Molecular Probes, Eugene, OR, USA). Slides were covered and left to sit overnight before imaging. Images were collected with a Zeiss LSM710 confocal microscope (Thornwood, NY, USA). PerkinElmer/Improvision Velocity 64-bit software (Waltham, MA, USA) was used to create a maximum projection, and Adobe Photoshop CS3 (San Jose, CA, USA) was used to merge the colors. MIAMI adult human stem cells were grown on fibronectin-coated cover slips. Image acquisition was performed with a Leica SP5 confocal microscope (Mannheim, Germany) using a 63 $\times$  oil-immersion 1.3 numerical aperture objective. Confocal acquisition parameters were determined at the beginning of the study, and the same parameters (e.g., gains, slit aperture, and laser intensity) were used for all the images. Confocal optical sections were 0.6  $\mu$ m thick. Field selection was performed using the 4', 6-diamidino-2-phenylindole (DAPI) channel to identify MIAMI cells. Adobe Photoshop (San Jose, CA, USA) was used to merge the colors.

### Fluorometry

Protein extracts were prepared from overnight *E. coli* cultures that were subsequently pelleted, incubated with BugBuster and Benzonase, and clarified by centrifugation as recommended by Novagen (Madison, WI, USA). Excitation and emission spectra were determined using a PTI QuantumMaster spectrofluorometer (Birmingham, NJ, USA) with 2-nm slits. The average integrated 1-sec samples detected ~ 10<sup>4</sup>–10<sup>6</sup> emitted photons. The data were collected using Felix (PTI, Birmingham,

NJ, USA) and analyzed using Excel (Microsoft, Redmond, WA, USA) and PRISM (GraphPad, La Jolla, CA, USA). Each spectrum was corrected for background, normalized to its peak value, and plotted.

#### Lentiviral Transduction

A total of  $2 \times 10^6$  293T cells were plated on 10 cm<sup>2</sup> dishes. The next day, 4  $\mu$ g of pDual-eGFP (or variants), 4  $\mu$ g of pHR'8.2 $\Delta$ R, and 0.4  $\mu$ g of pCMV-VSV-G were mixed with 24  $\mu$ L of Eugene 6 (Roche, Indianapolis, IN, USA) and 400  $\mu$ L of DMEM, incubated for 15–30 min, and then added to the 293T cells. Cells were incubated overnight in a BL2+ incubator. The next day, the medium was changed to MIAMI cell medium. The following day, media containing transducing particles were collected, filtered with a 0.45- $\mu$ m syringe filter, and added to growing MIAMI cells. MIAMI cells were incubated with transducing particles overnight, and then the media were changed to MIAMI cells media.

#### Flow Cytometry Analysis

About  $10^7$  cells were harvested, washed with phosphate buffered saline (PBS), and resuspended in DMEM<sup>2p</sup> (Evrogen, MC101, Moscow, Russia). Just before flow analysis, cells were vortexed and filtered. Samples were analyzed with the Accuri cytometer (Ann Arbor, MI, USA) using a blue laser and narrow bandpass filters. Data were analyzed using C-flow (Accuri, Ann Arbor, MI, USA).

## RESULTS AND DISCUSSION

#### GFPmut3\* Variants Created in the *E. coli* Genome

To change the color of GFPmut3\*, bacteria transiently expressing Beta were electroporated with mutagenic oligos encoding the desired substitution of one or more nucleotides near the chromophore sequences of the *gfpmut3\** gene. Recombinants were identified by a change in colony fluorescence or by allele-specific PCR. Protein extracts prepared from isolates of each recombinant class were analyzed by fluorescence spectroscopy (Fig. 1). We corroborated that changes in the chromophore region of GFPmut3\*(F<sub>64</sub>G<sub>65</sub>Y<sub>66</sub>...T<sub>203</sub>) produced spectral changes from green fluorescence. GFPmut3\*(F<sub>64</sub>G<sub>65</sub>W<sub>66</sub>...T<sub>203</sub>) produced a blue-shifted variant (Violeta). GFPmut3\*(F<sub>64</sub>T<sub>65</sub>W<sub>66</sub>...T<sub>203</sub>) and GFPmut3\*(L<sub>64</sub>T<sub>65</sub>W<sub>66</sub>...T<sub>203</sub>) produced cyan variants (Aqua and Mar, respectively). GFPmut3\*(F<sub>64</sub>G<sub>65</sub>Y<sub>66</sub>...Y<sub>203</sub>) produced a yellow variant (Amarillo), whereas GFPmut3\*(L<sub>64</sub>T<sub>65</sub>W<sub>66</sub>...Y<sub>203</sub>) produced a greener cyan variant (Bronze). We determined that these variants could be distinguished by microscopy (Fig. 1a) and flow cytometry (e.g., Supporting Information Fig. 2). Even the modestly shifted Aqua variant is easily resolved from GFPmut3\*, demonstrating the utility of oligo recombineering for producing useful variants for multicolor studies.

#### Recombineering Optimization

Initially, while creating the mutants using standard recombineering methods, the frequency of mutant recombinants was low

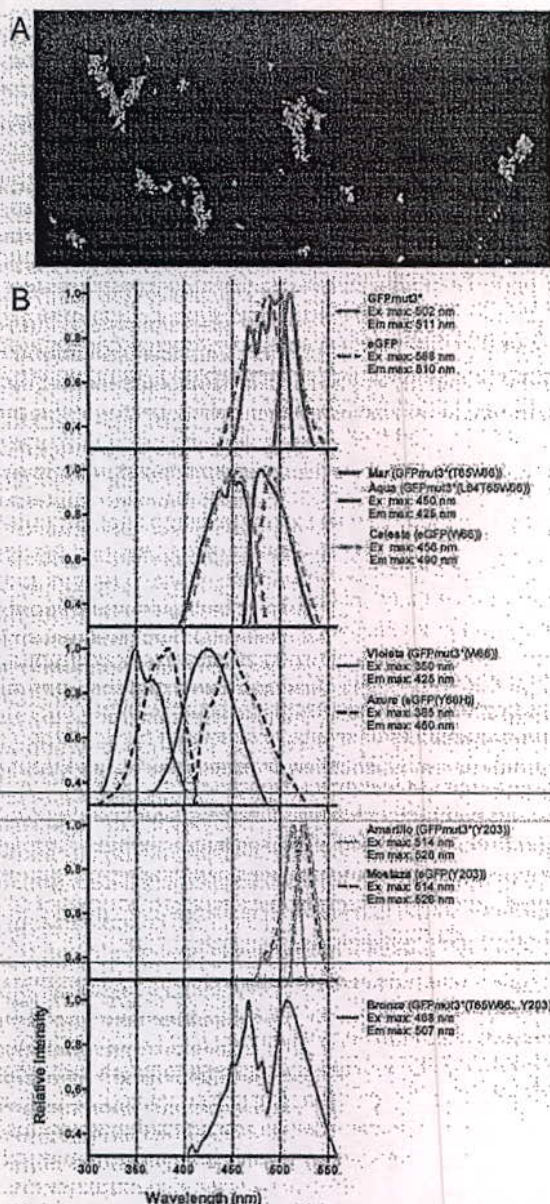


**Figure 1.** Fluorescent proteins created by *in vitro* (conventional) and *in vivo* (recombineering) site-directed mutagenesis. Mostaza and Azure FP were created by *in vitro* site-directed mutagenesis, whereas all others were created by recombineering. (A) Confocal image of Aqua, GFPmut3\*, and Amarillo *E. coli*. (B) Excitation and emission spectra of FP extracts.

(<0.1%). Therefore, we evaluated how to make the protocol more efficient. In our studies, we learned that the efficiency of mutagenesis was heavily influenced by the outgrowth time following electroporation, the oligo sequence, the structure of the heteroduplex recombination intermediate, and the transformation



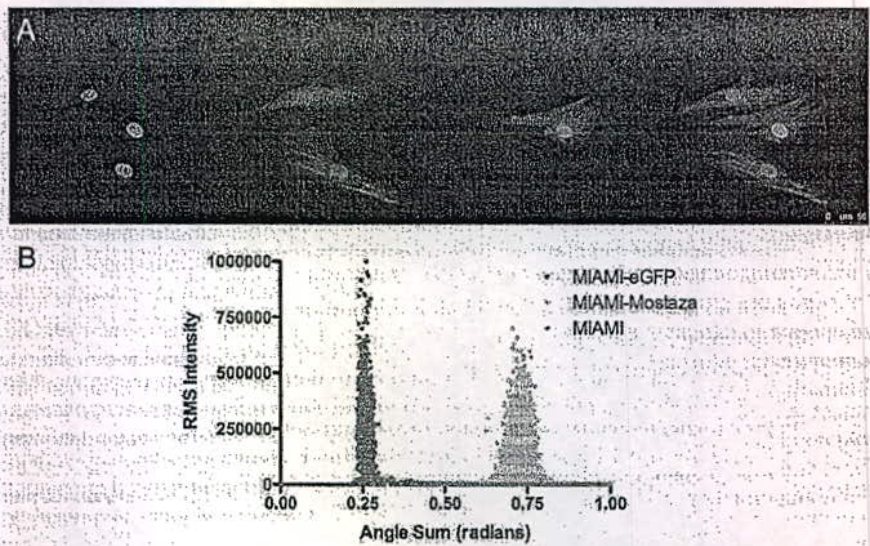


Figure 3. Discrimination of MIAMI human stem cells expressing eGFP and a Mostaza variant of eGFP. (A) Confocal image of MIAMI cell variants. The bar in white at the lower right corner corresponds to 50 μm. (B) Flow cytometry of MIAMI cell variants. Excitation was from a 488-nm laser. Fluorescence emission spectra were collected using 510 ± 7.5 nm and 540 ± 10 nm filters. Fluorescence intensity was transformed from Cartesian to polar coordinates. Each dot represents one cell.

bridize to the lagging-strand template produced ~ 70 times more mutant recombinants than oligos targeted to the leading-strand template (an example is shown in Fig. 2b). Therefore, we recommend using oligos complementary to the lagging-strand template.

The heteroduplex recombination intermediate contains one or more mispaired bases. DNA mismatch repair (MMR) can be a potent inhibitor of recombination, depending on the identity and number of mispaired bases in the heteroduplex (17). We evaluated inhibition of FP engineering by MMR using oligos that created different heteroduplex mispairs. The *mutS* gene was deleted to inactivate the MMR complex (18), and recombineering experiments were carried out in isogenic *MutS*<sup>+</sup> and *MutS*<sup>−</sup> strains (Fig. 2d and Supporting Information Table 3). The highest recombineering rates were achieved with single nucleotide mismatches when MMR was avoided. For example, the C•C mispair escapes MMR and is the most efficient intermediate for mutagenesis in *MutS*<sup>+</sup> strains. Interestingly, mispairs of two to nine nucleotides are equally good substrates if MMR is avoided (the median recombination frequencies are not different by Kruskal–Wallis ANOVA; *P* = 0.4366). We recommend oligos designed to produce the least MMR-correctable heteroduplex. Alternatively, one may inactivate the MMR system during mutagenesis and then recover the mutated gene into an *MMR*<sup>+</sup> strain to reduce the chance of picking up additional mutations from the “mutator” phenotype of *MMR*<sup>−</sup> strains.

The efficiency of introducing oligos into cells determines the amount of substrate available for recombineering. We saw that recombination frequencies fluctuated with the transformation ef-

Table 1  
Rules for high efficiency *in vivo* site-directed mutagenesis by recombineering

| Rule  | Advice                                                                                                                                             |
|-------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| One   | Use dividing cells to enrich for replication intermediates.                                                                                        |
| Two   | Use oligos complementary to the lagging-strand template.                                                                                           |
| Three | Use oligos that incorporate a C•C mismatch in the paired recombination intermediate. Alternatively, use a 2+ nucleotide mismatch or knock out MMR. |
| Four  | Plate transformants within 30–60 min of outgrowth.                                                                                                 |

iciency of individual cell preparations. We saw a positive correlation (by a one-tailed nonparametric correlation test; *P* = 0.0215) between the efficiency of plasmid uptake (to estimate transformation) and recombineering. We found that working with cells in early log phase improved both transformation and recombineering efficiencies.

**eGFP Variants Created in a Lentiviral Plasmid**

To extend the utility of FP engineering to mammalian cells, we modified *egfp* carried in a lentiviral plasmid propagated in *E. coli*. Recombineering *in vivo* or site-directed mutagenesis *in*

*vitro* was performed as described in Supporting Information Methods and FP variants identified by altered bacterial colony fluorescence. We obtained eGFP(L<sub>64</sub>T<sub>65</sub>H<sub>66</sub>...T<sub>203</sub>) with a pronounced blue spectral shift (Azure), eGFP(L<sub>64</sub>T<sub>65</sub>W<sub>66</sub>...T<sub>203</sub>) with cyan spectra (Celeste), and eGFP(L<sub>64</sub>T<sub>65</sub>Y<sub>66</sub>...Y<sub>203</sub>) with yellow spectra (Mostaza), as shown in Fig. 1. We generated lentiviral stocks of the engineered FP variants and used them to transduce human MIAMI stem cells. As shown in Fig. 3, MIAMI cells expressing Mostaza FP are easily distinguished from cells expressing eGFP by microscopy and flow cytometry. The differences allow excellent quantitation over several orders of magnitude in mixtures (Supporting Information Fig. 3).

## CONCLUSION

We have demonstrated that FP sequence changes easily introduced *in vivo* via recombineering produce significant spectral shifts. We showed that this method is useful for generating multicolor reporters and novel FPs. The Bronze variant looks especially promising for imaging studies, as the excitation maximum (468 nm) overlaps a common laser wavelength. This approach is not restricted to modifying FP genes in the *E. coli* genome, but also works for altering FP reporter genes in plasmid and viral sequences propagated in *E. coli*.

Using the optimized protocol, the efficiency of FP engineering is ~40% of transformed cells (Fig. 2c and Supporting Information Table 3). Therefore, the optimized protocol for *in vivo* mutagenesis via recombineering is nearly as efficient as *in vitro* site-directed mutagenesis but without size limits on target DNA molecules. A detailed protocol with explanations is provided in the Supporting Information Methods. The most critical parameters for successful mutagenesis via recombineering are summarized in Table 1.

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# Functional Characterization of the *S. cerevisiae* Genome by Gene Deletion and Parallel Analysis

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The functions of many open reading frames (ORFs) identified in genome-sequencing projects are unknown. New, whole-genome approaches are required to systematically determine their function. A total of 6925 *Saccharomyces cerevisiae* strains were constructed, by a high-throughput strategy, each with a precise deletion of one of 2026 ORFs (more than one-third of the ORFs in the genome). Of the deleted ORFs, 17 percent were essential for viability in rich medium. The phenotypes of more than 500 deletion strains were assayed in parallel. Of the deletion strains, 40 percent showed quantitative growth defects in either rich or minimal medium.

The budding yeast *S. cerevisiae* serves as an important experimental organism for revealing gene function. In addition to carrying out all the basic functions of eukaryotic cells, up to 30% of positionally-cloned genes implicated in human disease have yeast homologs (1). Determining the function of all yeast gene products will be an important step toward understanding their function in metazoans and lays the foundation for a more complete comprehension of cellular processes and pathways.

A powerful way to determine gene function is the phenotypic analysis of mutants missing the gene. Several genome-wide approaches have been proposed including genetic footprinting and random mutagenesis (2, 3). While genetic footprinting has the advantage that all genes can be tested for their contribution to fitness under a particular growth condition relatively quickly, it has the disadvantage that the mutant strains cannot be recovered. In addition, testing each additional condition is as time-consuming as the first. Random mutagenesis is relatively rapid, but the subsequent matching of phenotypes to genes is slower. In addition, with random

approaches a certain fraction of genes may be missed, even with oversampling. These limitations can be overcome by deleting each gene in the genome in a directed fashion and by marking each yeast gene with a molecular "barcode" that allows the phenotypes of the mutant strains to be assayed in parallel.

The precise deletion of yeast genes can be efficiently accomplished using a polymerase chain reaction (PCR)-mediated gene disruption strategy that exploits the high rate of homologous recombination in yeast (4). For this method, short regions of yeast sequence (~50 base pairs (bp)) identical to those found upstream and downstream of a targeted gene are placed at each end of a selectable marker gene through PCR. The resulting PCR product, when introduced into yeast cells, can replace the targeted gene by homologous recombination. For most genes, >95% of the resulting yeast transformants carry the correct deletion (5). In addition, this method can be modified so as to introduce two molecular barcodes (UPTAG and DOWNTAG) into the deletion strain. The barcodes or "tags" are unique 20-base oligomer (20-mer) sequences

that serve as strain identifiers (6, 7). We show that these barcodes allow large numbers of deletion strains to be pooled and analyzed in parallel in competitive growth assays. This direct, simultaneous, competitive assay of fitness increases the sensitivity, accuracy and speed with which growth defects can be detected relative to conventional methods.

To take full advantage of this approach and to accelerate the pace of progress, an international consortium was organized to generate deletion strains for all annotated yeast genes. Here, we report the construction of precise start-to-stop codon deletion mutants for 2026 ORFs (8).

Genes essential for viability in yeast, in particular those encoding proteins lacking human homologs, have been proposed to be the best targets for antifungal drugs. When spores from the 2026 heterozygous strains were germinated on YPD (yeast extract-peptone-dextrose) media at 30°C haploid deletants could not be recovered for 356 ORFs (see [www-sequence.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) for an exact list) (9). Despite the considerable interest in these genes as potential drug targets, only 56% of these ORFs had previously been shown to be essential for viability (10). Of the 2026 ORFs analyzed, 1620 were not essential for viability in yeast. For these one additional homozygous and two haploid deletants (Table 1) were also constructed.

A computational Smith-Waterman analysis indicated that 8.5% of the identified non-essential ORFs in the yeast genome have a closely related homolog elsewhere in the ge-

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nome ( $P < 1.0 \times 10^{-150}$ ), whereas only four (1%) of the essential genes (*PYK1*, *YDR341C*, *PRP22*, and *MYO2*) encoded proteins that were homologous to another predicted protein in the genome. The redundancy may be why more genes in the yeast genome are not essential. The essential genes were distributed fairly evenly across the chromosome but were slightly biased toward being located near other essential genes (60% of essential genes were within 5 kb of another essential gene, whereas 47% of nonessential genes were). Essential genes were generally not found within 50 kb of the telomeres (Fig. 1A). Essentials were also more heavily transcribed. Transcripts were detected for >99% of essential genes versus 90% of nonessential genes (11). The average number of transcripts per cell for all essential genes was 70% higher than for all nonessential genes. The functional classification of the essential genes versus the nonessential genes is shown in Fig. 1B.

The phenotypic analysis of the deletion strains, in particular those whose cognate protein is not essential to life, is a formidable task. The role of many genes will likely be manifested only under very specialized growth conditions, necessitating the examination of many different conditions. Previous work demonstrated that the barcodes allowed the relative abundance of their respective strains to be measured when 12 strains were grown competitively for many generations (6). The barcoding scheme thus has the potential to accelerate the phenotypic analysis of the deletion strains by allowing the growth rates of all strains to be assayed simultaneously. The first 558 homozygous deletion strains constructed were pooled (12) and grown in rich and minimal media for about 60 generations. During this time, aliquots were removed from the two pools. The tags were amplified, and hybridized to high-density arrays containing the tag complements (Fig. 2A) (13). The hybridization data were used to calculate the relative growth rates for each deletion mutant in the population (14). It was expected that the growth rate for each strain obtained independently with the UPTAG and DOWNTAG signals would agree. For strains in which both the UPTAG and DOWNTAG signal were at least threefold over background, the correlation for growth rates measured with the UPTAG and DOWNTAG were 0.97 in rich medium, and 0.94 in minimal medium (Fig. 2, B and C). The weakest correlations were observed for strains that were the most growth impaired (growth rate <0.6 of that of the wild type) because sufficient signal was detected only for first few time points.

As expected, the strains disappearing at the highest rates in minimal medium but which grew relatively normally in rich medi-

um (at >98% of the pool growth rate) included all of the strains carrying deletions in genes known to be essential for growth in minimal medium including *ade1* [tag average <0.50 in minimal (M); 0.98 in rich (R)]; *arg5,6* (<0.50, M; 0.98, R); *yer068c-a* (<0.50, M; 0.99, R; overlaps *arg5,6*); *ilv1* (<0.50, M; 1.01, R); *arg4* (<0.50, M; 1.0, R); *gcn4* (0.53, M; 0.98, R); *hom3* (0.54, M; 1.0, R); and *ade4* (0.56, M; 1.01, R). In addition, the *gyp1* (0.78, M; 0.99, R) deletant showed a minimal medium-specific growth defect (15). *GYP1* (*YOR070C*) is a GTPase

activating protein for Sec4p, a protein in the secretion pathway (16). Mutants of *lys7* also showed a growth defect in minimal medium but also grew somewhat slowly in rich medium (0.52, M; 0.88, R). Six strains exhibiting a specific growth defect in rich medium were also identified. These strains included *cin8* (0.80 R; 0.95, M); *erg6* (0.71, R; 0.96, M); *rpl39* (0.85, R; 0.98, M); *ym1193C-A* (0.85, R; 0.98, M; overlaps ribosomal protein *rpl36a*); *esc1* (0.83, R; 0.97, M) and *ym1013w* (0.78, R; 0.95, M).

Altogether, almost 40% of the deletants

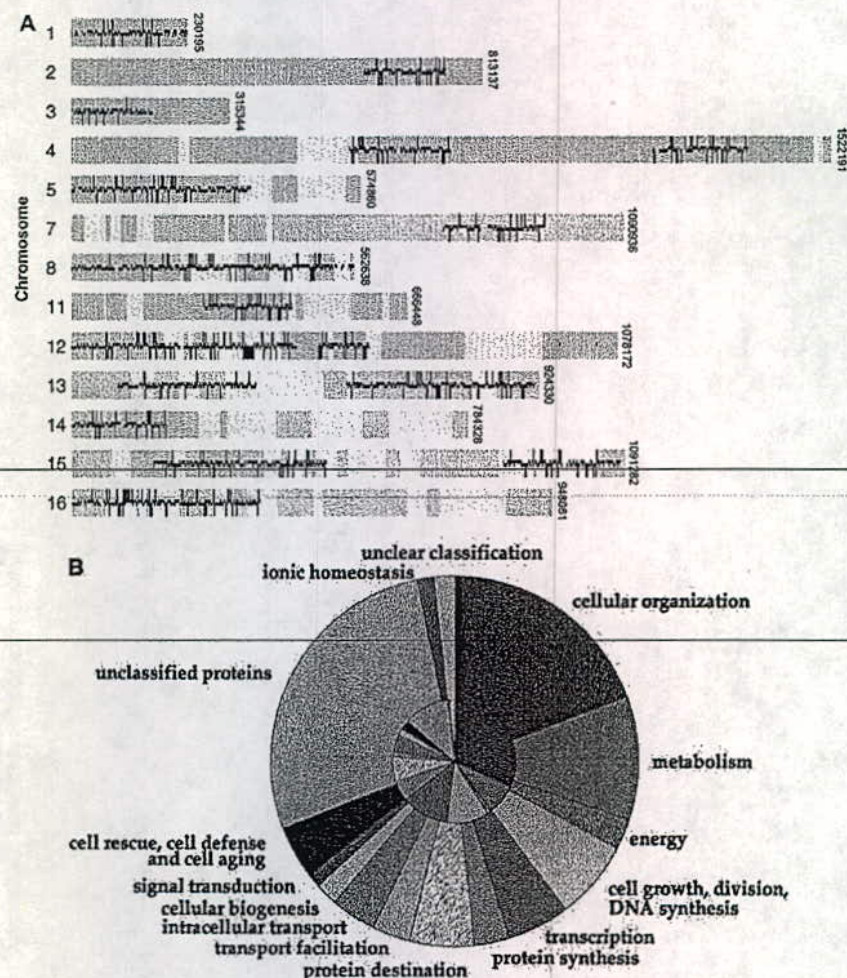


Fig. 1. (A) Genomic locations of 1620 nonessential (short black bars) and 356 essential genes (tall black bars). Deletions were generated in consecutive groups on multiple chromosomes. A lighter gray background indicates the location of chromosomal duplication blocks (23). For 15 of the 356 essential ORFs, a haploid null mutant had been previously described. These inconsistencies may be due to differences in strain background or in the conditions used for germination of spores. For example, the previously-constructed *yhr101c* (*big1*) and *ybr196c* (*pgi1*) null mutants show glucose sensitivity, and the *ybr256c* (*rib5*) deletion mutant requires a riboflavin supplement for viability (24). In several cases, haploid null mutants were reported to have slow-growth phenotypes [*ymr308c* (*PSE1*), *yol022c*, *ypl243c* (*srp68*), *ypl210c* (*srp72*), and *ydr353w* (*trr1*) (25)] or to be temperature sensitive [*ydr113c* (*pds1*) and *ygr216c* (*gpi1*) (26)]. In some cases, haploid deletions strains were constructed for genes previously determined to be essential by others [*HKR1*, *RNR1*, and *FUN9* (27)]. (B) Distribution of functional classes of essential (inner circle) and nonessential (outer circle) ORFs using criteria from the Munich Information Centre for Protein Sequences (MIPS) (10).

It is often assumed that if a gene is expressed under a particular set of conditions, then that gene is important for growth under those conditions. Deletion of the up-regulated gene would then be expected to cause a decrease in growth. These data offered a unique opportunity to correlate changes in gene expression with deletion phenotypes. The tran-

| Strain          | Genotype                                                                                                            | Reference |
|-----------------|---------------------------------------------------------------------------------------------------------------------|-----------|
| BY4730          | <i>MATa leu2Δo met15Δo ura3Δo</i>                                                                                   | (29)      |
| BY4739          | <i>MATα leu2Δo lys2Δo ura3Δo</i>                                                                                    | (29)      |
| BY4741          | <i>MATa his3Δ1 leu2Δo met15Δo ura3Δo</i>                                                                            | (29)      |
| BY4742          | <i>MATα his3Δ1 leu2Δo lys2Δo ura3Δo</i>                                                                             | (29)      |
| BY4743          | <i>MATa/α his3Δ1/his3Δ1 leu2Δo/leu2Δo met15Δo/MET15<br/>lys2Δo/LYS2 ura3Δo/ura3Δo</i>                               | (29)      |
| YD00000-YD09999 | <i>MATa orfΔ::kanMX4 his3Δ1 leu2Δo met15Δo ura3Δo</i>                                                               | This work |
| YD10000-YD19999 | <i>MATα orfΔ::kanMX4 his3Δ1 leu2Δo lys2Δo ura3Δo</i>                                                                | This work |
| YD20000-YD29999 | <i>MATa/α orfΔ::kanMX4/ORF his3Δ1/his3Δ1 leu2Δo/leu2Δo<br/>lys2Δo/LYS2 met15Δo/MET15 ura3Δo/ura3Δo</i>              | This work |
| YD30000-YD39999 | <i>MATa/α orfΔ::kanMX4/orfΔ::kanMX4 his3Δ1/his3Δ1<br/>leu2Δo/leu2Δo lys2Δo/LYS2 met15Δo/MET15<br/>ura3Δo/ura3Δo</i> | This work |

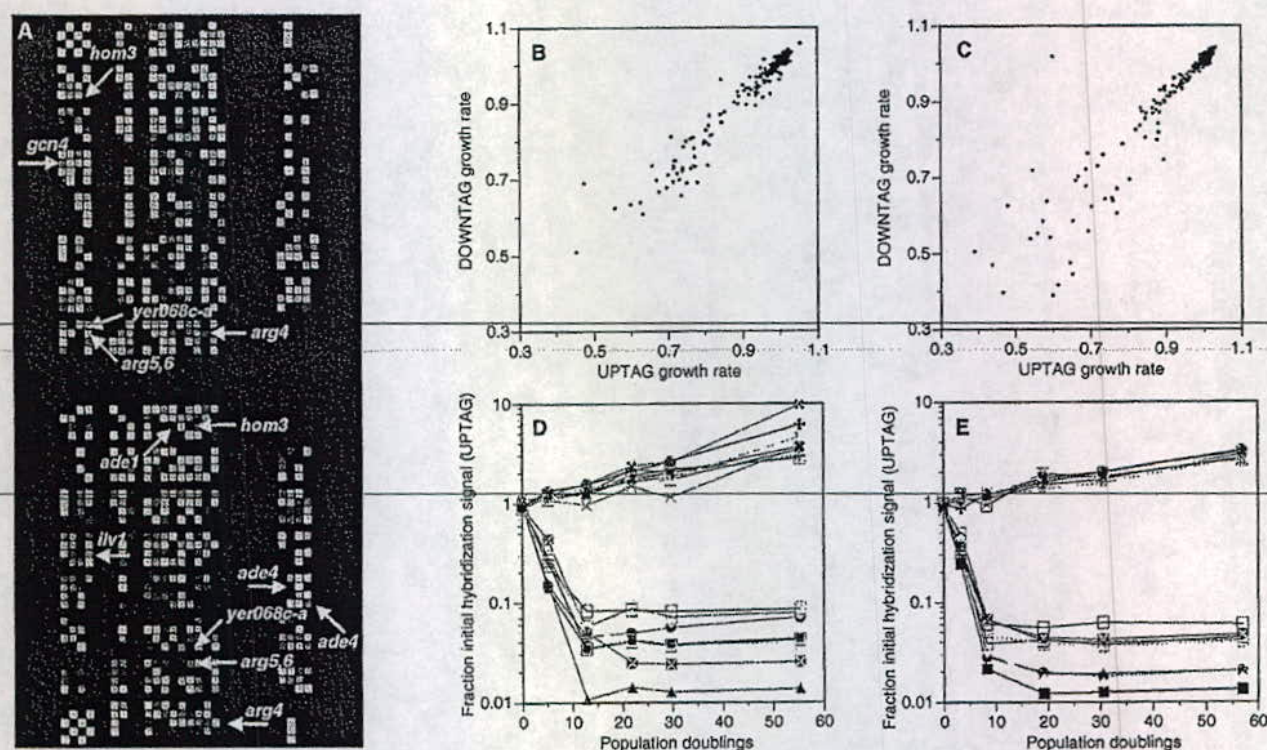


**Fig. 2.** Analysis of 558 homozygous diploid strains in rich and minimal medium. Pools were grown for ~60 generations in minimal medium (SD) supplemented with histidine, uracil, and leucine, or in rich medium (YPD) at 30°C. The batch-transfer method (2, 3) was used to ensure that the cell densities did not exceed  $4 \times 10^7$  cells/ml during the growth study. At least 500,000 cells were transferred during each dilution to avoid unequal representation of strains. Samples (approximately  $5 \times 10^7$  cells) were taken at six different time points for each growth study and genomic DNA was extracted. The two tags for each strain were amplified separately with biotin-labeled primers and were hybridized together to the arrays. (A) Two-color comparison of scanned images of high-density oligonucleotide arrays hybridized with fluorescently-labeled tags amplified from 558 strains grown for 0 (red channel) or 6 hours (green channel) in minimal medium. Approximately 4000 different features, each containing more than  $10^7$  20-mers of a specific sequence, were synthesized in a specific physical location on the array by photolithography and photosensitive oligonucleotide chemistry (6, 28). Only a portion of the array is shown. The features containing probes to the bar

codes for deletion strains that exhibit a growth defect in minimal medium, but not in rich medium, are labeled and indicated with an arrow. The array sequences were assigned sequentially to different deletion strains. A second-generation array has been designed, which contains enough tag complement sequences for every gene in the entire genome. [(B) and (C)] Correlation of growth rate data obtained with UPTAG and DOWNTAG sequences for strains grown in rich (B) and minimal (C) media. Data are shown for 331 strains (of the 401 strains that contained both an UPTAG and a DOWNTAG sequence) which had  $t = 0$  UPTAG and DOWNTAG hybridization signals that were both at least threefold over background. More frequent sampling during the initial growth period should improve the correlation. (D) Normalized hybridization intensity data for the 10 slowest-growing (*yer014c-a*, *rrn1*, *hem14*, *mot2*, *pfk2*, *rpl27a*, *yer044c*, *rp50a*, *ymr188c*, *yel029c*) and the 10 fastest-growing strains in rich medium, (E) Hybridization data for the 10 slowest-growing (*hem14*, *arg5,6*, *ade1*, *yer068c-a*, *ilv1*, *yer014c-a*, *yer044c*, *lys7*, *gcn4*, *rrn1*) and 10 fastest growing strains in minimal medium.

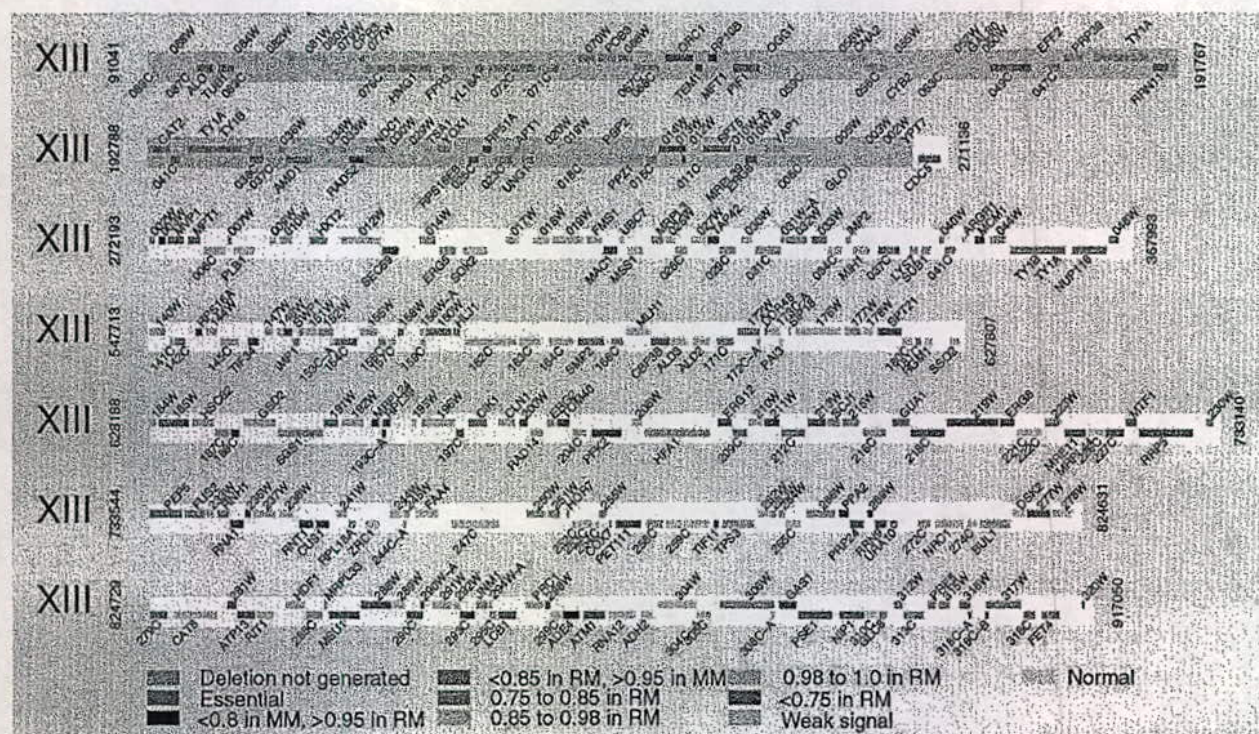


Fig. 3. Deletion map for 336 ORFs and the results of parallel phenotypic analysis for 226 ORFs on chromosome XIII. Data for additional chromosomes can be found at [www.sciencemag.org/feature/data/1040380.shl](http://www.sciencemag.org/feature/data/1040380.shl). Chromosome right arms are shown with white backgrounds and left arms

with gray. ORFs for which deletions were not generated (gray bars) resulted from failure during PCR or oligonucleotide synthesis (5.2%), failure for unknown reasons (4%), failure to pick unique primers (3.3%), or failure to generate deletions in all four strains (2.5%).

script abundance of all genes in the genome was measured in rich and minimal media to determine whether the set of genes whose inactivation produced a quantitative fitness defect in rich and minimal media would be the same set whose expression was induced under these growth conditions (18). Surprisingly little correlation existed between the growth study data and the expression data. Deletion of genes specifically induced in minimal media was no more likely to affect fitness in minimal medium than deletion of the uninduced genes. Of the genes showing a strong minimal medium growth defect, only *ARG4* and *ARG5,6* were significantly up-regulated (greater than twofold) in minimal medium relative to rich medium. Similarly, only one of the six genes that showed a rich medium-specific growth defect was up-regulated more than 2.5-fold in rich medium relative to minimal media. These data indicate the importance of multiple approaches in genome-wide functional analysis studies.

The results we present demonstrate that quantitative fitness data can be rapidly obtained under various conditions. Although the presence of the *KanMX4* gene has been shown to have no effect on the fitness of some deletion strains (19), it is theoretically possible that the encoded neomycin phosphotransferase could have an impact on a partic-

ular deletion strain. The composition of a pool of deletion strains and the conditions under which the pool was cultured could also have an effect on the observed fitness of the strains. Finally, the phenotypes of certain deletions strains might be complemented by factors released into the medium by other strains in the pool. Additional tests will be required to determine how frequently these artifacts will occur.

These results also show that thousands of deletants can be systematically made once the sequence of a genome is known. Several laboratories in Europe and North America are collaborating to finish construction of tagged deletions for all annotated *S. cerevisiae* ORFs within 1 year (20). Currently, more than three-quarters of the ORFs in the yeast genome have been deleted (5100 ORFs and more than 15,300 strains generated). Whereas a significant amount of work was expended to construct the strains, in contrast to other methods for generating functional data (2, 3), the strains provide a lasting resource. In addition, the availability of a consistent set of isogenic strains should provide a better way for researchers to compare their results with those of others, easing the task of curating the functional assignments that hitherto have been made in various strain backgrounds. Finally, while other efforts have been mount-

ed by a European consortium and others to generate deletion strains (21), the inclusion of barcodes significantly enhances the usefulness of the strains. The ability to assess thousands of strains quantitatively and in parallel will significantly decrease the amount of labor and materials needed for fitness screens (22) and increase the reliability of the data interpretation and functional classifications.

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8. To construct deletion strains, two long oligonucleotide primers are synthesized, each containing (3' to 5') 18 or 19 bases of homology to the antibiotic resistance cassette, *KanMX4* (U1, D1), a unique 20-bp tag sequence, an 18-bp tag priming site (U2 or D2), and 18 bases of sequence complementary to the region upstream or downstream of the yeast ORF

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being targeted (including the start codon or stop codon; see [http://sequence-www.stanford.edu/group/yeast/yeast\\_deletion\\_project/new\\_deletion\\_strategy.html](http://sequence-www.stanford.edu/group/yeast/yeast_deletion_project/new_deletion_strategy.html)). These 74-mers are used to amplify the heterologous KanMX4 module, which contains a constitutive, efficient promoter from a related yeast strain, *Ashbya gossypii*, fused to the kanamycin resistance gene, *nptII* (5). Because oligonucleotide synthesis is 3' to 5' and the fraction of full-size molecules decreases with increasing length, improved targeting is achieved by performing a second round of PCR using primers bearing 45 bases of homology to the region upstream and downstream of a particular ORF. Transformation with the PCR product results in replacement of the targeted gene upon selection for G418 resistance. The unique 20-mer tag sequences are covalently linked to the sequence that targets them to the yeast genome, creating a permanent association and genetic linkage between a particular deletion strain and the tag sequence. The use of two tags increases confidence in the analyses, and the redundancy is useful in case one of the tags carries a mutation or performs poorly in hybridization assays. To verify correct integration of the deletion cassette, genomic DNA was prepared from the resistant strains and used as template in PCR reactions using two primers common to the KanMX4 module (KanB (5'-CTGCAGCCGAGGAGCCGTAAT-3') and KanC (5'-TG-ATTTTGATGACGAGCGCTAAT-3')) and four ORF-specific primers (A, B, C, and D). A and D are from regions 200 to 400 bases upstream or downstream of the start codon, whereas B and C are from within the ORF (see [http://sequence-www.stanford.edu/group/yeast\\_deletion\\_project/confirmation.html](http://sequence-www.stanford.edu/group/yeast_deletion_project/confirmation.html)). For verification, both the A-KanB and the D-KanC PCR reactions were required to give the correct size product when analyzed by gel electrophoresis. If one of either the A-KanB or D-KanC reactions failed to yield a product, the identification of the correctly-sized AD product could suffice. In addition, haploid deletion strains were tested for the disappearance of the wild-type AB and CD products. All ORFs encoding proteins greater than 100 amino acids in size were initially selected for deletion. The deletion cassettes were designed to remove the entire coding sequence for a given ORF but to leave the start and stop codon intact. Although ~10% of ORFs in *S. cerevisiae* overlap one another, the positions of the deletions were not adjusted, nor was any attempt made to avoid essential genes, genes in which a previous deletion had been constructed, or genes with a well-defined function. Genes represented multiple times in the genome (telomeric ORF, T-elements) were usually not deleted as their targeted disruption would pose a challenge due to the conservation of upstream and downstream regions. Some smaller nonannotated ORFs (NORFs) will be deleted in the future. Transcripts from many of these NORFs have been detected in SAGE analysis, warranting their inclusion in the study [V. E. Velculescu et al., *Cell* 88, 243 (1997)]. All oligonucleotide primers (5 nmol scale) were synthesized on an automated multiplex oligonucleotide synthesizer [D. A. Lashkari, S. Hunnicke-Smith, R. M. Norgren, R. W. Davis, T. Brennan, *Proc. Natl. Acad. Sci. U.S.A.* 92, 7912 (1995)] in batches of 96 primers. Scripts were written to automate the selection of primers. Primer sequences and ORF locations were chosen from the Stanford Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>) at various times over a 2-year period. The KanMX4 cassette was PCR-amplified, and the resulting PCR products were sent to participating laboratories where 1 µg of PCR product was used to transform yeast by means of a variation on the standard lithium acetate procedure ([http://sequence-www.stanford.edu/group/yeast\\_deletion\\_project/protocols.html](http://sequence-www.stanford.edu/group/yeast_deletion_project/protocols.html)) in a 96-well format. Electronic records, accessible over the World-Wide Web, were kept for every strain constructed. MATa haploid strains were given record numbers of less than 10,000, MATα haploid strains were given record numbers between 10,000 and 20,000, the heterozygous diploid, between 20,000 and 30,000, and the homozygous diploid, greater than 30,000. Each record consists of primer sequence information, the results of the different diploid tests

that were performed, and notes about the phenotype. Data for completed strains are accessible from [www-sequence.stanford.edu/group/deletion/index.html](http://www-sequence.stanford.edu/group/deletion/index.html). These strains, frozen in 15% glycerol, can be obtained from Research Genetics (Huntsville, AL) or EUROSCARF (Frankfurt, Germany).

9. Four different types of strains, containing several genetic markers (Table 1), were generated for each ORF—two haploid strains, one of each mating type, and two diploid strains, heterozygous and homozygous for the deletion loci and kanamycin marker. The homozygous diploid was constructed by mating the two haploid strains, obtained from independent transformations. Typically, the heterozygous diploid and one of the haploid strains were obtained by direct transformation, while the other haploid strain and the homozygous diploid were obtained by sporulation and mating, respectively. Essential genes were identified by 2:2 segregation of viability in tetrads derived from the heterozygous diploid. Strains were sporulated by patching them on a fresh GNA plate (5% D-glucose, 3% Difco nutrient broth, 1% Difco yeast extract, 2% Difco Bacto agar) for 1 day at 30°C before transfer to liquid sporulation medium (1% potassium acetate and 0.005% zinc acetate supplemented with 0.1 mM uracil, 0.15 mM histidine-HCl, or 1.0 mM leucine as necessary). Sporulation cultures were incubated on a rollerwheel for 4 to 5 days at 25°C. If 2:2 segregation of viability was consistently observed in two independently-transformed heterozygous deletion strains, the gene was designated essential. All confirmed diploid strains obtained through mating were required to pass two of three tests: a diploid budding pattern, the ability to sporulate, and the inability to mate. In a few cases, essential genes overlapped other essential genes (20 pairs) or a gene whose viability status was unknown (four pairs), making it difficult to determine the cause of lethality.
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12. To construct the pools, each deletion strain was patched on YPD plates in the presence of 150 mg/liter G418. Approximately equal numbers of cells were harvested from the plate for each strain and combined. Aliquots of the pools were stored in the presence of 15% glycerol at -80°C.
13. The UPTAG and DOWNTAG sequences were separately amplified from genomic DNA by means of PCR using primers B-U2-comp (5' biotin-GTCGACCTGCAGCTAGC-3') and U1 (5'-GATGTCCAGAGGTCTCT-3'), or primers B-D2-comp (5'-biotin-CGAGCTCGAATTCATCG-3') and D1 (5'-CGGTGTCGGTCTCTAG-3'). In both cases, a twofold molar excess of biotinylated primers was used in the reactions. The amplified UPTAG and DOWNTAG sequences were combined and hybridized to high-density oligonucleotide arrays in 200 µl of 6×SSPE [1 M NaCl, 66 mM NaH<sub>2</sub>PO<sub>4</sub>, 6.6 mM EDTA, (pH 7.4)], containing 0.005% Triton X-100 (SSPE-T). 200 pmol U1, 200 pmol U2 (5'-CGTACGCTGCAGGTGAC-3'), 200 pmol D1, and 200 pmol primer D2 (5'-CGATGAATTCGAGCTCG-3'). The addition of complementary primers to the hybridization mix was shown to improve the signal-to-noise ratio [D. D. Shoemaker, thesis, Stanford University, Stanford, CA (1998)]. Samples were heated to 100°C for two min, and then cooled on ice before being applied to the array. Samples were hybridized for 1 hour at 42°C. The arrays were washed two times with six changes of 6×SSPE-T. The arrays were then stained at 42°C for 10 min with 6×SSPE-T containing 2 µg/ml phycoerythrin-streptavidin (Molecular Probes) and 1 µg/ml acetylated bovine serum albumin, washed two times with five changes 6×SSPE-T, and scanned at an emission wavelength of 560 nm using an Affymetrix GeneChip Scanner. Of the strains analyzed, 157 contained only a single tag sequence (UPTAG). Six of these strains were not detected in the hybridization mix. Of the strains represented with two-tag sequences, 98.5% exhibited either an UPTAG or DOWNTAG bar code hybridization signal that was greater than threefold over background. Sequencing of 186 deletion regions tags showed that 25% of mutations

in the tags or tag priming sites resulted in a nonfunctional tag that could not be amplified or detected by hybridization, or both. In only 1.1% of cases was a complete lack of hybridization signal not associated with a mutation in the tag or tag priming site. Mutations were most often found in the tags or tag priming sites (0.85% per base) and were less frequent in the regions of yeast homology (0.25% per base), most likely due to selection against the mutated PCR products during the recombination event or to the two-step PCR strategy.

14. Grids were aligned to the scanned images using the known feature dimensions of the array. The hybridization intensities for each of the elements in the grid were determined using the 75th percentile method in the Affymetrix GeneChip software package. Subsequent analysis of the hybridization intensities consisted of two steps: adjustment of data to achieve approximate equality of background and maximal signals on each array, and analysis of the decrease (or increase) of the UPTAG and DOWNTAG signal strength over time. Equalization of signal strength relied on the fact that for most array sites, the amount of tag DNA present did not vary over time. A consensus score for these sites was obtained from the first principal component of the logarithms of the signals. The logarithms of the hybridization intensity for each element on the array were linearly transformed to make each array's overall signal approximately equal to the consensus. The growth rate for each strain was determined by using the model  $\log_2(\text{signal}) = \max(a + b \cdot t, 0) + e$ , where  $a$  and  $b$  are model parameters,  $t$  represents the number of population doublings, and  $e$  is a random error term. The growth rate is calculated as  $1 + b$ . For strains that have dropped out of the pool ( $a + b \cdot t < 0$ ), the model describes the statistical distribution of background signals. When  $e$  is normally distributed, background signals have a lognormal distribution. This appeared to be roughly true in our data. However, we found that a small fraction of signals on each array are liable to have very high values, much larger than can be accounted for by a purely lognormal background model. To obtain a degree of robustness against these occasional outliers, we assumed that the error term  $e$  had a scaled  $t$  distribution with one degree of freedom. The use of this heavy-tailed distribution reduced the likelihood of false positive identifications of deficient strains due to occasional high signal levels at  $t = 0$ , at the expense of a possible reduction in the ability to detect marginally deficient strains.
15. The analysis was not as accurate for strains with growth rates of less than 0.5 that of the wild type, because generally only three data points were above background for these.
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17. A comprehensive study of chromosome V genes using genetic fingerprinting (3) provided an opportunity to validate the data: the results generally agreed, with a few exceptions. For example, of the 52 genes whose disruption had no effect on strain fitness under all conditions tested by genomic fingerprinting, we detected a growth defect in deletants *ye033w* (0.68, R; 0.83, M), *ye050c* (0.73, R; 0.68, M) and *ye028c* (0.79, R; 0.69, M). The observed phenotype for *ye033w* probably results from interference with a neighboring gene (*HYP2*, encoding translation initiation factor eIF-5A). In addition, the *hem14* deletant showed a strong growth defect in rich medium, while genomic fingerprinting revealed a salt-specific defect, but no defect in rich medium. Of the 11 genes that had been shown by genomic fingerprinting to have a severe growth defect, in three cases our deletants appeared to have no discernible phenotype (*nrf1*, *gda1*, *pci6*). These differences could be due to our use of diploid versus haploid strains, to the auxotrophies carried by our deletants, or to our using 30°C versus 25°C as the growth temperature. The effect of temperature is probably the cause of discrepancies for the *nrf1* strain, which grows slowly at 25°C but grows faster than normal at 36.5°C. Among other disparities, two (*YER082C* and *YEL026W*) of the 22 genes on chromosome V determined to be essential by tetrad

- analysis were wild-type by footprinting (perhaps because of cross-feeding in the Ty pool or because the products are required for germination) and the minimal medium-specific growth defects we detected for *hom3* and *liv1* mutants were not detected by genomic footprinting.
18. Cultures of BY4743 *hcc:KanMX4* were grown at 30°C in YPD or minimal media supplemented with histidine, uracil, lysine, methionine, and adenine. In the presence of G418, and were harvested at mid-log phase. cDNA was prepared from 20 µg of polyadenylated RNA from each sample, using a dT<sub>21</sub> primer and Superscript II reverse transcriptase (GibcoBRL), according to the manufacturer's recommendation. cDNA was fragmented using DNaseI (GibcoBRL), biotinylated using ddATP (NEN) and Terminal Transferase (Boehringer), and hybridized to yeast full-genome arrays (Affymetrix) as described in L. Wodicka et al. [Nature Biotechnol. 15, 1359 (1997)]. After scanning, the average signal from each array was normalized to the average signal strength of all eight chips.
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## Early Neocortical Regionalization in the Absence of Thalamic Innervation

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There is a long-standing controversy regarding the mechanisms that generate the functional subdivisions of the cerebral neocortex. One model proposes that thalamic axonal input specifies these subdivisions; the competing model postulates that patterning mechanisms intrinsic to the dorsal telencephalon generate neocortical regions. *Gbx-2* mutant mice, whose thalamic differentiation is disrupted, were investigated. Despite the lack of cortical innervation by thalamic axons, neocortical region-specific gene expression (*Cadherin-6*, *EphA-7*, *Id-2*, and *RZR-beta*) developed normally. This provides evidence that patterning mechanisms intrinsic to the neocortex specify the basic organization of its functional subdivisions.

The mammalian neocortex is organized into regionally distinct functional subdivisions. There are two proposed mechanisms for neocortical regionalization. The protocortex hypothesis postulates that thalamic afferent fibers play an important role in neocortical regional development (1). On the other hand, the protomap hypothesis (2) postulates that

regionalization of the neocortex is due to molecular differences within the neocortical ventricular zones (3–7). We investigated mice with a mutation of the *Gbx-2* gene (8, 9) that disrupts thalamic histogenesis, which in turn blocks formation of thalamocortical projections.

Analysis of *Gbx-2*, *Id-4*, and *Lef-1* expression showed that *Gbx-2*-deficient mice have abnormal thalamic development. *Gbx-2* expression in the dorsal thalamus (DT) could be detected in the subventricular (SVZ) and mantle zones (MZ) (10). In the *Gbx-2* mutant, *Gbx-2* mRNA expression was maintained in the SVZ but was lost in the MZ (Fig. 1, E and F), suggesting that the mutant is unable to produce normal DT postmitotic cells (*Gbx-2* transcripts were detected in the mutant because the 5' end of this gene has not been altered). This hypothesis was supported by the expression pattern of *Id-4*. The *Id-4* helix-loop-helix gene (11), which normally is expressed throughout the MZ of the DT, was not detectable in the DT of the *Gbx-2* mutant (Fig. 1, I and J). In addition to the apparent

defect in the MZ, there was ectopic expression of *Lef-1* in the mutant. At embryonic day (E) 14.5, the *Lef-1* high mobility group box transcription factor (12) was expressed in the MZ of the pretectum (PT) and DT. In the *Gbx-2* mutants, *Lef-1* expression in the PT was unaffected, whereas its expression in the MZ was lost in the DT, and ectopic periventricular expression was observed (Fig. 1, M and N). Thus, by E14.5, loss of *Gbx-2* function disrupted differentiation in most, or all, of the DT.

To verify the thalamic abnormalities, we studied thalamic anatomy at postnatal day (P) 0, the day when these animals die. Histological abnormalities in the P0 *Gbx-2* mutant thalamus were even more pronounced. *Gbx-2* expression marked the lateral dorsal (LD), centromedian (CM), dorsal lateral geniculate (DLG), and reuniens (Re) nuclei (Fig. 1G). Several other nuclei also expressed *Gbx-2*, including the ventral lateral, the midline, the anteromedial, the mediodorsal, and the medial geniculate (MG) nuclei (13). In the mutant, there was only weak *Gbx-2* expression, which was restricted to a periventricular region (see asterisk in Fig. 1H).

Some thalamic regions of the *Gbx-2* mutants retained *Id-4* and *Lef-1* expression. *Id-4* expression marked most nuclei in the DT and the reticular nucleus (RT) of the ventral thalamus (Fig. 1K). In contrast, the *Gbx-2* mutant had greatly reduced *Id-4* expression in most areas of the DT (Fig. 1L) but retained expression in the RT, which does not express *Gbx-2* (10). Limited *Id-4* expression was detectable in some dorsal thalamic areas that, on the basis of their location, may correspond to the ventrobasal complex (VB) and DLG nuclei (Fig. 1L). *Lef-1* expression marked specific DT subdivisions including the DLG, VB, ventromedial (VM), and posterior (Po) nuclei (Fig. 1O). In the *Gbx-2* mutant, *Lef-1* expression was lost from most of the MZ, but unlike the wild type, its expression was found near the ventricle (arrow in Fig. 1P). Some *Lef-1*

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# Differential Toxicities of Anticancer Agents among DNA Repair and Checkpoint Mutants of *Saccharomyces cerevisiae*<sup>1</sup>

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## ABSTRACT

Most cytotoxic anticancer agents damage DNA directly, interfere with DNA metabolism or chromosome segregation, and are particularly toxic in dividing cells. Although a considerable amount of information on the mechanisms of action of these agents is available, the molecular bases for selective tumor cell killing by chemotherapy are largely unknown. Many genetic alterations found in sporadic and hereditary cancers affect functions in DNA repair and cell cycle control and result in sensitivity to DNA damaging agents. We have therefore set out to determine the effects of these cancer mutations on sensitivity or resistance to various chemotherapeutic agents. Because most of the affected genes are well conserved among eukaryotes, we have carried out a comprehensive analysis of a panel of isogenic yeast strains, each defective in a particular DNA repair or cell cycle checkpoint function, for sensitivity to the Food and Drug Administration-approved cytotoxic anticancer agents. Widely different toxicity profiles were observed for 23 agents and X-rays, indicating that the type of DNA repair and cell cycle checkpoint mutations in individual tumors could strongly influence the outcome of a particular chemotherapeutic regimen.

## INTRODUCTION

The goal of chemotherapy is to selectively eliminate tumor cells. Identifying the molecular differences that exist between tumor and normal cells will aid in achieving this selectivity. Cancers accumulate a large number of genetic changes during progression toward malignancy due to an intrinsic genetic instability that should provide distinguishing characteristics to exploit therapeutically (1). However, because methods do not yet exist to comprehensively genotype mammalian cells, it has not yet been possible to catalogue these changes and correlate them with chemotherapeutic responses. However, comprehensive genotyping may not be necessary if specific lesions could be identified that create a vulnerability to chemotherapeutic agents. The very mutations that promote genetic instability would be expected to create such vulnerability because they cripple DNA repair functions or cell cycle checkpoints (2).

Contrary to current clinical practice, there is no reason to expect that the type of lesion creating genetic instability would necessarily correlate with the tissue of origin of a tumor (3). Indeed, lesions creating a genetic instability (for example, mismatch repair defects) occur in a wide variety of tumors from different tissues of origin (1). We propose, therefore, that identification of the particular mutations creating genetic instability in a tumor will be more predictive of the chemotherapeutic sensitivity of that tumor than will be its tissue of origin. In cases in which chemotherapy is effective, a conjunction of chemotherapeutic agent and tumor vulnerability may have been achieved fortuitously. Additionally, by using model organisms, de-

fects that lead to genetic instability could be used to identify new selective agents.

For this approach to work, one would need to correlate vulnerability to chemotherapeutic agents with particular DNA damage response defects. Here, we used yeast cells with defined genetic defects in DNA damage response elements to see whether currently used chemotherapeutic agents have specificity for individual DNA damage response defects. The agents for which such a specificity exists are candidates for correlating effectiveness in human cancer with defects in DNA damage response elements in those same tumors.

## MATERIALS AND METHODS

**Yeast Strains.** All strains used are haploid and in the A364a background. For treatment with X-rays and drugs, except pentostatin, the following strains were used (with the relevant genotype indicated in parentheses): yMP10381 (wild type), yMP10836 (*rad1Δ*), yMP10691 (*rad14::HIS3*), SP50248 (*mhl1::TRP1*), yMP11082 (*pms1::LEU2*), yMP10467 (*mgl1::URA3*), yMP10590 (*apn1::LEU2*), yMP10612 (*mgt1::LEU2*), 9170-Tx1a (*rad6::LEU2*), yMP10425 (*rad18::LEU2*), SP50280 (*rev1::LEU2*), yMP10382 (*rev3::LEU2*), yMP11406 (*rad50::hisG*), yMP10383 (*rad51::LEU2*), yMP10630 (*rad52Δ*), yMP10335 (*rad9::LEU2*), yMP10485 (*rad17::LEU2*), yMP10913 (*mec1Δ*), yMP10605 (*mec2Δ*), SP50252 (*mad1::LEU2*), yMP11372 (*mad3::LEU2*), and SP50265 (*sgs1::LEU2*). For treatment with pentostatin, all strains were as above except for SP50646 (*mgt1::kan*). All strains are of the genotype *MATa ade2 ade3-130 leu2 trp1 ura3-52 cyh2 SCR::URA3*, except for yMP10485 (*rad17::LEU2*) and yMP10636 (*rad1Δ*), which are *CYH2*<sup>+</sup>; yMP10630 (*rad52Δ*) and yMP11406 (*rad50::hisG*), which are *MATa*; and SP50646 (*mgt1::kan*), which is *MATa* and lacks *SCR::URA3*. *SCR::URA3* is a cassette consisting of two halves of *ADE3* separated by *URA3* for measuring sister-chromatid recombination (4). The alleles *rev1::LEU2*, *mad1::LEU2*, *sgs1::LEU2*, and *mgt1::kan* were generated by PCR fragment-directed gene replacement (5) using plasmids pJ252 or pJ250 (6) for amplification of the *LEU2* marker gene and pUCkanMX (7) for amplification of the *kan* gene, and they generally lack greater than 90% of the open reading frame. Strains harboring the PCR fragment-directed gene replacements *apn1::LEU2* and *mad3::LEU2* were obtained from Amanda Paulovich, and *mhl1::TRP1* was obtained from Brian Thornton. Strains harboring the point mutations *rad52-1* (8), *mec1-1* (9), and *mec2-1* (9) and the gene replacements *pms1::LEU2*, *rad18::LEU2*, *mgt1::LEU2*, *mgl1::URA3*, *rev3::LEU2*, *rad50::hisG*, *rad51::LEU2*, *rad14::HIS3*, *rad6::LEU2*, *rad1Δ*, *rad9::LEU2*, and *rad17::LEU2* were obtained from Amanda Paulovich.

**Drugs.** Chemotherapeutic drugs were obtained from the Developmental Therapeutics Program at the National Cancer Institute; cytarabine monophosphate was also obtained from Sigma. Drugs were stored at -80°C in aliquots and used only once. Camptothecin sodium salt and idarubicin solutions were prepared for each experiment.

**Toxicity Assays.** In preliminary experiments, appropriate concentration ranges were determined for each drug and each strain. Exponentially growing yeast cultures in complete minimal medium were diluted to  $7.4 \times 10^4$  cells/ml, and 135  $\mu$ l were dispensed into each well of flat-bottomed 96-well plates. Drugs were dispensed in seven 2-fold serial dilutions in 5% DMSO or 5% ethanol (thiotepa and lomustine) or in H<sub>2</sub>O (cisplatin, mitomycin, cytarabine, cytarabine phosphate, hydroxyurea, and fluorodeoxyuridine), and 15- $\mu$ l aliquots were added in triplicate to the yeast-containing wells. Fifteen  $\mu$ l of the appropriate solvent were also added in triplicate in control wells. Plates were incubated for 18 h at 30°C, and the *A*<sub>490</sub> of the cultures was read in a Bio-Tek Instruments EL340 microplate reader. Slowly growing strains, especially *rad6*

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and *rad52* mutants, were incubated until the  $A_{600}$  of control wells read at least 0.4 but no more than 0.8. For three agents,  $IC_{50}$  values were determined by measuring colony-forming units in the case of lomustine and cytarabine phosphate because this method resulted in greater sensitivity or in the case of mitoxantrone because the color of the drug interfered with the  $A_{600}$  reading. In this case, after incubation, aliquots from each well were plated onto rich medium agar plates. Sulfanilamide (0.1 mg/ml) was added to the growth medium for methotrexate and trimetrexate assays. Each experiment was carried out three times with yeast strains freshly streaked out from  $-80^{\circ}\text{C}$  stocks.<sup>4</sup>

**X-irradiation.** Yeast were grown in liquid culture, cell aggregates were dispersed by sonication, and serial dilutions of cells were plated onto YEPD plates. The plates were irradiated for times ranging from 10 to 400 s at a dose rate of 106 rads/s, from 10 to 400 s at 106 rads/s. After incubation at  $30^{\circ}\text{C}$  for 2 days, colony-forming unit were determined, and the percentage of surviving cells was calculated relative to unirradiated control plates.

## RESULTS

We have assembled a panel of isogenic yeast strains, each defective in a particular DNA damage repair or cell cycle checkpoint pathway. The damage repair pathways include nucleotide excision repair (*rad1* and *rad14*), mismatch repair (*mhl1* and *pms1*), base excision repair (*mag1* and *apn1*), direct reversal of  $O^6$ -alkylguanine (*mtf1*), error-free (*rad18*), error-prone (*rev1*, *rev3*), and error-free and error-prone damage (*rad6*) tolerance during DNA replication (also called postreplication repair or daughter-strand gap repair) and recombinational repair (*rad50*, *rad51*, and *rad52*). The roles of these genes in DNA repair have been reviewed extensively (10). Cell cycle checkpoints include the DNA damage checkpoint (*rad9* and *rad17*; Ref. 11), DNA damage and S-phase checkpoint (*mecl1* and *mec2/rad53*; Ref. 9), spindle assembly checkpoint (*mad1* and *mad2*; Ref. 12), and homologue of the genes mutated in Bloom's and Werner's syndromes (*sgs1*; Ref. 13).

There are 76 FDA<sup>5</sup>-approved anticancer agents, and of these, 44 are expected to be toxic to yeast. In preliminary experiments, we determined that 31 of these agents showed at least minimal toxicity in yeast. In two cases, cytarabine (NSC-63878) and topotecan (NSC-609699)/irinotecan (NSC-616348), we used analogues that were active yeast. The initial step in the activation of cytarabine in human cells is its conversion to cytarabine monophosphate. Because yeast lack the required deoxycytidine kinase, we used cytarabine monophosphate (NSC-99445), which is taken up efficiently and shows activity. Likewise, we used camptothecin sodium salt (camptothecin sodium, NSC-100880) in place of topotecan and irinotecan. Here we report the  $IC_{50}$  data for these agents in the damage response-deficient yeast strains. Fig. 1 shows the profiles of the alkylating and cross-linking agents. Fig. 2 shows the antimetabolites and nucleotide analogues. Finally, Fig. 3 shows topoisomerase poisons, X-rays, bleomycin, and actinomycin D. Most of the DNA repair and DNA damage checkpoint pathways are represented by more than one mutant in the strain panel. In a majority of the profiles (e.g., cisplatin; Fig. 1a), the various mutants representing a particular pathway show a similar sensitivity. This coherent response indicates that the observed sensitivity is a property of a pathway defect rather than a property of the individual mutant strain. Although many of the drugs have been studied previously in various yeast mutants (14, 15), this report is the first comprehensive examination of the relative sensitivities of damage response-defective yeast strains to anticancer agents.

The profiles are of three general types. First, there are agents showing selective toxicity, primarily in strains containing one path-

way defect. We define selective toxicity in a particular strain or pathway as a 10-fold or greater decrease in the  $IC_{50}$  relative to the next, most sensitive strain. We conclude that the selective agents cause a specific type of damage only requiring a subset of damage response functions for survival (e.g., mitoxantrone; Fig. 3b). Second, there are agents that show toxicity in many pathway defects indicating the introduction of multiple types of DNA damage (e.g., thiotepa, mitomycin C, hydroxyurea, and X-rays; Figs. 1, b and c, and 3c). Third, there are agents that lack selective toxicity between the various mutant strains and wild type. Such profiles indicate that although the agents are toxic, cell killing occurs by a mechanism that is probably independent of DNA damage (e.g., methotrexate; Fig. 2e).

**Selective Agents.** Five agents (cisplatin, cytarabine phosphate, camptothecin sodium, mitoxantrone, and idarubicin) showed a high degree of specificity.

Cisplatin (Fig. 1a) is specifically toxic to strains defective for the poorly understood Rad6/Rad18-controlled pathway of damage tolerance during S-phase. It is known, however, that Rad6 and Rad18 are required for the completion of S-phase when a damaged DNA template is present (i.e., daughter-strand gap repair; Ref. 16). Rad6- and Rad18-mediated survival does not require Rad50, Rad51, Rad52, Rad1, Rad14, Rev1, or Rev3 (17). The sensitivity of these strains reflects the additional role of nucleotide excision repair (Rad1 and Rad14), recombinational repair (Rad50, Rad51, and Rad52) and error-prone damage tolerance (Rev1, Rev3, and Rad6) in the repair of inter- and intrastrand DNA cross-links as well as mono-adducts formed by cisplatin. The importance of the Rad6 and Rad18 function is evidenced by the greater than 10-fold increase in sensitivity in *rad6* and *rad18* strains relative to the other sensitive strains (e.g., *rad50*). With the exception of the magnitude of the *rad6* and *rad18* sensitivity, the profiles for cisplatin and mechlorethamine are highly similar, indicating a common response to cross-links induced by platinum adduct formation and alkylative cross-linking. Thiotepa, which is not generally thought to create DNA cross-links, is also similar.

The nucleotide analogue cytarabine monophosphate (Fig. 2a) shows highly selective toxicity in the *sgs1* mutant strain. This profile does not resemble those of agents causing known types of DNA damage. Cytarabine inhibits DNA synthesis, is incorporated into DNA, and has also been reported to be recombinogenic in yeast (18). The *sgs1* mutant and the homologous Bloom's and Werner's syndrome defects in human cells cause hyperrecombination (19). It is therefore possible that the observed selectivity of cytarabine phosphate is due to an effect on recombination and that the Sgs1 protein is required for tolerance. We found that a *sgs1 rad52* double mutant is less sensitive to cytarabine phosphate compared to the *sgs1* single mutant, corroborating a possible role for recombination in the mechanism of toxicity (data not shown). We have observed differential growth inhibition of the *sgs1* mutant by adenine arabinoside phosphate and fludarabine phosphate, indicating that the arabinose moiety of these agents may be important for selectivity (data not shown). The profile of fludarabine (Fig. 2b) shows a greater sensitivity of the *rad50* and *mec2* mutants than that of cytarabine phosphate.

The selective category includes, in addition to cisplatin and cytarabine monophosphate, three topoisomerase poisons. These agents stabilize the covalent complex of topoisomerase and DNA ends during DNA relaxation and lead to single-strand breaks and DSBs for topo I and topo II poisons, respectively. The single-strand breaks caused by topo I poisons are converted into DSBs during DNA replication (20, 21). The camptothecin analogue, a topo I poison, kills DSB repair mutants with high specificity. The DNA damage checkpoint mutants also contribute to survival, albeit to a lesser degree (Fig. 3a).

The topo II poisons mitoxantrone (Fig. 3b) and idarubicin (Fig. 3c) also showed selectivity for DSB repair defects. It is noteworthy that

<sup>4</sup> Bar graphs for all 23 agents and X-rays can be obtained from our Web site: [http://www.fda.gov/cder/rdmt/yeast/yeast\\_project](http://www.fda.gov/cder/rdmt/yeast/yeast_project).

<sup>5</sup> The abbreviations used are: FDA, Food and Drug Administration; topo I, topoisomerase I; topo II, topoisomerase II; DSB, double-strand break.

cells deficient in the DNA damage checkpoint are not sensitized to these topo II poisons. This can be explained by the observation that topo I poison-induced damage requires ongoing DNA synthesis to create DSBs, thus largely limiting the damage to S-phase when DNA damage-induced and DNA synthesis checkpoints can contribute to cell survival. topo II poisons, in contrast, create DSBs throughout the cell cycle, with the majority occurring in mitosis, when the DNA damage-induced and synthesis checkpoints are no longer capable of protecting cells. Interestingly, two other topoisomerase poisons, daunorubicin and doxorubicin, show profiles that are nonselective, suggesting the presence of non-topo II-mediated cellular damage (Fig. 3, f and g).

**Broadly Selective Agents.** Nine agents (mitomycin C, thiotepea, lomustine, carmustine, streptozotocin, mechlorethamine, bleomycin, hydroxyurea, and X-rays) show selective toxicity in a large number of DNA damage response-defective strains. Most of these responses are consistent with the known mechanisms of action of the agents. Mag1 (3-methyladenine glycosylase) is involved in the removal of damage created by alkylation of adenine (22), and as expected, the *mag1* mutant is sensitive to thiotepea (Fig. 1b). The *rad1* and *rad14* strains are sensitive to agents that introduce DNA cross-links (e.g., cisplatin and mechlorethamine; Fig. 1, a and c). Thiotepea, however, is a non-cross-linking alkylating agent, and the sensitivity of these strains was unexpected (23). Lomustine (Fig. 1h) and carmustine (Fig. 1g) produce broad profiles; although both are capable of forming DNA cross-links (24), nucleotide excision repair-defective strains (e.g.,

*rad1* and *rad14*) were not sensitive. These differences demonstrate a continuum of pathway responses to cytotoxic drugs and underscore the importance of identifying the critical toxic lesions for agents that are capable of inducing different forms of DNA damage. Streptozotocin (Fig. 1d), a methylating agent, also yielded a broad profile, although the *mgt1* strain was particularly sensitive. This was expected, because the Mgt1 protein, the yeast homologue of human MGMT, is required for O<sup>6</sup>-methylguanine reversal (25, 26).

The ribonucleotide reductase inhibitor hydroxyurea (Fig. 2c) blocks DNA replication by depleting the dNTP pool. This is consistent with the observed sensitivity of the intra-S phase checkpoint-deficient *mec1* and *mec2* strains (9, 27). Additional DNA damage in the form of DSBs is indicated by the sensitivity of *rad50*, *rad51*, and *rad52* strains. This result is consistent with the observation that stalled replication forks result in DNA breaks (28). Since these breaks occur exclusively during S phase, the Rad9 and Rad17 functions may not be required, because the cell cycle would already be slowed down in a *MEC1*-dependent fashion due to a decreased level of dNTPs. X-ray treatment (Fig. 3c) results in a profile similar to the topo I poison camptothecin sodium (Fig. 3a), except for the strong requirement of the Rad6 and Rad18 proteins for survival. X-rays are often referred to as a DSB-causing agent, which is consistent with the strong sensitivity of the recombinational repair mutants. The sensitivity of *rad6* and *rad18* strains indicates that other forms of DNA damage are also present in irradiated cells as expected. The profiles of camptothecin

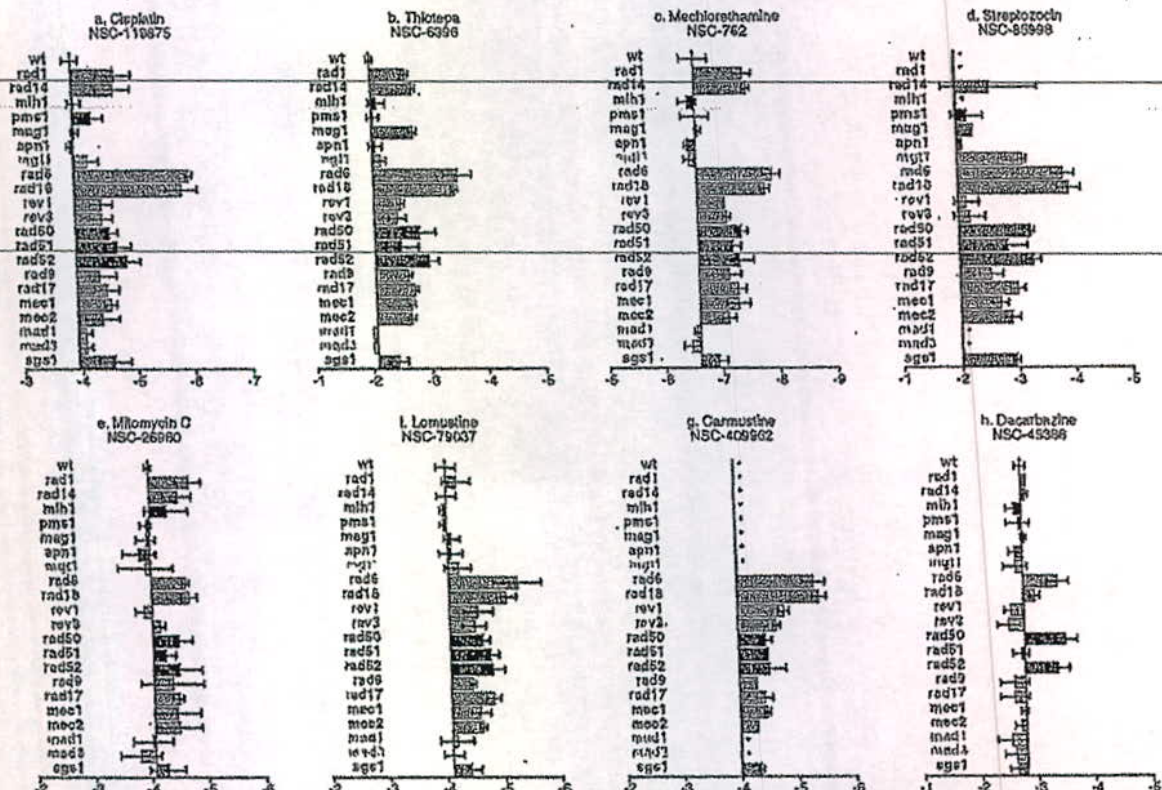


Fig. 1. Toxicity profiles of cytotoxic anticancer agents: the alkylating agents. The graphs show the IC<sub>50</sub> (log M) for each agent against the strain panel. The vertical line is set at the IC<sub>50</sub> of the wild-type strain. The strains are grouped and color-coded according to the DNA damage response pathway they represent. \*, an accurate IC<sub>50</sub> could not be obtained because of limiting solubility of the compound.

DIFFERENTIAL TOXICITIES OF ANTICANCER AGENTS

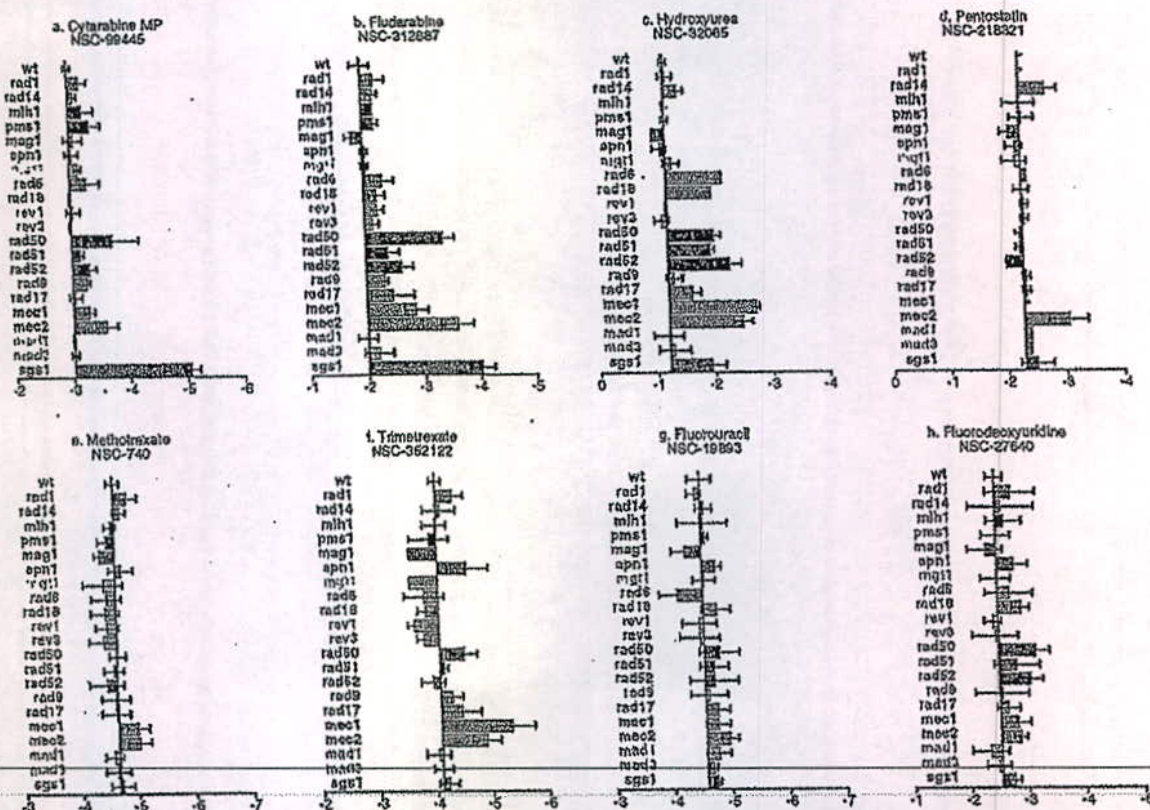


Fig. 2. Toxicity profiles of cytotoxic anticancer agents: the antimetabolites. The graphs show the  $IC_{50}$  (log M) for each agent against the strain panel. The vertical line is set at the  $IC_{50}$  of the wild-type strain. The strains are grouped and color-coded according to the DNA damage response pathway they represent. \*, an accurate  $IC_{50}$  could not be obtained because of limiting solubility of the compound.

sodium (Fig. 3a) and mitoxantrone (Fig. 3b) suggest that DSBs do not necessarily require Rad6 and Rad18 functions for repair or tolerance.

**Nonselective Agents.** Nine agents (methotrexate, trimetrexate, fluorouracil, fluorodeoxyuridine, pentostatin, dacarbazine, actinomycin D, daunorubicin, and doxorubicin), although toxic, showed only minor differences in sensitivity among the various mutant strains and the wild-type strain. These nonselective profiles, especially in the case of agents that block key steps in DNA synthesis (i.e., methotrexate, trimetrexate, fluorouracil, and fluorodeoxyuridine), are surprising because the induced damage must not require a DNA damage response for survival. Methotrexate (Fig. 2e) inhibits dihydrofolate reductase, resulting in a block in the biosynthesis of purines and thymidine. Our results suggest that the purine deficiency might affect RNA synthesis more strongly than DNA replication. Interestingly, the profile of the methotrexate analogue, trimetrexate (Fig. 1f) does show a moderate sensitivity of *mec1* and *mec2* strains, as would be expected for a block in DNA synthesis. Fluorouracil (Fig. 2g) and fluorodeoxyuridine (Fig. 2h) inhibit thymidylate synthase, resulting in a block in the synthesis of dTMP from dUMP. In this case, only DNA synthesis would be affected, and it is surprising that *mec1* and *mec2* strains are not more sensitive. Pentostatin (Fig. 2d) inhibits adenine deaminase, which is involved in posttranscriptional modification of RNA and is not expected to cause DNA damage (29). Dacarbazine, doxorubicin, daunorubicin, and actinomycin D (Fig. 1h and Fig. 3, f-h) showed very similar, virtually nonselective profiles. It is likely that these agents kill

yeast cells by a mechanism that does not require a DNA damage response for survival. Daunorubicin, doxorubicin, and actinomycin D, for example, are capable of generating free radicals, which target membranes in addition to DNA (30).

## DISCUSSION

The goal of this study has been to determine the cellular contexts that contribute to the sensitivity of cells to cytotoxic anticancer drugs. Although there is extensive information on the mechanisms of action for cancer drugs, there has been little progress in determining whether there are specific contexts (that is, molecular defects) that determine the selective killing of tumor cells over normal cells. To achieve a constant genetic context in which to assess various molecular defects, we chose to use a panel of isogenic yeast strains. The yeast mutants tested here were chosen because they affect the major pathways known to be required for survival in response to DNA damage and because they represent most of the pathway defects identified thus far that lead to genetic instability in cancer cells. It is possible that additional pathways responsible for genetic stability may be identified in the future. This may include Sgs1, the DNA damage response function of which is poorly understood.

We chose the nonbiological, nonhormonal, FDA-approved agents to assess the relative sensitivity of the yeast mutants and were able to obtain  $IC_{50}$  values for 23 of the 44 agents tested. The profiles shown

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DIFFERENTIAL TOXICITIES OF ANTICANCER AGENTS

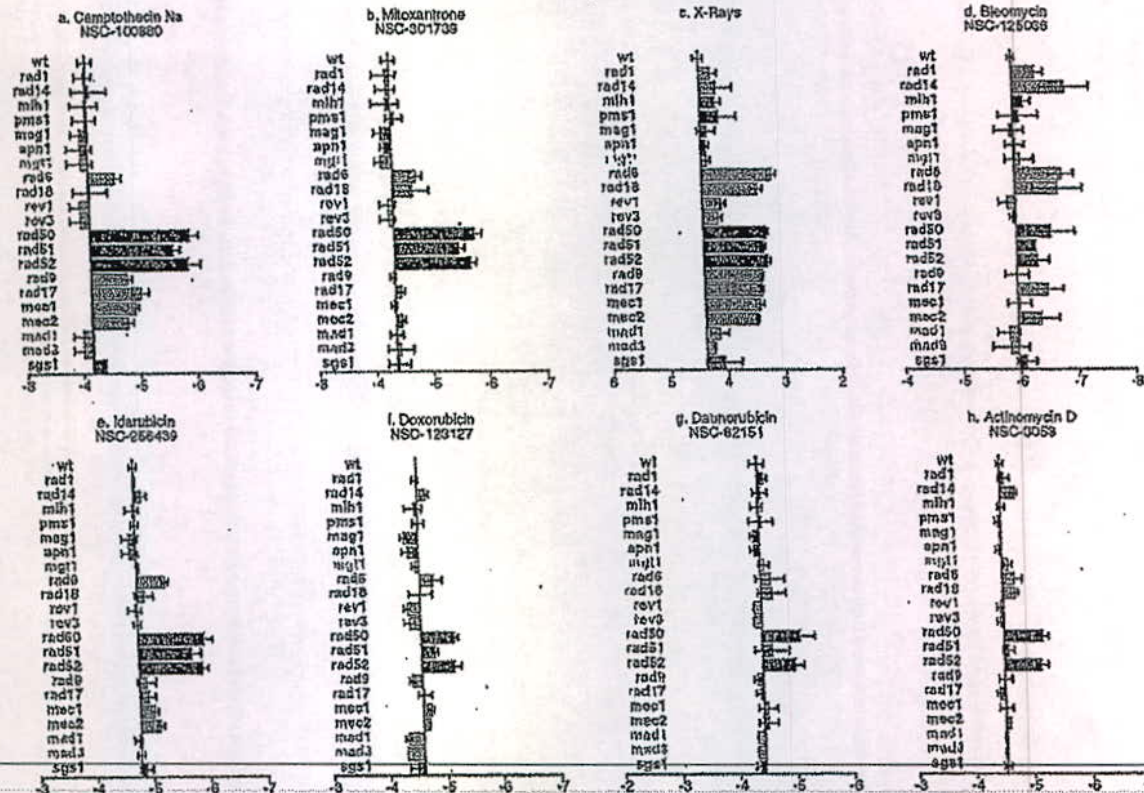


Fig. 3. Toxicity profiles of cytotoxic anticancer agents: topoisomerase poisons, X-rays, bleomycin, and actinomycin D. The graphs show the  $IC_{50}$  ( $\log M$  for compounds,  $\log k$  rad for X-rays) for each agent against the strain panel. The vertical line is set at the  $IC_{50}$  of the wild-type strain. The strains are grouped and color-coded according to the DNA damage response pathway they represent.

here emphasize the importance of damage response defects as the determinants of sensitivity to cytotoxic drugs and to X-rays. It is reasonable to expect, and our data support the idea, that molecular defects in the same pathway will have similar sensitivities (e.g., *rad50*, *rad51*, and *rad52* treated with mitoxantrone). Three patterns of cellular sensitivity emerged from these profiles: agents that selectively target only one or few damage response defects (e.g., cisplatin, mitoxantrone, and cytarabine phosphate); those that show a broad spectrum of toxicities (e.g., thiotepa or streptozocin); and those that show little or no enhanced killing in DNA damage response-defective strains. Our results underscore the notion that there are two broad modes by which chemotherapeutic agents act. One class of agents has activity against a broad range of tumors or cell types because these agents lack specificity for any particular cellular context (e.g., X-rays). The current emphasis of clinical trials, to obtain as large a response as possible in patients who are grouped only by the organ site of their cancer, favors agents that act broadly. These agents however, precisely because they target a wide spectrum of defects, have the highest probability of affecting normal, noncancerous tissues. The second class, chemotherapeutic agents with greater specificity, offers the potential to improve the therapeutic response by targeting a narrowly defined cellular context (i.e., a specific pathway defect). Each cancer is likely to harbor genetic defects that were important for the etiology of that particular cancer, and these defects are probably not shared by all cancers from the same organ site. Agents of high specificity and therapeutic advantage (for a precisely

defined class of tumors with specific genetic lesions) are almost certainly bypassed under current clinical trial procedures. The latter strategy, however, offers the greatest opportunity for selective targeting of cancer cells without killing normal cells.

The analysis of the FDA-approved cancer drugs using this isogenic panel of yeast addresses an important question in cancer therapy: do we need new drugs or better diagnostics? Agents, such as mitoxantrone, that selectively target a single pathway defect suggest that improved diagnosis may be efficacious. Cancers defective in DNA DSB repair may be hypersensitive to topo II poisons. The availability of better diagnostics that would allow the routine determination of pathway defects in individual tumors might greatly improve benefits from current chemotherapeutic agent. Conversely, the agents resulting in broad toxicities in multiple genetic backgrounds may lead to higher toxicity in noncancer cells. It is therefore important to identify agents with high specificity for unique genetic changes associated with cancers. Primary drug screens carried out in the context of a single genetic alteration may provide a valuable route to new selective drugs. Therapeutic agents could be screened for high toxicity in human cells carrying a particular damage response defect but low toxicity in isogenic wild-type control cells. This preliminary study has shown that many of the current cancer drugs (X-rays etc.) are relatively nonspecific and suggests that developing more specific therapeutics may be beneficial. At the same time some of the commonly used cancer drugs, such as cisplatin and the topoisomerase poisons, have significant specificity in their killing, and this provides strong evi-

dence that new molecular diagnostics could improve their utility. The results described in this communication pertain to the activity of anticancer agents in yeast, and it should be noted that the biology of yeast and mammalian cells differs in several important aspects. Foremost is the absence of programmed cell death in yeast. Although programmed cell death or apoptosis may not alter the intrinsic sensitivity of cells harboring specific defects to particular agents, it may alter the response of the cell to damage (*i.e.*, survival). In addition, the relative importance of alternative pathways for cellular response to cytotoxins can vary between the two systems. This is true for DNA DSB repair pathways, the major pathway of which in yeast is homologous recombination (represented by *rad50*, *rad51*, and *rad52* in this study), whereas in mammalian cells, nonhomologous end joining is a more important repair pathway (31). We expect, however, that sensitivity differences seen in yeast can, in many cases, be translated to mammalian cells with the same fundamental defect, such as a deficiency in DSB repair, regardless of the precise causative genetic defect. Finally, the examination of anticancer agents with non-DNA cellular targets in yeast can be limited by divergence of the target molecules in the two organisms. For example, the FDA-approved spindle poisons are not toxic to *Saccharomyces cerevisiae*, as these agents are not active against yeast tubulin; hence, it was not possible to include the spindle poisons in this study. Theoretically, the studies we performed in yeast could have been carried out using a human or rodent panel of matched pair cell lines. Although current limitations in generating a sizable panel (*e.g.*, 21 deletions in diploid cells) of cell lines make this approach impractical, future studies in mammalian cells will focus on cellular sensitivity to drugs in the context of specific DNA damage response alterations. As it becomes more feasible to perform similar studies using larger isogenic panels of mammalian cells, the general utility of this approach should be even more potent in defining the particular patients who should best respond to various cancer treatments.

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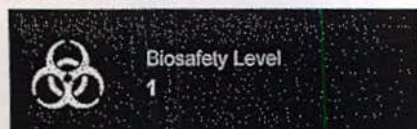
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Strain Designation: LS22  
Deposited Name: *Saccharomyces cerevisiae* Hansen, teleomorph  
Genotype: matdelta his3 ade2 can1 ura3 leu2 lys5 cyh2 ade3::GALHO rad9::TRP1



### Propagation

ATCC® Medium 1246; YEPD

Growth Conditions  
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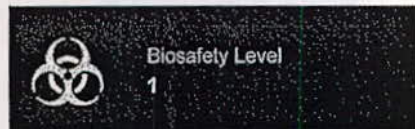
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Product Sheet

## *Saccharomyces cerevisiae* (ATCC® 90731™)

Please read this **FIRST**



### Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

### Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: *Saccharomyces cerevisiae* (ATCC® 90731™)



### Description

Strain Designation: LS24

Deposited Name: *Saccharomyces cerevisiae* Hansen, teleomorph

Genotype: matdelta his3 ade2 can1 ura3 lys5 cyh2 ade3::GALHO rad52::LEU2 rad9::TRP1

Product Description:

An ampoule containing viable cells (may include spores and mycelia) suspended in cryoprotectant.



### Propagation

The information recommended in this section is to assist users in obtaining living culture(s) for their studies. The recommendation does not imply that the conditions or procedures provided below are optimum. Experienced researchers may initiate the growth of a culture in their own way.

ATCC® Medium 1245: YEPD

### Growth Conditions

Temperature: 25.0°C

### Recommended Procedure

Frozen ampoules packed in dry ice should either be thawed immediately or stored in liquid nitrogen. If liquid nitrogen storage facilities are not available, frozen ampoules may be stored at or below -70°C for approximately one week. Do not under any circumstance store frozen ampoules at refrigerator freezer temperatures (generally -20°C). Storage of frozen material at this temperature will result in the death of the culture.

1. To thaw a frozen ampoule, place in a 2530 °C water bath, until just thawed (approximately 5 minutes). Immerse the ampoule just sufficient to cover the frozen material. Do not agitate the ampoule.
2. Immediately after thawing, wipe down ampoule with 70% ethanol and aseptically transfer 10 microliter (or any amount desired up to all) of the content onto a plate or broth with medium recommended.
3. Incubate the inoculum/strain at the temperature and conditions recommended.
4. Inspect for growth of the inoculum/strain regularly. The sign of viability is noticeable typically after 1-2 days of incubation. However, the time necessary for significant growth will vary from strain to strain.



### Notes

No special notes

Additional, updated information on this product may be available on the ATCC web site at [www.atcc.org](http://www.atcc.org).



### DNA Sequence

18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

```
TTGTTTCGCTAGACGCTCTCTTCTTATCGATAACGTTCCAATACGCTCAGTATAAAAAAGATTAGCCGC
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CGAATGCCCAAAGAAAAAGTTGCAAAGATATGAAAACCTCC
```



### References

References and other information relating to this product are available online at [www.atcc.org](http://www.atcc.org).



### Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

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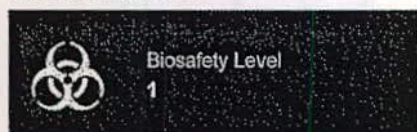
53



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**ATCC Warranty**

The viability of ATCC® products is warranted for 30 days from the date of shipment, and is valid only if the product is stored and cultured according to the information included on this product information sheet. ATCC lists the media formulation that has been found to be effective for this strain. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this strain. If an alternative medium formulation is used, the ATCC warranty for viability is no longer valid.

**Disclaimers**

This product is intended for laboratory research purposes only. It is not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at [www.atcc.org](http://www.atcc.org)

Additional information on this culture is available on the ATCC web site at [www.atcc.org](http://www.atcc.org).

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