The Journal of Microbiology, June 2002, p.134-139 Vol. 40, No. 2

Effects of Thiamine Pyrophosphate on the Inhibition of Self-splicing of Primary Transcripts of T4 phage Thymidylate Synthase Gene in the Presence of GTP

Hyun Joo Lee[1], Sung Joon Ahn[1], Sook Shin[2], and In Kook Park[1]*

[1]Department of Biology, Dongguk University, Seoul 100-715, Korea
[2]Department of Biology, Sahmyook University, Seoul 139-742, Korea

(Received April 1, 2002 / Accepted June 5, 2002)

Effects of GTP on the inhibition of self-splicing of primary transcripts of the phage T4 thymidylate synthase gene (*td*) by thiamine pyrophosphate and its analogs have been investigated. The order of the inhibitory efficiency for compounds tested was as follows: thiamine pyrophosphate > thiamine monophosphate > thiamine. Of all compounds examined, thiamine pyrophosphate was the most potent inhibitor. Increasing GTP concentration in splicing reaction tended to overcome the suppressive effects of self-splicing by thiamine pyrophosphate and its analogs. The inhibition by thiamine pyrophosphate was most sensitized to a higher concentration of GTP. It has been speculated that the key structural features in thiamine pyrophosphate and its analogs responsible for the inhibition of splicing may be a thiamine moiety in which the phosphorylation of 2-hydroxylethyl group on 5-position of thiazolium ring rendered further stimulation of inhibition in self-splicing reaction..

Key words: T4 phage, group 1 intron, thiamine pyrophosphate, GTP, self-splicing

The primary transcripts of T4 phage thymidylate synthase gene (*td*) self-splice *in vitro* in the absence of any protein factors or energy source (Chu *et al.*, 1994). Like the intron in the nuclear large rRNA gene of *Tetrahymena thermophila* (Cech *et al.*, 1983), the *td* intron is spliced from a precursor RNA *via* a series of the transesterification mechanism (Chu *et al.*, 1986).

The self-splicing reaction of group I introns has been known to be inhibited by a number of small molecules. The guanosine analogs deoxyguanosine and dideoxyguanosine (Bass and Cech, 1986), the amino acid arginine (Yarus, 1988) and the antibiotics streptomycin (von Ahsen and Schroeder, 1991), viomycin and di-β-lysylcapreomycin (Wank *et al.*, 1994) are competitive inhibitors of group I introns via guanidino groups, which compose a part of the cofactor guanosine. However, lysinomycin (Rogers and Davies, 1994) is a competitive inhibitor in spite of lack of guanidino groups.

Recently we demonstrated that the coenzymes FMN (Kim and Park, 2000) and NAD^+ (Park and Kim, 2001) inhibit splicing of group 1 intron RNA in a competitive and noncompetitive manner, respectively although they are devoid of guanidino groups in their structures. This is an unexpected finding in the light of their chemical structures totally unrelated to the cofactor guanosine.

In addition, it has been shown that some aminoglycoside antibiotics of neomycin, gentamycin, kanamycin and tobramycin (von Ahsen and Schroeder, 1991), tetracycline and pentamidine (Liu *et al.*, 1994), and spectinomycin (Park and Sung, 2000; Park *et al.*, 2000), which do not possess a guanidino group, are noncompetitve inhibitors of the group 1 intron splicing.

The thiamine pyrophosphate is synthesized from thiamine by an enzymatic transfer of pyrophosphate from ATP (Walsh, 1979). This coenzyme serves as a prosthetic group for enzymes such as decarboxylase and transketolase. Furthermore, thiamine pyrophosphate is thought to play an important role in the transmission of nerve impulses and may also be required for ion translocation reaction in stimulated neural tissue (Delvin, 1993).

Due to its biological importance as a major coenzyme in the cellular metabolism we undertook to look at how GTP affects the inhibition of self-splicing of primary transcripts of the phage T4 thymidylate synthase gene *in vitro* by thiamine pyrophosphate and its analogs.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strains TG1 and HB101 were obtained

* To whom correspondence should be addressed.
(Tel) 82-2-2260-3320; (Fax) 82-2-2275-8294
(E-mail) ikpark@dgu.ac.kr

from Amersham. M13mp8 phage was purchased from Bethesda Research Laboratories and pGEM-2 vectors were from Promega Corp.

Enzymes and chemicals

Restriction enzymes *Eco*RI and *Hind*III were obtained from New England Biolabs. [α-^{32}P]GTP (>400 Ci/mmol) was obtained from Amersham. Nucleoside triphosphates were obtained from Boehringer Mannheim. T7 RNA polymerase (20 U/μl) was obtained from United States Biochemical and RNasin (40 U/μl) and RQ1 DNase (1U/μl) from Promega Corp. Thiamine pyrophosphate, thiamine monophosphate, thiamine, thiochrome, GTP and $MgCl_2$ were obtained from Sigma Chemical Co.

Construction and preparation of recombinant plasmids

The cloning procedures were as described previously (Chu *et al.*, 1994). The pGEM recombinant containing 390 nt of the 5' exon 1, 1016 nt of the intron and 824 nt of the 3' exon 2 were kindly provided by Dr. Fred Chu. The pGEM recombinant plasmids were transformed into *E.coli* HB101 cells, propagated in the presence of ampicillin and amplified in the presence of chloramphenicol. The promoter alignment of the *td* fragment was determined by 0.8% agarose gel analysis of restriction fragments from pGEM-2 recombinant plasmids.

Synthesis of RNA by in vitro transcription

The pGEM recombinant plasmids were linearized with *Hpa*1 which cuts the *td* fragment once at 520 bp downstream of exon 2. Each linearized recombinant plasmid DNA was used as a template for *in vitro* transcription following deproteination by phenol extraction and ethanol precipitation. The transcription was performed at 30°C for 40 min in the transcription buffer (40 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM spermidine, 5 mM NaCl), 10 mM DTT, 1 U/ml RNasin, 0.5 mM of each rNTP, 5 μCi of [α-^{32}P] GTP, and 10 U of T7 RNA polymerase. The RNA synthesis was terminated by the addition of RQ1 DNase to destroy the DNA template. Following the transcription, the synthesized 2.23 kb primary transcript was purified free of proteins, ribonucleotides and salts by passage through a Nensorb^{20} cartridge (Du Pont). The bound RNA was eluted with 20% ethanol from the cartridge, followed by precipitation with 2 volumes of ethanol in the presence of 0.2 M sodium acetate. The RNA precipitate was washed three times with 70% ethanol to remove salts.

In Vitro self-splicing reaction

The splicing reaction buffer contained 40 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 μM GTP and 8 nM RNA. Varying concentrations of thiamine pyrophosphate and its analogs were added to the reaction buffer to examine their effects on the splicing. At the end of incubation, the reaction mixture was centrifuged briefly to collect the moisture, chilled on ice and 5 μl of the sample buffer (95% deionized formamide, 10 mM Na_2EDTA, 0.08% xylene cyanol, 0.08% bromophenol blue) was added. The spliced RNA products were electrophoresed in a 0.75 mm thick slab gel containing 4% polyacrylamide and 8 M urea in TBE buffer (0.1 M Trizma base, 0.1 M boric acid, 2 mM Na_2EDTA) and visualized by autoradiography without drying. Autoradiograms were scanned and integrated with a Hoefer image analyzer.

Effects of GTP on self-splicing inhibition by thiamine pyrophosphate and its analogs

For the determination of GTP effects on splicing, the reaction was performed by incubating precursor RNAs of the *td* intron RNA in the presence of varying concentrations of GTP (5, 20, 100, 500, 2000 and 4000 μM) at 5 mM Mg^2 in the presence of 1.6 mM thiamine pyrophosphate for 10 min at 58°C. The identical splicing reaction also was carried out for analogs except the concentrations used. The accumulated splicing products were determined from the densitometric analysis with Hoefer image analyzer. The relative splicing rate was expressed as the calculated ratio of the E1-E2 ligation product to the total spliced products (I-E2 + I-E1 + E1-E2 + CI + LI) plus the remaining pre-RNAs.

RNA Concentration

The RNA concentration was determined by the spectrophotometric method (Puglisi and Tonoco, 1989). The extinction coefficient was determined by hydrolyzing the RNA to nucleotides and by measuring the A_{260} value of the resulting mixture.

Results

Inhibition of td intron RNA splicing by thiamine pyrophosphate and its analogs

Precursor RNAs were incubated with varying concentrations (1-6 mM) of thiamine pyrophosphate under the normal splicing conditions to examine the effect of thiamine pyrophosphate on the self-splicing of the *td* intron RNA. As shown in Fig. 1A, the progressive inhibition by thiamine pyrophosphate is evident from the decreases in splicing products as a function of thiamine pyrophosphate concentration. At 1.75 mM the splicing activity was inhibited about 50% that of the normal splicing activity (Fig. 1B). Increasing the thiamine pyrophosphate concentration suppressed markedly the formation of I-E2 intermediate, E1-I ligation product, linear and circular introns and at 3 mM the splicing was completely abolished.

To determine the structure and self-splicing activity relationships in the interaction of thiamine pyrophosphate with the group 1 intron RNA, we screened a series of

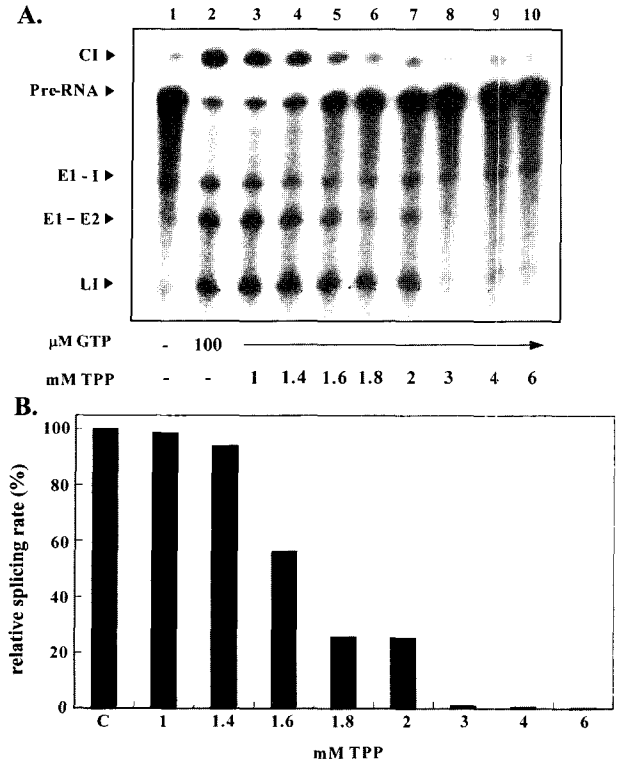

Fig. 1. The Inhibition of the self-splicing of *td* intron RNA by thiamine pyrophosphate. (A) Autoradiograms showing the splicing activity in the presence of 100 μM GTP and 5 mM $MgCl_2$ at varying concentrations (1-6 mM) of thiamine pyrophosphate. Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Splicing rates of the *td* intron RNA as a function of concentrations of thiamine pyrophosphate. The relative inhibition was expressed as the percentage reduction in the formation of E1-E2 ligation product in the presence of thiamine pyrophosphate with respect to that observed in the absence of thiamine pyrophosphate. C, normal splicing.

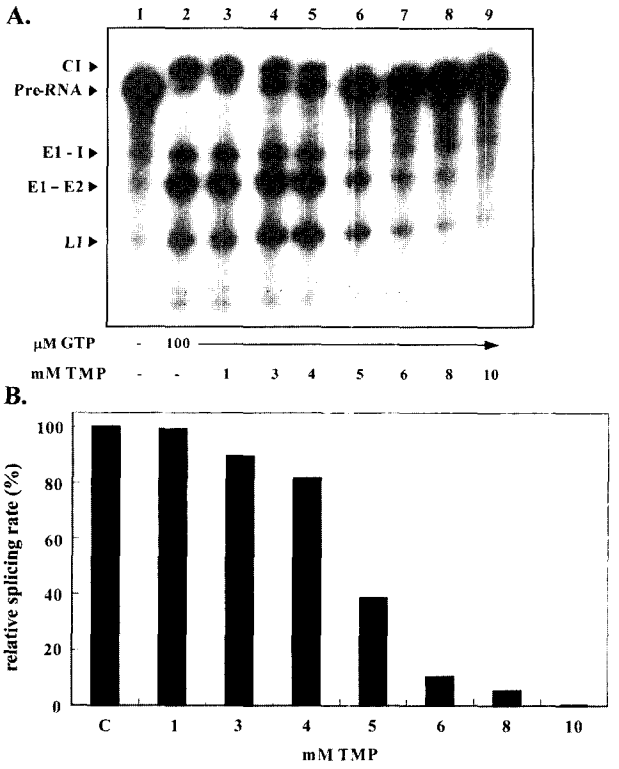

Fig 2. The inhibition of the self-splicing of *td* intron RNA by thiamine monophosphate. (A) Autoradiograms showing the splicing activity in the presence of 100 μM GTP and 5 mM $MgCl_2$ at varying concentrations (1-10 mM) of thiamine monophosphate (TMP). Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Splicing rates of the *td* intron RNA as a function of concentrations of thiamine monophosphate. The relative splicing rate was expressed as the percentage reduction in the formation of E1-E2 ligation product in the presence of thiamine monophosphate with respect to that observed in the absence of thiamine monophosphate. C, normal splicing.

structurally related members of thiamine pyrophosphate such as thiamine monophosphate and thiamine in splicing inhibition assays. Thiamine monophosphate at 3 mM started to inhibit self-splicing of *td* intron RNA and at 4.8 mM concentration about 50% inhibition was detected (Fig. 2B). At 10 mM no splicing occurred.

Thiamine without any phosphate groups present in both thiamine pyrophosphate and thiamine monophosphate did not exhibit any inhibitory actions on splicing activity up to 2 mM (Fig. 3B). However, at 4 mM thiamine started to show some inhibitions in splicing reaction. At 5.4 mM about 50% inhibition was obtained whereas at 10 mM no splicing reaction occurred. This demonstrates that phosphate groups in both thiamine pyrophosphate and thiamine monophosphate are involved in facilitating the suppression of splicing activity.

The order of the inhibitory efficiency for compounds tested was as follows: thiamine pyrophosphate > thiamine monophosphate > thiamine.

Effect of GTP on the splicing inhibition by thiamine pyrophosphate and its analogs

The splicing activity increased progressively as the concentration of GTP increased up to 500 μM at which the splicing activity was fully recovered, reaching almost the same level of the normal splicing activity (Fig. 4). Interestingly enough, the further increase of GTP concentration above 500 μM appeared to initiate to reduce the splicing reaction. At 2 mM GTP the splicing activity was markedly suppressed.

The effect of GTP on the inhibition of RNA splicing by thiamine monophosphate was quite different from that shown in thiamine pyrophosphate (Fig. 5). At 100 μM GTP about 70% splicing activity was recovered relative to that of the normal splicing. Unlike thiamine pyrophosphate, at 500 μM GTP the splicing activity was decreased rather than increased. Further increase of the concentration of GTP (4 mM) did not serve to alleviate the inhib-

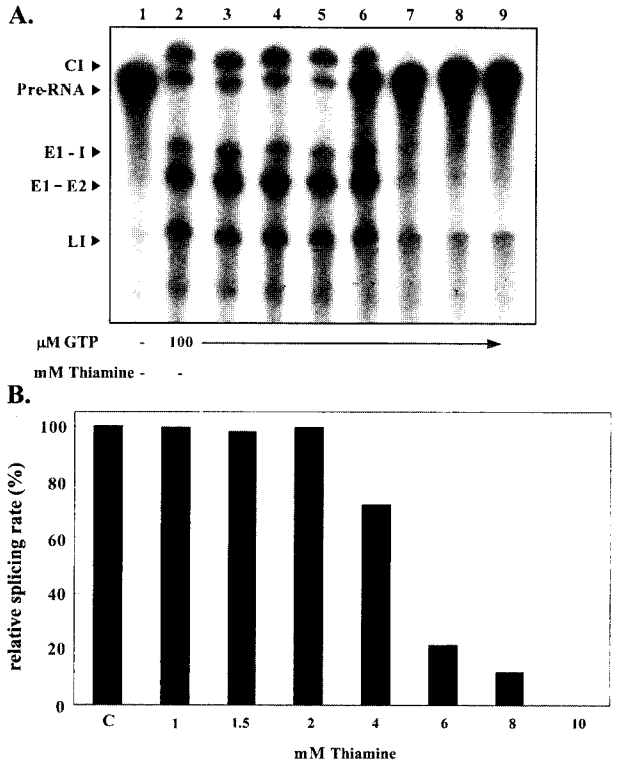

Fig. 3. The inhibition of the self-splicing of *td* intron RNA by thiamine. (A) Autoradiograms showing the splicing activity in the presence of 100 μM GTP and 5 mM $MgCl_2$ at varying concentrations (1-10 mM) of thiamine. Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Splicing rates of the *td* intron RNA as a function of concentrations of thiamine. The relative inhibition was expressed as the percentage reduction in the formation of E1-E2 ligation product in the presence of thiamine with respect to that observed in the absence of thiamine. C, normal splicing.

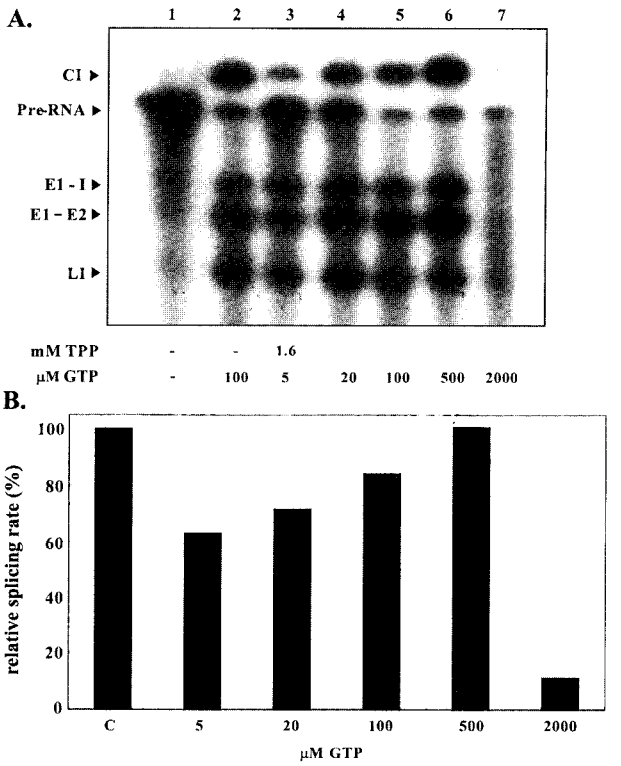

Fig. 4. Effect of GTP on the inhibition of *td* intron RNA splicing by thiamine pyrophosphate. (A) Autoradiograms showing the splicing activity in the presence of varying concentrations (5-4000 μM) of GTP at 4 mM thiamine pyrophosphate. and 5 mM $MgCl_2$. Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Relative splicing activities of the *td* intron RNA as a function of GTP concentrations. The relative splicing rate was expressed as the percentage of the formation of E1-E2 ligation product in each reaction to that of he normal splicing reaction. C, normal splicing; TPP, thiamine pyrophosphate.

itory actions by thiamine monophosphate.

Fig. 6 shows the effect of GTP on the inhibition of RNA splicing by thiamine. At low 5 μM GTP the splicing activity was only recovered 20% that of normal splicing activity. Increasing GTP concentration up to 100 and 500 μM led to the recovery of splicing about 73 and 71%, respectively. However, at 2000 μM GTP the splicing activity was rather markedly suppressed about 22% that of normal splicing activity.

Discussion

Although thiamine pyrophosphate does not contain a guanidino group in the structure, it interferes with the self-splicing of the *td* intron RNA. A similar observation was also made with the coenzymes FMN and NAD which do not possess a guanidino residue and interfere with the first step of the self-splicing of the *td* intron RNA as competitive and noncompetitive inhibitors, respectively (Kim and Park, 2000; Park and Kim, 2001). Like FMN, NAD^+ demonstrates the structural specificity in inhibiting the splicing for the *td* intron RNA.

In fact, the coenzyme NAD^+ inhibited the first step of the transesterification for the self-splicing of the *td* intron RNA in a noncompetitive manner. The first step inhibition was mostly observed with molecules with guanidino residues such as streptomycin (von Ahsen *et al.*, 1991), arginine (Yarus, 1988), guanosine analogs (Bass and Cech, 1986), and tuberactinomycin peptides (Wank *et al.*, 1994). This is due to the competition with the binding of the guanosine cofactor for the G-binding site located in the core of the intron RNA (von Ahsen and Schroeder, 1991).

In addition, increasing GTP concentration in splicing reaction tended to overcome the suppressive effects of self-splicing by thiamine pyrophosphate and its analogs (Figs. 4-6). Similar observations were also made with the inhibition by coenzymes FMN (Kim and Park, 2000) and

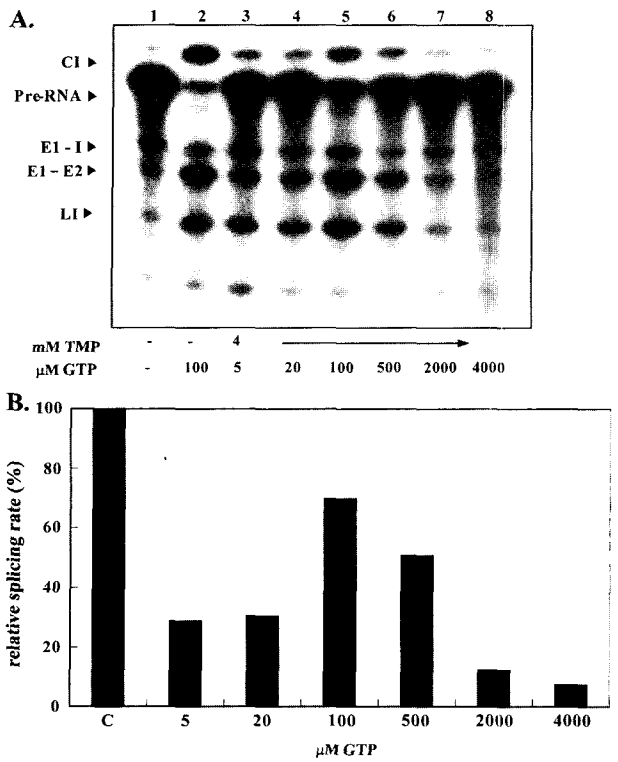

Fig. 5. Effect of GTP on the inhibition of *td* intron RNA splicing by thiamine monophosphate. (A) Autoradiograms showing the splicing activity in the presence of varying concentrations (5-4000 μM) of GTP at 4 mM thiamine monophosphate. and 5 mM $MgCl_2$. Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Relative splicing activities of the *td* intron RNA as a function of GTP concentrations. The relative splicing rate was expressed as the percentage of the formation of E1-E2 ligation product in each reaction to that of the normal splicing reaction. C, normal splicing; TMP, thiamine monophosphate.

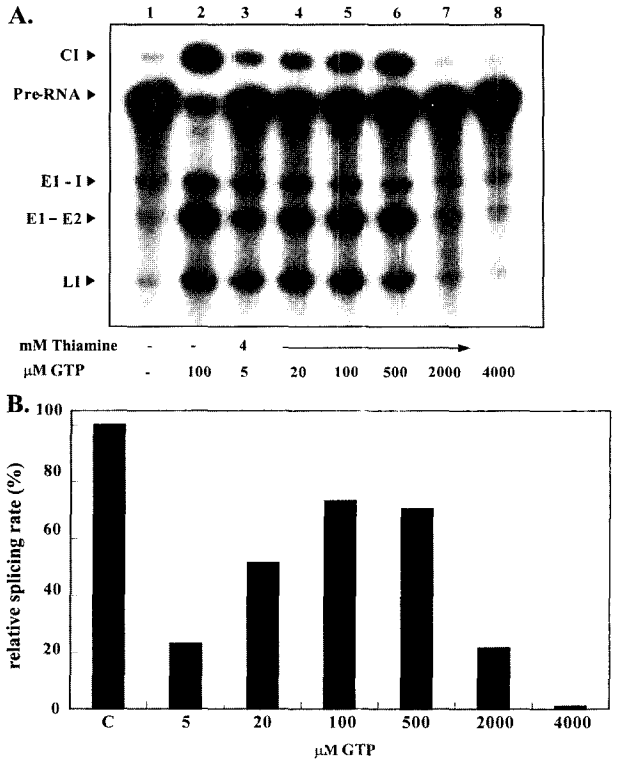

Fig. 6. Effect of GTP on the inhibition of *td* intron RNA splicing by thiamine. (A) Autoradiograms showing the splicing activity in the presence of varying concentrations (5-4000 μM) of GTP at 4 mM thiamine and 5 mM $MgCl_2$. Lane 1, unspliced pre-RNA; lane 2, normal splicing; E1-I, intron-exon 1; LI, linear intron; CI, circular intron; E1-E2, exon 1-exon 2 ligation product. (B) Relative splicing activities of the *td* intron RNA as a function of GTP concentrations. The relative splicing rate was expressed as the percentage of the formation of E1-E2 ligation product in each reaction to that of the normal splicing reaction. C, normal splicing.

NAD^+ (Park and Kim, 2001). An inclusion of a high concentration of GTP in splicing reaction served to counteract the inhibitory actions of splicing by these two coenzymes. This strongly implies a possibility that both thiamine pyrophosphate and its analogs may compete with a binding site of cofactor guanosine in self-splicing reaction (Bass and Cech, 1986). To clarify the catalytic interactions of thiamine pyrophosphate with guanosine in self-splicing reaction the kinetic studies should be carried out.

The inhibitory action of thiamine pyrophosphate at 1.75 mM concentration on the splicing activity also appeared to be dependent on the Mg^{2+} concentration (data not shown). A preliminary study showed that increasing concentrations of Mg^{2+} alleviated the inhibition of the splicing activity by thiamine pyrophosphate, suggesting a possible ionic interaction of thiamine pyrophosphate with Mg^{2+} in splicing reaction.

A very similar observation was also made with the coenzyme NAD^+ (Park and Kim, 2001) whose splicing inhibition turned out to be weakened by gradually enhancing the concentration of Mg^{2+} ions in splicing reaction. Besides its role as a catalytic cofactor, Mg^{2+} is also thought to be involved in the proper folding and general electrostatic interactions to shield the phosphodiester backbone of the group 1 intron RNA (Grosshans and Cech, 1989; Celander and Cech, 1991).

Thus the inhibitory effect of thiamine pyrophosphate appeared to be dependent on not only the GTP concentration but also the Mg^{2+} concentration. This suggests that thiamine pyrophosphate may possibly interact with other specific sites within the intron RNA structure which could be Mg^{2+}-binding sites although its inhibitory mechanism should be further explored.

Of all analogs tested, thiamine pyrophosphate was the most inhibitory. Interestingly enough, the absence of additional phosphate group of thiamine pyrophosphate somehow induced a less inhibitory potency although its functional role has not been clearly understood yet.

It was quite an unexpected finding that the coenzyme thiamine pyrophosphate inhibited the self-splicing of the *td* intron RNA although it does not possess a guanidino group. The examination of the thiamine pyrophosphate-intron RNA complex by the footprinting or the NMR experiments could promote to identify the specific functional groups involved in the interaction of thiamine pyrophosphate with the intron RNA.

Acknowledgments

We thank Dr. Fred K. Chu for his generous gift of the pGEM recombinant plasmids. This work was supported by Korea Research Foundation Grant (KRF-2000-015-DP0378).

References

Bass, B. and T.R. Cech. 1986. Ribozymes inhibitors: deoxyguanosine and dideoxyguanosine are competitive inhibitors of self-splicing of the *Tetrahymena* ribosomal RNA precursor. *Biochemistry* 25, 4473-4477.

Cech, T.R., N.K. Tanner, I. Tinoco, Jr., B.R. Weir, M. Zuker, and P.S. Perlman. 1983. Secondary structure of the *Tetrahymena* ribosomal RNA intervening sequence : Structural homology with fungal mitochondrial intervening sequences. *Proc. Natl. Acad. Sci. USA* 80, 3903-3907.

Celander, D.W. and T.R. Cech. 1991. Visualizing the higher order folding of a catalytic RNA molecule. *Science* 251, 401-407

Chu, F.K., G.F. Maley, F. Maley, and M. Belfort. 1994. An intervening sequence in the thymidylate synthase gene of bacteriophage T4. *Proc. Natl. Acad. Sci. USA* 81, 3049-3053.

Chu, F.K., G.F. Maley, D.K.West, M. Belfort, and F. Maley. 1986. Characterization of the intron in the phage T4 thymidylate synthase gene and evidence for its self-excision from the primary transcript. *Cell* 45, 157-166.

Delvin, T.M. 1993. Biochemistry with clinical correlations, 3rd ed., Wiley-Liss, Inc., New York.

Grosshans, C.A. and T.R Cech. 1989. Metal ion requirements for sequence-specific endoribonuclease activity of the *Tetrahymena* ribozyme. *Biochemistry* 28, 6888-6894.

Kim, J.Y. and I.K Park. 2000. The flavin coenzymes : a new class of group 1 intron inhibitors. *Biochim. Biophys. Acta.* 1475, 56-61.

Liu, Y., R.R. Tidwell, and M.J. Leibowitz. 1994. Inhibition of *in vitro* splicing of group 1 intron of *Pneumocystis carinii*. *J. Euk. Microbiol.* 41, 31-38.

Park I.K. and J.S. Sung. 2000. K^+ and Mg^{2+} ions promote the self-splicing of the *td* intron RNA inhibited by spectinomycin. *Biochim. Biophys. Acta.* 1492, 94-99.

Park, I.K. and J.Y. Kim. 2001. NAD^+ inhibits the self-splicing of the group 1 intron. *Biochem. Biophys. Res. Commun.* 281, 206-211.

Park, I.K., E.H. Lim, and S. Shin. 2000. Spectinomycin inhibits the self-splicing of the group 1 intron RNA. *Biochem. Biophys. Res. Commun.* 269, 574-579.

Puglisi, J.D. and I. Tinoco. Jr. 1989. Absorbances melting curves of RNA. *Methods in Enzymology* 180, 304-325.

Rogers, J. and J. Davies. 1994. The pseudodisaccharides: a novel class of group 1 introns by competition with the guanosine substrate. *Nucleic Acids Res.* 24, 4983-4988.

von Ahsen, U. and R. Schroeder. 1991. Streptomycin inhibits splicing of group I introns by competition with the guanosine substrate. *Nucleic. Acids Res.* 19, 2261-2265.

von Ahsen, U., J. Davies, and R. Schroeder. 1991. Antibiotic inhibition of group 1 ribozyme function. *Nature* 19, 2261-2265.

Walsh, C. 1979. Enzymatic Reaction Mechanisms, pp. 683-684, Freeman W.H. and Co., San Francisco.

Wank, H., J. Rogers, J. Davies, and R. Schroeder. 1994. Peptide antibiotics of tuberactinomycin family as inhibitors of group 1 intron RNA splicing. *J. Mol. Biol.* 236, 1001-1010.

Yarus, M. 1988. A specific amino acid binding site composed of RNA. *Science* 240, 1751-1758.