Inhibition of c-Ha-ras Gene Expression by Hammerhead Ribozymes Containing a Stable C(UUCG)G Hairpin Loop

Makoto KOIZUMI, Hiroyuki KAMIYA, and Eiko OHTSUKA*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-ku, Sapporo 060, Japan.

Received February 24, 1993

Catalytic RNAs recognize specific sequences of RNA and cleave at a specific site. In this study, we designed hammerhead ribozymes with a thermodynamically stable loop of the sequence 5'C(UUCG)G3' to prevent the aggregation of ribozymes with hammerhead structures. The cleavage activities of these ribozymes were examined using a synthetic pentadecamer with the sequence for the c-Ha-ras mRNA mutated at codon 12 (GGU → GUU). For *in vivo* studies, we constructed a plasmid which expressed a highly active ribozyme targeted against the mutated c-Ha-ras mRNA. When this ribozyme-encoding gene and the activated c-Ha-ras gene were cotransfected into NIH3T3 cells, morphologically normal cells were obtained. We also determined that the expression of the c-Ha-ras gene was inhibited in these cells. These results show that ribozymes containing this stable hairpin loop are useful for the regulation of specific gene expression *in vivo*.

Keywords antisense RNA; catalytic RNA; gene therapy; RNA secondary structure

Recently, new antisense RNA technology has been employed to block the expression of a specific gene.^{1,2)} In this technique, antisense RNA, which has sequences complementary to the mRNA of a target gene, hybridizes to this mRNA and inhibits protein synthesis without affecting other cellular genes. Ribozymes can also be used as regulators for gene suppression.^{1,2)} A class of hammerhead ribozymes, found in viroids, transcripts of new satellite DNA, and in the satellite RNAs of viruses,^{3–6)} has been shown to catalyze the sequence-specific cleavage of RNA. The catalytic domains of this type of hammerhead ribozyme consist of 3 stems and 13 conserved nucleotides. From mutagenesis experiments, 11 of 13 conserved nucleotides and a nucleotide at the cleavage site (A, C, or U) were shown to be important to the catalytic activity.^{7–11)} Furthermore, we were able to design ribozymes that cleaved target RNA at a specific position (X=A, C, or U) by recognizing sequences of 9–15 nucleotides, such as (N)_nUX(N)_n.^{11,12)} The designed ribozymes have been shown to hydrolyze the mutated c-Ha-ras mRNA without cleaving the wild type of mRNA.¹²⁾ We have also reported that the expression of ribozyme-encoding genes, which were targeted against the mutated c-Ha-ras mRNA, and were cloned into a mammalian expression vector, inhibited the transformation of NIH3T3 cells induced by the mutated c-Ha-ras gene.¹³⁾

On the other hand, several reports indicate problems in ribozyme design.^{12,14,15)} One of the difficulties in the efficient cleavage of substrates by ribozymes arises from the formation of unfavorable structures in either the ribozymes or the substrates, which can cause a considerable decrease in catalytic activity (Figs. 1 and 2). Feder and Uhlenbeck showed that aggregation of the substrate caused low catalytic efficiency.¹⁴⁾ Heus *et al.*¹⁵⁾ and our lab¹²⁾ have found several unfavorable secondary structures of hammerhead ribozymes that interfere with the formation of the ribozyme-substrate complex, due to a mispairing between the 5' substrate binding region and the 3' substrate binding region (Fig. 2b), or between the hairpin region and the substrate binding region (Figs. 2c and 2d).

To prevent the aggregation of ribozymes with hammer-

head ribozymes between the hairpin region and the substrate binding region, we introduced a stable hairpin loop, 5'C(UUCG)G3', into a ribozyme (shown in Fig. 1b). This loop, which has been found in the mRNA of T4 phage, and in the ribosomal RNA of *Escherichia coli*,^{16–18)} has an unusually high melting temperature.^{16,18)} Ribozymes containing this loop are thought to hybridize to the substrate and to form an exact hammerhead-type structure, without forming inactive conformations. The cleavage activities of these stable ribozymes for the mutated c-Ha-ras mRNA were measured *in vitro* and *in vivo*.

Materials and Methods

Synthesis of Oligonucleotides Oligoribonucleotides were synthesized by the phosphoramidite method using 5'-O-dimethoxytrityl-2'-O-tert-butylidimethylsilyl nucleosides 3'-O-phosphoramidite (American Bionetic, Inc.) on a DNA/RNA synthesizer, Applied Biosystems model 391.¹⁹⁾ Deprotection and purification of synthetic oligoribonucleotides were performed with modifications of the procedures of refs. 20 and 21. The protected oligoribonucleotide was cleaved from the support (1 μmol scale) using conc. NH₄OH-EtOH (3:1, v/v, 2 ml). The solution was evaporated to dryness. An ammonia/EtOH solution (5 ml) was added and the mixture heated at 55 °C for 16 h. The reaction mixture was evaporated to dryness, then 1 M tetrabutylammonium fluoride in THF (1 ml, Aldrich) was added, and the solution was stirred for 18 h. Triethylammonium acetate (0.1 M, 5 ml) was added, and the solution was chromatographed on a preparative C-18 reverse phase column (Waters/Millipore) with a linear gradient of 5–40% CH₃CN in 50 mM triethylammonium bicarbonate. The fractions containing the 5'-dimethoxytritylated product were evaporated and repeatedly evaporated with H₂O. HCl (0.01 N, pH 2.0) was added, then the solution was stirred for 1 h at room temperature, and neutralized with 0.1 N NH₄OH. The solution was washed with AcOEt, and was evaporated. The deprotected oligoribonucleotide was purified by reverse phase HPLC (column: YMC A-324, 10 mm i.d. × 300 mm length, Yamamura Chemical Laboratories) with a linear gradient of CH₃CN in 0.1 M triethylammonium acetate (pH 7.0), and by anion-exchange HPLC (column: TSK gel DEAE-2SW, 4.6 mm i.d. × 250 mm length, Tosoh) with a linear gradient of HCOONH₄ in 20% CH₃CN.

Oligodeoxyribonucleotides encoding ribozymes and primers for PCR reactions were also synthesized by the phosphoramidite method using a DNA/RNA synthesizer, Applied Biosystems model 391.²²⁾

RNA Cleavage Reaction *in Vitro* The 5'-labeled substrate (5'-GCG CUG UUG GUG UAG-3') was dissolved in 25 mM MgCl₂, 40 mM Tris-HCl (pH 7.5), and 20 mM NaCl, and was incubated with the ribozyme (ribozyme 1 or 2) in the same solution at 37 °C. The reaction was stopped by the addition of 50 mM EDTA (pH 8.0), and was analyzed by

Fig. 1. Secondary Structures of Hammerhead Ribozymes, Which Targeted the Activated c-Ha-ras mRNA with GUU (Indicated by Bold Letters)

a) Secondary structure of ribozyme 1 with the substrate (see refs. 12, 13). b) Ribozyme 2, containing the unusually stable loop 5'C(UUCG)G3'. c) Ribozyme 3, which has been thought to form an unfavorable structure.¹²⁾

Fig. 2. Unfavorable Secondary Structures of Hammerhead Ribozymes

a) Normal hammerhead structure. Hairpin regions in the ribozyme are indicated by H and H'. Substrate binding regions in the ribozyme are represented by B and B'. b—d) Proposed unfavorable secondary structures of hammerhead ribozymes.

homochromatography using Homo-mix III.²³⁾ Cleavage rates were estimated by quantitating the radioactivity of the products with a Bio-image analyzer BAS-2000 system (Fuji Film).

Construction of Plasmids The construction of plasmids pRSV-rv12neo, pRSVΔneoCS, and pRZ1Δneo was described previously.^{24,25)} Plasmid pRZ4Δneo was constructed by the ligation of two oligodeoxynucleotides (upper; 5'-CGA TCT ACA CCC TGA TGA GCC GCT TCG GCG GCG AAA CAG CGC G-3'; lower; 5'-TCG ACG CGC TGT TTC GCC GCC GAA GCG GCT CAT CAG GGT GTA GAT-3') into *Cla*I, *Sal*I-digested pRSVΔneoCS, as shown in Fig. 3.^{13,24)}

Isolation of Morphologically Normal Cells Plasmid pRZ4Δneo (2 μg) was cotransfected with plasmid pRSV-rv12neo (10 ng) into NIH3T3 cells (approximately 8×10^5 cells) using the calcium phosphate methods described previously,¹³⁾ and the transfected cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum. Stable transformants were selected after 24 h in 600 mg/l G418 (0.534 potency, Gibco Laboratories) medium. During this culture, islands of morphologically normal cells were observed, transferred into other wells by pipetting, and further grown in DMEM containing 5% calf serum.

Polymerase Chain Reaction (PCR) Analysis of DNA Genomic DNA from the isolated transformants was analyzed by the PCR method, as

described previously.^{13,26)}

Northern Hybridization Total RNAs from cells were prepared using RNazol (RNA isolation reagent, Cinna Biotech). Total RNAs (20 μg per lane) were separated by a 1% agarose gel containing formaldehyde as described previously.^{13,27)} Transfer of RNAs from the gel to a nitrocellulose membrane filter was carried out with $20 \times$ SSC. The filter was baked at 80 °C in a vacuum oven. The filter was prehybridized in $5 \times$ SSC/50 mM sodium phosphate (pH 6.7)/2 × Denhardt solution/1% SDS/50% deionized formamide/100 μg/ml salmon sperm DNA at 42 °C for 8 h. Random primed labeled probes (1×10^6 cpm/μg) were added, and the filter was incubated at 42 °C for 17 h. The filter was washed in $0.2 \times$ SSC/0.5% SDS at 60 °C for 20 min. The filter was analyzed by a Bio-image analyzer BAS-2000 system.

Results

Design and *in Vitro* Catalytic Activities of Hammerhead Ribozymes Containing a Thermodynamically Stable Loop The secondary structures of hammerhead ribozymes targeted against the c-Ha-ras mRNA mutated at codon 12

(GGU → GUU) are shown in Fig. 1. We have shown that ribozymes of type 1 distinguished a single mutation in the c-Ha-ras mRNA, both *in vitro* and *in vivo*.^{12,13} A hairpin loop in ribozyme 1 was substituted with the thermodynamically stable loop 5'C(UUGG)G3' as shown in Fig. 1b (ribozyme 2: T_m value of a ribozyme with this 5'C(UUCG)G3' loop was about 90°C. Koizumi *et al.*, in preparation), because this ribozyme is predicted to more easily form a ribozyme-substrate complex.

The catalytic activities of these ribozymes were compared using a synthetic pentadecamer with a sequence for the mutated c-Ha-ras mRNA. Kinetic parameters obtained by Hanes-Woolf plots are shown in Table I. The substrates were cleaved at the expected position by ribozyme 2. Ribozyme 2, containing the stable loop, had a slightly smaller K_m value but exhibited a similar cleavage activity to that of ribozyme 1. Ribozyme 1 has been shown to have higher activity than ribozyme 3 (Fig. 1c and Table I), which was thought to form an unfavorable structure as described previously.¹² Because ribozyme 2 with the stable 5'C(UUCG)G3' loop had similar cleavage activity to ribozyme 1, which formed an exact hammerhead-type structure,¹² ribozyme 2 with this stable loop, as well as ribozyme 1, could prevent unfavorable structures, as shown in Figs. 2c and 2d.

Inhibition of Mutated c-Ha-ras Gene Expression by the Stable Ribozyme *in Vivo* To examine the effects of a ribozyme containing the stable loop in NIH3T3 cells, which were transformed by the single mutated c-Ha-ras gene,^{24,29} plasmid pRZ4Δneo was constructed by ligation of synthetic oligodeoxynucleotides encoding ribozyme 2, as shown in Fig. 3. The ribozyme was expressed under the control of a long terminal repeat (LTR) of the Rous sarcoma virus (RSV). NIH3T3 cells were cotransfected with pRZ4Δneo and pRSV-rv12neo, which contain the c-Ha-ras gene mutated at codon 12 (GGU → GUU) and a *neo* marker

under the control of the RSV LTR and the SV40 early promoter, respectively. Twenty-four hours after the cotransfection, the NIH3T3 cells were cultured in DMEM containing G418 to select stable transformants with pRSV-rv12neo. Four morphologically normal colonies, growing as monolayers, were observed in 31 stable, G418 resistant cell lines (Fig. 4 and Table II). On the other hand, when pRSV-rv12neo and pRZ1Δneo (in which the ribozyme 1-encoding gene was inserted as described previously¹³), were cotransfected, morphologically normal colonies were also found with a similar frequency, as shown in Table II. However, no flat cells were observed in 16 stable transformants cotransfected with pRSV-rv12neo and pRSVΔneoCS.

To detect the presence of the ribozyme-encoding gene and the c-Ha-ras gene, genomic DNA from each of the flat colonies was subjected to PCR amplification by using specific primers for the ribozyme sequences and for the c-Ha-ras gene.¹³ The ribozyme-encoding gene was detected in all colonies; however, the mutated c-Ha-ras gene was lost in two of the four flat colonies, derived from the NIH3T3 cells transfected with pRZ4Δneo and pRSV-rv12neo (data not shown).

In order to examine the expression level of the c-Ha-ras mRNA, total RNAs from the morphologically normal colonies were analyzed by Northern hybridization and were compared to those from malignant colonies with abnormal, spindly shapes. The c-Ha-ras mRNA was detected in *ras*-transfected cells (lane 3 in Fig. 5). The levels of c-Ha-ras mRNA from the morphologically normal cells (clones 7, 11, 103, and 106) were drastically reduced, and were not detectable (lanes 1, 2, 5, and 6 in Fig. 5).

TABLE I. Kinetic Data for Ribozymes 1–3

Ribozyme	Cleavage percentage (%) ^a	K_m (μM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (M ⁻¹ ·min ⁻¹)
1	90	1.1	0.12	1.1×10^5 (100%)
2	95	0.63	0.08	1.3×10^5 (118%)
3	28	N.D.	N.D.	N.D.

a) The substrate (1.4 μM) was treated with the ribozyme (1.6 μM) in the solution described in Materials and Methods for 2 h at 37°C (see ref. 12). N.D.: not determined.

Discussion

Hammerhead ribozymes, which comprise 30–40 nucleotides (A, G, C, and U), do not require the presence of any modified nucleotides for their catalytic RNA hydrolysis activity. Therefore, these ribozymes can be synthesized by a T7 RNA polymerase transcription system *in vitro*, and genes which encode ribozymes can be designed and expressed in some cell lines. Based on the results of an *in vitro* mutagenesis of tobacco ringspot virus satellite RNA, Haseloff and Gerlach have reported that ribozymes catalyzed the cleavage of chloramphenicol acetyltransferase (CAT) mRNA.³⁰ Cameron and Jennings have examined the inhibition of CAT expression in COS1 cells by a ribozyme-encoding gene cloned into a mammalian expres-

Fig. 3. Structures of Plasmids pRZ4Δneo and pRSV-rv12neo

Plasmid pRSV-rv12neo was constructed as described previously.¹³ Plasmid pRZ4Δneo encodes a sequence for ribozyme 2, targeted to the c-Ha-ras mRNA mutated at codon 12, as shown in Fig. 1b.

Fig. 4. Morphology of NIH3T3 Cells Cotransfected with the Mutated c-Ha-ras Gene and the Ribozyme-Encoding Gene

a) Normal NIH3T3 cells. b) Malignant cells transfected with the mutated c-Ha-ras gene. c, d) Morphologically normal colonies cotransfected with the c-Ha-ras gene and the ribozyme 1-encoding gene (clones 7 and 11, respectively). e, f) Morphologically normal colonies cotransfected with the c-Ha-ras gene and the ribozyme 2-encoding gene (clones 103 and 106, respectively).

TABLE II. Morphology of NIH3T3 Cells Transfected with the Mutated c-Ha-ras Gene and the Ribozyme-Encoding Gene

Morphology	pRSV-rv12neo pRZ1Δneo	pRSV-rv12neo pRZ4Δneo	pRSV-rv12neo pRSVΔneoCS ^{a)}
Malignant	27	28	16
Normal	4 ^{b)}	4 ^{b)}	0

a) Plasmid pRSVΔneoCS does not contain the ribozyme-encoding gene. b) The mutated c-Ha-ras gene was lost in half of these colonies.

sion vector.³¹⁾ A modified tRNA gene, with ribozyme sequences inserted into the anticodon loop, was constructed to be expressed in *Xenopus* oocytes.³²⁾ Saxena and Ackerman have also reported that injected ribozymes cleaved the α -sarcin domain of the 28S rRNA in *Xenopus* oocytes.³³⁾ Sarver *et al.* have shown that ribozymes reduced the levels of human immunodeficiency virus (HIV) *gag*-1 transcripts in CD4⁺ HeLa cells infected with HIV.³⁴⁾ In prokaryotic cells, ribozymes have eliminated the HIV-1 integrase RNA, and thereby have prevented the synthesis of HIV-1 integrase.³⁵⁾ Recently, the inhibition of oncogene expression by anti-*ras* and anti-*fos* ribozymes has been reported.^{36,37)}

In this study, we designed a ribozyme with a highly stable hairpin, 5'C(CUUG)G, in order to prevent the aggregation of ribozymes with hammerhead structures. This stable ribozyme could cleave a specific RNA, both *in vitro* and *in vivo*. This ribozyme seemed to have a slightly higher affinity for the substrate, but it did not have an improved k_{cat} value. The k_{cat}/K_m value indicates that the stabilization of the ribozyme loop might slightly enhance the catalytic activity

Fig. 5. a) Northern Analysis of the c-Ha-ras mRNA from NIH3T3 Cells Cotransfected with the c-Ha-ras Gene and the Ribozyme-Encoding Gene

The mutated c-Ha-ras gene (582 bp) was used as the probe.

b) Ethidium Bromide-Strained Gel Prior to RNA Transfer and Hybridization

The position of 28S and 18S rRNAs from cells are shown. Lanes 1 and 2, morphologically normal colonies cotransfected with the c-Ha-ras gene and the ribozyme 1-encoding gene (clones 7 and 11, respectively); lane 3, *ras*-transfected NIH3T3 cells; lane 4, normal NIH3T3 cells; lanes 5 and 6, morphologically normal colonies cotransfected with the c-Ha-ras gene and the ribozyme 2-encoding gene (clones 103 and 106, respectively).

of ribozymes. Based on these and previous results, a general secondary structure of a ribozyme containing a stable loop is shown in Fig. 6. If the target RNA has a UA, UC, or UU sequence, a ribozyme can be designed to cleave the target RNA at the 3' position of this sequence. Introduction of a stable hairpin loop in a hammerhead ribozyme is advantageous for the design of RNA endonucleases, which

Fig. 6. Secondary Structure of a Ribozyme Containing the Stable Loop 5'C(UUCG)G3' and Its Target RNA

Bold and plain letters indicate a designed ribozyme and its substrate, respectively. N or N' can be A, G, C, or U. X can be A, C, or U. The cleavage site is indicated by an arrow.

sequence-specifically cleave target RNA, and suppress the expression of a target gene, because these ribozymes can prevent misspairing in the ribozyme and are expected to be resistant to nucleases.

Acknowledgements This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

References

- 1) C. Helene, J.-J. Toulme, *Biochim. Biophys. Acta*, **1049**, 99 (1990).
- 2) E. Uhlmann, A. Peyman, *Chem. Rev.*, **90**, 543 (1990).
- 3) J. M. Buzayan, W. L. Gerlach, G. Bruening, *Nature* (London), **333**, 349 (1986).
- 4) C. J. Hutchins, P. D. Rathjen, A. C. Forster, R. H. Symons, *Nucleic Acids Res.*, **14**, 3627 (1986).
- 5) L. M. Epstein, J. G. Gall, *Cell*, **48**, 535 (1987).
- 6) A. C. Forster, R. H. Symons, *Cell*, **49**, 211 (1987).
- 7) O. C. Uhlenbeck, *Nature* (London), **328**, 596 (1987).
- 8) M. Koizumi, S. Iwai, E. Ohtsuka, *FEBS Lett.*, **228**, 228 (1988).
- 9) C. C. Sheldon, R. H. Symons, *Nucleic Acids Res.*, **17**, 5679 (1989).
- 10) D. E. Ruffer, G. D. Stormo, O. C. Uhlenbeck, *Biochemistry*, **29**, 10695 (1990).
- 11) M. Koizumi, S. Iwai, E. Ohtsuka, *FEBS Lett.*, **239**, 285 (1988).
- 12) M. Koizumi, Y. Hayase, S. Iwai, H. Kamiya, H. Inoue, E. Ohtsuka, *Nucleic Acids Res.*, **17**, 7059 (1989).
- 13) M. Koizumi, H. Kamiya, E. Ohtsuka, *Gene*, **117**, 179 (1992).

- 14) M. J. Fedor, O. C. Uhlenbeck, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1668 (1990).
- 15) H. A. Heus, O. C. Uhlenbeck, A. Pardi, *Nucleic Acids Res.*, **18**, 1103 (1990).
- 16) C. Tuerk, P. Gauss, C. Thermes, D. R. Groebe, M. Gayle, N. Guild, G. Stormo, Y. Aubenton-Carafa, O. C. Uhlenbeck, I. Tinoco, Jr., E. N. Brody, L. Gold, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1364 (1988).
- 17) C. R. Woese, S. Winker, R. R. Gutell, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 8467 (1990).
- 18) G. Varani, C. Cheong, I. Tinoco, Jr., *Biochemistry*, **30**, 3280 (1991).
- 19) K. K. Ogilvie, N. Usman, K. Nicoghossian, R. J. Cedergren, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 5764 (1988).
- 20) S. A. Scaringe, C. Francklyn, N. Usman, *Nucleic Acids Res.*, **18**, 5433 (1990).
- 21) G. Slim, M. J. Gait, *Nucleic Acids Res.*, **19**, 1183 (1991).
- 22) N. D. Sinha, J. Biernat, J. McManus, H. Köter, *Nucleic Acids Res.*, **12**, 4539 (1984).
- 23) E. Jay, R. Bambara, R. Padmanabhan, R. Wu, *Nucleic Acids Res.*, **1**, 331 (1974).
- 24) H. Kamiya, K. Miura, N. Ohtomo, T. Koda, M. Kakinuma, S. Nishimura, E. Ohtsuka, *Jpn. J. Cancer Res.*, **80**, 200 (1989).
- 25) K. Miura, Y. Inoue, H. Nakamori, S. Iwai, E. Ohtsuka, M. Ikehara, S. Noguchi, S. Nishimura, *Jpn. J. Cancer Res.*, **77**, 45 (1986).
- 26) R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich, *Science*, **239**, 487 (1988).
- 27) J. Sambrook, E. F. Fritsch, T. Maniatis, "Molecular Cloning, A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, Chapt. 7.
- 28) K. J. Hertel, A. Pardi, O. C. Uhlenbeck, M. Koizumi, E. Ohtsuka, S. Uesugi, R. Cedergren, F. Eckstein, W. L. Gerlach, R. Hodgson, R. H. Symons, *Nucleic Acids Res.*, **20**, 3252 (1992).
- 29) C. J. Tabin, S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, E. H. Chang, *Nature* (London), **300**, 143 (1982).
- 30) J. Haseloff, W. L. Gerlach, *Nature* (London), **334**, 585 (1988).
- 31) F. H. Cameron, P. A. Jennings, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9139 (1989).
- 32) M. Cotten, M. L. Birnstiel, *EMBO J.*, **8**, 3861 (1989).
- 33) S. K. Saxena, E. J. Ackerman, *J. Biol. Chem.*, **265**, 17106 (1990).
- 34) N. Sarver, E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, J. J. Rossi, *Science*, **247**, 1222 (1990).
- 35) M. Sioud, K. Drlica, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 7303 (1991).
- 36) K. J. Scanlon, L. Jiao, T. Funato, W. Wang, T. Tone, J. J. Rossi, M. Kashani-Sabet, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10591 (1991).
- 37) M. Kashani-Sabet, T. Funato, T. Tone, L. Jiao, W. Wang, E. Yoshida, B. I. Kashifinn, T. Shitara, A. M. Wu, J. G. Moreno, S. T. Traweck, T. E. Ahlering, K. J. Scanlon, *Antisense Res. Dev.*, **2**, 3 (1992).