Phosphorothioate-containing RNAs show mRNA activity in the prokaryotic translation systems in vitro

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ABSTRACT
Phosphorothioate-containing RNAs were generated by transcription of colphage T7 DNA using the Sp diastereomers of ribonucleoside 5'-O-(1-thiotriphosphates) and T7 RNA polymerase. RNAs in which a single nucleotide was substituted by the corresponding nucleoside phosphorothioate functioned as mRNA in the cell-free translation systems prepared from Escherichia coli and from an extreme thermophilic bacterium, Thermus thermophilus. This substitution increased the efficiency of protein synthesis by stabilizing the mRNAs in these systems. As the proportion of substituted nucleotides was increased, their mRNA activity was decreased accordingly. As judged from the analysis by SDS-polyacrylamide gel-electrophoresis, the proteins synthesized using phosphorothioate-containing mRNAs as template were identical to those obtained with unsubstituted mRNAs. However, larger proteins which were barely detectable when unsubstituted mRNA was used were well represented when phosphorothioate-RNA was used instead. The advantages in using the phosphorothioate-mRNAs in the in vitro translation systems are discussed.

INTRODUCTION
One of the problems encountered in using translational systems reconstructed in vitro, is the instability of the mRNA. As mRNA is easily degraded by nucleases the translational efficiency is very often not satisfactory for preparing proteins in amounts sufficient for structural and functional analysis as well as their utilization. Thus, the stabilization of mRNA against degradation would greatly increase the usefulness of cell-free translation systems.

Phosphorothioate-containing nucleic acids in which one of the oxygens in the internucleotidic linkage is replaced by sulfur have found considerable application in molecular biology (1). The phosphorothioate groups can easily be introduced into DNA and RNA as the Sp diastereomers of dNTPaS and NTPaS, respectively, are readily accepted as substrates by DNA as well as RNA polymerases (2). Several reports have described that the presence of these phosphorothioate groups increases the stability of oligodeoxynucleotides, DNA and RNA (2). In particular it has been shown that phosphorothioate-containing polyribonucleotides are considerably more stable against RNases and other nucleases than the unmodified polymers and that this effect can result in an enhanced biological activity (3).

It stands to reason, therefore, to explore the possibility of incorporating phosphorothioate groups into mRNA and to examine the consequences of their presence with respect to stabilization against nucleases and to template properties in translational systems. Moreover our previous studies have suggested the usefulness of protein synthesis system of T. thermophilus for such a study (4,5). Thus, we decided to examine the effect of phosphorothioate substitution in the T. thermophilus and E. coli translation systems.

MATERIALS AND METHODS
Materials
T7 RNA polymerase was purified from its overproducing strain of E. coli kindly provided by Dr. J. Studier, according to the method reported previously (6,7). The Sp diastereomers of the four nucleoside 5'-O-(1-thiotriphosphates) were synthesized chemically as described previously (8), or purchased from Amersham, Japan. Radioactive amino acids, [U-14C]-amino acids (protein hydrolysate, mixture of 16 amino acids except for asparagine, glutamine, tryptophan cysteine; 2.11 GBq/milliatom) and [35S] methionine (55.5 TBq/mmol), were purchased from Amersham, Japan. Pyruvate kinase from Bacillus stearothermophilus was a gift from Dr. H. Sakai, the University of Tokyo. Sephadex G-50 was purchased from Pharmacia, RNase-free DNase I, NTPs, pyruvate kinase from rabbit muscle and phosphoenolpyruvate from Boehringer Mannheim, T7 coliphage DNA, spermidine, spermine and phosphodiesterase from Sigma, RNase inhibitor from human placenta and agarose for gel electrophoresis from Takara, cold amino acids from Wako Chemicals, and nucleoside P, from Seikagaku Kogyo and acrylamide for gel electrophoresis from Bio-Rad.

The components for the cell-free protein synthesis system
Components used for the in vitro translation were prepared from E. coli A 19 (9) or T. thermophilus HB 27 (10) by the same procedures as reported previously (4).

The tRNA fraction was prepared according to the Zubay's method (11), as reported previously (4).
Enzymatic synthesis of RNAs by T7 RNA polymerase

The enzymatic synthesis of the phosphorothioate-containing RNAs was performed according to the method by Sampson and Uhlenbeck (12) with a slight modification, where T7 coliphage DNA was used as a DNA template. The reaction mixture (total volume, 50 μl) contained 40 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1 mM spermidine, 100 units of RNase inhibitor of human placenta, 1 μg of T7 phage DNA and 2 mM each of nucleoside 5'-triphosphates or any of the Sp-NTPs. The reaction was initiated by addition of 1 μg of T7 RNA polymerase, and was carried out at 42°C for 60 minutes. It was terminated by addition of 5 μl of 500 mM EDTA (pH 8.0). The template DNA was removed by treatment with RNase-free DNase I. The resulting solution was applied onto a Sephadex G-50 gel-filtration column, and the fractions in the void volume were collected. It was stored at −20°C until use. By these procedures, RNAs could be separated from the template DNA as well as the free nucleotides. The synthesized RNAs were analyzed by 1% agarose gel electrophoresis and staining with ethidium bromide to determine their chain lengths.

In vitro translation systems

The reaction mixture used for T. thermophilus translation assays (total volume 25 μl) consisted of 50 mM Tris-HCl (pH 7.5), 60 mM NH₄Cl, 5 mM Mg(CH₂COO)₂, 6 mM 2-mercaptoethanol, 3 mM spermine, 1 mM ATP, 0.2 mM GTP, 5 mM phosphoenolpyruvate, 0.08 μg pyruvate kinase of Bacillus stearothermophilus, 8 μg RNA polymerase, 100 μg protein equivalent of S100 fraction and 0.5 μg S30 fraction. The S30 fraction was preincubated at 65°C for 7 min in the presence of 1 mM ATP, 0.2 mM GTP, 5 mM phosphoenolpyruvate and 0.08 μg pyruvate kinase for 35S amino acids. The reaction was started by the addition of 2 μg of T7 RNA polymerase, and was terminated by addition of 2 μg of T7 RNA polymerase, and was carried out at 42°C for 60 minutes. It was terminated by addition of 5 μl of 500 mM EDTA (pH 8.0). The template DNA was removed by treatment with RNase-free DNase I. The resulting solution was applied onto a Sephadex G-50 gel-filtration column, and the fractions in the void volume were collected. It was stored at −20°C until use. By these procedures, RNAs could be separated from the template DNA as well as the free nucleotides. The synthesized RNAs were analyzed by 1% agarose gel electrophoresis and staining with ethidium bromide to determine their chain lengths.

Analysis of the synthesized proteins

The reaction mixtures used for the translation assays were analyzed by 12% SDS polyacrylamide gel electrophoresis under reduced conditions (13).

RESULTS

Enzymatic synthesis of phosphorothioate-containing RNAs

T7 coliphage DNA consists of 39,936 base-pairs with 17 promoters and one termination site for T7 RNA polymerase. The length of RNAs synthesized in vitro by T7 RNA polymerase is

![Transcriptional efficiency (%)](attachment:transcriptional_efficiency.png)

Fig. 1. Relative efficiencies of the synthesis of RNAs for transcription of T7 DNA with T7 RNA polymerase using NTPs and NTPs as substrates. The amounts of RNAs, purified by gel filtration as described in Materials and Methods, were estimated by measuring the absorbance at 260 nm. The efficiency of each RNA product was calculated assuming a 100% efficiency for the unsubstituted RNAs. The substituted nucleotides are shown under each bar. N represents unsubstituted RNAs.

![Electrophoretic analysis of phosphorothioate RNAs](attachment:electrophoretic_analysis.png)

Fig. 2. Electrophoretic analysis of phosphorothioate RNAs. 3 μg each of unsubstituted (N), single phosphorothioate-substituted (A, U, G, C) and four phosphorothioate-substituted (S) RNAs were analyzed by 1% agarose gel-electrophoresis and stained with ethidium bromide. Asterisks indicate the bands corresponding to the RNAs predicted from the sequence of T7 DNA (13). RNA length was estimated by using an RNA marker (0.24-9.5 kb RNA Ladder purchased from BRL).
expected to range between 500 and 36,000 nucleotides (14). Transcription of such long RNA molecules by T7 RNA polymerase using NTPαS as substrates has not been reported so far. Thus, the transcriptional efficiency of these RNA molecules was first examined.

Fig. 1 shows the efficiency with which variously substituted RNAs are synthesized from T7 DNA by T7 RNA polymerase in a period of 1 hour, estimated by measuring the amount of RNA products purified by Sephadex G-50 column chromatography. When only one NTP was replaced by the corresponding NTPαS, RNAs were synthesized almost with an equal efficiency as unsubstituted RNAs. With pyrimidine nucleoside phosphorothioates as substrates the amounts of RNA produced were about 96% of that with all NTPs as substrates, whereas with the purine nucleoside phosphorothioates it was 84 - 91%. When the four NTPs were substituted by the corresponding NTPαS, the efficiency gradually decreased to 33%, the value observed when all four NTPs were replaced by NTPαS. In these reactions, 16 - 48 ng of the substituted RNAs could be obtained in 50 µl reaction mixtures, sufficient amounts for examining their mRNA activities in the in vitro translation systems.

Analysis of the phosphorothioates incorporated into RNAs

T7 DNA dependent RNA polymerase incorporates the Sp-NTPαS with inversion of configuration at phosphorus, thus yielding an internucleotidic phosphorothioate linkage of the Rp configuration (1,15). Snake venom phosphodiesterase cleaves the Rp phosphorothioate linkage (1). As expected, digestion of aliquots of the phosphorothioate transcripts with this enzyme generated the nucleoside 5'-phosphorothioates in combination with the nucleoside 5'-phosphates as detected by HPLC analysis (data not shown). This analysis confirms that the phosphorothioates have indeed been incorporated.

Characterization of the synthesized RNAs

The chain-lengths of the phosphorothioate RNAs were analyzed by agarose gel electrophoresis. They were in the range from several hundred to ten thousand nucleotides in all cases as shown in Fig. 2. Their electrophoretic patterns were similar to that of the unsubstituted RNAs. In addition, RNA bands corresponding to the sizes predicted from the DNA sequence of the T7 coliphage (14) (marked by asterisks) were detected in all cases. This analysis shows that neither the incorporation process was prematurely terminated nor were there any unusual pause sites.

Protein synthesis directed by the phosphorothioate RNAs

The mRNA activity of phosphorothioate RNAs was examined in the in vitro translation systems of T. thermophilus and E. coli. The results obtained with phosphorothioate RNA in which only one nucleotide was replaced by a phosphorothioate are reported in Fig. 3 a and b. In the T. thermophilus system each of the four phosphorothioate RNAs was used more efficiently as template than the unsubstituted RNA.

However, there are differences. Phosphorothioate substitution in pyrimidine nucleotides enhanced mRNA activity by a factor of approximately two, whereas that of purine nucleotides showed only a slight enhancement. The situation was quite different in the E. coli system, where the phosphorothioate substitutions of guanosine and uridine showed a slight enhancement of mRNA activities but that of adenosine had no effect, and that of cytosine resulted in a somewhat lower activity than the unsubstituted RNAs.

When two, three and all four nucleotides in RNAs were replaced by phosphorothioate ribonucleotides, the mRNA activity was scarcely detected using [14C] labeled amino acids. To increase the sensitivity of the system [35S] methionine was used for the assay. Incorporation of [35S] radioactivity was indeed detectable at higher concentration of RNA even when all four nucleotides were replaced by phosphorothioates (data not shown).

Stability of phosphorothioate RNAs

The stabilities of RNAs in both translation systems were determined. After preincubation of the RNAs in the translation reaction mixture without amino acids for various time periods,
Fig. 4. Relative stabilities of RNAs in the translation system of *T. thermophilus* (a) and *E. coli* (b). The RNAs were preincubated in the reaction mixture without amino acids for the times incubated, and following the addition of amino acids to the reaction mixture, the incorporation of radioactivity into hot TCA-insoluble material was determined after reaction for 7 min. The symbols are the same as in Fig. 3.

The protein synthesis reaction was initiated by addition of amino acids. The amount of protein synthesis was determined after reaction for 7 minutes. The decrease in incorporation of [14C] amino acid mixture was taken as a measure for the degradation of the RNAs (Fig. 4).

Approximately 80% of the unsubstituted RNAs were degraded during the first 5 min of preincubation in the *T. thermophilus* system, but only between 5 and 45% of the RNAs with a single nucleotide substitution depending on the nucleotide phosphorothioate present. The relative remaining activity was the highest in the RNA with G-substitution and the lowest with U-substitution in the order G > C > A > U. After preincubation these differences disappeared but the phosphorothioate RNAs were still more active than the unsubstituted RNAs.

In the *E. coli* systems, the stabilizing effect of the phosphorothioate substitution was not as pronounced. The relative remaining activity of the unsubstituted RNA was 33% in the 5 min preincubation, whereas those of the single substituted RNAs were between 33—62% in the order of A > G > C > U, the U-substitution showing no stabilization effect in this systems.

Electrophoretic analysis of synthesized protein

The proteins synthesized in the translation systems of *T. thermophilus* and *E. coli* were analyzed by SDS-polyacrylamide gel-electrophoresis (Fig. 5). The molecular weights of the products obtained with the phosphorothioate RNAs were very similar to those obtained with the unsubstituted RNAs in both systems. Several proteins synthesized in both systems (the bands with asterisks in Fig. 5) are similar to those known to be expressed in *E. coli* cells infected by T7 coliphage (14). These results strongly suggest that the translation system of this extreme thermophilic bacterium is equivalent to that of *E. coli* as had previously been demonstrated by Ohno-Iwashita et al. (16). The similar sizes of the proteins synthesized by either the unsubstituted RNA (lane N) or the substituted RNAs (lanes A, U, G and C) also suggests that phosphorothioate RNAs are translated in a similar way as the unsubstituted RNAs in these systems, including both proper initiation and termination in the translation process.

In the *T. thermophilus* system some proteins produced by the phosphorothioate RNAs are more distinct than those produced by the unsubstituted RNAs. In particular, the bands of the largest proteins (around 40 kDa) were scarcely detected with the unsubstituted RNA, but can clearly be seen with all the substituted RNAs (Fig. 5a). RNAs substituted with pyrimidine nucleoside phosphorothioates (lanes U and C in Fig. 5a) produced a larger amount of proteins than those with purine nucleoside phosphorothioates (lanes A and G). Any of the phosphorothioate RNAs led to the production of more protein than the unsubstituted RNA. These results are consistent with the results shown in
The introduction of the phosphorothioates can have at least two effects. First, there will be an inhibitory effect on nucleases. It has been shown previously that RNase A, RNase T1, and calf serum nucleases are inhibited by the presence of phosphorothioate groups in alternating and homopolyribonucleotides (24,25). In addition, there is ample evidence that the presence of phosphorothioates in oligodeoxyxynucleotides increases their stability against nucleases (26).

That nucleases indeed are responsible for low translational activity was shown in an experiment with unmodified RNA in the E. coli system where plasmid RNase inhibitor was added. The translational activity was indeed increased about twofold (data not shown). This inhibitor, however, is not active in the T. thermophilus system. Thus, the observation in the T. thermophilus system that the phosphorothioate-RNAs prepared with CTPoS and UTPoS directed protein synthesis more efficiently than those prepared with GTPoS and ATPoS might be taken as an indication that pyrimidine-specific RNases are either more prevalent or inhibited better by phosphorothioates than those cleaving at purine nucleotides.

However, a second effect has to be considered. Milligan and Uhlenbeck (21) have found that the binding of R17 coat protein to its RNA is weakened by the presence of phosphorothioate groups in 3 out of 21 positions and enhanced by that in one position. Results reported by Buzayan et al. (18) demonstrate that autocatalytic cleavage of satellite RNA of tobacco ringspot virus is reduced by a phosphorothioate substitution at one particular position. Similar observations were made by Waring (22) for group I intron splicing. All these results suggest that a few phosphate groups in a given RNA might be more susceptible to changes introduced by a phosphorothioate group than others leading to interference with the proper functioning of the RNA. The translational process involves numerous interactions of the mRNA with proteins and other RNAs. Thus, the lower template activity of the purine phosphorothioate-RNAs could be caused by a particular weakening of an interaction which does not occur with the pyrimidine phosphorothioates. At present no distinction between these two possibilities can be made.

Gel analysis of the mixture of proteins produced with various RNAs in the T. thermophilus system shows that in the presence of the monophosphorothioate-RNAs a larger amount of proteins is produced in agreement with the radioactive incorporation data. This is particularly evident for the proteins with larger molecular weights. Thus proteins with a molecular weight >40 kDa are barely visible when unmodified RNA is the template but are very pronounced with all the phosphorothioate-RNAs. This clearly is an advantage in using the phosphorothioate-RNAs in the T. thermophilus system.

Moreover, the T. thermophilus system has generally some advantages in comparison with E. coli system for the in vitro protein synthesis (4,5) such as stability for longer time, easier control of temperature and greater resistance toward nucleases.

Recently a continuous flow system using an ultrafiltration membrane for the in vitro protein synthesis has been developed (27,28) which allows to obtain proteins on a preparative scale. We expect that the use of phosphorothioate-RNAs described in this report will be useful in constructing such a flow system on the basis of the T. Thermophilus translation system.

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REFERENCES