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Effects of Divalent Cations on the Splicing of Phage T4 Thymidylate Synthase Intron RNA

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Effects of divalent cations such as Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2} on splicing activity of phage T4 thymidylate synthase intron RNA have been investigated. At the concentration of 0.5 mM Mn^{2+} in the absence of Mg^{2+}, a very small amount of pre-RNA was cleaved into ligation products (E1-E2) but no circular or linear intron was produced. As the concentration of Mn^{2+} was increased from 1 to 5 mM, the pre-RNA was completely hydrolyzed. In the presence of 5 mM Mg^{2+}, both the linear intron and circular intron were produced but no E1-E2 ligation product was produced. At both 3 and 5 mM Mn^{2+}, the RNA was hydrolyzed completely as observed with no Mg^{2+} being present. In the case of Zn^{2+}, even at 0.5 mM concentration, the pre-RNA was completely hydrolyzed. This observation suggested that Zn^{2+} facilitates RNA hydrolysis more rapidly than Mn^{2+} does. At 5 mM Ca^{2+}, the RNA was not hydrolyzed and remained intact as a primary transcript.

T4 phage thymidylate synthase gene(*td*), the first intron-containing prokaryotic protein encoding gene, contains a group I intron (Chu et al., 1984). Similar to the *Tetrahymena thermophila* large rRNA precursor (Cech, 1987), the *td* precursor RNA can undergo self-splicing *in vitro* in the absence of any protein factors or energy source (Burke et al., 1986). When a U · G pair was changed to a U · C pair in the 5' splice site of P1 stem of the *td* intron, using *in vitro* oligodeoxyribonucleotide-directed mutagenesis the activity of thymidylate synthase *in vivo* was totally lost whereas the wild type retained normal activity (Shin and Park, 1993). The enzyme activity was about 32% of that of the wild type when U at 12 position was substituted to C. The deletion of P2 led to a complete loss of the enzyme activity (Park, 1992).

A very similar loss of enzyme activity was observed with substitutions at the 5'-end (U18G) and at the 3'-end (A29C) of P2 stem. This suggested the importance of maintenance of the intact stem structure of intron RNA in splicing.

Metal ions have been implicated to play very important roles in catalytic mechanisms of ribozymes such as the proper folding of active structures and the catalysis at the active site (Piccirilli et al., 1993; Park et al., 1995; Sung et al., 1995a, b; Sung and Park, 1996).

In addition, monovalent ions are capable of affecting the conformation of guanosine binding site, thereby reducing intramolecular repulsion within intron and enhancing the affinity for GTP (Michel et al., 1989).

Thus, metal ions are essential for the splicing reaction of ribozymes and ribozymes are recognized as metalloenzymes (Pyle, 1993).

We thus examined the effects of various divalent metal ions such as Mg^{2+}, Mn^{2+}, Ca^{2++}, and Zn^{2+} on the splicing activity of T4 phage intron RNA.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli strains TG1 and HB101 were obtained from Amersham. M13mp8 phage was purchased from Bethesda Research Laboratories and pGEM-1 and pGEM-2 vectors were from Promega Corp.

Enzymes and chemicals

Restriction enzymes *Eco*RI and *Hin*dIII were obtained from New England Biolabs. [α-^{32}P]GTP (> 400Ci/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 SP6 RNA polymerase (15 U/μl), RNasin (40 U/μl) and RQ1 DNase (1 U/μl) from Promega Corp.

Construction and preparation of recombinant plasmids

The 2.85 kb *Eco*RI fragment containing the T4 *td*

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gene was originally cloned from strain T4-alc4 (Chu et al., 1984). The *td*-containing fragment was inserted into M13 phage to yield M13 *td* in the *Eco*RI site. For subcloning of *td*, M13 recombinant phage was infected into *E. coli* TG1 cells. The M13 *td* gene was extracted from the phage-infected *E. coli* TG1 culture. Cell pellet was suspended in 0.7 ml of STET buffer (50 mM Tris-HCl, pH 8.0, 50 mM Na_2EDTA, 5% triton X-100, 8% sucrose) and lysed by boiling for 2 min in the presence of 1mg of lysozyme. After the lysate was spun to pellet host DNA, the supernatant was mixed with an equal volume of isopropanol for precipitation at -20℃. The *td* gene insert in M13 mutant replicative form was excised with *Hin*dIII and *Eco*RI, followed by ligation into *Eco*RI site of pGEM-1 or *Hin*dIII - *Eco*RI site of pGEM-2. The pGEM recombinant plasmids 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-1 and pGEM-2 recombinant plasmids.

Thymidylate synthase assay

Thymidylate synthase activity was measured by the ^3H release method of Roberts (1966). Aliquots of the sample were incubated with substrate mixture containing 100 mM Tris, pH 7.1, 10 mM formaldehyde, 1 mM L-tetrahydrofolate, 200 mM mercaptoethanol, 20 mM Na-ascorbate, 50 mM NaF, 100 mM $MgCl_2$ and 0.1 mM [5-^{3}H]dUMP(1×10^5 cmp/nmol). After 30 min at 37℃, the reaction was terminated by addition of Norit A in 2% TCA. Following centrifugation in microfuge for 2 min, 100 µl of supernatant were counted in 4 ml of aquasol (New England Nuclear).

Synthesis of RNA by in vitro transcription

The pGEM recombinant plasmids were linearized with *Hpa*I which cuts the *td* fragment once at 520 bp downstream of exon 2 and then incubated with DNase-free RNase at 37℃ for 15 min. Each linearized recombinant plasmid DNA was used as template for *in vitro* transcription following deproteination by phenol extraction and ethanol precipitation. The transcription was performed at 30℃ for 50 min in the transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 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. RNA synthesis was terminated by the addition of RQ1 DNase to destroy the DNA template. Following transcription, the synthesized RNA was isolated free of proteins, ribonucleotides and salts by passage through a Nensorb^{20} cartridge (Du Pont). 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 and 0.1 mg/ml of yeast tRNA as carrier. The RNA precipitate was washed with 70% ethanol, dissolved in a volume of nuclease-free water equal to that of the original transcription mixture, and the radioactivity of primary transcript was achieved by counting in liquid scintillation counter (Beckman, LS 6000).

In vitro self-splicing reaction

Typical splicing reaction buffer contained 40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 100 µM GTP. Aliquots (5 µl) containing 50,000 cpm of radioactive RNA were incubated at 58℃ with varying concentrations of divalent cations either in the presence of or in the absence of 5 mM $MgCl_2$ for 10 min as indicated in the figure legends. At the end of incubation, the reaction was centrifuged briefly to collect moisture, chilled on ice and 5 µl of 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 5% polyacrylamide and 8 M urea in TBE buffer (0.1 M Trizma base, 0.1 M boric acid, 2 mM Na_2EDTA). After the gels were dried onto filter paper under vacuum, autoradiography was performed by exposing to X-ray film at -70℃. Autoradiograms were scanned and integrated with a Hoefer densitometer (GS 300) using the GS 365W program. The extent of reaction at a given time was determined by scanning the remaining pre-RNA.

Metal contamination precautions

Whenever possible, plasticware was used instead of glass. All reagent containers were soaked in 10% nitric acid for 24-48 h and then thoroughly rinsed with water from the Millipore purification system.

Results and Discussion

The splicing of *td* intron RNA occurs by an autocatalytic mechanism resembling that of many group I introns (Cech, 1987). As shown in Fig. 1, most of the predicted secondary structures have been implicated as essential determinants for the self-splicing of group I introns. Like protein enzymes that act on nucleic acids, group I intron and other catalytic RNAs show strong requirements for metal ions as cofactors in these reactions (Symons, 1989).

The alteration of *td* intron by site-directed mutagenesis and its consequent effect on thymidylate synthase activity *in vivo* are shown in Table 1. As expected, no change in enzyme activity was observed with the wild type (3.85 nmol/mg protein). When part

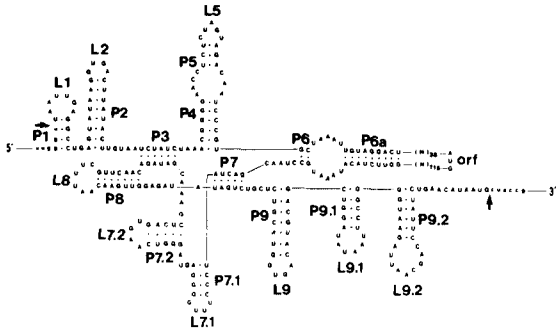

Fig. 1. Proposed secondary structure of phage T4*td* intron RNA. Arrows indicate the 5'-end and 3'-end splice sites. Numerical values are numbered from the 5' end of the intron. Intron bases are in upper case letter and exon bases in lower case letters.

of a plausible core structure encompassing the hairpin structures such as P2, 1/2P6/ P6a/ORF and P6/P6a/ORF were deleted, the enzyme activity was completely abolished. This indicates the defectiveness of enzyme synthesis as a result of the mutation. Similar observations were also made with *Tetrahymena* rRNA in which mutations in P2, P3 and P7 hairpin structures result in the reduced splicing activity (Burke et al., 1986). This supports the notion that the maintenance of the intact hairpin structure within the core structure of the intron is crucial for the normal splicing.

The effects of varying concentrations of $MgCl_2$ on splicing activity of wild type and P2 deletion mutant RNAs was shown in Fig. 2. There was no splicing observed at 1 mM $MgCl_2$ but splicing started to take place at 3 mM $MgCl_2$. The maximum splicing occurred at 5 mM $MgCl_2$ while higher concentration of Mg^{2+} at 15 mM resulted in decreasing the splicing activity rather than promoting the splicing activity. Interestingly, pre-RNA was found to be completely hydrolyzed at 30 mM $MgCl_2$. Similarly, the inhibition of RNA cleavage at high Mg^{2+} concentration was also observed with hapatitis delta virus genomic DNA (Rosenstein and Been, 1990). Unlike the wild type in P2 deletion mutant, there was no splicing observed at 3 mM $MgCl_2$ but reduced and incomplete splicing with some ligation product (E1-E2) and no linear intron (LI) at 5 mM $MgCl_2$. Raising the Mg^{2+} concentration to 15 mM appeared to activate slightly P2 deletion mutant RNA to restore some splicing

Table 1. Effect of mutation of *td* intron gene on thymidylate synthase activity *in vivo*

Intron	Thymidylate synthase activity (nmol/mg protein)
Wild type	3.85
P2 deletion	0
1/2 P6/ P6a/ ORF deletion	0
P6/ P6a/ ORF deletion	0

ORF; open reading frame.

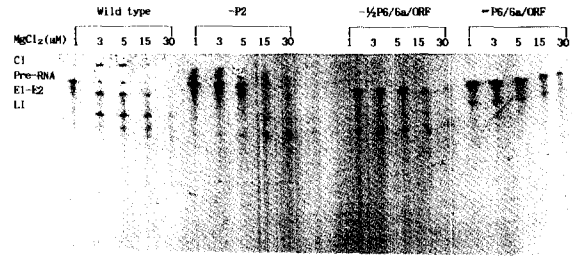

Fig. 2. Dependence of *in vitro* splicing of wild type and mutant RNAs on Mg^{2+} concentration. *In vitro* splicing reaction was carried out at 58℃ for 15 min. The spliced RNA products were analyzed in 5% acrylamide-8 M urea slab gel electrophoresis and visualized by autoradiography. CI, circular intron; Pre-RNA, primary transcript; E1-E2, ligation product; LI, linear intron.

activity. As in the case of the wild type, a complete hydrolysis of mutant RNA occurred at 30 mM $MgCl_2$. The mechasism by which a higher concentration of Mg^{2+} led to a complete hydrolysis of *td* intron RNA is still not known.

The splicing profile of *td* intron RNA in the presence of respective divalent cations at 5 mM concentrations was shown in Fig. 3. In the presence

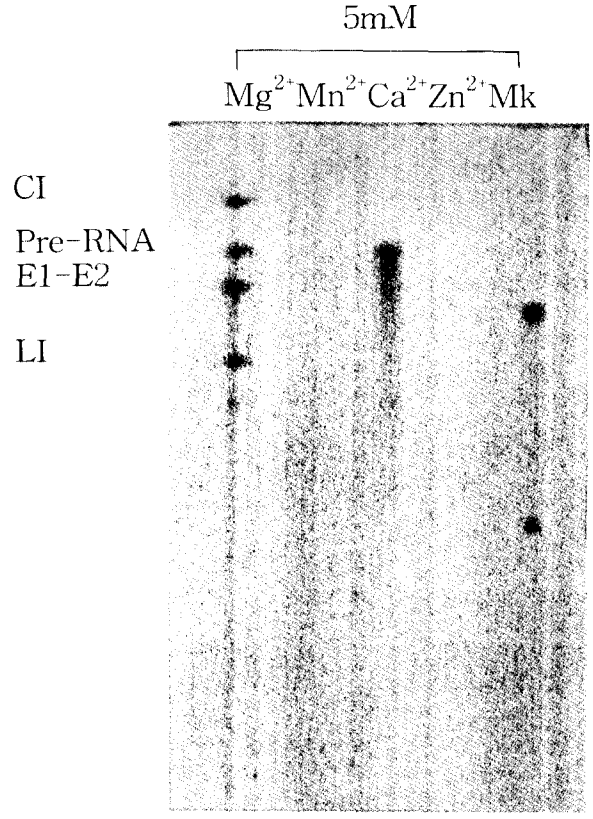

Fig. 3. Effect of various divalent ions on *in vitro* splicing. *In vitro* splicing reaction was carried out at 58℃ for 15 min. The spliced RNA products were analyzed in 5% acrylamide-8M urea slab gel electrophoresis and visualized by autoradiography. CI, circular intron; Pre-RNA, primary transcript; E1-E2, ligation product; LI, linear intron The RNA size markers (1.4kb and 0.56kb) were synthesized from pSP-λ marker DNA.

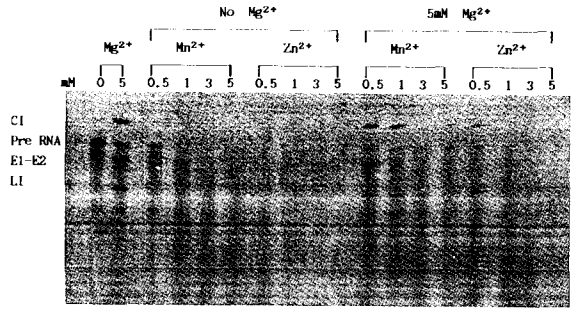

Fig. 4. Effect of varying concentrations of Mn^{2+} and Zn^{2+} on *in vitro* splicing in the presence and the absence of Mg^{2+}. *In vitro* splicing reaction was carried out at 58℃ for 15 min. The spliced RNA products were analyzed in 5% acrylamide-8M urea slab gel electrophoresis and visualized by autoradiography. CI, circular intron; Pre-RNA, primary transcript; E1-E2, ligation product; LI, linear intron.

of 5 mM Mg^{2+} the normal splicing occurred. However, pre-RNA was completely hydrolyzed either in the presence of 5 mM Mn^{2+} or 5 mM Zn^{2+}. At 5 mM Ca^{2+} the pre-RNA did not undergo splicing but remained almost intact. In *Tetrahymena* intron, Mn^{2+} can substitute for Mg^{2+} in splicing reaction and Zn^{2+} can not (Cech et al., 1986). While Ca^{2+} has no activity by itself, it acts to reduce a portion of Mg^{2+} requirement (Grosshans and Cech, 1989) This proposed two classes of metal binding sites for splicing: one class that plays specific structural roles or is directly involved in active site chemistry, and another class that promotes the global folding of the RNA (Latham and Cech, 1989).

Effects of varying concentrations of Mn^{2+} and Zn^{2+} on *in vitro* RNA splicing of *td* intron in the presence and absence of Mg^{2+} were illustrated in Fig. 4.

No splicing occurred in the absence of Mg^{2+} ion but the normal splicing occurred in the presence of 5 mM Mg^{2+} ion. At the concentration of 0.5 mM Mn^{2+} in the absence of Mg^{2+}, a very small amount of pre-RNA was cleaved into ligation product (E1-E2) but no circular and linear introns were produced. As the concentration of Mn^{2+} was increased from 1 to 5 mM the splicing did not take place to any extent and the pre-RNA was completely hydrolyzed. In the presence of 5 mM Mg^{2+}, however, the pre-RNA at 0.5 mM Mn^{2+} was cleaved into a very small amount of ligation product (E1-E2), and linear and circular introns.

At 1 mM Mn^{2+}, both linear intron and circular intron were produced but no E1-E2 ligation product was produced. At both 3 and 5 mM Mn^{2+}, the RNA was hydrolyzed completely as observed with no Mg^{2+} being present. This demonstrates that Mg^{2+} concentration at 5 mM was not sufficiently high enough to prevent RNA hydrolysis caused by 3 mM or 5 mM Mn^{2+}. For the cleavage reaction of human hapatitis delta virus ribozyme, Mn^{2+} was the most effective divalent ion at concentrations below 1 mM (Suh et al., 1993). However, the nonspecific cleavage occurred when the concentration of Mn^{2+} was increased higher than 1 mM. In contrast, Mn^{2+} behaves very similarly to Mg^{2+} in cleavage activity of *Tetrahymena* ribozyme (Grosshans and Cech, 1989). Likewise, the hammerhead ribozyme can utilize Mg^{2+}, Mn^{2+}, and Ca^{2+} for both structural and catalytic functions (Dahm and Uhlenbeck, 1991). All these results suggested that the roles of Mn^{2+} in cleavage reaction vary with type of ribozymes. On the other hand, *td* intron RNA appears to be more susceptible to the degradation induced by Mn^{2+} compared to other ribozyme.

In the case of Zn^{2+}, even at 0.5 mM concentration, the pre-RNA was completely hydrolyzed. This observation suggested that Zn^{2+} facilitates RNA hydrolysis more rapidly than Mn^{2+} does. At 0.5 mM Zn^{2+}, a very small amount of both the linear and circular introns was produced but ligation product (E1-E2) was not produced at all.

At the concentration of Zn^{2+} higher than 1 mM, the pre-RNA was completely hydrolyzed, suggesting the inability of Mg^{2+} to overcome the degradation of RNA caused by Zn^{2+}. Like *td* intron RNA, Zn^{2+} at 0 - 10 mM did not show any cleavage activity of *Tetrahymena* ribozyme in the absence of Mg^{2+} (Davies et al., 1982). Very similar observation was also made with the human hapatitis delta virus. However, in the hammerhead ribozyme, Zn^{2+} can partially fulfill the catalytic ion requirement only in the presence of spermidine (Dahm and Uhlenbeck, 1991).

Thus, it can be concluded that Mn^{2+} and Mg^{2+} display quite unique functions in the splicing reaction of *td* intron RNA and that the catalytic roles of divalent cations vary with kinds of ribozymes.

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