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Re: Expediente No. 06.027.688  
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Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 21 de septiembre de 2010, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 09762 notificado por fijación en lista el 17 de junio de 2011, relacionado con el concepto técnico de fondo No. 1618, me permito informar lo siguiente:

Acompañamos copia informal de los artículos

- CZAUDERNA F et al: "Structural Variations and stabilizing modifications of synthetic s1RNAs in mammalian cells" Nucleic Acids research, Oxford University, Press, Surrey, GB. Vol. 31, no. 11, 1 June 2003 (2003-06-01), pages 2705-2716, XP002270732. ISSN: 305-1048.
- HAMADA M. et al "Effects of RNA interference in gene expression (RNA1) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of sirnas" Antisense & nucleic Acids drug Development, Mary Ann Liebert, INC, New York, US, Vol. 12, no. 5., October 2002 (2002-10), pages 301-309, XP009006637. ISSN 1087-2906.

Con estos artículos se proporciona cabal respuesta al requerimiento exigido por el señor Examinador con base en lo manifestado en el artículo 46 de la Decisión 486.

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# Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells

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## ABSTRACT

Double-stranded short interfering RNAs (siRNA) induce post-transcriptional silencing in a variety of biological systems. In the present study we have investigated the structural requirements of chemically synthesised siRNAs to mediate efficient gene silencing in mammalian cells. In contrast to studies with *Drosophila* extracts, we found that synthetic, double-stranded siRNAs without specific nucleotide overhangs are highly efficient in gene silencing. Blocking of the 5'-hydroxyl terminus of the anti-sense strand leads to a dramatic loss of RNA interference activity, whereas blocking of the 3' terminus or blocking of the termini of the sense strand had no negative effect. We further demonstrate that synthetic siRNA molecules with internal 2'-O-methyl modification, but not molecules with terminal modifications, are protected against serum-derived nucleases. Finally, we analysed different sets of siRNA molecules with various 2'-O-methyl modifications for stability and activity. We demonstrate that 2'-O-methyl modifications at specific positions in the molecule improve stability of siRNAs in serum and are tolerated without significant loss of RNA interference activity. These second generation siRNAs will be better suited for potential therapeutic application of synthetic siRNAs *in vivo*.

## INTRODUCTION

The term RNA-mediated interference (RNAi) was initially introduced by Fire and co-workers (1) to describe the observation that injection of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* can block expression of genes highly homologous in sequence to the delivered dsRNA. RNAi is evolutionarily conserved among eukaryotes and it appears to have an essential role in protecting the genome against invasion by pathogens such as viruses, which generate dsRNA molecules upon activation and replication (2). Most recently two independent studies demonstrated that the yeast RNAi machinery is required for the formation and

maintenance of heterochromatin during mitosis and meiosis, indicating additional functions for RNAi (3,4). In the past, repression of genes by long dsRNAs has been less successful in mammalian cells with the exception of embryonic cells (5,6). Use of short (<30 nt) synthetic dsRNAs allowed for sequence-specific gene silencing yet avoided the non-selective toxic effects of long dsRNAs in differentiated mammalian cells (7). This discovery triggered a much wider interest in the RNAi phenomenon since it provides a new avenue for loss of function studies in somatic cells of vertebrates. Initial studies with these small interfering RNAs (siRNAs) demonstrated that the duplex must have 2 or 3 nt overhanging 3' ends for efficient cleavage destruction of the target mRNA (8). The 3' overhangs can be generated by cleavage of long dsRNA into siRNAs by a multidomain RNase III-like enzyme, known as Dicer (9). In a subsequent step the siRNA associates with the RNAi-induced silencing complex (RISC), which is then guided to catalyse the sequence-specific degradation of the mRNA (9-11).

RNAi induced by synthetic siRNAs is transient, and in mammalian cell culture systems re-expression of the target mRNA usually occurs after a few days (12,13). Therefore an improvement in the intracellular and extracellular stability of the effector molecules will be one crucial aspect for the successful *in vivo* application of synthetic siRNAs. A variety of chemical modifications, including terminal and internal modifications (e.g. 2'-O-modification or phosphorothioate linkages), have been tested to see whether they influence RNAi inducing activity (7,8,13-15). However, these studies did not adequately assess the effect of the different modifications on sensitivity of these molecules to siRNA-degrading serum-derived nucleases.

In the present work we define the minimal structural requirements of siRNAs in mammalian cells and test a series of chemical modifications to improve the stability of siRNAs for future *in vivo* applications. To determine differences in potency, we measured the inhibition of expression of several targets, including PTEN, p110 $\beta$  and Akt1, which are all members of the phosphatidylinositol (PI) 3-kinase pathway (16,17). Surprisingly, we found no overhang dependence of the siRNA duplexes in HeLa cells, which is in contrast to observations made in the *Drosophila* system (15). We have established the minimal required size for functional siRNAs in HeLa cells and have developed functionally active siRNA

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molecules with increased nuclease resistance. Our results provide a basis for the further development of synthetic siRNA molecules with improved characteristics, including higher resistance to serum-derived endonucleases.

## MATERIALS AND METHODS

### Synthetic siRNAs and GeneBlocs

Synthetic siRNAs were purchased from BioSpring (Frankfurt, Germany). The oligoribonucleotides were resuspended in RNase-free TE to a final concentration of 50  $\mu$ M. In the case of bimolecular siRNA molecules, equal aliquots (100  $\mu$ M) were combined to a final concentration of 50  $\mu$ M. For the formation of duplexes the siRNAs were incubated at 50°C for 2 min in annealing buffer (25 mM NaCl, 5 mM MgCl<sub>2</sub>) and were cooled down to room temperature. The PTEN-specific GeneBlocs used have the schematic structure cap-nnnnnnnNNNNNNNNnnnnnn-cap, as published previously (16), where cap represents an inverted deoxy abasic modification, n stands for 2'-O-methyl ribonucleotides (A, G, U or C) and N represents phosphorothioate-linked deoxyribonucleotides (A, G, T or C).

### Cell culture and transfections

The particular HeLa cell line used in the experiments presented was a gift from M. Gossen (MDC, Berlin, Germany) and was grown in Eagle's minimum essential medium with 2 mM L-glutamine, Earle's balanced salt solution, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% fetal bovine serum (FBS). Synthetic siRNA and antisense GeneBloc transfections were carried out in 96-well or 10 cm plates (at 30–50% confluency) by using cationic lipids such as Oligofectamine (Invitrogen, Carlsbad, CA) or NC388 (Atugen, Berlin, Germany) as reported previously (16). HeLa cells were transfected by adding a pre-formed 5 $\times$  concentrated complex of siRNAs and lipid in serum-free medium to cells in complete medium. The total transfection volume was 100  $\mu$ l for cells plated in 96-wells and 10 ml for cells in 10 cm plates. The final lipid concentration was 0.8–1.2  $\mu$ g/ml depending on cell density; the siRNA concentration is indicated in each experiment.

### Antibodies and immunoblotting

Cell lysates were prepared and aliquots of the cell extracts containing equal amounts of protein were analysed by immunoblotting as described previously (16,17). The murine monoclonal anti-p110 $\alpha$  antibody has been described (18). Rabbit polyclonal anti-Akt and anti-phospho-Akt (S473) antibodies were obtained from Cell Signalling Technology. The murine monoclonal anti-PTEN antibody was from Santa Cruz Biotechnology.

### Quantitation of mRNA by Taqman analysis

The RNA of cells transfected in 96-wells was isolated and purified using the Invisorb RNA HTS 96 kit (InVitec GmbH, Berlin, Germany). Inhibition of targeted mRNA expression was detected by real time RT-PCR (Taqman) analysis using 300 nM 5' forward primer, 300 nM 3' reverse primer and 100 nM Fam-Tamra-labelled Taqman probe. The gene-specific primer sequences can be obtained on request. The

reaction was carried out in 50  $\mu$ l and assayed in an ABI PRISM 7700 Sequence detector (Applied Biosystems) according to the manufacturer's instructions under the following conditions: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

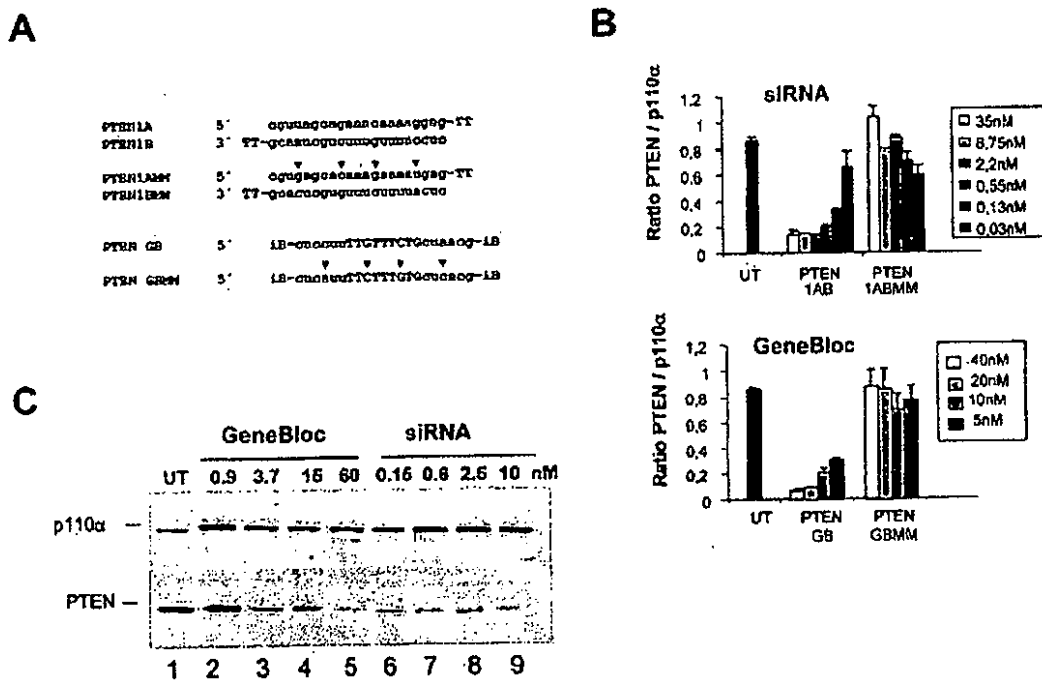
### Nuclease resistance assay

For the stability assay 5  $\mu$ l of (2.5  $\mu$ M) siRNA was incubated in 50  $\mu$ l of fetal bovine serum for 15 or 120 min at 37°C. The solution was extracted with phenol and siRNA was precipitated with ethanol and separated on 10% polyacrylamide gels followed by ethidium bromide staining. Equal amounts of siRNA before serum incubation (0 min) was extracted with phenol in parallel and loaded as an input control.

## RESULTS

### Comparison of synthetic siRNAs and antisense molecules in HeLa cells

As most published studies with siRNA in mammalian cells have involved the knock-down of ectopically or abundantly expressed genes, we first set out to demonstrate that synthetic siRNAs can be efficient tools in reducing endogenous mRNA and protein levels. For this purpose we designed and analysed siRNA molecules specific for the tumour suppressor PTEN. For comparison and as a positive knock-down control, we employed in parallel conventional antisense molecules containing the same nucleotide sequences. The antisense molecules, here called GeneBlocs, consisted of nine internal deoxyribonucleotides for activation of RNase H flanked by six 2'-O-methyl-modified ribonucleotides, whereas the siRNAs used consisted of two 21mer ribonucleotides with two deoxythymidine nucleotides at the 3' termini (Fig. 1A). The double-stranded siRNA as well as the single-stranded GeneBlocs are identical in length (21mer + terminal modifications). To control for non-specific transfection effects, we included mismatch sequences as negative controls. HeLa cells were transfected with increasing amounts of these molecules and the PTEN mRNA level was analysed 24 h later by real time PCR (Taqman). The maximal reduction of PTEN mRNA normalised to the mRNA of p110 $\alpha$ , one of the catalytic subunits of PI 3-kinase, was reached with 2.2 nM siRNA and 20 nM GeneBloc (Fig. 1B). To verify the result on the mRNA level, we analysed protein reduction induced by antisense GeneBloc and siRNA transfection in a second set of experiments. HeLa cells were transfected with increasing amounts of these molecules and cell lysates were analysed 48 h later by immunoblot analysis using PTEN-specific antibodies. p110 $\alpha$  protein level served as a loading control (Fig. 1C). Maximal PTEN protein knock-down was detected with 2.5 nM siRNA and 15 nM GeneBloc. These experiments demonstrate that siRNA molecules can efficiently reduce mRNA and protein levels of endogenous genes. Furthermore, these siRNAs can be more efficient in mediating mRNA reduction when compared to conventional antisense molecules directed against the same target sequence. The observed differences in efficacy may be due to different mechanisms of target recognition and/or degradation and may reflect the involvement of more efficient catalytic steps in the case of RNAi.



**Figure 1.** mRNA and protein knock-down of endogenous PTEN and induced by transfection of siRNA or GeneBloc (GB, antisense) in HeLa cells. (A) The sense (A) and antisense (B) strands of siRNA targeting human PTEN mRNA are shown in comparison to the corresponding GeneBloc (antisense molecules). siRNAs were synthesised with 2 nt deoxythymidine (TT) 3' overhangs. GeneBlocs representing the third generation of antisense oligonucleotides with inverted abasic (iB) and modifications (see Materials and Methods). The sequences of the respective mismatch controls (MM) containing 4 nt changes are shown. (B) Reduction of PTEN mRNA expression in siRNA- and GeneBloc-transfected HeLa cells. HeLa cells were transfected with the indicated amounts of siRNA and GeneBloc as described. After 24 h, RNA was prepared and subjected to real time RT-PCR (Taqman) analysis to determine PTEN mRNA levels relative to p110 $\alpha$  mRNA levels. Each bar represents triplicate transfections ( $\pm$  SD). (C) Inhibition of PTEN protein expression analysed by immunoblot. The cells were harvested 48 h after transfection of the indicated amounts of GeneBlocs or siRNAs. Cell lysates were separated by SDS-PAGE and analysed by immunoblotting using anti-PTEN and anti-p110 $\alpha$  antibody. The amount of p110 $\alpha$ , a catalytic subunit of PI 3-kinase, was used as a loading control. Control cell extract from untransfected HeLa cells (UT) were loaded in the left lane.

### No overhang requirements of siRNA duplexes in mammalian cells

The structural-functional relationship of siRNAs has been extensively studied biochemically using *Drosophila melanogaster* embryo lysates (7,15). Using this system it has been demonstrated that duplexes with 2 nt 3' overhangs were the most efficient triggers of mRNA degradation (8,15,19). To test whether a similar dependence is true for mammalian cells, we transfected PTEN siRNA molecules with 3' or 5' overhangs or without overhangs into HeLa cells. Surprisingly, we did not observe a more efficient knock-down of target RNA with siRNAs containing 3' overhangs when compared to blunt molecules or those with 5' overhangs (Fig. 2A). Similar results were obtained for the second target p110 $\beta$  (Fig. 2C). To verify the observed mRNA knock-down, we performed an immunoblot analysis using PTEN-specific antibodies (Fig. 2B). In these experiments the reduction of PTEN protein expression as well as the downstream phosphorylation of Akt1 kinase, a consequence of PTEN protein inhibition (16,17), was very similar with the different siRNA duplexes. We concluded from these data that 3' overhangs on synthetic siRNAs are not essential for RNAi in HeLa cells.

### Duplex length requirements of siRNA duplexes in mammalian cells

We next examined the effects of duplex length variations on siRNA activity. For these experiments we used the Akt1 kinase as a target. Duplexes of 19 nt length were highly efficient in reducing Akt1 mRNA levels independent of the nature (deoxyribonucleotides or ribonucleotides) of the 3' overhang (Fig. 3A, compare molecules 1AB, 2AB, 3AB and 4AB). This result is consistent with our observation that a 3' overhang appears not to be crucial for siRNA function in HeLa cells. Next we reduced the duplex length to 17 nt (Fig. 3A, molecule 5AB). This molecule did show a dramatically reduced silencing activity, suggesting that active siRNA duplexes must have a minimal length (~19 nt), which is in agreement with experiments assessing activity of siRNA molecules with different duplex lengths in *Drosophila* extracts (15). This minimal duplex length for active siRNA molecules might be explained mechanistically by two different requirements. First, a minimal base pairing between the antisense siRNA and the target mRNA may be obligatory or, second, incorporation into the RISC requires a minimal length of the siRNA duplex. To address this question we synthesised and

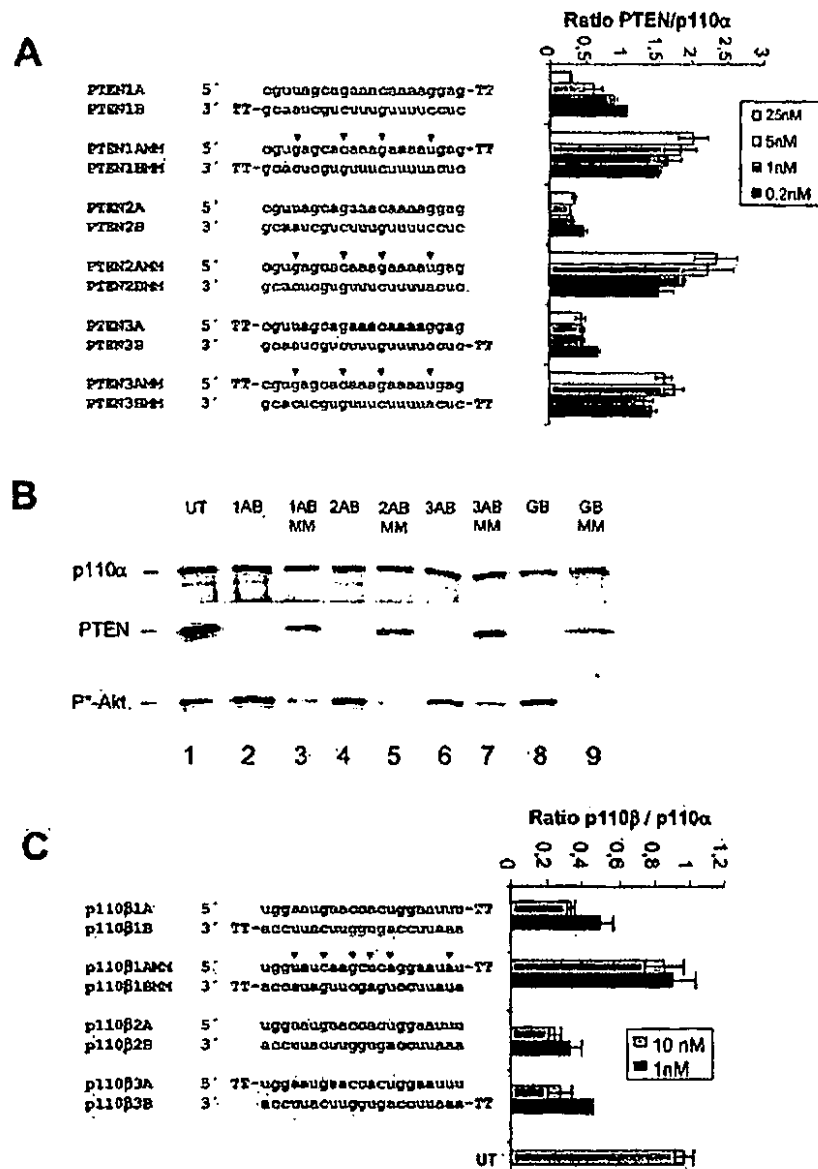
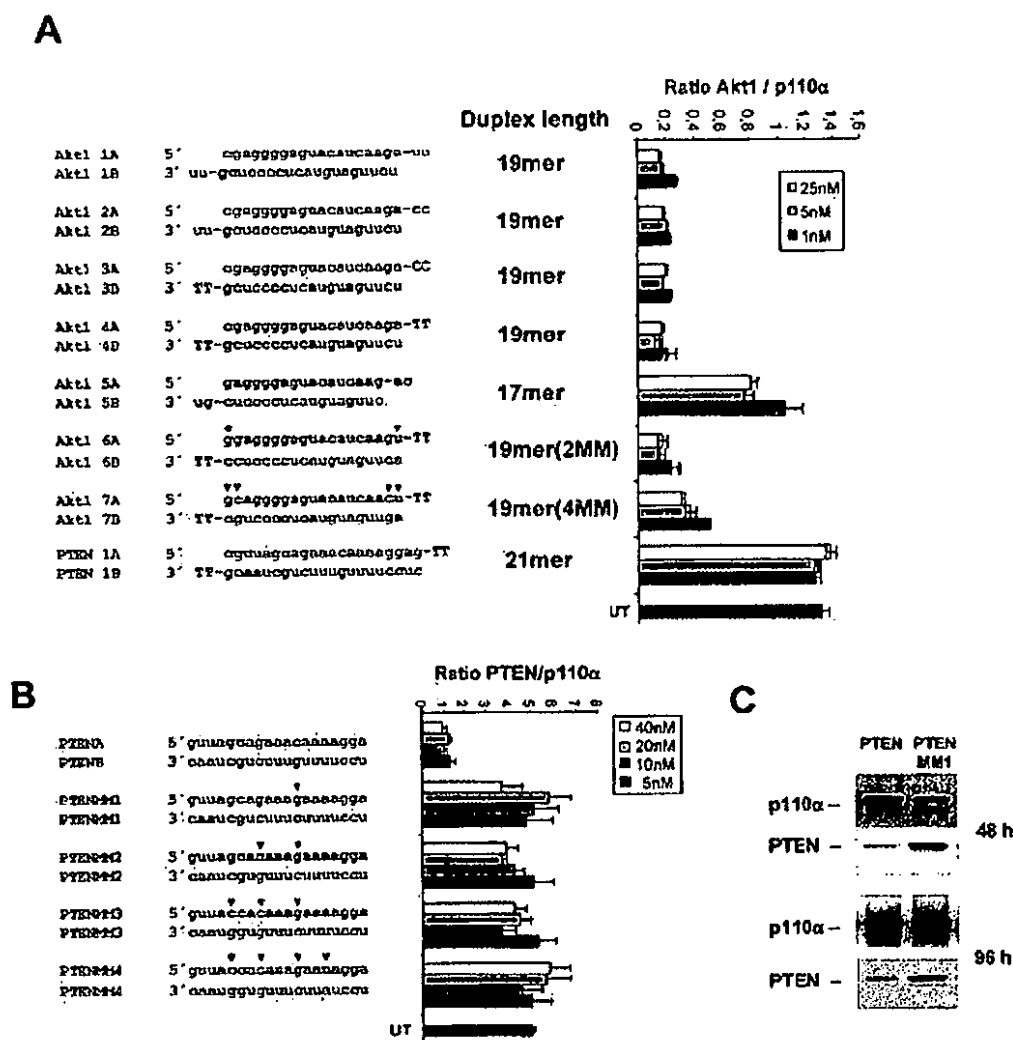


Figure 2. siRNA duplexes with a 3' or 5' overhang or without overhang (blunt) are equally potent in mediating gene silencing in HeLa cells. (A) Inhibition of PTEN mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules. The sequences and different terminal structures of the siRNAs molecules are shown on the left. Mutations in the mismatch molecules are indicated by arrowheads. Samples were analysed in parallel for the level of PTEN mRNA expression 24 h after transfection by real time RT-PCR (Taqman) analysis. PTEN mRNA levels are shown relative to the mRNA levels of p110 $\alpha$ , which served as internal reference. Each bar represents triplicate transfections ( $\pm$  SD). (B) Inhibition of PTEN protein expression by use of siRNAs with different terminal structures. The cells were harvested 48 h after transfection of the indicated siRNAs (30 nM) (lanes 2–7) or GeneBlocks (30 nM) (lanes 8 and 9). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting using anti-p110 $\alpha$ , anti-PTEN or anti-phospho-Akt antibody. The amount of p110 $\alpha$  was used as a loading control and control cell extracts from untransfected HeLa cells (UT) were loaded in lane 1. (C) Inhibition of p110 $\beta$  mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules. p110 $\beta$  mRNA levels are shown relative to the mRNA levels of p110 $\alpha$ , which served as internal reference. The ratio of p110 $\beta$ /p110 $\alpha$  mRNA of untransfected HeLa cells is shown at the bottom (UT). Each bar represents triplicate transfections ( $\pm$  SD).

transfected 19 nt long siRNA duplex molecules with one and two terminal mutations (CG and UA inversion) relative to the wild-type sequence (Fig. 3A, molecules 6AB and 7AB). Both

molecules, even the molecule with a stretch of only 15 nt base pairing to the target mRNA, were functional in reducing the Akt1 mRNA level. We concluded from this result that the



**Figure 3.** Duplex length requirement and tolerance for mutation in siRNAs in HeLa cells. (A) Inhibition of Akt1 mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules. The sequences, lengths and different terminal structures (3' deoxyribonucleotides in upper case letters) of the siRNA molecules are shown on the left. The nucleotide changes in the mismatch siRNA molecule are indicated by arrowheads. Samples were analysed in parallel for the level of Akt1 and p110 $\alpha$  mRNA expression 24 h after transfection by real time RT-PCR (Taqman) analysis. The mRNA levels of p110 $\alpha$  served as internal reference. Each bar represents triplicate transfections ( $\pm$  SD). (B) Inhibition of PTEN mRNA expression in HeLa cells transfected with the indicated amounts of siRNA molecules. The sequences of the corresponding siRNA molecules are shown on the left. (C) Inhibition of PTEN protein expression analysed by immunoblot. The cells were harvested 48 or 96 h after transfection of the indicated siRNAs (30 nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting as described previously. The positions of PTEN and p110 $\alpha$ , which was used as a loading control, are indicated on the left.

duplex length itself, but not the base pairing of the antisense siRNA with the target mRNA, seems to determine the minimal length of functional siRNAs. These data suggest that the length of the double-stranded helix is an important determinant for incorporation into the RISC complex. Since the introduced mismatches at the terminal ends of the siRNA duplexes had little effect on RNAi, we wanted to analyse the effect of mismatches located in the centre of the molecule (Fig. 3B). For these experiments we used 19 nt long blunt siRNAs directed against PTEN mRNA. The sequence changes

in one siRNA strand were compensated by complementary changes in the other strand to avoid disrupting duplex formation. A siRNA with only one point mutation in the centre of the molecule was severely compromised in its ability to reduce mRNA and protein expression levels (Fig. 3B and C). This result indicates that the RNAi machinery is highly discriminative between perfect and imperfect base pairing between target mRNA and siRNA in the centre of the duplex. The dependence on a perfect complementarity between target and siRNA has been investigated for RNAi before (13–15, 19).

### Effects on RNAi and siRNA stability of 5' and 3' terminal modifications

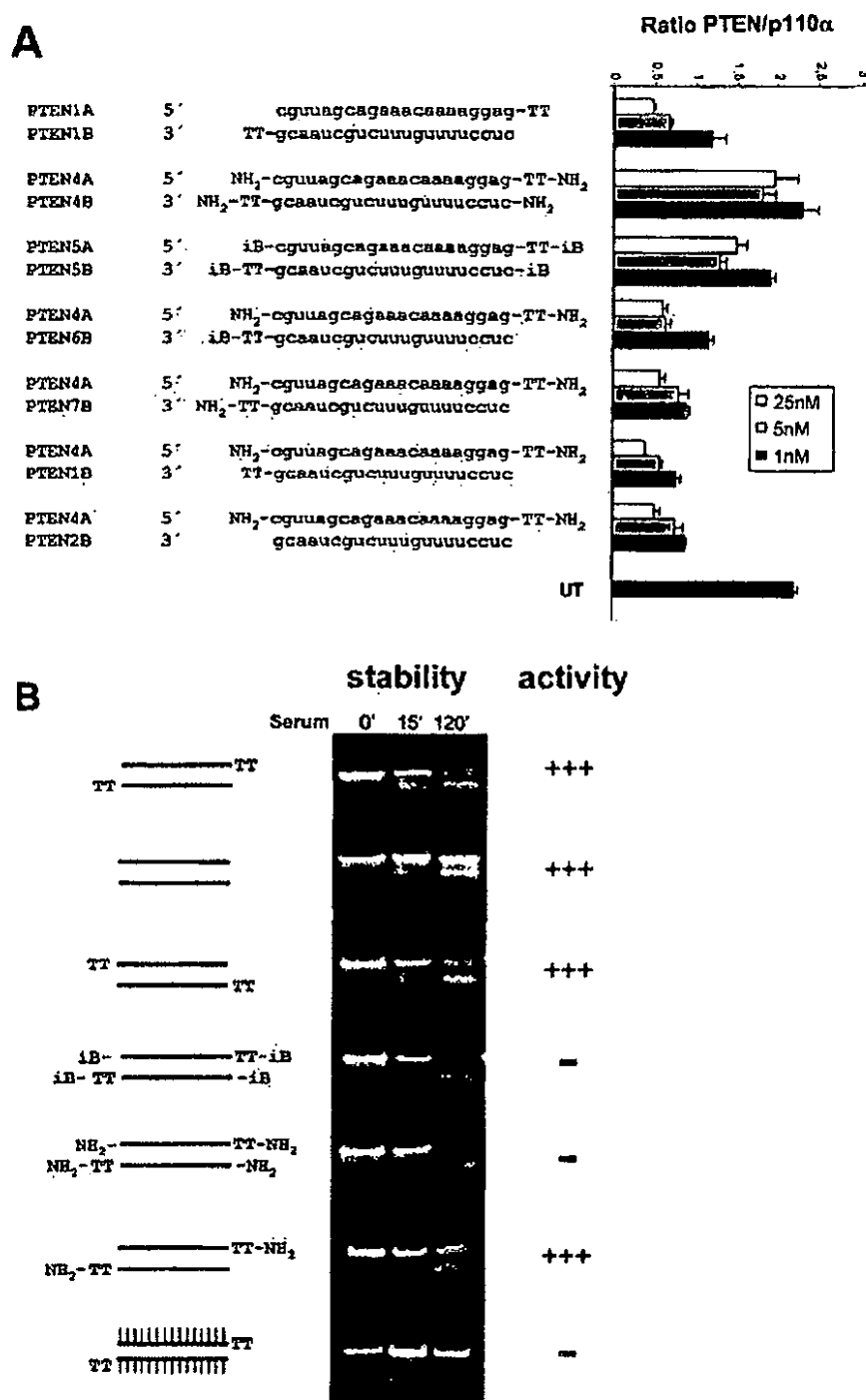
Having established the minimal structural requirements of siRNA duplexes, we wanted to test whether chemical end modifications can lead to more stabilised, active siRNA molecules. As a starting point we have used an inverted deoxy abasic (iB) modification (for details and structure see 16) and an amino end modification (NH<sub>2</sub>) (amino group with 6-carbon linker at the terminal phosphate; for details see 20). Similar end modifications have been successfully used to stabilise conventional antisense molecules and ribozymes (21) and are generally considered to protect therapeutic nucleic acids against serum-derived exonuclease activities (22). RNAi induction by siRNA duplexes with an NH<sub>2</sub> or iB modification was dramatically reduced when all four termini were modified (Fig. 4A, compare molecule 1AB with 4AB and 5A5B). However, siRNA molecules which were modified only at the terminus of the sense strand showed no reduction in gene silencing activity (Fig. 4A, molecule 2B4A). Even siRNA molecules, which had no end protection at the 5'-end of the antisense strand but on all other termini, showed no reduction in RNAi activity (Fig. 4A, molecules 6B4A and 7B4A). This result is in agreement with recently published results demonstrating the importance of the 5'-hydroxyl group of the antisense strand for RNAi (20,23). In order to test whether the terminal modifications were capable of increasing stability in serum, we incubated the different siRNA duplexes at 37°C in calf serum, followed by separation on 10% polyacrylamide gels. Surprisingly we were not able to detect any increase in stability or nuclease resistance (Fig. 4B). Neither the NH<sub>2</sub> nor the iB end modification stabilised the siRNA duplexes significantly. This result suggests that siRNA molecules are degraded predominantly by serum-derived endonucleases. To test this hypothesis we synthesised siRNA duplex containing internal 2'-O-methyl ribonucleotides at all positions, a commonly used modification to increase stability of RNA-containing molecules (24). This internally modified molecule showed extreme resistance against degradation in our serum incubation assay (Fig. 4B, siRNA molecule at the bottom), and therefore might have better pharmacodynamic properties *in vivo*.

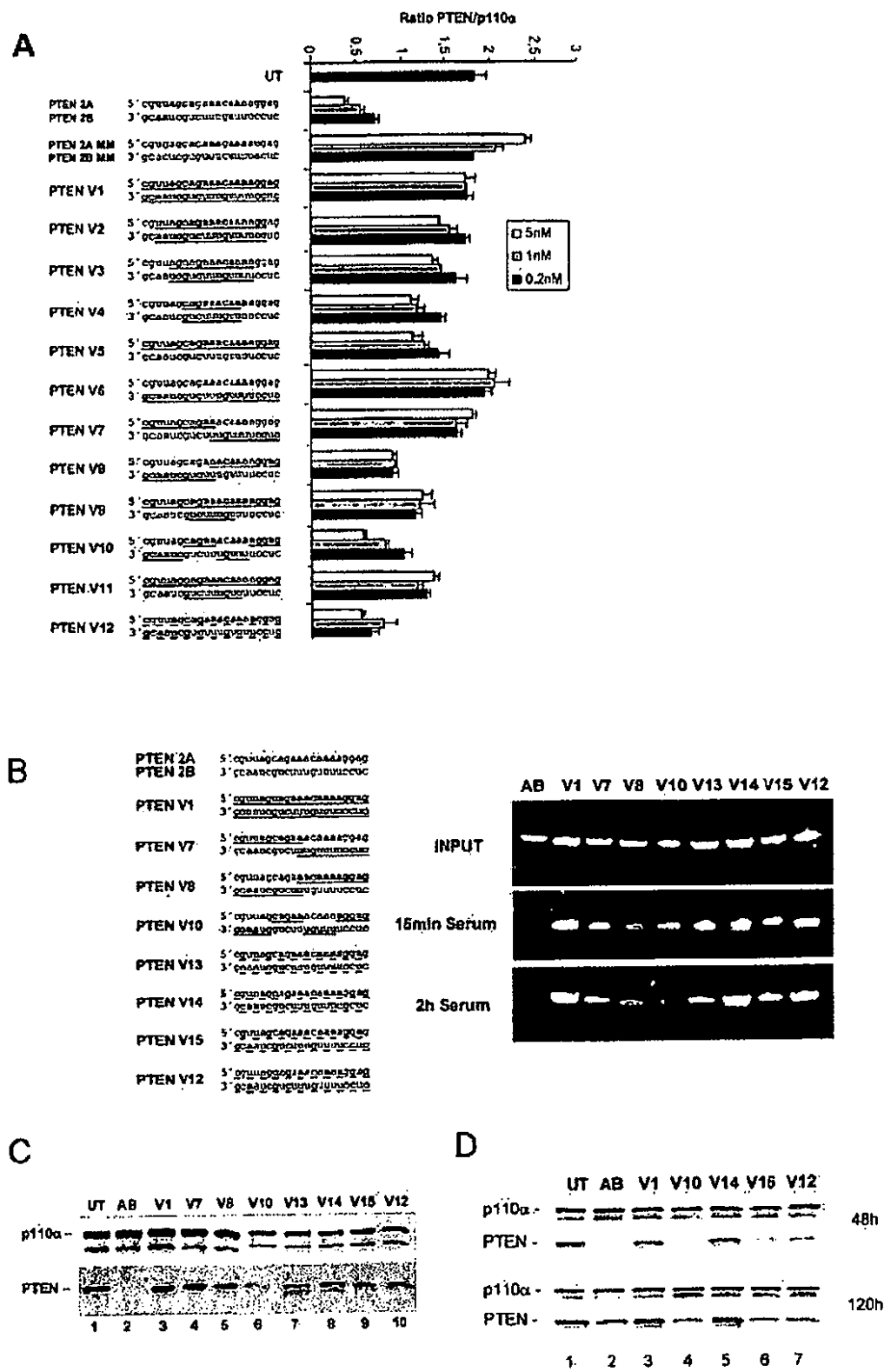
### Synthetic siRNA molecules with specific internal 2'-O-methyl modification mediate RNAi and have increased stability in serum

To identify synthetic siRNA molecules which have increased stability, but are also able to efficiently induce RNAi, we tested a series of molecules with 2'-O-methyl residues at different positions (Fig. 5). siRNA molecules with either one or both strands consisting of 2'-O-methyl residues were not able to induce RNAi in our mammalian system (Fig. 5A, molecules V2, V5 and V6). Similar results were observed with 2'-O-methyl-modified siRNAs in *Drosophila* lysates employing a luciferase-based RNAi assay (15). However, the decrease in activity was less pronounced when only parts of the strands were modified. This result is in agreement with a recent study employing siRNAs, which were not modified in the core but contained different internal modifications at the terminal nucleotides of both strands (14). Interestingly, a molecule with an unmodified antisense strand (lower) and a

completely modified sense strand was significantly more active when compared to the reversed version (Fig. 5A, compare molecules V5 and V6). This result suggests again that the antisense strand of the siRNA seems to be more critical and sensitive to modification. The most efficient molecules in reducing PTEN mRNA had only stretches of modification leaving the 5'-end unmodified or were modified on alternating positions on both strands (Fig. 5A, molecules V10 and V12). At this point it was crucial to analyse the stability and activity of partially 2'-O-methyl-modified siRNA molecules in more detail. To demonstrate nuclease resistance we incubated the different siRNA versions in serum followed by polyacrylamide gel electrophoresis. As shown before, blunt-ended siRNA molecules with unmodified ribonucleotides were very rapidly degraded whereas a complete substitution with 2'-O-methyl nucleotides mediated resistance against serum-derived nucleases (Fig. 5B, compare molecule AB with V1). siRNA molecules with partial 2'-O-methyl modification also showed an increased stability when compared to unmodified siRNAs. Especially, molecules with alternating modifications on both strands showed a significant improvement in stability (Fig. 5B, molecules V13, V14, V15 and V12). More importantly, transfection of three of these molecules into HeLa cells did result in a significant down-regulation of PTEN protein expression (Fig. 5C, lanes 6, 9 and 10). Detection of p110 $\alpha$  protein by immunoblot was used as a loading control. In this RNAi activity assay we observed an unexpected preference for molecules which were modified at every second nucleotide beginning with the most 5' terminal nucleotide of the antisense strand (molecules V15 and V12). Molecules which contained modifications beginning with the second nucleotide at the 5' end of the antisense strand were more stable but had a strongly reduced activity in gene silencing (molecules V13 and V14). This result points towards highly specific interactions between the involved enzymes and precise nucleotides in the siRNA duplex. Taken together our data demonstrate that 2'-O-methyl modifications at particularly selected positions in the siRNA duplex can increase nuclease resistance and do not necessarily abolish RNAi completely.

An increased stability of synthetic siRNAs should primarily have implications for *in vivo* application, e.g. mouse models or in therapeutic applications of siRNA. Nevertheless we wanted to analyse whether the introduced modification can also lead to an extended protein knock-down in cell culture systems. In order to demonstrate this effect, we transfected HeLa cells transiently for 6 h using our cationic lipids with different versions of PTEN-specific siRNAs. The lipid siRNA complex was then washed away and the PTEN protein knock-down was analysed 48 and 120 h later. Generally knock-down experiments without continuous transfection of siRNAs are complicated due to rapid growth of untransfected cells in this time period resulting in a very transient knock-down (13). However, here we were able to demonstrate a prolonged PTEN protein knock-down with siRNA molecules stabilised by the described 2'-O-methyl modifications. At 48 h post-transfection the unmodified siRNA (AB) shows the biggest reduction in PTEN protein levels; however, at 120 h post-transfection the reduction in PTEN protein expression is superior with the siRNAs stabilised by alternating 2'-O-methyl modifications (Fig. 5D, compare lane 2 with lanes 4,





6 and 7). At this point we do not know whether this increase in stability will also improve the pharmacodynamic properties of these molecules, and it is very likely that further modifications and improvements in delivery are necessary to enable the use of synthetic siRNAs *in vivo*.

It was puzzling that not all siRNA molecules with alternating 2'-O-methyl modifications on both strands were functional. As mentioned above, the starting nucleotide position of the alternating modification seems to be important. To test this preference in more detail, we synthesised and tested for functionality two additional series of siRNAs, one specific for the kinase Akt1, the other specific for p110 $\beta$ , one of the two catalytic subunits of PI 3-kinase. Here we used shorter, only 19 nt long, siRNA duplexes either without any or with 2'-O-methyl modification on every second nucleotide (Fig. 6). Using Akt1 as a target we observed an efficient protein knock-down as well as a dramatic reduction in phospho-Akt levels with blunt, unmodified siRNAs (Fig. 6A, right panel). From the different versions of molecules with modifications on every second nucleotide, only one efficiently mediated RNAi (Fig. 6A, molecule V5). This siRNA molecule contained an antisense strand which was modified at the most terminal 5' and 3' nucleotides. The sense strand started with the unmodified nucleotides at the most terminal positions, resulting in a structure in which the modified and unmodified ribonucleotides of both strands are facing each other. As expected, this molecule was also protected against serum-derived nucleases (Fig. 6B, molecule V5). Interestingly, a very similar 19 nt long siRNA molecule (V4) with modifications beginning at the second nucleotide of the antisense strand showed no RNAi activation in our assay. Molecule V6, in which the modified nucleotides of the antisense strand face modified nucleotides on the sense strand, was also inactive in this experiment. An identical series of 19 nt long siRNA molecules specific for p110 $\beta$  confirmed these observations (Fig. 6C). Again, the similarly modified siRNA molecule (V5) was the most active, as indicated by reducing Akt phosphorylation, which is indicative of a reduced PI 3-kinase activity due to reduced p110 $\beta$  levels (16,17). The reduced activity of the V6 molecule might be explained by reduced duplex stability since the same structure was active in the PTEN knock-down experiment with 21mer siRNAs. It is well documented that 2'-O-methyl modification on both strands facing each other will reduce the stability of nucleic acid duplexes (25). However, the difference between the activity of siRNA molecules V4 and V5 (Fig. 6B and C) in these experiments is probably not due to differences in duplex stability since the number of base pairings of modified and unmodified nucleotides is identical. This difference in activity

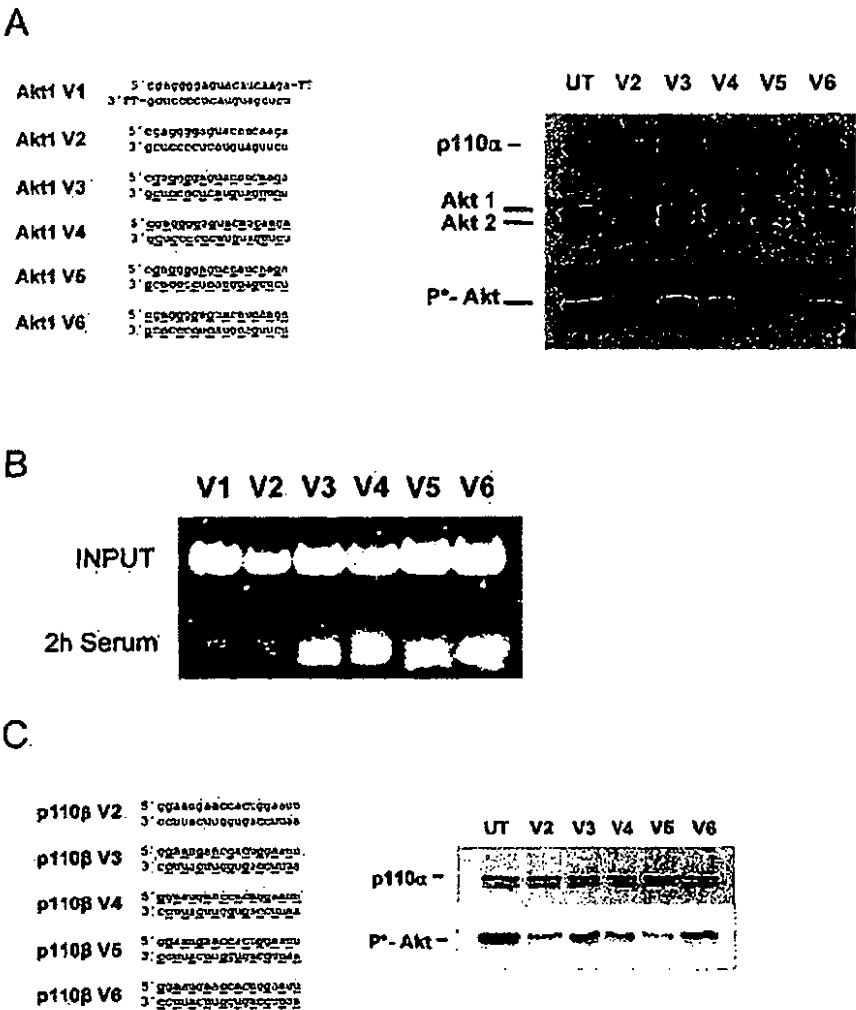
might be due to specific requirements of the interacting proteins involved in the degradation of the target mRNA. Interestingly, our data demonstrate that the most terminal nucleotides of the antisense strand can be modified at the 2'-hydroxyl group without significant loss of silencing activity.

Taken together the presented differences in the potency of different siRNA chemistries suggest that not only an alternating design principle but also the specific nucleotide positions of the internal 2'-O-alkyl modifications in the siRNA duplex are crucial to balance beneficial stabilising effects with activity reducing effects.

## DISCUSSION

siRNAs represent a new tool for functional knock-down studies in mammalian cells (7). In contrast to long dsRNA, siRNAs do not activate the dsRNA-dependent protein kinase response (8,26) and are thus better suited to phenotypic loss of function studies. In order to facilitate the usage of this new class of gene knock-down tools as potential therapeutic nucleic acids, we have analysed the minimal structural requirements of siRNA function in HeLa cells. A comprehensive study on the functional anatomy of siRNAs in *Drosophila* has been published by Elbashir and colleagues (15). In this study an *in vitro* RNAi assay with *D.melanogaster* embryo lysates was used to measure the silencing activity of different types of siRNAs. Here we have used cationic delivery vehicles to transfect different siRNA molecules of various chemistries into HeLa cells and have quantitated the gene silencing activity by Taqman analysis. Our analysis reveals some striking differences in the structural requirements for siRNAs between the *Drosophila* system and the mammalian system. For instance, in *Drosophila* extracts it seems to be obligatory to use siRNAs with 3' overhangs (15) whereas no particular overhang requirement was observed in the HeLa cell system (Fig. 2). Previously, *Drosophila* microinjection experiments with phosphorylated siRNAs, lacking 3' overhangs, showed moderate activity at high concentrations (19). At this point we cannot exclude the formation of a 3' overhang by an intracellular nuclease activity such as Dicer in HeLa cells. However, a potential processing of these 19 nt long siRNAs would lead to molecules which are most likely too short to be functional (see below). Regarding the minimal length for functional siRNA duplexes in mammalian cells, we concluded that efficient RNAi can be achieved with blunt 19 nt long siRNA duplexes in mammalian cells. There are at least three different factors which may influence the minimal length requirement: (i) a minimal length of the double-stranded siRNA duplex which has to be

Figure 5. (Opposite) siRNA molecules with distinct 2'-O-methyl ribonucleotide modifications show increased stability in serum and mediate protein knock-down in HeLa cells. (A) RNAi activity of siRNA molecules with various 2'-O-methyl ribonucleotide modifications. Inhibition of PTEN mRNA expression in HeLa cells transfected with the indicated amounts of modified siRNA molecules. 2'-O-methyl ribonucleotide modifications are underlined and indicated by bold letters in the sequence on the left. Samples were analysed in parallel for the level of PTEN mRNA as described before. (B) Polyacrylamide gel electrophoresis of modified and unmodified siRNA molecules after incubation in serum. The PTEN siRNA sequence and the position of the 2'-O-methyl ribonucleotide modifications (underlined and bold letters) are shown on the left. (C) Inhibition of PTEN protein expression analysed by immunoblot. The cells were harvested 48 h after transfection of the indicated siRNAs (30 nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting as described previously. The positions of PTEN and p110 $\alpha$ , which was used as a loading control, are indicated at left. (D) siRNA molecules with distinct 2'-O-methyl ribonucleotide modifications mediate a prolonged protein knock-down. Inhibition of PTEN protein expression analysed by immunoblot. The cells were harvested 48 or 120 h after transfection of the indicated siRNAs (30 nM). Cell extracts were separated by SDS-PAGE and analysed by immunoblotting as described previously. The positions of PTEN and p110 $\alpha$ , which was used as a loading control, are indicated on the left.



**Figure 6.** siRNA molecules with distinct 2'-O-methyl ribonucleotide modifications specific for Akt1 and p110β mRNA show increased stability in serum and mediate protein knock-down in HeLa cells. (A) The Akt1-specific siRNA sequence, the position of the 2'-O-methyl modifications (underlined and bold letters) and the integrity of the indicated siRNA molecules after incubation in serum are shown. (B) Inhibition of Akt1 protein expression as well as Akt phosphorylation analysed by immunoblot after transfection of the indicated siRNAs (30 nM). (C) Inhibition of the phosphorylation of the downstream kinase Akt1 by 2'-O-methyl-containing p110β-specific siRNAs. The positions of the phosphorylated Akt1 and p110α, which was used as a loading control, are indicated on the left.

recognised by the RISC complex or other protein complexes involved; (ii) a minimal stretch of homologous base pairing between the target mRNA and the antisense strand of the siRNA duplex to ensure specific recognition; (iii) a minimal siRNA length to guarantee stable duplex formation. It seems very likely that factors (ii) and (iii) will be dependent on the particular sequence composition (e.g. GC content). However, from our data we conclude that the duplex length itself represents one critical factor below a duplex length of 19 bp since a 17 bp siRNA duplex was inactive, even in the presence of additional target-specific overhangs (Fig. 3A). A 19 bp long siRNA duplex with up to four mismatches (with respect to the target) at the terminal positions still mediated efficient gene

silencing, suggesting that the base pairing with the target mRNA is not the most decisive factor for determining the minimal length. The result that 19 nt long siRNA with only a 15 nt target-specific stretch (two mismatches on either side) was able to mediate a significant knock-down may have general implications for target specificity and off-target effects of siRNAs. In contrast, a single nucleotide inversion in the centre of the siRNA duplex resulted in a complete loss of gene silencing, suggesting that a longer uninterrupted stretch of base pairing with the target mRNA is necessary. Several reports published on the issue of sequence specificity of target recognition have shown that single mutations within the centre of a siRNA duplex are more discriminating than mutations

located at the 5'- and 3'-ends (13–15). More recently it has been suggested that even single-stranded antisense siRNA molecules, preferably phosphorylated at the 5'-hydroxyl, can be sufficient to mediate RNAi *in vitro* as well as in cell culture systems (14,27,28). We have attempted to repeat these studies in HeLa cells using our siRNA transfection protocol followed by Taqman or immunoblot analysis. So far we have not been able to demonstrate significant knock-down with single-stranded siRNA molecules either with or without phosphorylated 5' termini (data not shown). Currently we do not know whether this discrepancy may be due to a difference in cell lines or transfection protocols used in these experiments.

One major drawback of the usage of siRNAs is its transient nature owing to the limited stability and availability of the synthetic RNA-based molecules. Using conventional transient transfection protocols gene expression is inhibited for a maximum period of 2–4 days (14,29). Therefore, we reasoned that it will be beneficial to develop more stable siRNA molecules, especially for potential *in vivo* application of siRNAs, where sustained delivery will be one major obstacle. Our data on siRNA stability in serum suggest that unmodified siRNAs are for the most part substrates for endonucleases and to a lesser extent substrates for exonucleases. Consequently, chemical modifications at the termini of the RNA strands which are known to abolish exonuclease activity do not significantly improve stability of siRNAs in serum (Fig. 4B). Initial experiments with internal 2'-O-alkyl modifications have demonstrated that complete substitution of all nucleotides abolished RNAi activity completely (15). Partial substitution with up to a total of six modifications in the form of either methylation or thiolation of the backbone has recently been reported to reduce silencing activity only marginally in HaCat cells (14). According to our data it is essential to balance the effects of 2'-O-alkyl modifications on stability against the negative effects on activity, which makes it important to be aware of various design principles. First, modifications at the 5'-hydroxyl group of the antisense strand should be avoided since end modifications (-NH<sub>2</sub> or inverted abasic) at this position abolish RNAi inducing activity (Fig. 4A). This result is consistent with a proposed *in vivo* phosphorylation step of the 5' terminus necessary for siRNA activity (23). Second, alternating 2'-O-alkyl modification but not blocks of 2'-O-alkyl modification lead to significant resistance against serum-derived nucleases without significant loss of RNAi activity (Fig. 5). Third, 2'-O-methyl-modified siRNAs are more active when the incorporated modifications are on every second nucleotide, facing a non-modified nucleotide. This may indicate critical changes in duplex stability since it is known that 2'-O-methyl modifications stabilise base pairing with non-modified nucleotides but can destabilise the duplex when incorporated on both strands (25). A fourth feature arises from the observation that siRNAs in which the alternating modifications at the 2'-hydroxyl group start with the most terminal nucleotides of the antisense strand are more efficient than siRNAs which begin this modification at the second nucleotide of the antisense strand (Fig. 6, compare molecules V3 and V5). Taken together the results suggest that the specific structural requirements for RNAi inducing activity are at least partially distinct from the substrate requirements of serum-derived endonucleases. In

summary, we conclude that specific internal modifications at the 2'-hydroxyl group are suitable to improve the stability of synthetic siRNAs without significant loss of activity, and thus may also help to advance the use of siRNAs in *in vivo* experiments.

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## Effects on RNA Interference in Gene Expression (RNAi) in Cultured Mammalian Cells of Mismatches and the Introduction of Chemical Modifications at the 3'-Ends of siRNAs

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### ABSTRACT

The highly specific posttranscriptional silencing of gene expression induced by double-stranded RNA (dsRNA) is known as RNA interference (RNAi) and has been demonstrated in plants, nematodes, *Drosophila*, and protozoa, as well as in mammalian cells. The suppression of expression of specific genes by chemically synthesized 21-nucleotide (21-nt) RNA duplexes has been achieved in various lines of mammalian cells, and this technique might prove to be a valuable tool in efforts to analyze biologic functions of genes in mammalian cells. In order to investigate the utility of potential modifications that can be introduced into small interfering RNAs (siRNAs) and also to study their functional anatomy, we synthesized different types of siRNA targeted to mRNA of Jun dimerization protein 2 (JDP2). Our detailed analysis demonstrated that siRNAs with only one mismatch, relative to the target, on the antisense strand had reduced RNAi effect, whereas the corresponding mutation on the sense strand did not interfere with the RNAi. Moreover, one 2-hydroxyethylphosphate (hp) substitution at the 3'-end of the antisense strand but not of the sense strand also prevented RNAi, whereas a related modification at the 3'-end of either strand, using 2'-O,4'-C-ethylene thymidine (eT), which is a component of ethylene-bridge nucleic acids (ENA), completely abolished RNAi. These results support the hypothesis that the two strands have different functions in RNAi in cultured mammalian cells and indicate that their chemical modification of siRNAs at the 3'-end of the sense strand exclusively is possible, without loss of RNAi activity, depending on the type of modification. Because modification at the 3'-end of the antisense strand by hp or eT abolished the RNAi effect, it appears possible that the 3'-end is recognized by the RNA-induced silencing complex (RISC).

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## INTRODUCTION

**R**NA INTERFERENCE (RNAi) is a highly specific mechanism of posttranscriptional silencing of gene expression that is triggered by double-stranded RNA (dsRNA). Posttranscriptional gene silencing (PTGS) by dsRNA has been demonstrated in plants, nematodes, *Drosophila*, protozoa, and also in mammalian cells (Akashi et al., 2002; Caplen et al., 2001; Elbashir et al., 2001a; Fire et al., 1998; Fire, 1999; Hammond et al., 2001b; Howard et al., 1999; Matzke et al., 2001; Miskuita and Paterson, 1999; Miyagishi and Taira, 2002; Napoli et al., 1990; Sharp, 2001; van der Krol et al., 1990; Zamore, 2001). It is likely that such responses to dsRNA have been conserved in various eukaryotes to prevent the transposition and replication of foreign nucleic acids (such as transposons or viruses) (Baulcombe, 1999; Grant, 1999; Plasterk, 2002; Ratcliff et al., 1999; Stark et al., 1998). The biologic pathway of RNAi remains to be fully clarified, but recent genetic and biochemical analysis using *Caenorhabditis elegans* and extracts prepared from *Drosophila melanogaster* have resulted in putative models for RNAi at the molecular level.

It appears that dsRNA is cleaved first into RNAs of ~21–25 nucleotides (nt) in length, which are referred to as small interfering RNAs (siRNAs). Dicer, a member of the ribonuclease III family (Bernstein et al., 2001), catalyzes these endonucleolytic cleavages. The siRNAs form a multicomponent nuclease complex known as an RNA-induced silencing complex (RISC) (Hammond et al., 2001a), and the siRNAs function as guide RNAs, directing the complex to the target mRNA (Nykanen et al., 2001). The targeted mRNA is recognized by protein factors within the RISC and cleaved by the nuclease within RISC (Hammond et al., 2000).

The phenomenon of RNAi has been exploited as a powerful tool in reverse-genetic studies of *C. elegans* (Fraser et al., 2000; Gonczy et al., 2000). Until recently, the application of RNAi to differentiated mammalian cells was problematic because of the activation by long dsRNAs (>30 base pairs [bp]) of two enzymes that are related to interferon (IFN) responses: (1) 2',5'-oligoadenylate synthetase (2',5'-OAS), which increases the concentration of 2',5'-oligoadenylate that activates RNase L, a sequence-nonspecific RNase (Silverman, 1997), and (2) protein kinase PKR, which, in its active form, phosphorylates initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), shutting down protein synthesis (Clemens and Elia, 1997). A specific RNAi effect has been achieved, however, using a duplex of 21-nt RNAs that form a 19-bp region, which is small enough to bypass IFN responses in differentiated cultured cells (Elbashir et al., 2001b). Thus, RNAi has the potential to become a valuable tool for analysis of the biologic functions of genes in mammalian cells.

We describe here an assessment of the activity of siRNAs targeted to a member of the activated protein 1 (AP-1) family of transcription factors, namely, Jun dimerization protein 2 (JDP2) (Pitt et al., 2001), as well as of the chemical and sequence requirements of sense and antisense strands of siRNA duplexes for RNAi in lines of cultured mouse cells. We found that a single mismatch (relative to the target) on the antisense strand but not on the sense strand reduced the RNAi effect by siRNAs. Our data are consistent with previously reported results obtained with extracts of *D. melanogaster* (Elbashir et al., 2001c). Furthermore, a single 2-hydroxyethylphosphate substitution at the 3'-end of the antisense strand but not of the sense strand also prevented RNAi. These results support the hypothesis that the two trigger strands have different function in RNAi.

## MATERIALS AND METHODS

### Construction of reporter plasmid

A plasmid encoding JDP2 and luciferase (JDP2-luc-expressing plasmid) for luciferase assays was constructed as follows. A fragment, which encoded JDP2, was amplified by PCR with pCR3.1-jdp-2 (unpublished observations) as template and primers 5'-AAA AAA AAG CTT ATG ATG CCT GGG CAG ATC CCA GAC C-3' and 5'-AAA AAA AAG CTT CCT TCT TGT CCA GCT OCT CCA GCA GT-3' (unpublished observations). After digestion of products of PCR with *Hind*III, fragments were ligated into the *Hind*III site of pGL3 (Promega, Madison, WI), which encoded firefly luciferase.

### Preparation of RNA

A modified nucleoside 3'-O-phosphoramidite unit, 5'-O-(4,4'-dimethoxytrityl)-2'-O,4'-C-ethylene thymidine 3'-O-(2-cyanoethyl) phosphoramidite and ethylene glycol-attached CPG were prepared as reported previously (Koizumi et al., 1997; Morita et al., 2002). RNA oligonucleotides were synthesized by an automated synthesizer (model 394, Applied Biosystems, Foster City, CA). Newly synthesized RNAs were deprotected and purified by electrophoresis on a denaturing polyacrylamide gel (Scaringe et al., 1990). After elution from the gel, the siRNAs were desalted by passage through a NAP-10 column (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with elution in RNase-free water and dried under a vacuum. For annealing of siRNAs, 20  $\mu$ M single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) at 95°C for 1 minute, cooled to 70°C for 1 minute, and then slowly cooled to 37°C over the course of 60 minutes.

### Culture and transfection of cells

Mouse RAW264.7 and NIH3T3 cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air in DMEM supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin (all from Life Technologies, Inc., Rockville, MD). Transfection of siRNAs for gene targeting was performed using Lipofectamine™ 2000 (Life Technologies, Inc.) with 20.8 nM siRNA duplex, sense or antisense RNA per well. Stably transformed RAW264.7 cells were transfected twice with an interval of 15 hours.

### Assays of luciferase activity

Twenty-four hours before transfection, NIH3T3 cells ( $2 \times 10^5$ /ml) were transferred to 12-well plates (1 ml per well). They were transfected, using Lipofectamine 2000, according to instructions for transfection of adherent cell lines. For luciferase assays, cells in individual wells were transfected with 200 ng JDP2-luc-expressing plasmid and the indicated amounts of siRNA duplex, sense or antisense RNA, together with 20 ng of a second reporter plasmid, pRL-SV40 (Promega), which encoded luciferase from *Renilla*. After 24 hours, cell extracts were prepared in Passive Lysis Buffer (Promega). The activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega) according to the manufacturer's protocol. Results were confirmed by at least three independent transfection experiments with two cultures each and are expressed as the average from six experiments as mean  $\pm$  SD.

### Antibodies

Actin-specific antibodies were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), and hemagglutinin-specific (HA-specific) antibodies were obtained from Roche Diagnostics K.K. (Tokyo, Japan). For Western blotting, horseradish peroxidase (HRP)-linked antibodies against rabbit IgG (Amersham Pharmacia Biotech) and against rat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as second antibodies.

### Western blot analysis

Transfected cells, grown in 12-well plates, were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS in PBS) supplemented with complete protease inhibitor cocktail (Roche Diagnostics K. K.) to yield whole cell extracts. Concentrations of protein were determined with a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Individual samples containing equal amounts of protein

(10–15 µg) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10%–20% gradient gel, and the proteins on the gel were subsequently electrotransferred to a polyvinylidene difluoride membrane. After the membrane had been blocked with Block Ace (Dainippon Pharmaceuticals Co.), the membrane was incubated first with the indicated antibodies and then with peroxidase-conjugated second antibodies. Bands of immunoreactive protein were detected with an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

## RESULTS

### Design of siRNA targeted to gene for JDP2 and estimation of effects of siRNA on gene expression

In this study, we selected the gene for JDP2 as the target gene for assessment of the effects of siRNA in cultured mammalian cells. The targeted sequence in the gene for JDP2 (DDBJ/GenBank accession number AB077438, version GI: 2833639) was arbitrarily chosen as the sequence from position 300 to position 320 relative to the codon for initiation of translation. To ascertain whether targeting of siRNA was able to induce JDP2-specific gene silencing, we constructed a plasmid that expressed a JDP2-luciferase fusion protein (JDP2-luc) so that we could monitor the effect of siRNA in terms of luciferase activity. We cotransfected cells with siRNA and the JDP2-luc-expressing plasmid. In controls, sense or antisense RNA strands were used for transfections instead of an siRNA duplex.

We found that double-stranded siRNA suppressed expression of the gene for JDP2-luc by about 50%, as compared with the sense-strand control, whereas the antisense-strand control reduced expression by only ~20% (Fig. 1). These results indicated that the siRNA silenced expression of the gene for JDP2-luc specifically. In general, in attempting to exploit antisense technology, it has been very difficult to select accessible target sites (Kawasaki et al., 2002; Krupp and Guir, 2000; Patzel and Sczakiel, 1998; Warashina et al., 2001). In fact, when we targeted the same site in the gene for JDP2 with a tRNA-driven hammerhead ribozyme known to be functional when accessible sites are targeted (Kuwabara et al., 1998a,b, 2001; Tanabe et al., 2000), we were unable to detect any meaningful suppression (data not shown). However, our first trial with an arbitrarily chosen target site resulted in significant (~50%) suppression by siRNA, demonstrating the power of this technology. We have not yet searched for more accessible sites, and it is likely that with more trials, higher levels of suppression should be possible.

We next used siRNAs to transfect cells that stably expressed a gene for HA-tagged JDP2 (JDP2-HA) to esti-

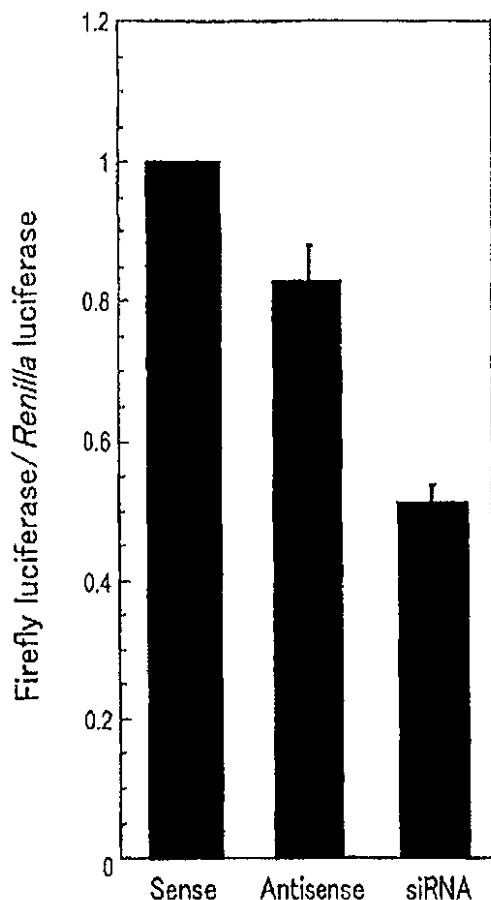


FIG. 1. RNA interference by siRNA duplexes targeted against a gene for JDP2 in NIH3T3 cells. NIH3T3 cells were cotransfected with 200 ng of a JDP2-luc-expressing plasmid and 136 ng of siRNA duplex or the sense or antisense RNA strand, together with 20 ng of reporter plasmid pRL-SV40, which encoded *Renilla* luciferase for reference and normalization. Columns and bars show mean results and SD, respectively.

mate the effects of siRNA on expression of a stable transgene. We had to use this strategy because we were unable to detect endogenous JDP2 with the available antibodies, most probably because of the low level of expression of this protein. Measuring the level of the chimeric JDP2-HA protein, we were able to estimate the effects of siRNA on the level of endogenous JDP2. We produced a line of RAW264.7 cells that stably expressed JDP2-HA by transfecting RAW264.7 cells with a plasmid that encoded JDP2-HA. These newly prepared cells were transfected with siRNA. Compared with cells transfected with only the sense or the antisense strand, siRNA-transfected

cells expressed significantly lower levels of JDP2-HA (Fig. 2). In fact, the specificity and extent of suppression of the overexpressed and the endogenous JDP2 were significantly greater than we had anticipated from the model study for which results are shown in Figure 1. Thus, the siRNA used in this study should be useful for future functional analysis of the biologic roles of endogenous JDP2.

#### Effects of 3'-end modifications on activity of siRNA

We tested two types of modification of the phosphate-sugar backbone for their effects on gene suppression by siRNAs. Replacement of single or double overhanging 3'-nucleotides by 2'-deoxyribonucleotides does not prevent RNAi (Elbashir et al., 2001a,b). This observation prompted us to examine the effects of replacement of such overhanging nucleotides with novel moieties, such as 2'-O,4'-C-ethylene thymidine (eT) and 2-hydroxyethyl phosphate (hp) (Fig. 3A,B). Such modifications

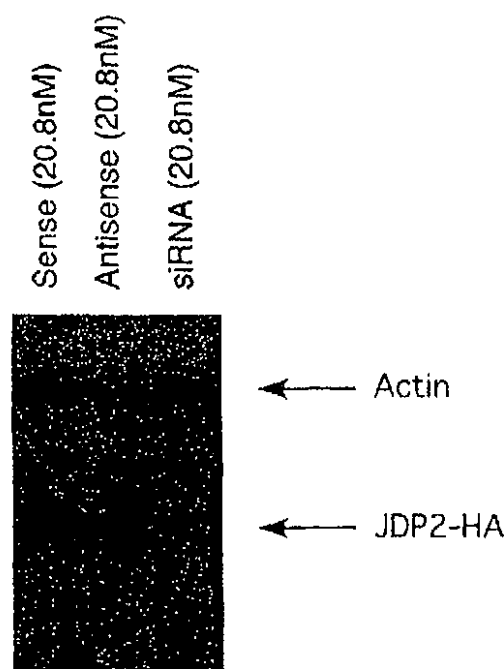
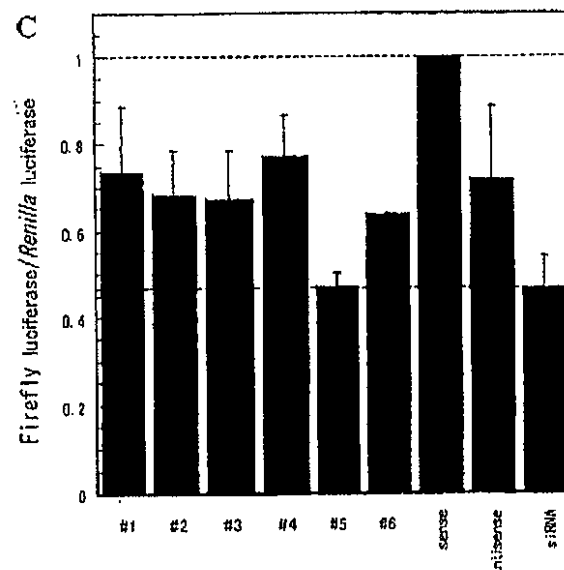
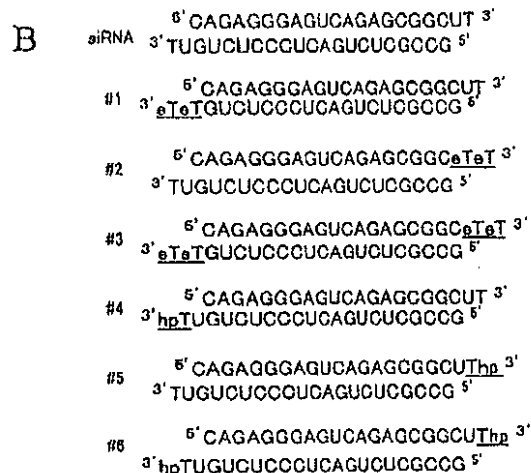
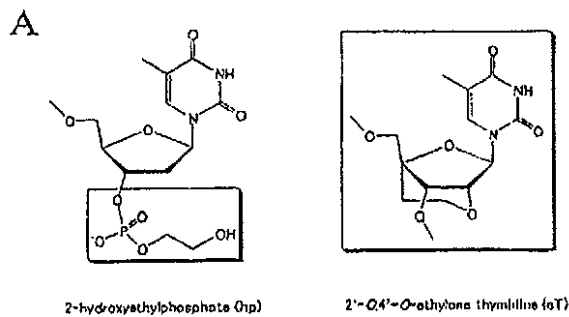


FIG. 2. The effect of transfection with an siRNA duplex targeted to the gene for JDP2 cells that stably expressed a plasmid that encoded JDP2-HA. RAW264.7 cells that expressed stably JDP2-HA were transfected with siRNA targeted against the gene for JDP2. Western blotting was performed to confirm the specificity of siRNA, with actin-specific antibodies (1/500) and HA-specific antibodies (1/500). Protein (15  $\mu$ g) was loaded in each lane.

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are favorable in the case of DNA (Koizumi et al., 1997; Morita et al., 2002), and they might also be useful for elucidation of relationships between the structure and the activity of modified siRNAs.

Replacement of 2-nt 3' overhangs with eT, even when



the modification involved only the sense strand, abolished RNAi. After replacement of a 1-nt 3' overhang on the sense strand with hp (No. 5), the activity of RNAi was retained, but similar modification of the antisense strand (No. 4) diminished the RNAi effect, reaching to the level of antisense RNA effect (Fig. 3C). Therefore, the nature of the chemical modification at the 3'-end of the sense strand determines if the RNAi effect is retained (compare No. 5 and siRNA in Fig. 3C). However, we did not detect an enhanced RNAi effect after introduction of the exonuclease-resistant hp siRNA (No. 5) when we examined the effect 4 days after transfection (data not shown) rather than 24 hours of transfection (Fig. 3C).

#### Effects of mismatches on activity of siRNA

To assess the requirement for sequence specificity in target recognition by sense and antisense strands, we introduced sequence mismatches into the paired segments of siRNA duplexes and also into individual sense and antisense strands (Fig. 4A). The effects of these changes were quantified by luciferase assays in NIH3T3 cells. As shown recently by others (Elbashir et al., 2001c), one or several mismatches (with reference to the target sequence) introduced into the paired segments of siRNA duplexes (Nos. 11–14 in Fig. 4A) and one or several mismatches within antisense strands (Nos. 15–18 in Fig. 4) significantly reduced the RNAi effects. Limited RNAi effects by these mismatches were not completely in accord with the findings of more recent studies, possibly because of the different conditions used (Holen et al., 2002).

In contrast, limited mismatches in the middle region of the sense strand in the siRNA duplex did not reduce the extent of RNAi (Fig. 4B, left). Specifically, compared with the parent siRNA, Nos. 7 and 8 siRNAs, with one and two mismatches, had a similar RNAi effect. Introduction of larger numbers of mismatches gradually reduced the extent of RNAi, as in the case, for example, of Nos. 9 and 10, with three and five mismatches, respec-

**FIG. 3.** Effects of chemical modifications on the activity of siRNA. (A) Schematic RNA backbone and the modifications tested in this study. (B) Sequences of the chemically modified siRNAs. (C) Effects of chemical modification on the activity of siRNA in NIH3T3 cells. Cells were cotransfected with 200 ng of the JDP2-luc-expressing plasmid and 214 ng of siRNA duplex (25 nM) or the sense or antisense RNA strand, together with 20 ng of the reporter plasmid pRL-SV40 that encoded *Renilla* luciferase for reference and normalization. The values were normalized to *Renilla* luciferase activity. The results were confirmed by at least three independent transfection experiments with two cultures each. Columns and bars show mean results and SD, respectively.

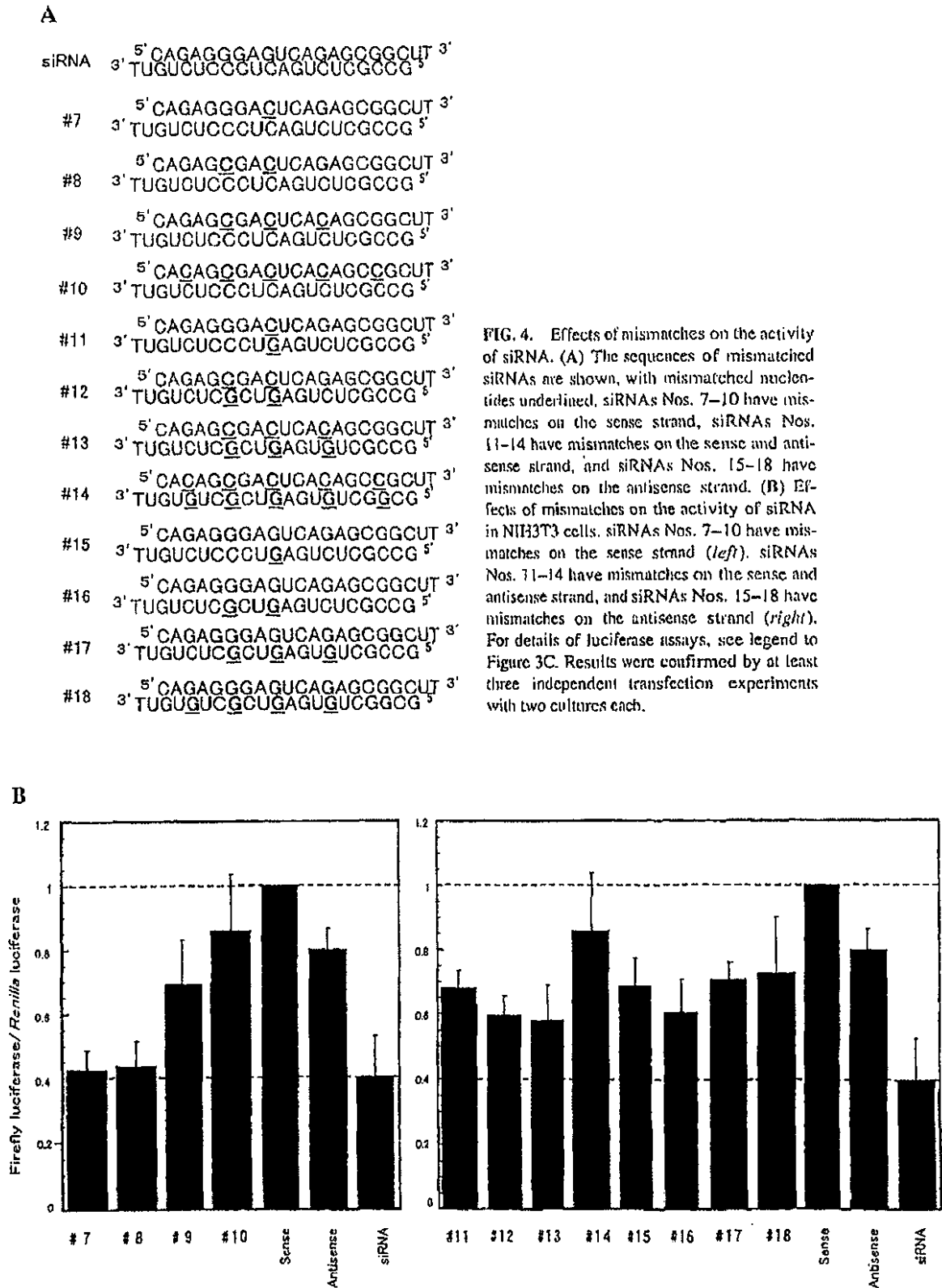


FIG. 4. Effects of mismatches on the activity of siRNA. (A) The sequences of mismatched siRNAs are shown, with mismatched nucleotides underlined, siRNAs Nos. 7–10 have mismatches on the sense strand, siRNAs Nos. 11–14 have mismatches on the sense and antisense strand, and siRNAs Nos. 15–18 have mismatches on the antisense strand. (B) Effects of mismatches on the activity of siRNA in NIH3T3 cells. siRNAs Nos. 7–10 have mismatches on the sense strand (left), siRNAs Nos. 11–14 have mismatches on the sense and antisense strand, and siRNAs Nos. 15–18 have mismatches on the antisense strand (right). For details of luciferase assays, see legend to Figure 3C. Results were confirmed by at least three independent transfection experiments with two cultures each.

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tively. We obtained very similar results with RAW264.7 cells (data not shown).

## DISCUSSION

siRNAs repress the expression of target genes and have considerable potential as tools for analysis of gene function and clinical therapy (Elbashir et al., 2001b), particularly when they can be expressed by vectors (Brummelkamp et al., 2002; Lee et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Tuschl, 2002; Yu et al., 2002). In this study, we examined the RNAi effect of a chemically synthesized siRNA that was targeted to a gene for JDP2, and we also examined the effects on siRNA activity of chemical modification and of mismatches on the sense and antisense RNA strands in cultured mammalian cells. We confirmed some previous observations made in *C. elegans*, *Drosophila*, and extracts of *D. melanogaster* (Boutla et al., 2001; Elbashir et al., 2001c; Parrish et al., 2000) using cultured mammalian cells and obtained new information about the effects of mismatches and of chemical modification.

Our results demonstrate that RNAi is unaffected by limited mismatches within the sense strand (Fig. 4, Nos. 7 and 8), but chemical modification of the 3'-end of the antisense strand dramatically reduced the extent of RNAi (Fig. 3B,C, Nos. 1 and 4). However, modification at the 3'-end of the sense strand is tolerated depending on the nature of the chemical modification (Fig. 3B,C, Nos. 2 and 5). These modifications might affect the incorporation of siRNA into the RISC and probably do not affect target recognition by the antisense strand, as the sequence of 3' overhangs does not appear to influence the binding or recognition step (Elbashir et al., 2001c). Alternatively, it remains a possibility that 5' phosphorylation of siRNA duplexes, which was shown to be required for RNAi effect, might be reduced by 3' modification (Nykanen et al., 2001). Experiments using a series of siRNAs with mismatched sense strands suggested that the incorporation of siRNA into the RISC can tolerate limited structural changes within the sense strand, such as mismatches of up to 2 nt and chemical modification of the 3'-end by hp.

Our transfection experiments demonstrated the effective and specific reduction of expression of a transgene that encoded JDP2 in a line of mouse macrophages, RAW264.7. Thus, our siRNA should be a useful tool in attempting to characterize the functional significance of endogenously expressed JDP2. To our knowledge, this is the first report that demonstrates by chemical modifications that the sense and antisense strands have different functions in RNAi in cultured mammalian cells.

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