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A Method for Screening Antisense Oligodeoxyribonucleotides Effective for mRNA Translation-Arrest[1]

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A transcription and translation coupled reticulocyte lysate system was established for rapid screening of antisense oligodeoxyribonucleotides (ODNs) to determine which are most effective for mRNA translation-arrest. A plasmid containing the target cDNA under the control of the T7 (or SP6) promoter was added to the lysate system in the presence of the T7 (or SP6) RNA polymerase, RNase H, and the antisense ODN under test. Transcription and translation were accomplished in a one-tube reaction. Translation-arrest caused by antisense ODN was evaluated in terms of the amounts of *de novo*-synthesized, [^{35}S]-methionine or [^{35}S]cysteine labeled target protein measured by gel electrophoresis and autoradiography. The properties of this system and optimal reaction conditions for use in antisense ODN screening were determined. Our method is simpler and more rapid than other *in vitro* screening methods.

Key words: antisense oligonucleotide, *de novo* synthesis, *in vitro* screening, transcription and translation coupled system, translation-arrest.

In recent years, many laboratories have successfully suppressed expression of target genes through the use of antisense oligodeoxyribonucleotides (ODNs) (*1-3*). Due to their high specificity of action, antisense ODNs are potentially useful for studying cellular and viral gene functions and as therapeutic agents for viral infection and cancer (*3-6*). Problems in the use of antisense ODN, such as insufficient stability and rate of uptake of ODN are being solved by utilizing oligonucleotide analogues or ODN conjugates (*3, 5, 7, 8*). However, to determine the ODN sequences which are most effective for arresting translation of a target mRNA is still important and difficult.

Due to the presence of secondary structure in mRNA and possible effects of the translation process, no rules have been established for prediction of the regions or sequences most effective in translation-arrest. The effectiveness of antisense sequences may be dramatically different (*9-11*), even for sequences which are in tandem array (*12*). Two methods for *in vitro* screening of effective antisense ODNs have been described: (i) antisense ODN-target mRNA hybridization assay (*9, 13*), and (ii) antisense ODN-target mRNA hybridization followed by translation assay (*6, 14*). However, the first method does not reflect the process of translation arrest caused by antisense ODN. In the second method, production and translation of the target mRNA are achieved by different systems and the procedure is quite inconvenient. In this report, we use a modified transcription-translation coupled reticulocyte lysate system for screening of effective antisense ODNs. This method is rapid, convenient and mimics the *in vivo* translation occurring in mammalian cells.

MATERIALS AND METHODS

Materials—The *in vitro* transcription-translation coupled system used for the assay was a rabbit reticulocyte lysate-based T_NT^{TM} system purchased from Promega (Madison, WI). Fifteen-meric antisense ODNs were custom-made by DNAFax (Taiwan, Republic of China). RNasin nuclease inhibitor and pGEM-3zf(+) plasmid were purchased from Promega. [^{35}S]Methionine (1,175 Ci/mmol) and RNase H were purchased from Amersham (Amersham, UK) and GibcoBRL (Gaithersburg, MD), respectively.

Construction of an In Vitro Transcription Vector-pGEM-E6—The E6 gene of the type 16 human papillomavirus (HPV-16) was obtained by polymerase chain reaction using HPV-16 plasmid as a template. The 5′-end primer and 3′-end primer were 5′-CTGAATTCAAGAGA-ACTGCAATGTTTCA-3′ and 5′-CTGGATCCTTACAGC-TGGGTTTCTCTAC-3′, respectively, which amplified a 468-bp fragment containing 12 nucleotides upstream of the initiation codon to the end of the E6 stop codon. The sequences GAATTC and GGATCC underlined are *Eco*RI and *Bam*HI restriction sites that were introduced into the primers for cloning purposes. After restriction digestion,

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Abbreviations: ODN, oligodeoxyribonucleotide; SDS, sodium dodecyl sulfate.

the E6 DNA fragment was inserted into pGEM-3zf(+) vector at the same restriction sites. Using this construct, *in vitro* expression of the E6 gene was driven by the T7 promoter of pGEM-3zf(+).

Stability of ODN in T_NT Reticulocyte Lysate System—Fifteen nanograms of an antisense oligomer was 5′-end labeled using [γ-^{32}P]ATP (6,000 Ci/mmol) (NEN, Du Pont) and T4 polynucleotide kinase. It was then mixed with excess unlabeled oligomer (final concentration 62.5 μM) and 1 μl of the mixed oligomers was added to the T_NT reaction mixture (see below). The reaction mixture was incubated at 37°C for different periods of time. The integrity of ODN was analyzed by electrophoresis in a 20% polyacrylamide-7 M urea gel followed by autoradiography.

In Vitro Translation-Arrest Experiment—The standard reaction mixture of an *in vitro* translation-arrest assay for antisense ODNs was as follows: T_NT lysate 12.5 μl, T_NT reaction buffer 1 μl, amino acid mixture minus methionine 0.5 μl, T7 RNA polymerase 0.5 μl (the above reagents were supplied in the T_NT kit), RNasin ribonuclease inhibitor (40 U/μl) 0.5 μl, [^{35}S]methionine (1,175 Ci/mmol) 1 μl, RNase H (0.5 U/μl) 1 μl, pGEM-E6 DNA (1 μg/μl) 1 μl, antisense ODN (62.5 μM) 1 μl, nuclease-free water 6 μl. The reaction was incubated at 37°C for 50 min. An equal volume of 2×SDS loading buffer (2% SDS, 10% β-mercaptoethanol, 0.06 M Tris-Cl, pH 6.8, 20% glycerol, and 0.01% bromophenol blue) was added, followed by boiling for 8 min. The *de novo*-synthesized proteins were electrophoresed in a 10% SDS-polyacrylamide gel. The gel was dried and autoradiographed using a phosphor screen (Molecular Dynamics, CBL, CA).

RESULTS AND DISCUSSION

To rapidly screen for the most effective antisense ODNs in translation-arrest, a transcription-translation coupled reticulocyte lysate-based system was used. This system directly assayed the effects of antisense ODNs on translation products in a one-tube reaction. The HPV-16 E6 gene was chosen as a model to demonstrate the feasibility of this approach. An *in vitro* transcription plasmid of E6, pGEM-E6, was first constructed. Since the 5′-terminus of the E6 sequence was inserted into the *Eco*RI site of the pGEM-3zf(+) vector localized at a site only 5 nucleotides downstream from the transcription initiation site of the T7 promoter, the possible effects of the vector sequence on the secondary structure of the E6 transcript were minimized. Five 15-meric normal ODNs complementary to different regions of the E6 mRNA were synthesized (see Table I). The effectiveness of these sequences for translation-arrest was tested in a commercial preparation of transcription-translation coupled reticulocyte lysate-based system (the T_NT^{TM} system as described in "MATERIALS AND METHODS"). The stability of ODNs in this system was first determined by incubating a labeled ODN in the T_NT^{TM} reaction mixture and analyzing the mixture by gel electrophoresis. The result, shown in Fig. 1, indicates that most of the ODN was still intact after incubation for 50 min at 37°C.

To evaluate whether the T_NT^{TM} system was suitable for the monitoring of translation-arrest caused by antisense ODNs, the experiments shown in Fig. 2 were conducted.

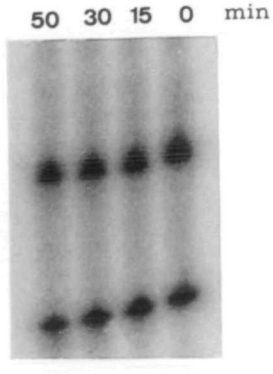

Fig 1. **Stability of ODN in the T_NT reticulocyte lysate system.** The upper bands represent labeled ODNs and the lower bands indicate unincorporated [γ-^{32}P]ATP.

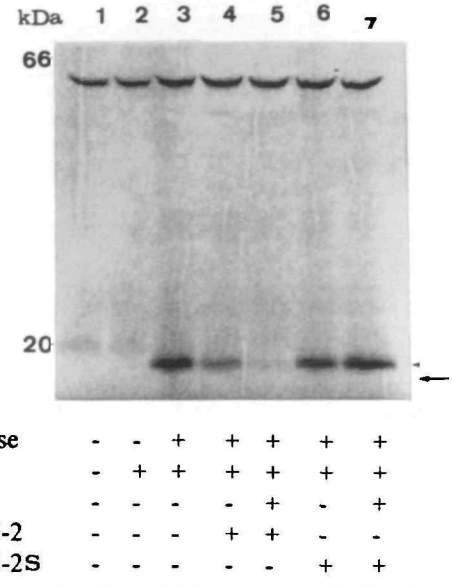

Fig. 2. ***In vitro* production of E6 protein (18 kDa) in the presence or absence of different reaction components.** The antisense ODN E6-2 and control ODN E6-2S were used in this experiment. "+" and "−" indicate the presence or absence of the reaction components, respectively. The arrow indicates the *de novo* synthesized E6 protein.

TABLE I. **Sequences of antisense oligomers complementary to different regions of E6 mRNA.**

ODNs	Sequence	Complementary to the region	Nucleotide position[a]	G+C content
E6-1	GGTCCTGAAACATTG	Initiation region	−2-13	0 47
E6-2	GTCGCTCCTGTGGGT	Coding region	11-25	0.66
E6-3	GTGGTAACTTTCTGG	Coding region	26-40	0.6
E6-4	GTCATATACCTCACG	Coding region	118-132	0.47
E6-5	CACCGACCCCTTATA	Coding region	381-395	0.53
E6-2S	GCTGCTCTCGTGGTG	Scrambled sequence of E6-2		0.66

[a] "+1" was the "A" of the initiation codon of the E6 mRNA.

The results indicate that the 18-kDa E6 protein was produced in the absence of the E6 antisense ODN (Fig. 2, lane 3). The addition of the antisense ODN, E6-2, did result in an inhibition of the E6 protein production (Fig. 2, lane 4). On the other hand, E6-2S, a scrambled sequence of E6-2, had no effect at all (Fig. 2, lane 6). RNase H, which digests the RNA sequence in an RNA-DNA duplex, has been reported to play an important role in translation-arrest induced by antisense ODNs (*15, 16*). Although RNase H usually exists in dividing cells, RNase H activities are generally low in commercial preparations of rabbit reticulocyte lysate (*15*). To mimic a more realistic *in vivo* situation, exogenous RNase H was added to the T_NT^{TM} reaction mixture. The addition of exogenous RNase H augmented the inhibitory effect, but only in the presence of an antisense ODN (see Fig. 2; compare lanes 4 and 5 with lanes 6 and 7). The results imply the formation of duplexes of ODN and target mRNA in this system. Besides the 18-kDa E6 protein, there was a 50-kDa protein produced even in the absence of the transcription plasmid and the T7 RNA polymerase (Fig. 2, lane 1). Apparently, the 50-kDa protein was system-borne, translated from mRNA originally existing in the reticulocyte lysate. However, this protein was undetectable if [^{35}S]cysteine was used instead of [^{35}S]methionine (data not shown).

Antisense ODNs complementary to different regions of the E6 mRNA (see Table I) were next tested for their effectiveness in translation-arrest by the modified T_NT^{TM} method described above. Antisense ODNs E6-2 and E6-4 suppressed protein production to 90% (Fig. 3A, lanes 4 and 6, respectively). However, E6-1, E6-3, and E6-5 ODNs were much less effective (Fig. 3, lanes 3, 5, and 7). The decreased effectiveness of E6-1, which was complementary to the translation initiation site of the E6 gene, was consistent with the *in vivo* study of Storey *et al.* (*17*). They found that antisense ODN complementary to the initiation region of E6 only caused a slight reduction in E6 synthesis in the cervical epithelial tumor cell line, CaSki. To prove that the inhibitory effects of the antisense ODNs were not caused by a suppression of transcription, the E6 mRNA synthesized in each reaction mixture containing different ODNs was monitored. There were no significant differences in the synthesis of E6 mRNA among these reactions, except for the control, in which no pGEM-E6 was added (see Fig. 3B).

To elucidate the dose-response relation and the specificity of the antisense ODNs, we added to the assay system different amounts of antisense ODN E6-2, and an unrelated plasmid, pGEM-DAg, which expressed the small delta antigen (24 kDa) of the hepatitis delta virus regulated by the T7 promoter. A dose-dependent decrement of E6 protein production was found (Fig. 4A, lanes 3 to 6 as indicated by the arrow). However, E6-2 had little effect on the expression of the delta antigen (Fig. 4A, lanes 3 to 6, arrowhead). Similarly, increasing amounts of E6-2S had no effect on the expression of the E6 protein (Fig. 4B, lanes 1 to 4). This result indicates a high sensitivity and specificity of antisense ODN in translation-arrest in this system. Furthermore, the inclusion of an unrelated plasmid, such as the small delta antigen-expressing plasmid, could be used as an internal control to normalize possible individual quantitative differences in the assay. Thus, percentage of inhibition resulting from a specific ODN could be calculated more accurately as follows:

$$100-\frac{\text{ratio of target protein to control protein in the presence of antisense ODN}}{\text{ratio of target protein to control protein in the absence of antisense ODN}}\times 100$$

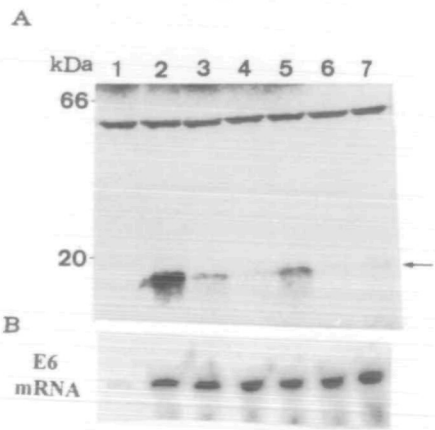

Fig. 3. **Screening of effective antisense ODNs by T_NT system.** A: The translation-arrest caused by different ODNs was monitored. Lane 1, no pGEM-E6, lane 2, no antisense ODN; lanes 3 to 7, reaction mixture containing 2 5 μM antisense ODN E6-1, E6-2, E6-3, E6-4, or E6-5, respectively Each reaction contained 20 U/ml RNase H. The arrow indicates the *de novo*-synthesized E6 protein. B: The *de novo*-synthesized E6 mRNA in each reaction was monitored In this experiment, however, [α-^{32}P]ATP was added instead of [^{35}S]methionine Each reaction product was then run into a 5% denaturing polyacrylamide gel followed by autoradiography.

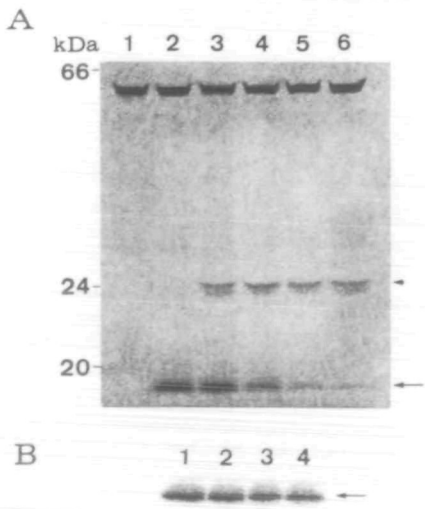

Fig. 4. **Specificity and dose-response relation of antisense ODN.** A: The reaction mixture contained 1 μg of pGEM-E6, 0.5 μg of pGEM-DAg, and different amounts of E6-2 (lanes 3-6). Lane 1, no DNA; lane 2, only pGEM-E6 was added; lane 3, no antisense ODN; lane 4, 0.3 μM E6-2; lane 5, 1.25 μM E6-2, lane 6, 2.5 μM E6-2. The arrowhead shows the delta antigen of hepatitis delta virus. The arrow indicates the E6 protein. B: The reaction mixture contained 1 μg of pGEM-E6 and different amounts of E6-2S. Lane 1, no antisense ODN; lane 2, 1.25 μM E6-2S; lane 3, 2.5 μM E6-2S; lane 4, 5 0 μM E6-2S.

TABLE II. **Synergistic inhibitory effect caused by antisense ODNs, E6-2, and E6-3.**[a]

	ODN added (μM)				
E6-2	1.25	–	2.5	–	1.25
E6-3	–	1.25	–	2.5	1.25
Inhibition (%)[b]	80	50	90	65	92

[a]E6-2 and E6-3 are in tandem array (see nucleotide positions in Table I). [b]The percentage inhibition shown is the mean value of duplicated experiments.

The sequences of E6-2 and E6-3 were in tandem array (see nucleotide positions in Table I). ODNs E6-2 and E6-3, when added simultaneously, cooperated to exert a synergistic effect on translation-arrest (see Table II). This observation may allow us to reduce the effective dosage of antisense ODNs.

In conclusion, the modified T_NT^{TM} reticulocyte lysate system is a rapid and convenient method for screening antisense ODNs effective for translation-arrest. This method is useful when antibodies against the target protein are not available or when there are no clear phenotypic characteristics suitable for monitoring.

REFERENCES

1. Van der Krol, A.R., Mol, J.N.M., and Stuitje, A.R. (1988) Modulation of eukaryotic gene expression by complementary RNA and DNA sequences. *BioTechniques* **6**, 958-976
2. Stein, C.A. and Cohen, J.S. (1988) Antisense oligodeoxynucleotides as inhibitors of gene expression: A review. *Cancer Res.* **48**, 2659-2668
3. Stein, C.A. and Cheng, Y.C. (1993) Antisense oligonucleotides as therapeutic agents—Is the bullet really magic? *Science* **261**, 1004-1012
4. McManaway, M.E., Neckers, L.M., Loke, S.L., al-Nasser, A.A., Redner, R.L., Shiramizu, B.T., Goldschmidts, W.L., Huber, B.E., Bhatia, K., and Magrath, I.T. (1990) Tumor-specific inhibition of lymphoma growth by an antisense oligodeoxynucleotide. *Lancet* **335**, 808-811
5. Kulka, M., Wachsman, M., Miura, S., Fishelevich, R., Miller, P.S., Ts'o, P.O.P., and Aurelian, L. (1993) Antiviral effect of oligo (nucleoside methylphosphonates) complementary to the herpes simplex virus type 1 immediate early mRNAs 4 and 5. *Antiviral Res.* **20**, 115-130
6. Wakita, T. and Wands, J.R. (1994) Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. *J. Biol. Chem.* **269**, 14205-14210
7. Lin, S.B., Huang, S.S., Choo, K.B., Chen, P.J., and Au, L.C. (1995) Inhibition of α-fetoprotein production in a hepatoma cell line by antisense oligonucleotide analogues. *J. Biochem.* **117**, 1100-1104
8. Boutorin, A.S., Gus'kova, L.V., Ivanova, E.M., Kobetz, N.D., Zarytova, V.F., Ryte, A.S., Yurchenko, L.V., and Vlassov, V.V. (1989) Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3′-terminus and their interaction with DNA within mammalian cells. *FEBS Lett.* **254**, 129-132
9. Goodchild, J., Carroll, E. 3d, and Greenberg, J.R. (1988) Inhibition of rabbit β-globin synthesis by complementary oligonucleotides: Identification of mRNA sites sensitive to inhibition. *Arch. Biochem. Biophys.* **263**, 401-409
10. Chiang, M.Y., Chan, H., Zounes, M.A., Freier, S.M., Lima, W.F., and Bennett, C.F. (1991) Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.* **266**, 18162-18171
11. Woolf, T.M., Melton, D.A., and Jennings, C.G.B. (1992) Specificity of antisense oligonucleotides *in vivo*. *Proc. Natl. Acad. Sci. USA* **89**, 7305-7309
12. Roth, G., Curiel, T., and Lacy, J. (1994) Epstein-Barr viral nuclear antigen 1 antisense oligodeoxynucleotide inhibits proliferation of Epstein-Barr virus-immortalized B cells. *Blood* **84**, 582-587
13. Boiziau, C., Thuong, N.T., and Toulme, J.J. (1992) Mechanism of the inhibition of reverse transcription by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* **89**, 768-772
14. Minshull, J. and Hunt, T. (1986) The use of single-stranded DNA and RNase H to promote quantitative 'hybrid arrest of translation' of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. *Nucleic Acids Res.* **14**, 6433-6451
15. Walder, R.Y. and Walder, J.A. (1988) Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* **85**, 5011-5015
16. Larrouy, B., Blonski, C., Boiziau, C., Stuer, M., Moreau, S., Shire, D., and Toulme, J.J. (1992) RNase H-mediated inhibition of translation by antisense oligodeoxynucleotides: Use of backbone modification to improve specificity. *Gene* **121**, 189-194
17. Storey, A., Oates, D., Banks, L., Crawford, L., and Crook, T. (1991) Anti-sense phosphothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16. *Nucleic Acids Res.* **19**, 4109-4114