J. Microbiol. Biotechnol. (2006), **16**(3), 355–359

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Enhanced *In Vitro* Protein Synthesis Through Optimal Design of PCR Primers

AHN, JIN-HO[1], JEONG-MI SON[2], MI-YEON HWANG[2], TAE-WAN KIM[2], CHANG-KIL PARK[3], CHA-YONG CHOI[1,2], AND DONG-MYUNG KIM[3]*

[1]*Interdisciplinary Program for Biochemical Engineering and Biotechnology, College of Engineering, Seoul National University, Seoul 151-742, Korea*
[2]*School of Chemical and Biological Engineering, College of Engineering, Seoul National University, Seoul 151-742, Korea*
[3]*Department of Fine Chemical Engineering and Chemistry, Chungnam National University, Daejeon 305-764, Korea*

Received: May 11, 2005
Accepted: July 30, 2005

Abstract The functional stability of mRNA is one of the crucial factors affecting the efficiency of *in vitro* translation. As the rapid degradation of mRNA in the cell extract (S30 extract) causes early termination of the translational reactions, extending the mRNA half-life will improve the productivity of the *in vitro* protein synthesis. Thus, a simple PCR-based method is introduced to increase the stability of mRNA in an S30 extract. The target genes are PCR-amplified with primers designed to make the ends of the transcribed mRNA molecule anneal to each other. When compared with normal mRNA, the mRNA with the annealing sequences resulted in an approximately 2-fold increase of protein synthesis in an *in vitro* translation reaction. In addition, sequential transcription and translation reactions in a single tube enabled direct protein expression from the PCR-amplified genes without any separate purification of the mRNA.

Key words: *In vitro* protein synthesis, *in vitro* translation, coupled transcription/translation, linked transcription/translation, polymerase chain reaction, chloramphenicol acetyltransferase, mRNA stability

With the rapid progress of various genome sequencing projects, a huge number of cDNAs and ORFs have already been cloned for protein expression. However, the use of *in vivo* bacterial expression for the preparation of recombinant proteins has a limited throughput because of the complicated steps of cloning, cell growth, and product isolation and purification. As a result, relying on conventional *in vivo* expression technology is augmenting the discrepancy between the amounts of genetic information available and their translation products, thereby increasing the demand for more parallel and rapid methodologies for translating the fast-accumulating genetic information into protein molecules [1, 2, 6].

In vitro protein synthesis has thus emerged as an effective alternative to overcome the limitations of conventional *in vivo* gene expression technology [7], since *in vitro* protein synthesis does not depend on cell growth, and proteins can be prepared with a minimal process time and laboratory set-ups. However, the current protocols for *in vitro* protein synthesis rely mostly on plasmid templates, meaning that cells still need to be grown to clone and amplify plasmids [12]. Yet, such time- and labor-intensive steps for plasmid preparation can be avoided when using PCR-amplified DNAs as the protein synthesis template. Thus, with the rapid preparation of expression templates, PCR-based *in vitro* protein synthesis can provide an excellent platform for providing protein molecules to various post-genomic applications. Nonetheless, practical application of this strategy is only possible when sufficient amounts of proteins are produced. Although several reports already exist that describe successful expression of proteins from PCR-amplified templates [4, 14], the productivity of *in vitro* synthesis is generally very low when PCR products are used as the expression templates. Accordingly, the current study attempted to increase the efficiency of *in vitro* protein synthesis from PCR-amplified genes to provide a high-throughput method for protein preparation.

Maintaining the stability of the functional integrity of mRNA is one of the essential factors in the efficient generation of proteins in an *in vitro* protein synthesis system, and many efforts have been made to enhance the stability of mRNA during protein synthesis. Reported methods include

*Corresponding author
Phone: 82-42-821-5899; Fax: 82-42-823-7692;
E-mail: dmkim@cnu.ac.kr

the chemical modification of mRNA molecules, making them less susceptible to nucleolytic digestion. For example, phosphorothioate mRNA has been shown to be more resistant to exonuclease activities [16, 17], and Verheijen *et al.* [18] reported that the chemical modification of the 2'-hydroxyl group of mRNA substantially increased its stability. However, despite the substantial resistance to exonucleolytic digestion provided by these approaches, the procedures are highly expensive and complicated, and their inclusion with *in vitro* protein synthesis procedures is not very straightforward. Therefore, the present study presents a method for stabilizing mRNA through the addition of stabilizing sequence elements, which is compatible with simple PCR techniques.

Since all identified bacterial RNases only exhibit 3'→5' exonuclease activity against single-stranded RNA, PCR primers are designed that provide the amplified target genes with complementary 5'- and 3'-flanking sequences in anticipation that the ends of the transcribed mRNA will then anneal to each other to form a double-stranded stem structure. As expected, the introduction of the complementary sequences led to a more than 2-fold increase of protein synthesis in an *in vitro* translation reaction. Furthermore, the *in vitro* protein synthesis procedures were also simplified by sequential transcription and translation reactions without the separate preparation of purified mRNA. In such a "linked" transcription and translation reaction, the presence of the complementary sequences also significantly increased the final amount of synthesized proteins.

MATERIALS AND METHODS

Materials

The ATP, GTP, UTP, CTP, creatine phosphate, and *E. coli* total tRNA mixture were all purchased from Roche Applied Science (Indianapolis, IN, U.S.A.), whereas the L-[U-^{14}C]leucine (11.9 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). The other reagents were purchased from Sigma, and the T7 RNA polymerase and S30 extract were prepared as previously described [3, 8].

Preparation of DNA and RNA

The coding region of the bacterial chloramphenicol acetyltransferase was PCR-amplified from the plasmid pK7CAT using the sense and antisense primers given in Table 1. The PCR products were purified using a PCR-clean-up kit (Genenmed, Seoul, Korea) prior to use in the protein expression. The amount of each PCR product was determined using a spectrophotometer and agarose gel electrophoresis [5, 9]. All the mRNAs used in this study were prepared using a commercial *in vitro* transcription kit from Promega (Madison, WI, U.S.A.). The secondary structure of the transcribed mRNA was predicted using RNAdraw (v1.1) software [11].

In Vitro Translation Reaction

The standard reaction mixture (15 µl) for the *in vitro* translation of the mRNA consisted of the following components: 57 mM Hepes/KOH pH 8.2, 1.2 mM ATP, 0.85 mM GTP, 1.7 mM dithiothreitol, 80 mM ammonium acetate, 0.17 mg/ml *E. coli* total tRNA mixture (from strain MRE 600), 34 µg/ml l-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 0.5 mM each of 20 amino acids, 0.01 µM [^{14}C]leucine, 0.3 U/ml creatine kinase, 67 mM creatine phosphate, 1 µg of purified mRNA, and 4 µl of S30 extract. The protein synthesis was conducted by incubating the reaction mixture at 37°C for 3 h, and then the synthesized protein was quantified by measuring the TCA-precipitable radioactivities, as described earlier [8].

Coupled Transcription and Translation Reaction

In the experiments for the coupled transcription and translation, plasmids or PCR-amplified genes were used as templates in place of the purified mRNA. In addition to the reaction components for the *in vitro* translation described above, the reaction mixture also contained 33 µg/ml T7 RNA polymerase and 0.85 mM each of UTP and CTP to support the transcription of the DNA templates.

Linked Transcription and Translation Reaction

In certain experiments, the transcription and translation reactions were conducted sequentially. First, the plasmid template or PCR-amplified DNA was incubated in a reaction mixture containing the same components as in the coupled transcription and translation reaction except for the S30 extract. After 2 h of incubation for transcription, the reaction was supplemented with a 0.27 volume of S30 extract to initiate the translation reaction and then incubated again for 3 h.

Table 1. Oligonucleotide primers used in the present study.

Oligonucleotide	Orientation	Sequence (5' to 3')
T7P -15UP	Sense	TCGATCCCGCGAAATTAATACGACTCACTATAGG
Control	Antisense	CAGCTTCCTTTCGGGCTTTGTTA
15A	Antisense	TTTTTTTTTTTTTTTCAGCTTCCTTTCGGGCTTTGTTA
15G	Antisense	CCCCCCCCCCCCCCCCAGCTTCCTTTCGGGCTTTGTTA
5'-3'C	Antisense	GGGAGACCACAACGGCAGCTTCCTTTCGGGCTTTGTTA

Analysis of mRNA

The samples were isolated, extracted twice with phenol: chloroform:isoamylalcohol (25:24:1), and precipitated with three volumes of ethanol. The pellet was then vacuum dried and resuspended in nuclease-free water. RNase-free DNase was added to the resuspended RNA and the mixture incubated for 15 min at 37°C. The DNase extraction was performed with phenol/chloroform/isoamylalcohol and re-extraction with chloroform/isoamylalcohol (24:1), followed by precipitation with three volumes of ethanol. The RNA was then vacuum dried and resuspended in water, and the samples analyzed by denaturing electrophoresis on a 2% formaldehyde agarose gel and the RNA molecules stained with ethidium bromide.

Measurement of the Enzymatic Activity of CAT

The enzymatic activity of the synthesized chloramphenicol acetyltransferase (CAT) was determined by the spectrophotometric procedures described by Shaw [15]. After diluting a sample 1,000-fold with water, 2 μl of the diluted sample was added to a 96-well plate containing 178 μl of a prewarmed assay mixture (100 mM Tris-Cl, pH 7.8; 0.1 mM acetyl-CoA; 0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid [DTNB]; 0.1 mM chloramphenicol), and then incubated at 37°C for 30 min.

RESULTS AND DISCUSSION

To date, two major exonucleases [ribonuclease (RNase II) and polynucleotide phosphorylase (PNPase)] have been identified in bacteria, both working in the 3' to 5' direction [15]. These enzymes require a single-stranded extension of RNA for the initiation of exonucleolytic degradation and cannot degrade RNA that contains a tightly folded 3'-terminus. Thus, to reduce the exonuclease-catalyzed degradation of mRNA during *in vitro* protein synthesis, PCR primers were designed to make both termini of the transcribed mRNA anneal to each other (Table 1). Specifically, the antisense primer added 15 nucleotides (hereafter termed "the annealing sequence") to the 3' end of the CAT sequence, where the additional sequence was complementary to the 5' untranslated region (refer to Fig. 1). Thereafter, the PCR-amplified DNA with or without the annealing sequence was transcribed *in vitro*, and then the purified mRNA was translated *in vitro*, as described in Materials and Methods. As shown in Fig. 2, the mRNA containing the annealing sequence facilitated a substantially higher translation

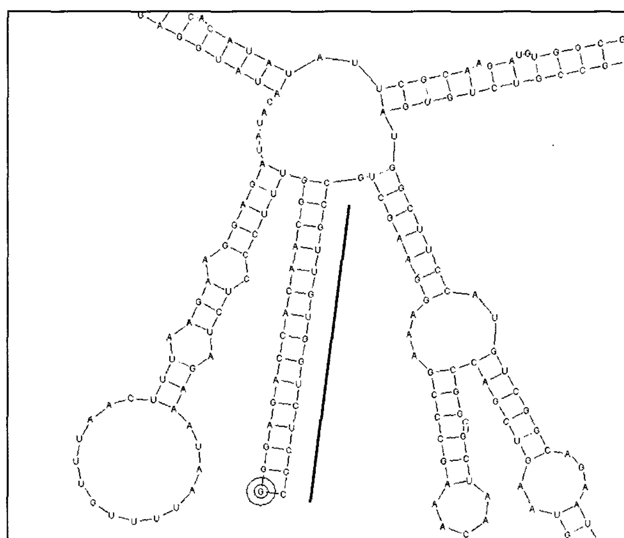

Fig. 1. Secondary mRNA structure of CAT transcript calculated using the RNAdraw (v1.1) program.

The 5'-end of the mRNA is indicated by a circle, whereas the straight line indicates the predicted 5'-3' annealing region.

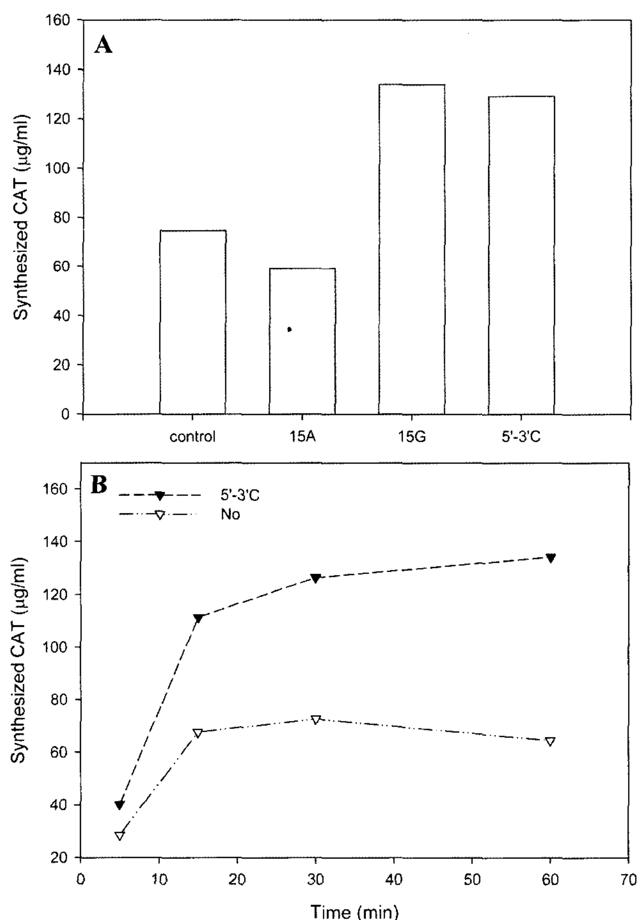

Fig. 2. Effect of a 3'-annealing sequence on the efficiency of *in vitro* translation; final amount of translated protein (**A**) and time course of *in vitro* translation (**B**).

One μg of mRNA was incubated for 3 h in a reaction mixture for *in vitro* translation at 37°C. Fifteen μl samples were withdrawn at the given time points and the TCA-insoluble radioactivity was measured as described in Materials and Methods.

efficiency than the control mRNA. A time course analysis of the protein synthesis demonstrated that the elongation rate and longevity of the translation reaction were both enhanced when using the self-annealing mRNA. As the same amounts of mRNA were used in all the experiments, this result indicates that the efficiency of the *in vitro* translation was enhanced by the introduction of the annealing sequence, most likely through the complementary base-pairing of the 5' and 3' ends.

Whereas an *in vitro* translation reaction requires a separate purification of the mRNA, protein synthesis can be directed by DNA templates in a coupled transcription/translation reaction. Since the proteins are synthesized by the translation of *in situ* generated mRNA molecules, a coupled transcription/translation can provide more convenience and speed. Therefore, an attempt was also made to directly express the PCR products in a coupled transcription/translation reaction. The PCR-amplified DNA was incubated in a reaction mixture containing CTP, UTP, and T7 RNA polymerase, in addition to the reaction mixture for the *in vitro* translation (see Materials and Methods). As shown in Fig. 3, the coupled transcription/translation reaction with the PCR-amplified CAT sequence yielded approximately 60 μg/ml of protein. However, contrary to the results of the *in vitro* translation experiments, the presence of the annealing sequence did not significantly increase the protein synthesis. During the coupled transcription/translation reaction, ribosomes started to be loaded onto the mRNA molecules before the completion of the transcription. Therefore, it would appear that the loaded ribosomes interfered with the base-pairing of the 5'- and 3'-termini of the mRNA, thereby eliminating the stabilizing effect of the complementary sequences in the coupled transcription/translation reaction.

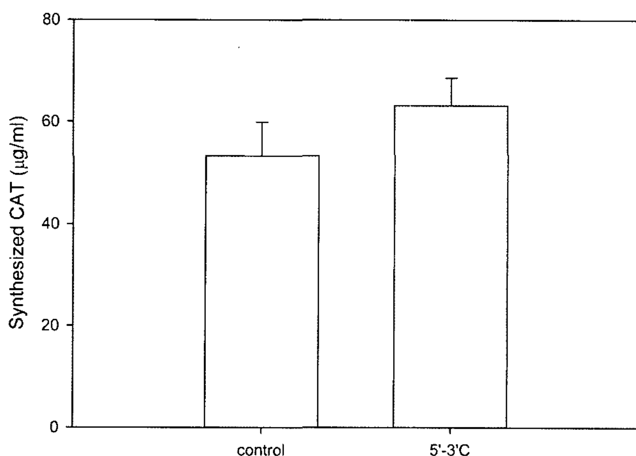

Fig. 3. Effect of the 3'-annealing sequence on the efficiency of coupled transcription/translation.
The reaction mixture was incubated for 3 h at 37°C. Fifteen μl samples were withdrawn and the TCA-insoluble radioactivity measured.

This problem was solved when conducting the transcription and translation reactions sequentially in the same reaction tube. After incubating the reaction mixture for the coupled transcription/translation without the S30 extract (meaning only the transcription reaction took place) for 2 h, the reaction tube was supplied with the extract and incubated for a further 3 h. In such a "linked" transcription and translation reaction that facilitated the self-annealing of the mRNA molecules, the presence of the annealing sequence likely enhanced the protein synthesis. The final amount of active CAT

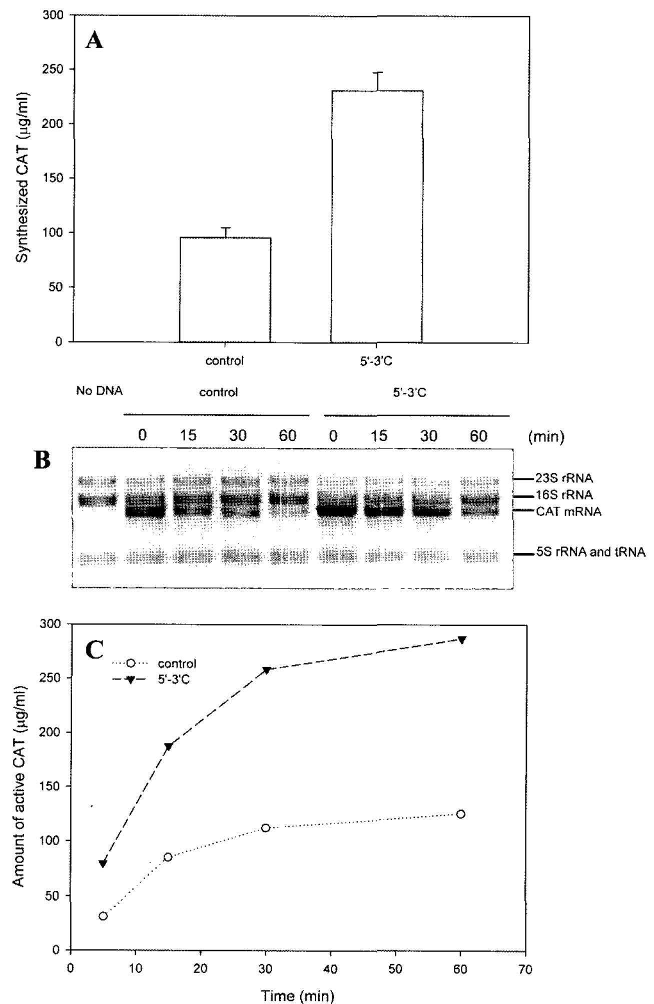

Fig. 4. Effect of the 3'-annealing sequence on translational efficiency in a "linked" transcription/translation system.
After incubating the reaction mixture for the coupled transcription/translation without the S30 extract for 2 h, the reaction tube was supplied with the extract and incubated for a further 3 h. **A.** Fifteen μl samples were withdrawn and the TCA-precipitable radioactivity measured as described. **B.** Twenty μl samples were withdrawn from the incubations at the indicated time point for an electrophoretic analysis of the mRNA. The isolated samples were analyzed by denaturing electrophoresis on a 2% formaldehyde agarose gel and the RNA molecules stained with ethidium bromide. **C.** The time-course increase of CAT activity was monitored following the protocols of Shaw, as described in Materials and Methods.

synthesized from the PCR-amplified DNA reached 280 μg/ml (Fig. 4).

Yoshizawa *et al.* [19] previously reported that the mRNA stability during *in vitro* protein synthesis is enhanced when using mini-hairpin DNA. Yet, this method has serious limitations, as an excessive amount of mini-hairpin DNA is required to completely "block" the mRNA molecules, affecting its economical efficiency. In addition, as denaturation and annealing steps are required prior to the translation reaction, the cell-free synthesis procedures are quite complicated. More recently, Lee and Cohen [10] reported that the addition of a poly(G) tail to the 3'-termini of the mRNA substantially enhances its nucleolytic stability. The present study also repeated such experiments, as shown in Fig. 3, and indeed the presence of a poly(G) tail led to a substantial increase in CAT synthesis. In contrast, the addition of poly(A) did not affect the protein synthesis, implying nucleotide-specific effect from the 3'-tails. However, the enhancement resulting from the poly(G) tail did not exceed the method proposed in the present study.

Furthermore, it is believed that the use of an annealing sequence can find more general applications, due to several technical reasons. For example, when the target DNA contains patches of G-rich sequences, the PCR-amplification of poly(G)-flanked DNA can be problematic owing to the nonspecific annealing of the primers, whereas the use of an annealing sequence can solve this problem.

To further improve the productivity of PCR-based cell-free protein synthesis, various terminal sequences that can provide more robust resistance to nuclease attack are currently under investigation. Consequently, it is expected that the results will make a significant contribution to the establishment of a versatile *in vitro* protein synthesis system, while also offering a fundamental insight into the protein synthesis mechanism.

REFERENCES

1. Betton, J. M. 2004. High throughput cloning and expression strategies for protein production. *Biochimie* **86:** 601–605.
2. Braun, P. and J. LaBaer. 2003. High throughput protein production for functional proteomics. *Trends Biotechnol.* **21:** 383–388.
3. Davanloo, P., A. H. Rosenberg, J. J. Dunn, and F. W. Studier. 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **81:** 2035–2039.
4. DiTursi, M. K. W., J. H. Chung, M. R. Newman, and J. S. Dordick. 2004. Simultaneous *in vitro* protein synthesis using solid-phase DNA template. *Biotechnol. Prog.* **20:** 1705–1709.
5. Heo, M. S., J. H. Kim, S. H. Park, G. J. Woo, and H. Y. Kim. 2004. Detection of genetically modified maize by multiplex PCR method. *J. Microbiol. Biotechnol.* **14:** 1150–1156.
6. Kang, T. J., H. K. Song, J. H. Ahn, C. Y. Choi, and H. Joo. 2003. Optimization of programmed suppression in a cell-free protein synthesis system with unnatural amino acid S-(2-nitrobenzyl)cysteine. *J. Microbiol. Biotechnol.* **13:** 344–347.
7. Katzen, F., G. Chang, and W. Kudlicki. 2005. The past, present and future of cell-free protein synthesis. *Trends Biotechnol.* **23:** 150–156.
8. Kim, D. M., T. Kigawa, C. Y. Choi, and S. Yokoyama. 1996. A highly efficient cell-free protein synthesis system from *Escherichia coli*. *Eur. J. Biochem.* **239:** 881–886.
9. Kim, T. K., H. D. Shin, M. C. Seo, J. N. Lee, and Y. H. Lee. 2003. Molecular structure of PCR cloned PHA synthase genes of *Pseudomonas putida* KT2440 and its utilization for medium-chain length polyhydroxyalkanoate production. *J. Microbiol. Biotechnol.* **13:** 182–190.
10. Lee, K. and S. N. Cohen. 2001. Effects of 3′ terminus modifications on mRNA functional decay during *in vitro* protein synthesis. *J. Biol. Chem.* **276:** 23268–23274.
11. Matzura, O. and A. Wennborg. 1996. RNAdraw: An integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Comput. Appl. Biosci.* **12:** 247–249.
12. Mergulhao, F. J. M., G. A. Monterio, J. M. S. Cabral, and M. A. Taipa. 2004. Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* **14:** 1–14.
13. Nierlich, D. P. and G. J. Murakawa. 1996. The decay of bacterial messenger RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **52:** 153–216.
14. Rungpragayphan, S., H. Nakano, and T. Yamane. 2003. PCR-linked *in vitro* expression: A novel system for high-throughput construction and screening of protein libraries. *FEBS Lett.* **540:** 147–150.
15. Shaw, W. V. 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Meth. Enzymol.* **43:** 737–755.
16. Tang, J. Y., J. Temsamani, and S. Agrawal. 1993. Self-stabilized antisense oligodeoxynucleotide phosphorothioates: Properties and anti-HIV activity. *Nucleic Acids Res.* **21:** 2729–2735.
17. Tohda, H., N. Chikazumi, T. Ueda, K. Nishikawa, and K. Watanabe. 1994. Efficient expression of *E. coli* dihydrofolate reductase gene by an *in vitro* translation system using phosphorothioate mRNA. *J. Biotechnol.* **34:** 61–69.
18. Verheijen, J. C., A. M. van Roon, N. J. Meeuwenoord, H. R. Stuivenberg, S. F. Bayly, L. Chen, G. A. van der Marel, P. F. Torrence, *et al.* 2000. Incorporation of a 4-hydroxy-N-acetylprolinol nucleotide analogue improves the 3'-exonuclease stability of 2'-5'-oligoadenylate-antisense conjugates. *Bioorg. Med. Chem. Lett.* **10:** 801–804.
19. Yoshizawa, S., T. Ueda, Y. Ishido, K. Miura, K. Watanabe, and I. Hirao. 1994. Nuclease resistance of an extraordinary thermostable mini-hairpin DNA fragment, d(GCGAAGG), and its application to *in vitro* protein synthesis. *Nucleic Acids Res.* **22:** 2217–2221.