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Role of Mg^{2+} in RNA Splicing of T4 *td* Intron

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The splicing activity of T4 phage *td* intron RNA has been examined with various Mg^{2+} ions such as $MgCl_2$, $MgSO_4$ and magnesium acetate using various splicing conditions such as different incubation time and temperature. The maximum splicing of *td* intron RNA occurred at the concentration of 5 mM $MgCl_2$. Raising the Mg^{2+} concentration up to 15 mM appeared to promote P2 deletion mutant to overcome the loss of some splicing activity. In both wild type and mutant, a complete hydrolysis of RNA occurred at 30 mM $MgCl_2$. $MgSO_4$ and magnesium acetate exhibited the rate and pattern of RNA splicing identical to $MgCl_2$. The optimal splicing conditions involve the incubation of RNA with 5 mM $MgCl_2$ at 58°C for 15 min. The results suggest that Mg^{2+} may play a key role in the catalytic mechanism of *td* intron RNA.

Key words: T4 phage *td* intron, Mg^{2+} ion, RNA splicing

T4 phage thymidylate synthase gene(*td*), the first intron-containing procaryotic protein encoding gene, contains a group I intron (3). Similar to the *Tetrahymena thermophila* large rRNA precursor, the *td* precursor RNA can undergo self-splicing *in vitro* in the absence of any protein factors (1, 11). The self-splicing of *td* intron is achieved by a series of transesterification reaction. The first involves a guanosine added to the 5′ end of the intron, releasing the 5′ exon; the second involves the ligation of the 5′ and 3′ exons, releasing the intron as a linear molecule; the third joins the 3′ nucleotide of the intron to a nucleotide near the 5′ end of the intron to form a circular intron molecule, releasing an oligonucleotide containing the added guanosine (2). Because protein factors are not required in this reaction, it has been postulated that the RNA can assume a critical conformation enabling it to undergo self-splicing, site specific autocatalytic cleavage and ligation (9).

Like protein enzymes, catalytic RNAs or ribozymes have been shown to need divalent metal ions essential for RNA splicing (19). It has been suggested that metal ions have two important roles in RNA splicing activity. Metal ions are not only important in determining the tertiary structure of RNA but also they provide reactive groups for RNA splicing at the active catalytic site (7). In particular, RNA splicing of *Tetrahymena* intron is essentially dependent on the presence of Mg^{2+} or Mn^{2+} ions which are likely to play an important structural and catalytic role (10). In the present study we report the role of Mg^{2+} ion in RNA splicing of T4 phage *td* intron.

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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 from Promega Biotec.

Enzyme and chemicals

Restriction enzyme *Eco*RI and *Hin*dIII were obtained from New England Biolabs. [α-^{32}P]GTP (>400 Ci/mmol) was obtained from Amersham. Nucleoside triphosphate was 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 Biotec.

Construction and preparation of recombinant plasmids

The 2.85 kb *Eco*RI fragment containing the T4 *td* gene was originally cloned from strain T4-alc4. The *td*-containing fragment was inserted into M13 phage to yield M13 *td* in the *Eco*RI site. For subcloning of *td*, M13 recom-

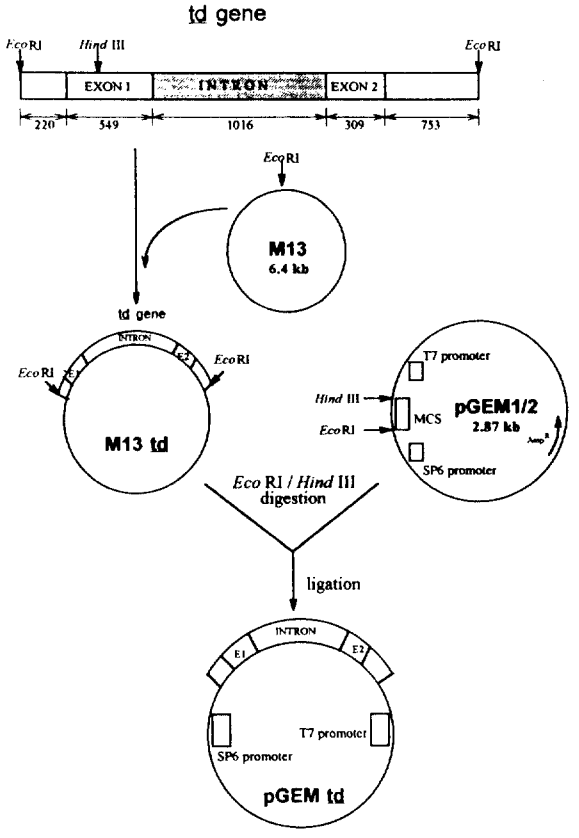

Fig. 1. Construction of pGEM-1/2 containing the T4 *td* gene. *Eco*RI-*td* fragment was cloned into M13mp8. M13 *td* recombinant and pGEM-1/2 plasmid were digested with *Eco*RI and linearized byproducts were subjected to ligation by DNA ligase. Ligated recombinant pGEM-*td* was used for the measurement of RNA splicing *in vitro*.

binant phage was infected into *E. coli* TG1 cells. The M13 *td* gene was extracted from 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_2 EDTA, 5% triton X-100, 8% sucrose) and lysed by boling for 2 min in the presence of 1 mg 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 (Fig. 1). 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.

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 (4). 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 SP6 or T7 RNA polymerase. RNA synthesis was terminated by the addition of RQ1 DNase to destroy the DNA template. After transcription, 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 at −20℃. 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 stored at −70℃.

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 concentration of $MgCl_2$ for the different incubation time and with 5 mM $MgSO_4$ and magnesium acetate at 58℃ 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℃.

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 throughly rinsed with water from the Millipore purification system.

Results and Discussion

The splicing of *td* intron RNA occurs by an autocataly-

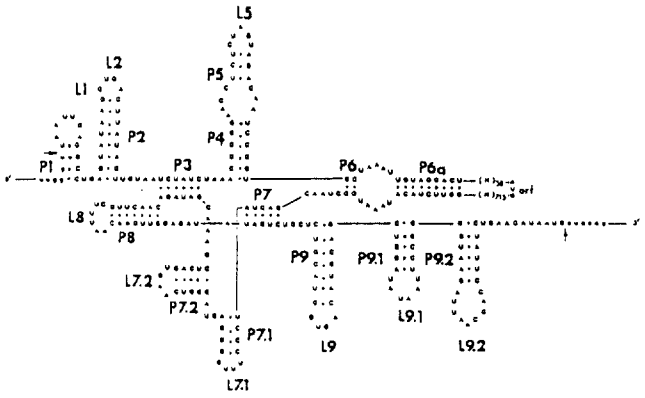

Fig. 2. Proposed secondary structure of phage T4 *td* intron RNA. Arrows indicate the 5'end and 3'end splice sites. Numerical values are numbered from the 5'end of the intron. Intron bases are in upper case letters and exon bases in lower case letters.

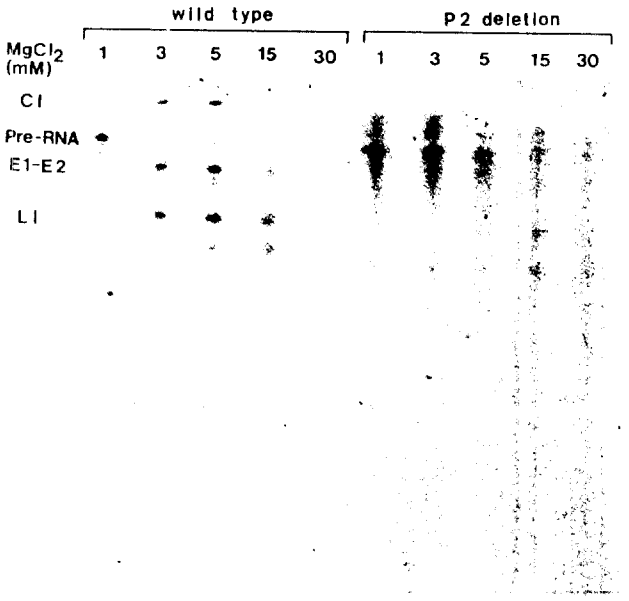

Fig. 3. Effect of varying concentrations of $MgCl_2$ on splicing activity of wild type and P2 deletion mutant T4 *td* RNAs. [α-^{32}P]labeled td primary transcript was incubated for 15 min at 58℃. $MgCl_2$ concentrations of 1, 3, 5, 15 and 30 mM were tested in the wild type and mutant *td* splicing reaction containing 100 μM GTP.

tic mechanism resembling that of many group I introns (8). As illustrated in Fig. 2 the proposed hypothetical secondary structure of *td* intron RNA is comprised of 9 paired segments ranging from P1 to P9.1, some of which are stem-loop structures. Most of the predicted secondary structures have been implicated as essential determinants for the self-splicing activity intrinsic group I RNAs (12).

In the current study one deletion mutation (−P2) was created using site-directed mutagenesis to determine whether increasing the concentration of Mg^{2+} in RNA splicing reaction can suppress the defect in splicing of mutant RNA (17). The effect of varying concentrations of $MgCl_2$ on splicing activity of wild type and P2 deletion mutant RNAs was shown in Fig. 3. In order to examine the minimum concentration of Mg^{2+} necessary for RNA splicing, incubation was performed at 58℃ for 15 min. 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 the hepatitis delta virus genomic DNA (16). Unlike the wild type 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 activity. As in the case of the wild type, a complete hydrolysis of mutant RNA occurred at 30 mM $MgCl_2$. The mechanism by which a higher concentration of Mg^{2+} led to a complete hydrolysis of *td* intron RNA is still not known.

In *Tetrahymena* intron Mn^{2+} can substitute Mg^{2+} but not Ca^{2+}, Zn^{2+}, Co^{2+}, or Pb^{2+} (5). The reversal of intron cyclization requires a Mg^{2+} optimum of 10 mM (18) while the L-21 *Sca*I form of the *Tetrahymena* intron requires 2 mM Mg^{2+} for RNA splicing (14). While Ca^{2+} does not have any activity by itself, it alleviates a portion of the Mg^{2+} requirements. It has been shown that the Mg^{2+} optima are much higher for RNase P RNA (13). The maximum splicing of hairpin ribozyme derived from tobacco ringspot virus satellite RNA was observed at a concentration of 10 mM $MgCl_2$ (6). Among the cations tested in the current study, Mg^{2+} was found to be the optimal cation for the cleavage of *td* intron RNA. Unlike *Tetrahymena* intron rRNA Ca^{2+} did not have any alleviating effect on Mg^{2+} requirement for *td* intron RNA but instead resulted in almost complete hydrolysis of *td* intron RNA (data not shown).

Some discrepancy in optimal concentrations of Mg^{2+} requirement for RNA splicing among different ribozymes may be derived from different splicing reaction conditions including the different mechanism of RNA folding (20).

The splicing activity of T4 phage *td* intron RNA has been tested with various Mg^{2+} ions such as $MgSO_4$ and magnesium acetate under the different incubation time and temperature (Fig. 4). The better splicing reaction

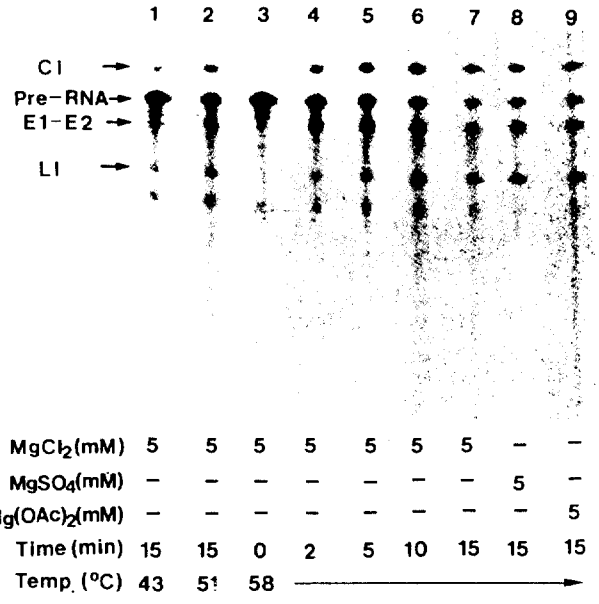

Fig. 4. Splicing activity of *td* intron RNAs when incubated with various Mg^{2+} ions under the different incubation time and temperature. [^{32}P]labeled *td* primary transcript was incubated for different time intervals indicated in the lower panel with 5 mM $MgCl_2$ at 43℃ (lane 1), 51℃ (lane 2) and 58℃ (lanes 3-7). In lanes 8 and 9, 5 mM $MgSO_4$ and 5 mM magnesium acetate were used in splicing reaction instead of 5 mM $MgCl_2$, respectively.

occurred at 51℃ compared to 43℃ when the pre-RNA was incubated with 5 mM $MgCl_2$ for 15 min (Fig. 4, lanes 1 and 2). Thus, the incubation temperature at 58℃ higher than 51℃ was used to determine the optimal incubation time for splicing. The results showed that 15 min incubation time resulted in three distinct cleavage products of RNA such as circular intron, exon1-exon2 ligation product and linear intron along with a small amount of pre-RNA remaining (Fig. 4, lane 7). The pre-RNA underwent no splicing at zero time incubation and remained almost intact without any cleavage (Fig. 4, lane 3). However, the splicing started to occur beginning from 2 min incubation time (Fig. 4, lane 4). When $MgSO_4$ and magnesium acetate replaced $MgCl_2$ as metal ion source there were no changes observed in the rate and pattern of RNA splicing (Fig. 4, lanes 8 and 9). This suggests that sulfate ion, chloride ion and acetate ion did not exert any impact on the cleavage reaction of *td* intron RNA. It appears that Mg^{2+} ion may have special catalytic roles in stabilizing the structure of RNA by neutralizing the negative charge of RNA which may lead to facilitate the nucleophilic attacks of guanosine or GTP (15). By interacting with adjacent functional nucleotides, Mg^{2+} may bridge separate RNA strands and initiate to induce the proper folding of RNA structure. Overall these results imply that the catalytic roles of Mg^{2+} may vary with the kinds and origin of RNA even among the same group I RNAs and the optimal metal ion concentration will be dependent on other reaction conditions that may affect the active structure of RNA.

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