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Effects of K^+ Ion on *in vitro* RNA Splicing of T4 Phage Thymidylate Synthase Gene

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The effects of K^+ ion on the activity of RNA splicing of T4 phage thymidylate synthase gene have been investigated. The splicing activity was stimulated within the range of 5 to 20 mM concentration of KCl. When the concentration of KCl in the splicing reaction was brought to 100 or 200 mM a small amount of the exonI-intron product (1.4 kb) was formed with large proportion of primary RNA transcript not undergoing splicing. This observation strongly suggests that there may exist some kinds of interferences with transesterification at the first step of splicing. Overall it can be concluded that K^+ ion exhibits very unique roles in RNA splicing of *td* gene depending on its concentration.

Key words: T4 phage *td intron*, K^+ 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 and energy source (1, 7). 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 dinucleotide 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 site specific autocatalytic cleavage and ligation (5).

Metal ions have been suggested 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 (14, 15, 16). In the fifth intron of *COB* gene of yeast mitochondria K^+ ion was found to be more effective in inducing the RNA splicing than other monovalent ions tested (13). 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 (10). Since some monovalent ions have been believed to be involved in the stabilization of RNA structure and its subsequent cleavage reaction (9), we decided to look at whether K^+ ion produces any stimulatory or inhibitory functions in RNA splicing of T4 phage thymidylate synthase gene. In the present study we demonstrate that K^+ ion plays very unique roles in splicing depending on its concentration.

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 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 Biotec.

Construction and preparation of recombinant plasmids

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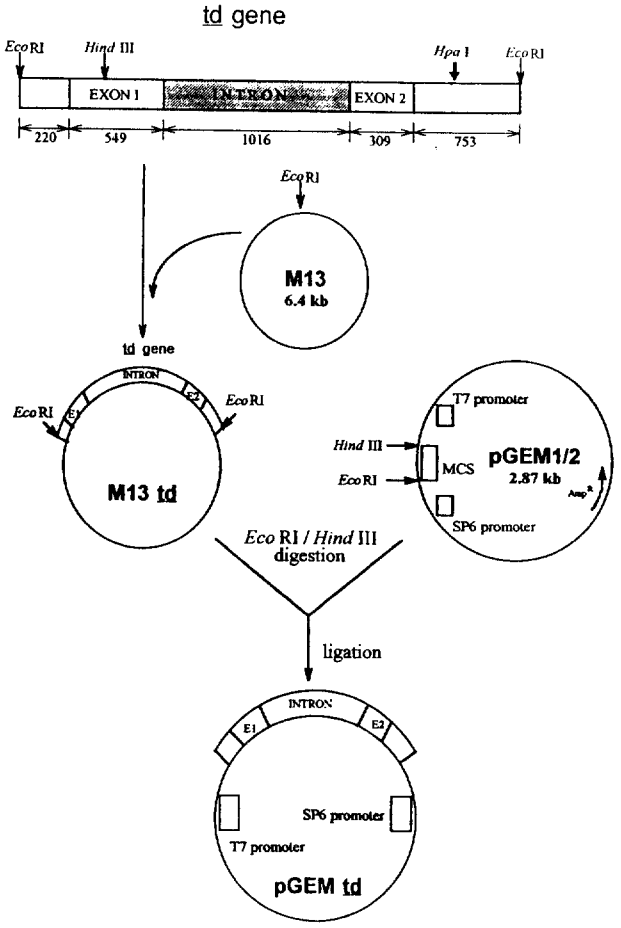

Fig. 1. Construction of pGEM-1/2 containing the T4 *td* gene. *Eco*RI-*td* fragment was cloned into M13mp8. M13 *td* recombinant and pGEM-1/2 plasmid were digested with *Eco*RI and linearized byproducts were subjected to ligation by DNA ligase. Ligated recombinant pGEM-*td* was used for the measurement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

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 recombinant 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_2EDTA, 5% triton X-100, 8% sucrose) and lysed by boiling 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 T7 RNA polymerase. RNA synthesis was terminated by the addition of RQ1 DNase to destroy the DNA template. Following 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. 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 KCl in the presence 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_2 EDTA). 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 remained pre-

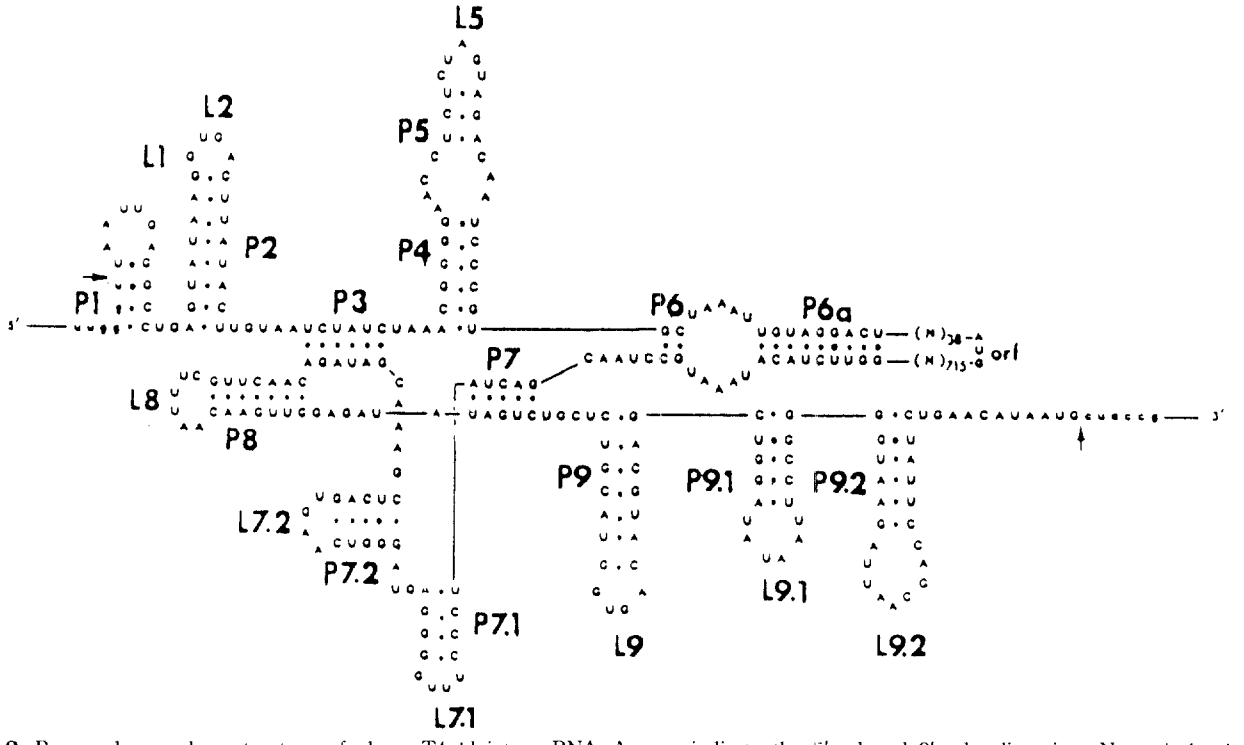

Fig. 2. Proposed secondary structure of phage T4 *td* intron RNA. Arrows indicate the 5'end and 3'end splice sites. Numerical values are numbered from the 5'end of the intron. Intron bases are in upper case letters and exon bases in lower case letters.

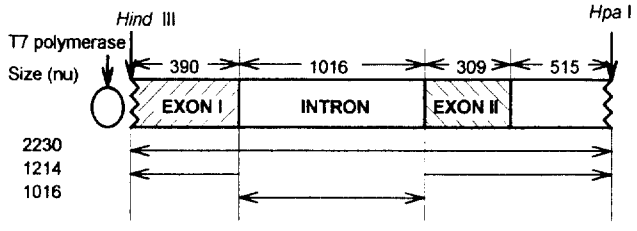

Fig. 3. Organization of T4 *td* primary transcript of recombinant pGEM-*td* plasmid.

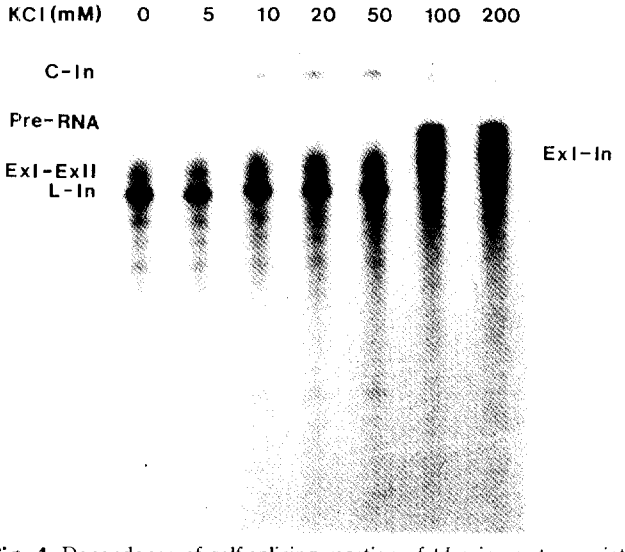

Fig. 4. Dependence of self-splicing reaction of *td* primary transcript on concentration of K^+. *In vitro* splicing reaction was carried out for 10 min at 58°C with varying concentrations of KCl as indicated in the presence of 5 mM $MgCl_2$. The spliced RNA products were analyzed in 5% polyacrylamide-8 M urea gel electrophoresis and visualized by autoradiography. Abbreviation: 2.23-kb Pre-RNA, *td* precursor RNA; ExI-ExII, 1.21-kb ligated exon product; ExI-In, 1.4-kb exonI-intron product; C-In and L-In, circular and linear forms of intron, respectively.

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. 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 (8). The 2.23 kb primary transcript of pGEM recombi-

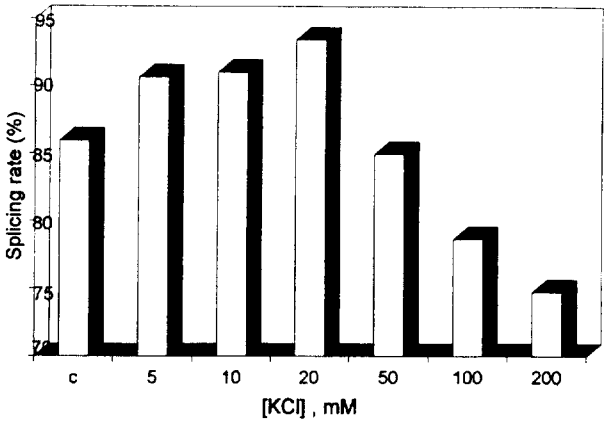

Fig. 5. Rates of self-splicing of *td* primary transcript as a function of concentration of K^+. The extent of splicing reaction was determined by scanning the remained pre-RNA. The splicing rate(%) represents the ratio of $F=([\text{pre-RNA}]_t/[\text{pre-RNA}]_0)$. C shows the rate of splicing at 5 mM $MgCl_2$ in the absence of KCl.

nant plasmid containing *td* gene was generated by T7 RNA polymerase. As schematically shown in Fig. 3, the *td* gene is cleaved into two unequal parts (exon I and exon II) by a 1016 base pair intron, and 1214 base pair of ligated exon is produced.

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 (17).

The dependence of splicing reaction of *td* primary RNA transcript on the concentration of KCl was shown in Fig. 4. The varying concentrations of KCl were incubated with *td* primary RNA transcript prior to the addition of 5 mM $MgCl_2$. In the control splicing reaction without KCl the RNA splicing occurred normally (lane 1). When the concentrations of KCl increased gradually from 5 to 20 mM (lanes 2, 3 and 4) the splicing reaction was stimulated, resulting in the formation of more ligation products.

In fact the maximum splicing activity occurred at 20 mM KCl (Fig. 5, lane 4) which corresponded to approximately 8% higher than that of control splicing reaction (Fig. 5, lane 1). In the presence of 100 mM or 200 mM KCl, however, a small amount of the exonI-intron product (1.4 kb) was observed instead of exonI-II ligation product and a large proportion of primary RNA transcript still did not undergo splicing (Fig. 4, lane 5 and 6). In contrast, in the case of the splicing reaction of the fifth intron of *COB* gene of yeast mitochondria the K^+ ion at 100 mM was observed to be more effective in inducing the splicing than other monovalent ions such as Na^+, NH_4^+, Rb^+ and Cs^+ tested (13). The rate of splicing was found to be inversely related to the hydration number of ion (11, 12). The fact that the presence of exonI-intron was observed strongly suggests some kind of interferences with transesterification at the first step of splicing (5). At the concentration of KCl higher than 50 mM the splicing rate declined dramatically (Fig. 5, lanes 5, 6 and 7). On the other hand, in the self-splicing reaction of ribozyme derived from *Neurospora* VS RNA K^+ ion at 50 mM stimualtes the reaction in the presence of 5 mM $MgCl_2$ only but cannot replace Mg^{2+} ion (6). The additional requirement for Mg^{2+} strongly suggests that K^+ ion may act as structural counterions, facilitating folding of the RNA and/or be directly involved in the splicing mechanism. This implies that the splicing activity varies with different concentrations of K^+ ion in splicing reaction. Unlike other ribozymes the K^+ ion exhibits very unique function in splicing activity of *td* intron RNA. Thus it can be concluded that the catalytic roles of K^+ ion may differ with kinds of ribozymes and in particular *td* intron RNA exhibits K^+ ion's concentration dependent splicing activity.

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