RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells

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Abstract Various types of synthetic oligonucleotide duplexes against the *Photinus luciferase* gene were tested on their induction of the sequence-specific RNA interference (RNAi) activity in transfected human cells. Results indicate that RNA duplexes with ribonucleotide 3′ overhangs rather than those with deoxyribonucleotide 3′ overhangs induce more efficient RNAi activity, and that sense-stranded DNA/antisense-stranded RNA hybrids induce a moderate RNAi activity. These results suggest that there is a difference in the potential of oligonucleotide duplexes to be RNAi mediators, i.e. short interfering RNAs (siRNAs), between human RNAi and invertebrate RNAi. The data further show that different siRNAs induce different levels of RNAi. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: RNA interference; Synthetic oligonucleotide duplex; Cultured human cell

1. Introduction

RNA interference (RNAi) is the process of sequence-specific posttranscriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes. This intriguing phenomenon has been found in various species including flies, worms, protozoa, vertebrates and higher plants (reviewed in [1–4]). The majority of studies on the molecular mechanism underlying RNAi activity have been conducted using *Drosophila* and *Caenorhabditis elegans*, and demonstrated that 21–25-nucleotide (nt) RNA fragments, referred to as short interfering RNAs (siRNAs), are essential sequence-specific mediators of RNAi [5–7], and that siRNAs are generated from long dsRNAs by digestion with an RNase III-like nuclease, Dicer [8,9]. It is also noteworthy that although the mechanism underlying RNAi activity has not been completely elucidated, RNAi has already become a powerful reverse genetic method for suppressing the expression of a gene of interest in various species (reviewed in [3,10]).

In mammalian RNAi, it was initially thought that RNAi might occur only in oocytes [11] and preimplantation embryos [12]. Mammalian cells in general possess a rapid and non-specific RNA degradation pathway involving the sequence-non-specific RNase, RNase L [13], and a rapid translation inhibition pathway involving the interferon-inducible, dsRNA-activated protein kinase, PKR, both of which can be activated by long dsRNAs (>30 bp) [14–16]. Thus, these rapid responses to long dsRNAs may mask the sequence-specific RNAi activity in mammalian cells [17] except in undifferentiated cells [18,19] as well as differentiated cells that possibly lack PKR [20]. Elbashir et al. [21] have recently demonstrated that synthetic 21-nucleotide siRNA duplexes can specifically inhibit the expression of cognate genes in cultured mammalian cells. RNAi induction with synthetic oligonucleotide duplexes seems to have paved the way for molecular analyses of mammalian RNAi and for its possible application to mammalian reverse genetic studies [22].

In this study, I investigated the effects of various types of oligonucleotide duplexes targeting the exogenous reporter gene, *Photinus luciferase*, on the suppression of the expression of the gene, i.e. RNAi, and examined the properties of the allowable oligonucleotide duplexes for being RNAi mediators in mammalian cells.

2. Materials and methods

2.1. Cell culture

HeLa and NTera2D1 cells were grown at 37°C in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies) in a 5% CO_2-humidified chamber.

2.2. Preparation of oligonucleotide duplex

Oligonucleotides synthesized in this study are indicated in Table 1. All the oligonucleotides including the GL3 oligonucleotides [21] were obtained from GENSET OLIGOS. Since siRNA duplexes with 2-nt 3′ overhangs appeared to be the most efficient inducers of RNAi [21,23], the synthesized oligonucleotides possessed a ribo-uridine or deoxythymidine dinucleotide sequence at the 3′ ends; thus, the resultant duplexes contain 2-nt 3′ overhangs (Table 1). For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 μM each) were mixed together in annealing buffer (30 mM HEPES pH 7.0, 100 mM potassium acetate, and 10 mM magnesium acetate), heat-denatured at 90°C for 3 min, and annealed at 37°C overnight.

2.3. Transfection and luciferase assay

The day before transfection, cells were trypsinized, diluted with the fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^{-5} cells/well in 0.5 ml of the culture medium). Cotransfection with reporter plasmids and synthetic oligonucleotides was carried out using a DOSPER liposomal transfection reagent (Roche) as described by the manufacturer. Before cotransfection, the culture medium was replaced with 0.5 ml of OPTI-MEM I (Life Technologies), and to each well, 0.25 μg of pGL3-Control plas-

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Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; dsRNA, double-stranded RNA; PKR, interferon-inducible, dsRNA-activated protein kinase; nt, nucleotide

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mid (Promega), 0.05 μg of phRL-TK plasmid (Promega), and 0.24 μg of oligonucleotide duplexes were applied. Cells were incubated for 4 h at 37°C. After the 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37°C was carried out. Cell lysate was prepared 24 h after transfection and luciferase expression assay was carried out using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

3. Results and discussion

3.1. RNAi induction by various types of synthetic oligonucleotide duplexes

In order to realize efficient RNAi induction by synthetic oligonucleotide duplexes in mammalian cells, it is important to know the properties of the duplexes conferring a strong RNAi activity. Here I constructed various types of synthetic oligonucleotide duplexes against the *Photinus luciferase* gene, introduced the duplexes together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a phRL-TK plasmid carrying the *Renilla luciferase* gene as a control into cultured human cells, and examined the effect of the duplexes on the suppression of the expression of *Photinus* luciferase, i.e. on the induction of RNAi.

Fig. 1 shows the results of dual-luciferase assay. When the GL3 and GL3UU duplexes, which are identical except for their 3′ ends, i.e. containing 2-nt deoxy-thymidine (TT) and 2-nt ribo-uridine (UU) 3′ overhangs, respectively, were introduced together with the reporter plasmids into HeLa cells, a human epithelial cell line, results indicate that there is a difference in the degree of silencing of the *Photinus luciferase* gene between these duplexes: the GL3UU duplex appears to induce a stronger RNAi activity than the GL3 duplex. Similar results were also obtained when NTera2D1 cells, a human embryonal carcinoma cell line, were used. In a previous study [21], the GL2 and uGL2 duplexes, which, like the GL3 and GL3UU duplexes, possessed the same dsRNAs with TT and UU 3′ overhangs, respectively, appeared to induce similar levels of RNAi activity in mammalian cells. The differences in outcome of the induction by the duplexes having TT and UU 3′ overhangs between the previous and present studies might be attributed to the differences in the target sites (genes) and/or in the culture and cell conditions between the studies. Since the data described above were reproducible in this study, I further analyzed the difference in the RNAi induction between the GL3 and GL3UU duplexes in the present system.

When the sense- or antisense-stranded elements of the GL3 and GL3UU duplexes were swapped, the resultant sense-stranded GL3/antisense-stranded GL3UU (ssGL3/asGL3UU) and ssGL3UU/asGL3 duplexes appeared to induce a similar level of RNAi activity to that of the GL3UU and GL3 duplexes, respectively, in HeLa cells (Fig. 1). Similar results, except for the ssGL3UU/asGL3 duplex, were obtained when NTera2D1 cells were used; the RNAi induction level by the ssGL3UU/asGL3 duplex appeared to be lower than that by the GL3 duplex in NTera2D1 cells. The difference in profiles of the RNAi induction by the ssGL3UU/asGL3 duplex between HeLa and NTera2D1 cells may reflect the difference in the contribution of cellular proteins associated with siRNAs to induction of RNAi between these cell lines.

I also constructed DNA/RNA hybrids, the ssDNA/asGL3 and ssDNA/asGL3UU hybrids, and examined their effects on RNAi induction. Interestingly, results indicated that the ssDNA/asGL3UU hybrid induced a moderate level of RNAi activity in either HeLa or NTera2D1 cells, whereas the ssDNA/asGL3 hybrid induced little or no RNAi activity in both cells (Fig. 1). In addition, the ssDNA/asGL3UU hybrid appears to induce a more efficient RNAi activity than the GL3 duplex. These results suggest that the nature of RNA in the antisense-stranded elements of the duplexes is important for mediating RNAi. It should also be pointed out that in *C. elegans*, DNA/RNA hybrids appear to hardly induce RNAi activity [6]. Therefore, these observations suggest that there is a difference in the potential of oligonucleotide duplexes to be RNAi mediators in human cells and those in *C. elegans*. It is of great interest to determine the difference in the oligonucleotide duplexes having potential for being RNAi mediators between human and invertebrate cells; to elucidate it, further extensive studies must be conducted.

The above observations indicate the possibility that RNA/DNA hybrids composed of sense RNA oligomers and antisense DNA oligomers that terminate in ribonucleotides at 3′ ends may induce RNAi to some degree in mammalian cells. To clarify this possibility, two DNA–RNA mixed oligomers corresponding to asGL3UU were synthesized (Table 1): one is GL3DNA-UU that is 19-nt DNA linked with 2-nt RNA at the 3′ end, and the other is GL3DNA3R-UU that is 16-nt DNA linked with 5-nt RNA at the 3′ end. The GL3DNA-UU and GL3DNA3R-UU oligonucleotides were annealed with the ssGL3UU oligonucleotide and the resultant RNA/DNA hybrids together with reporter plasmids were introduced into

Table 1
Synthesized oligonucleotides in this study

Name	Sequence	T_m (°C)
ssGL3[a]	5′-rCUUrArCrGrCUrGrArGUrArCUUrCrGrATT-3′	46
asGL3[a]	5′-UrCrGrArArGUrArCUrCrArGrCrGUrArArGTT-3′	46
ssGL3UU	5′-rCUUrArCrGrCUrGrArGUrArCUUrCrGrAUU-3′	46
asGL3UU	5′-UrCrGrArArGUrArCUrCrArGrCrGUrArArGUU-3′	46
ssDNA	5′-CTTACGCTGAGTACTTCGATT-3′	46
GL3DNA-UU	5′-TCGAAGTACTCAGCGTAAGUU-3′	46
GL3DNA3R-UU	5′-TCGAAGTACTCAGCGTrArArGUU-3′	46
ssLa2	5′-rGrGrArArGrArCrGrCrCrArArArArArCrAUrAUU-3′	51
asLa2	5′-UrAUrGUUUUUrGrGrCrGUrCUUrCrCUU-3′	51
ssLa21	5′-rArCrCrGrCUrGrGrArGrArGrCrArArCUrGrCUU-3′	58
asLa21	5′-rGrCrArGUUrGrCUrCUrCrCrArGrCrGrGUUU-3′	58
ssLa100	5′-rGrCrArGUUrGrCrGrCrCrCrGrCrGrArArCrGUU-3′	68
asLa100	5′-rCrGUUrCrGrCrGrGrGrCrGrCrArArCUrGrCUU-3′	68

'r' prefixed to G, A, and C represents ribonucleotide, and U indicates ribo-uridine.
[a]These oligonucleotides were used in a previous study [21].

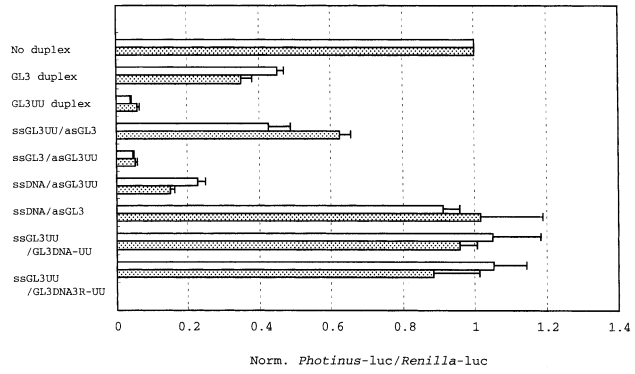

Fig. 1. RNAi activities induced by various types of oligonucleotide duplexes. Various oligonucleotide duplexes together with pGL3-control and phRL-TK plasmids carrying *Photinus* and *Renilla luciferase* reporter genes, respectively, were cotransfected into either HeLa or NTera2D1 cells. Twenty-four hours after transfection, cell lysate was prepared and dual-luciferase assay was carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of duplexes are normalized to the ratio obtained for a control in the absence of duplex (No duplex). Open and dotted bars indicate the data of HeLa and NTera2D1 cells, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

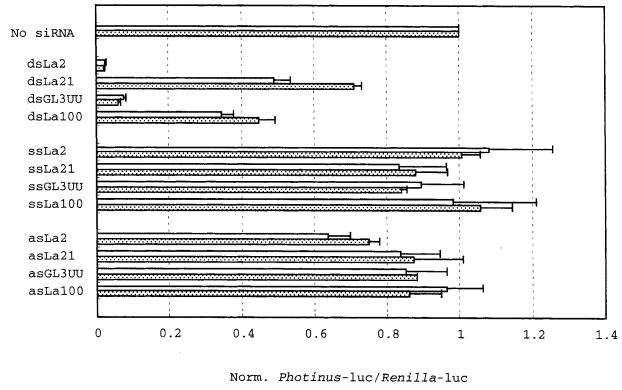

Fig. 3. Different RNAi activities induced by different siRNAs. Synthetic siRNAs together with pGL3-control and phRL-TK plasmids were cotransfected into either HeLa or NTera2D1 cells. Prefixes attached to the name of siRNA, i.e. ds, ss and as, represent double-stranded (duplex), and sense and antisense single-stranded siRNAs, respectively. Twenty-four hours after transfection, cell lysate preparation and dual-luciferase assay were carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 1. Open and dotted bars indicate the data of HeLa and NTera2D1 cells, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

either HeLa or NTera2D1 cells, and the expression of luciferase was then examined. Results show that little or no RNAi activity was induced by these hybrids in either HeLa or NTera2D1 cells (Fig. 1). In this study, therefore, the GL3UU and ssGL3/asGL3UU duplexes appear to induce the most efficient RNAi activity of all the duplexes examined. This suggests that a double-stranded form of RNA and ribonucleotide 3′ overhangs, particularly those on the antisense single-stranded side, are important for maximizing the efficiency of synthetic oligonucleotide duplexes to induce RNAi in mammalian cells. As for the importance of the ribonucleotide 3′ termini on the antisense single-stranded side of the duplexes, it may be associated with RNA-dependent RNA polymerase activity [24].

3.2. SiRNA-dependent RNAi activity

I examined if there were any differences in the RNAi activity among various siRNA duplexes that recognized different sequences in a cognate gene. To address the question, I constructed three new 21-nt RNA duplexes against the *Photinus luciferase* gene other than the GL3UU duplex, and tested the effects of these four RNA duplexes on the induction of RNAi in either HeLa or NTera2D1 cells using the same procedure as described above. The sequences of the RNA oligomers synthesized as siRNAs are shown in Table 1, and their positions on the target gene, the *Photinus luciferase* gene, are shown in Fig. 2. Note that the synthesized RNA oligomers possess a ribo-uridine dinucleotide sequence at the 3′ ends to maximize the efficiency of the oligomers for induction of RNAi; thus, the resultant RNA duplexes contain 2-nt ribo-uridine 3′ overhangs. Fig. 3 shows the results of the dual-luciferase assay. Interestingly, a difference in the degree of silencing of the *Photinus* luciferase gene was observed among the RNA duplexes used: the La2, La21, GL3UU, and La100 duplexes at a concentration of 40 nM decreased the *Photinus* luciferase ex-

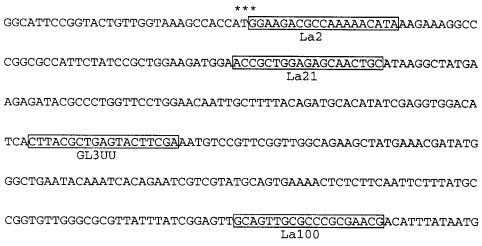

Fig. 2. Nucleotide sequence of part of pGL3-control plasmid. Sequence of pGL3-control plasmid from nucleotide positions 251–610 is presented (the nucleotide positions are based on the numbering system used in the technical manual provided by the manufacturer). The sequences homologous to synthetic siRNAs used are indicated by boxes, and the name of the siRNA is indicated under the box. Asterisks indicate the first methionine codon of the *Photinus luciferase* gene.

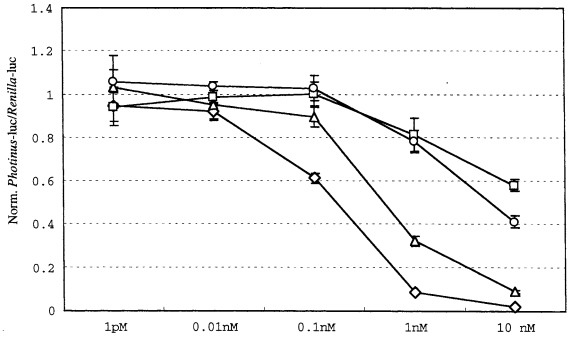

Fig. 4. Dose-dependent inhibition of target luciferase in HeLa cells. The pGL3-control and phRL-TK plasmids were cotransfected with an increasing amount of each siRNA duplex, from 1 pM to 10 nM. Twenty-four hours after transfection, dual-luciferase assay was carried out. Data are presented as normalized ratios of target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity as in Fig. 1. Open diamond, square, triangle and circle indicate the ratios for La2, La21, GL3UU and La100 duplexes, respectively.

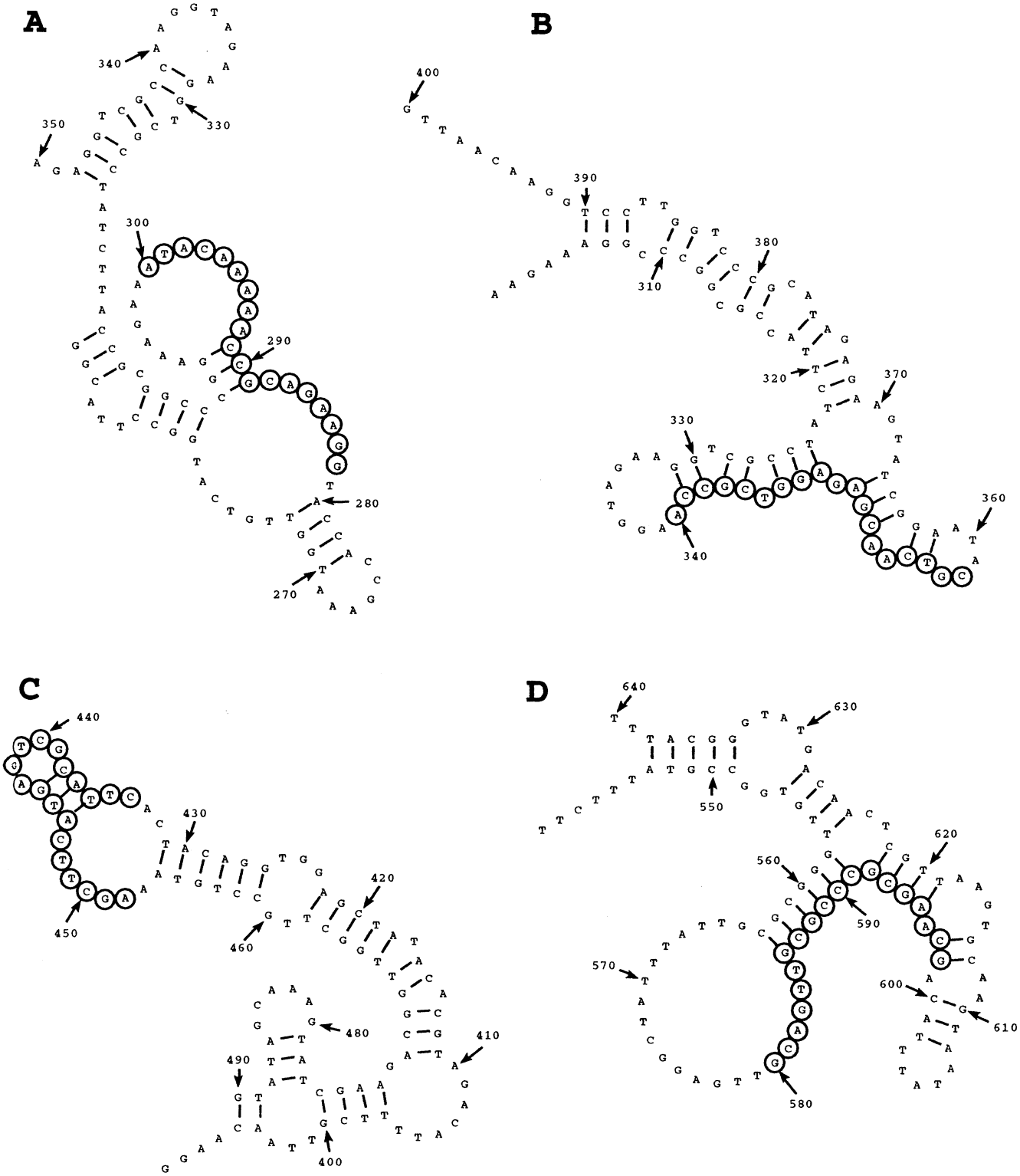

Fig. 5. Predicted secondary structures. The nucleotide sequences of pGL3-control plasmid from nucleotide positions 251–350 (A), 301–400 (B), 391–490 (C) and 541–640 (D) are presented. The sequences were analyzed by the GENETYX-MAC 8.0 computer program. Putative hydrogen bonds are indicated by solid bars. Figures and arrows indicate the nucleotide position based on the numbering system used in the technical manual provided by the manufacturer. The sequences homologous to La2 (A), La21 (B), GL3UU (C) and La100 (D) siRNAs used in this study are indicated by circles.

pression level up to 2.3%, 48.9%, 7.5%, and 34.4%, respectively, in HeLa cells. Because similar results were also observed in NTera2D1 cells, the results may be common to various human or mammalian cells.

I further examined the effects of various doses of the RNA duplexes on the *Photinus* luciferase expression in the transfected cells. As shown in Fig. 4, results indicate that a dose-dependent, sequence-specific RNAi activity occurs in either HeLa or NTera2D1 cells (data not shown). In addition, it appears that the La2 and GL3UU duplexes can inhibit the *Photinus* luciferase expression at a concentration as low as 0.1 nM in this system, whereas the La21 and La100 duplexes slightly suppress the expression at the same concentration. Together with the above observations (Fig. 3), these results suggest that the La2 and GL3UU duplexes induce a relatively stronger RNAi activity than the La21 and La100 duplexes.

The evidence presented above suggests that a siRNA-dependent RNAi activity occurs in cultured human cells; it possibly occurs in various mammalian cells including human cells. It is of great interest to determine the reason why such a difference in the RNAi activity induced by various siRNAs duplexes occurs. I first examined whether there was any association between RNAi activity and either the sequence similarity or melting temperature (T_m) of the siRNA used, but no significant association was observed between them. For other possible explanations, the following are conceivable: (i) there may be some difference in the affinity between cellular siRNA-binding protein(s) [25] and each of the siRNA duplexes used; (ii) the secondary and/or tertiary structures of target mRNA may participate in the induction of distinct RNAi activities. As for the latter possibility, when the nucleotide sequence of *Photinus luciferase* was analyzed using the GENETYX-MAC 8.0 computer program, the target sequence of either La21 or La100 siRNA in the *luciferase* gene was predicted to form a stem structure with 12 hydrogen bonds, whereas the La2 and GL3UU sequences were predicted to form a few hydrogen bonds in them (Fig. 5). To elucidate whether the predicted structures can be actually formed in the RNA transcript, and whether the RNA structures are indeed associated with the RNAi activity, more extensive studies are required to be conducted.

Finally, although the molecular mechanism underlying the siRNA-dependent RNAi activity is still unknown, the data presented here may provide significant information concerning synthetic siRNAs as a novel tool for mammalian reverse genetic studies. Selection of a suitable siRNA for the efficient suppression of the expression of a gene of interest is necessary.

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