ing synthetic phosphorothioated antisense ODNs, synthetic siRNAs, and synthetic ribozymes. In this system, we measured firefly and Renilla luciferase activities in the same sample, and suppressive effects on the expression of firefly luciferase were standardized by reference to the expression of Renilla luciferase, which allowed accurate evaluation of the suppressive effects. We selected six target sites within the coding region of the firefly gene for luciferase. Each target sequence included the NUX triplet motif at the center of the sequence, as this is the motif at which cleavage by the hammerhead ribozyme occurs. N can be any residue, and X can be C, U, or A. The length of each arm of the ribozyme was set at 7–9 nt.

In the case of RNAi, the cleavage site for a target RNA has been identified as being 11 nt downstream of the target position that is complementary to the 3′-most nucleotide of the antisense strand of the siRNA (Elbashir et al., 2001a,b). We selected six sites for siRNAs that overlapped the cleavage site as the ribozyme (the cleavage sites were not necessarily identical, but the annealing regions overlapped each other). A length of 21 nt and a 2-nt 3′-overhang were chosen for the siRNA because this form of siRNA was shown to be most effective with *Drosophila* lysate *in vitro* (Elbashir et al., 2001b).

In our antisense experiments, we used 20-nt phosphorothioate-modified oligonucleotides. However, as we used ribozymes as controls for the stability of siRNAs, we did not chemically modify the ribozymes. We used the naked RNAs. Details of the various sense and antisense siRNAs and antisense ODNs are shown in Figure 1.

Suppressive effects of antisense ODNs

In the first experiment, we examined the effects of antisense ODNs against the gene for firefly luciferase. We cotransfected HeLa S3 cells with a series of antisense ODNs, the firefly luciferase expression vector, and the Renilla luciferase expression vector, using various cationic transfection lipids, including Lipofectin™, Lipofectamine, Lipofectamine 2000, FuGENE 6, and Effectene™. The luciferase assays gave poor results because the activities of Renilla luciferase, used as an internal control, were too low or were unstable when we used antisense ODNs at 200 nM or higher concentrations for transfections. High doses of ODNs might have an inhibitory effect on the efficiency of transfection of plasmids, or they might act nonspecifically to block protein synthesis.

Oligofectamine appeared to be a much better reagent for transfection of antisense ODNs. It is a recently developed low-toxicity reagent that was optimized for delivery of ODNs. We continued to use FuGENE 6 for the transfection of plasmids. We obtained greatly improved luciferase activities, with stable values and low toxicity. Using this procedure, we evaluated the effects of antisense ODNs targeted to six sites in the firefly gene for luciferase. As shown in Figure 2, cotransfections with antisense ODNs that corresponded to site 1, site 2, site 3, site 5, and site 6 of the firefly gene for luciferase plus the firefly luciferase expression vector and the Renilla luciferase expression vector caused decreases in firefly luciferase activity of 59%, 55%, 57%, 23%, and 65% respectively. The antisense ODN targeted to site 4 had stronger suppressive activity (87%) without any negative effects on Renilla luciferase activity, compared with the negative control (Fig. 2C).

Effects of RNAi on expression of firefly gene for luciferase

We transfected cells with siRNAs using the Lipofectamine 2000 reagent. This lipid allows cotransfection of HeLa S3 cells with siRNAs and plasmids at high efficiency (Elbashir et al., 2001a; Miyagishi and Taira, 2002). We examined the suppressive activities of siRNAs that corresponded to the six sites in the firefly gene for luciferase and found that the siRNAs directed against site 1 and site 4 strongly suppressed the luciferase activity (92% and 95%), whereas the corresponding val-

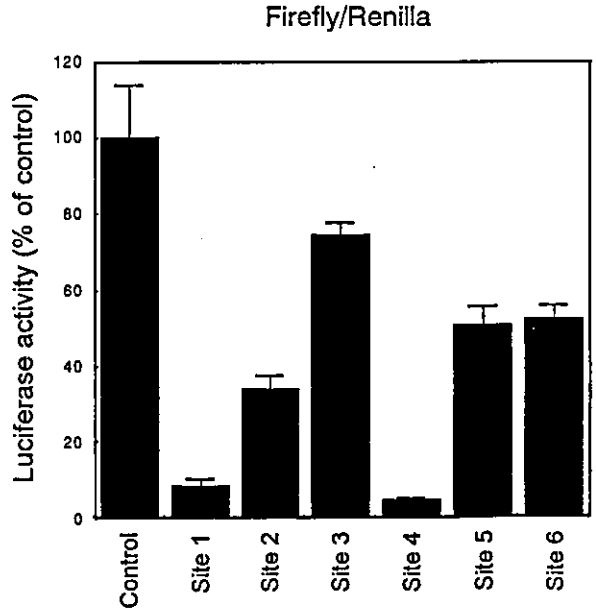
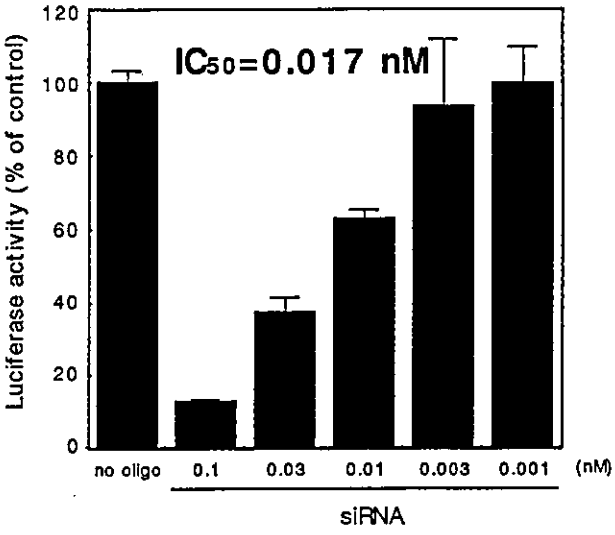


FIG. 3. Effects of siRNAs targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and 1 nM siRNA directed against individual target sites in the firefly gene for luciferase or with control siRNA. Values were normalized by reference to Renilla luciferase activity. Values are means ± SEM of results from three transfections in each case.

ues for site 2, site 3, site 5, and site 6 were much lower (66%, 25%, 49%, and 48%, respectively) (Fig. 3). The results demonstrated the positional dependence of RNAi, as was also the case for the antisense ODNs. Although the correlation coefficient between the results for antisense ODNs and siRNAs was low (0.42), it is of interest that the highest efficacy was observed at the same position (site 4), even though the two systems are quite different. The low correlation between the antisense ODN and siRNA may originate from the small difference of target sites for siRNA and ODN at single nucleotide level (especially at site 1 and site 2).



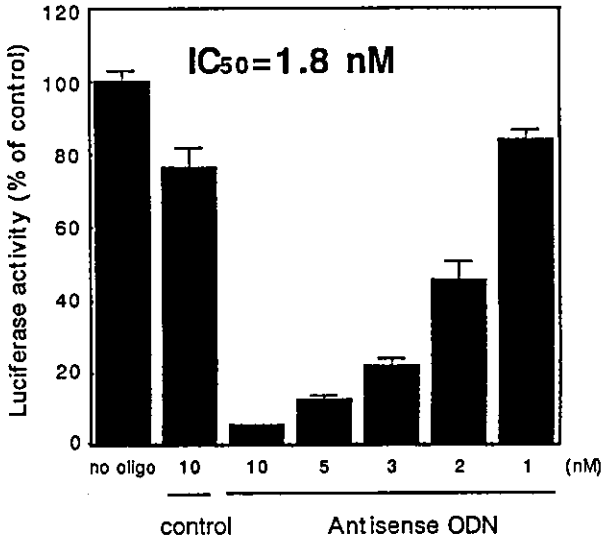


FIG. 4. Dose dependence of siRNAs and antisense ODNs on luciferase activity. HeLa S3 cells were transfected with 30 ng Renilla luciferase expression vector (pRL-RSV), 30 ng firefly luciferase expression vector (pGL3), and the indicated amounts of siRNA or antisense ODN directed against site 4 in the firefly gene for luciferase or with control siRNA or control antisense ODN. Values are means ± SEM of results from three transfections in each case.

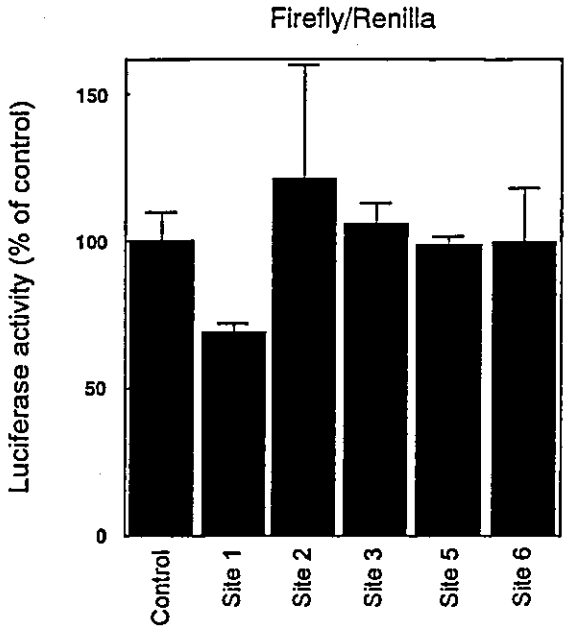


FIG. 5. Effects of ribozymes targeted to the firefly gene for luciferase on luciferase activity in HeLa S3 cells. Values were normalized by reference to Renilla luciferase activity. Values are means ± SEM of results from three transfections in each case.

To compare the suppression efficacy of two methods, we performed dose-response experiments of the siRNA and the ODN on the suppression effect of luciferase gene expression at site 4. Under this experimental condition, the inhibitory concentration of 50% reduction for luciferase activity (IC_{50}) for the siRNA and the ODN is 0.017 nM and 1.8 nM, respectively (Fig. 4). It is important to note that the IC_{50} value of the siRNA was about 100-fold lower than that of the antisense ODN.

Suppressive effects of ribozymes

We examined the effects of unmodified ribozymes directed against the six sites in the firefly gene for luciferase. We demonstrated previously that ribozymes function effectively in cells when transcribed as tRNA constructs under the control of a pol III promoter (Kato et al., 2001; Koseki et al., 1999; Tanabe et al., 2000). However, in the present study, in order to compare the effects of concentration as an index of stability in cells between siRNAs and ribozymes, we introduced naked RNA ribozymes directly into cells, in the same way as we had introduced antisense ODNs and siRNAs, in order to evaluate directly the activities of siRNAs and ribozyme. Ribozymes

were synthesized by T7 polymerase, purified by electrophoresis, and introduced into HeLa S3 cells with the firefly luciferase expression vector and the Renilla luciferase expression vector under the same conditions as those used for siRNA, with the exception of significantly higher final concentrations (100 nM; note that the concentration of siRNA was 1 nM; with Lipofectamine 2000 reagent) (Fig. 5). Even at these higher concentrations, we observe no significant reduction in the expression of firefly luciferase (data not shown).

DISCUSSION

The dual-luciferase system allows the accurate evaluation of gene-suppressive effects and has been used widely in analyses of the mechanism of RNAi (Elbashir et al., 2001a,b; Miyagishi and Taira, 2002). Nonetheless, there are only a few reports of application of the system to analysis of the effects of antisense ODNs (Faria et al., 2001). In present study, we established a system that allowed us to evaluate antisense effects accurately by use of different cationic lipids for antisense ODNs and reporter plasmids. Our protocol allowed us to compare site-dependent effects between antisense ODNs and siRNAs.

Analysis of the effects of the siRNAs targeted to six sites in the firefly gene for luciferase revealed that positional effects were not well correlated with positional effects on the efficacy of antisense ODNs, but both an siRNA and an antisense ODN were most effective when directed against site 4. This result implies that a major factor in site dependence of the effect of siRNA might be accessibility to the target mRNA, as is also the case for antisense ODNs and ribozymes (Kawasaki et al., 2002; Kawasaki and Taira, 2002; Krupp and Gaur, 2000; Warashina et al., 2001). It is possible that the effects of siRNA and ODN might share some common features. However, we could not find any significant correlation between structures of mRNA, as predicted by Mfold (Zuker and Jacobson, 1998), and the suppressive effects of siRNAs. However, more experimental data about the accessibility of antisense ODN to target RNA *in vitro* and *in vivo* are needed for more precise estimations of such a relationship.

In our experiment with naked ribozymes, we failed to detect suppressive activity even at a concentration of ribozyme of 100 nM during transfection, despite the apparent repressive activity of siRNA under similar conditions at a concentration of 1 nM. This result was most likely due to the poor resistance to nuclease of naked single-stranded ribozymes in cells. By contrast, siRNAs, which are 21–23-nt RNA duplexes with a 2-nt or 3-nt 3′-overhang, are considered to be much more resistant than ribozymes to nucleases. Indeed, we showed previously that an siRNA duplex was significantly more stable in cells than the cognate single-stranded sense or antisense RNA, with transcription under the control of the identical promoter in each case (Miyagishi and Taira, 2002). Double-stranded siRNAs appear to be protected by RISC from attacks by RNases, whereas ssRNA and ribozymes are not protected by such proteins in cells. Thus, constitutive expression of ribozymes from vectors is required for the effective exploitation of ribozymes.

In our comparative analysis of three major methods for suppression of gene expression, we found a low but significant correlation between the effects of siRNA and antisense ODN. Our results led to the hypothesis that one of the major factors responsible for the dependence of the activity of siRNA on its target site is accessibility to the target mRNA. This working hypothesis should help us focus on such effects and help us to clarify the mechanisms of positional effects in the suppression of gene expression.

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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

RNA 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; Misquitta 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 2α (eIF2α), 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) (Piu 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 GCT 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 μM 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_2 in air in DMEM supplemented with 10% heat-inactivate (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 (2×10^5/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 ± 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/GeneBank 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 Gaur, 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-

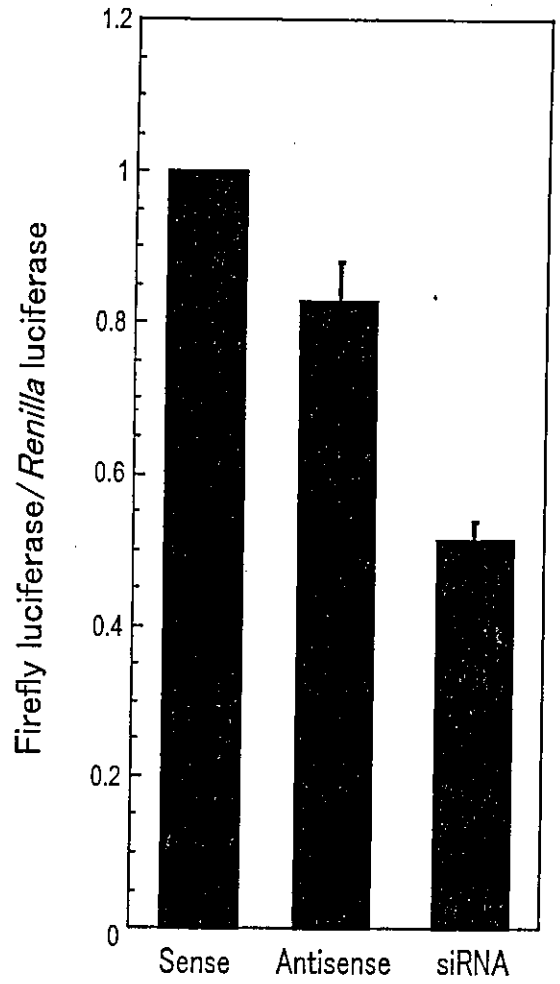


FIG. 1. RNA interference by siRNA duplexes targeted against a gene for JDP2 in NIH3T3 cells. NIH3T3 cells were cotransfected with 200 ng of a JDP2-luc-expressing plasmid and 136 ng of siRNA duplex or the sense or antisense RNA strand, together with 20 ng of reporter plasmid pRL-SV40, which encoded *Renilla*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

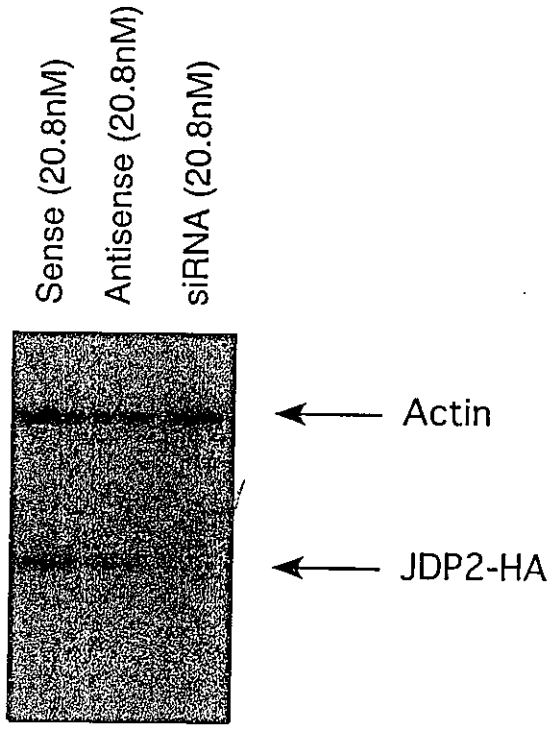
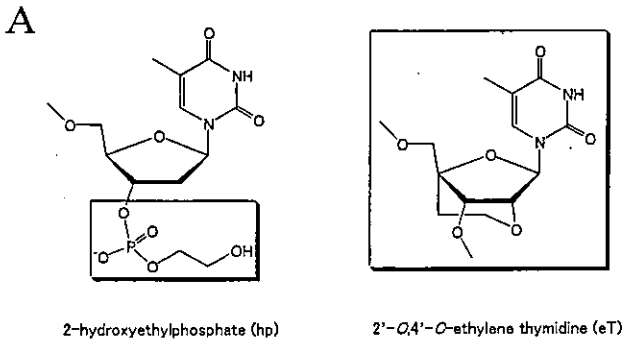


FIG. 2. The effect of transfection with an siRNA duplex targeted to the gene for JDP2 cells that stably expressed a plasmid that encoded JDP2-HA. RAW264.7 cells that expressed stably JDP2-HA were transfected with siRNA targeted against the gene for JDP2. Western blotting was performed to confirm the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

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′ overhand 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).



B

siRNA 5′ CAGAGGGAGUCAGAGCGGCUT 3′
3′ TUGUCUCCCUCAGUCUCGCCG 5′

#1 5′ CAGAGGGAGUCAGAGCGGCUT 3′
3′ eTeTGUCUCCCUCAGUCUCGCCG 5′

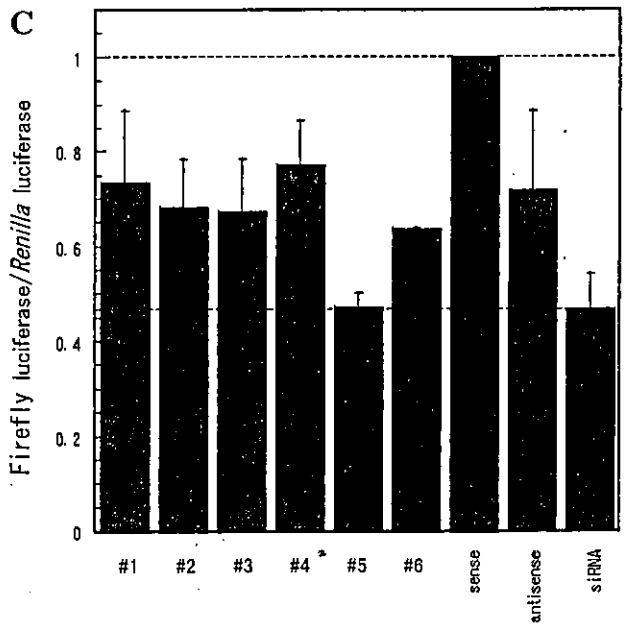
#2 5′ CAGAGGGAGUCAGAGCGGCeTeT 3′
3′ TUGUCUCCCUCAGUCUCGCCG 5′

#3 5′ CAGAGGGAGUCAGAGCGGCeTeT 3′
3′ eTeTGUCUCCCUCAGUCUCGCCG 5′

#4 5′ CAGAGGGAGUCAGAGCGGCUT 3′
3′ hpTUGUCUCCCUCAGUCUCGCCG 5′

#5 5′ CAGAGGGAGUCAGAGCGGCUThp 3′
3′ TUGUCUCCCUCAGUCUCGCCG 5′

#6 5′ CAGAGGGAGUCAGAGCGGCUThp 3′
3′ hpTUGUCUCCCUCAGUCUCGCCG 5′



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-

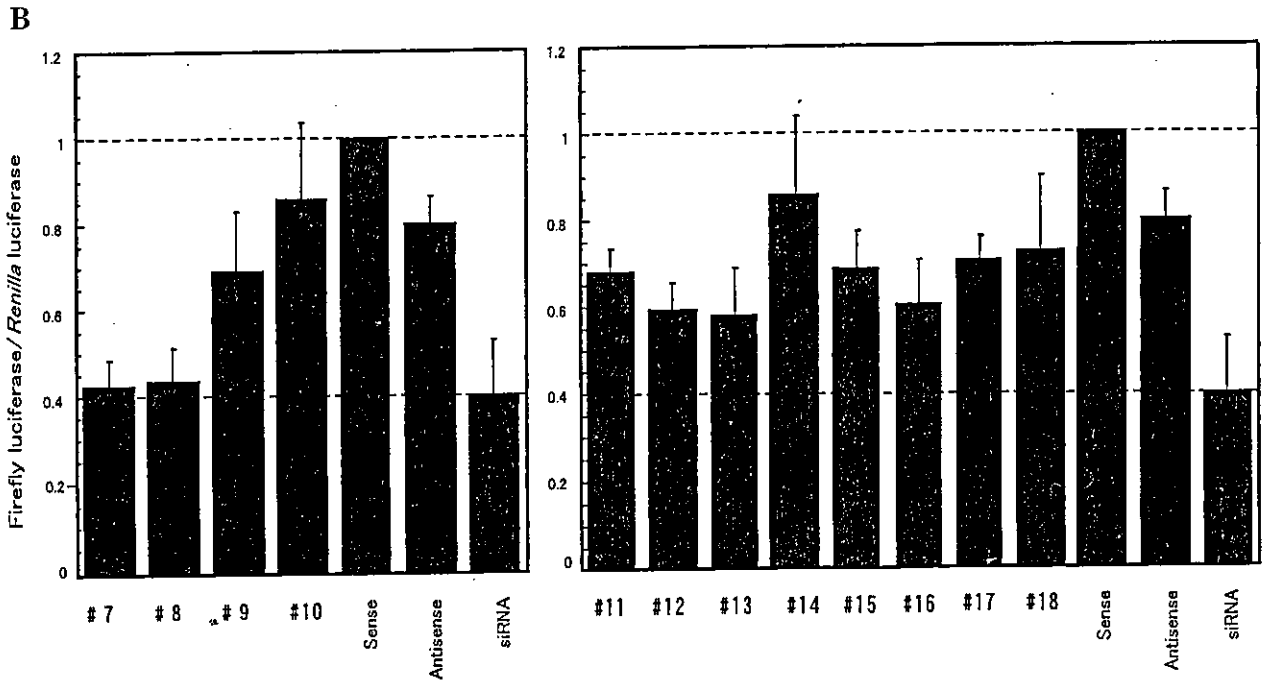
FIG. 3. Effects of chemical modifications on the activity of siRNA. (A) Schematic RNA backbone and the modifications tested in this study. (B) Sequences of the chemically modified siRNAs. (C) Effects of chemical modification on the activity of siRNA in NIH3T3 cells. Cells were cotransfected with 200 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.

A

siRNA	5' CAGAGGGAGUCAGAGCGGCUT 3' 3' TUGUCUCCCUCAGUCUCGCCG 5'
#7	5' CAGAGGGACUCAGAGCGGCUT 3' 3' TUGUCUCCCUCAGUCUCGCCG 5'
#8	5' CAGAGCGACUCAGAGCGGCUT 3' 3' TUGUCUCCCUCAGUCUCGCCG 5'
#9	5' CAGAGCGACUCACAGCGGCUT 3' 3' TUGUCUCCCUCAGUCUCGCCG 5'
#10	5' CACAGCGACUCACAGCCGCUT 3' 3' TUGUCUCCCUCAGUCUCGCCG 5'
#11	5' CAGAGGGACUCAGAGCGGCUT 3' 3' TUGUCUCCCUGAGUCUCGCCG 5'
#12	5' CAGAGCGACUCAGAGCGGCUT 3' 3' TUGUCUCGCUGAGUCUCGCCG 5'
#13	5' CAGAGCGACUCACAGCGGCUT 3' 3' TUGUCUCGCUGAGUGUCGCCG 5'
#14	5' CACAGCGACUCACAGCCGCUT 3' 3' TUGUGUCGCUGAGUGUCGGCG 5'
#15	5' CAGAGGGAGUCAGAGCGGCUT 3' 3' TUGUCUCCCUGAGUCUCGCCG 5'
#16	5' CAGAGGGAGUCAGAGCGGCUT 3' 3' TUGUCUCGCUGAGUCUCGCCG 5'
#17	5' CAGAGGGAGUCAGAGCGGCUT 3' 3' TUGUCUCGCUGAGUGUCGCCG 5'
#18	5' CAGAGGGAGUCAGAGCGGCUT 3' 3' TUGUGUCGCUGAGUGUCGGCG 5'

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.

B



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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