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Suppressive Effects of Divalent Cations on Self-splicing Inhibition by Spectinomycin of Group 1 Intron RNA

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Effects of divalent cations on self-splicing inhibition by the antibiotic spectinomycin of the phage T4 thymidylate synthase intron (*td*) have been investigated. Ca^{2+} ion at 1 mM concentration suppressed splicing inhibition of spectinomycin by 10% and 50 μM Co^{2+} ion also suppressed splicing inhibition of spectinomycin by 10%. Mg^{2+} ion at 6 mM concentration decreased splicing inhibition of spectinomycin by 42% while Mn^{2+} ion decreased the splicing inhibition by 10%. Zn^{2+} ion at 10 uM concentration lowered the splicing inhibition by spectinomycin of 15%. Of all divalent cations tested, Mg^{2+} ion was the most effective in suppressing splicing inhibition by specinomycin whereas Ca^{2+} ion was the least effective. The results suggest that spectinomycin may interact with specific and functional Mg^{2+}-binding sites within intron RNA that lead to a displacement of Mg^{2+} essential for catalytic activity.

Key words: Spectinomycin, divalent cations, T4 phage, *td* intron RNA

T4 phage thymidylate synthase gene (*td*), the first intron-containing procaryotic protein encoding gene, contains a group intron (4). Similar to the *Tetrahymena thermophilia* large rRNA precursor, the *td* precursor RNA can undergo self-splicing *in vitro* in the absence of any protein factors and energy source. The self-splicing of the *td* intron is achieved by a series of transesterification reactions (1).

Like protein enzymes, catalytic RNAs or ribozymes have been shown to need divalent metal ions essential for RNA splicing (16). 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 provide reactive groups for RNA splicing at the active catalytic core site (3). For group ribozymes, Mg^{2+} has been shown to participate in RNA folding, substrate binding, and catalysis of phosphotransesterification (8). The *Tetrahymena thermopohilia* L-21 ScaI ribozyme utilizes Mg^{2+} to catalyze a site specific endonuclease reaction analogous to the first step of rate increase in RNA substrate binding, one or more decrease in the rate of substrate dissociation, and two are involved in the chemical step (10). Mg^{2+}, Mn^{2+}, Ca^{2+}, and Sr^{2+} efficiently supported the self-cleavage reactions of the genomic human delta virus ribozyme, and Cd^{2+}, Ba^{2+}, Co^{2+}, Pb^{2+} and Zn^{2+} were also able to support the reaction to some extent. In our previous studies we found that monovalent cations such as K^+ and Na^+ exerted the inhibition of self-splicing *td* intron RNA at a concentration ranging from 30 mM to 200 mM in the presence of 5 mM Mg^{2+}, and Na^+ was more effective in inhibiting the splicing reaction than K^+ (15).

Spectinomycin (Fig. 1) is an aminoglycoside antibiotic produced by *Streptomyces spectabilis* and contains the aminocyclitol actinamine (13). This antibiotic selectively inhibits protein synthesis in cells and inhibit polypeptide synthesis *in vitro* by binding to and acting on the 30S ribosomal subunit. Spectinomycin causes the dissociation of cell-free initiation complexes prepared with either the 30S or 70S ribosome (12). Both

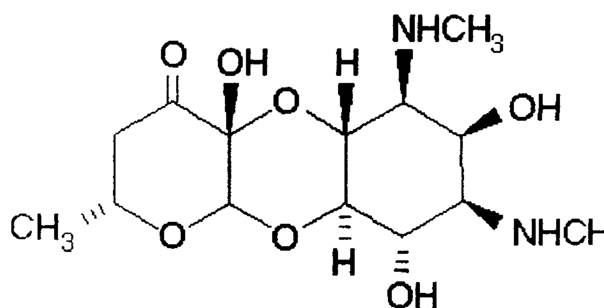

Fig. 1. The chemical structure of spectinomycin.

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the initiation factor and the Mg^{2+}-induced processes are subject to inhibition by spectinomycin. Furthermore, spectinomycin differs from streptomycin and other aminoglycoside antibiotics in that it is bacteriostatic instead of bactericidal and does not cause any detectable misreading of polynucleotides (7).

Here we describe the effects of divalent cations on the self-splicing inhibition of the *td* intron RNA by spectinomycin.

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-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 U.S. Biochemical and RNAsin (40 U/μl) and RQ1 DNase (1U/μl) from Promega Corp.

Construction and preparation of recombinant plasmids

The cloning procedures were as described previously (4). The pGEM recombinant plasmids 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*I 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 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, 5μCi 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 2.23 kb primary transcript was purified free of proteins, ribonucleotides and salts by the passage through a Nensorb 20 cartridge (Dupont). 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 divalent cations were added to the splicing reaction buffer in the presence of spectinomycin to examine their effects on 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 was added (95% deionized formamide, 10 mM Na_2EDTA) and visualized by autoradiography without drying. Autoradiograms were scanned and integrated with a Hoefer image analyzer. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity. Three separate experiments were performed to determine the relative splicing activity.

RNA measurement

RNA concentration was determined by the spectrophotometric method. The extinction coefficient was determined by hydrolyzing the RNA to nucleotides and by measuring the absorbance at 260 nm of the resulting mixture.

Results and Discussion

To examine the effect of divalent cations on the self-splicing inhibition by spectinomycin of the *td* intron RNA, pre-RNAs were incubated with varying concentrations of metal ions such as $MgCl_2$, $CaCl_2$, $CoCl_2$, $MnCl_2$ and $ZnCl_2$ under normal splicing conditions. Since pre-RNA was almost completely hydrolyzed at 1 mM of Ca^{2+}, Mn^{2+} and Zn^{2+} (11), we tested a lower concentration range of Ca^{2+} (0.05~1 mM), Co^{2+} (1~50 μM), Mn^{2+} (0.5~10 μM) and Zn^{2+} (0.5~10 μM) for the splicing activity. The effect of Ca^{2+} on self-splicing inhibition by spectinomycin is shown in Fig. 2. As the concentration of added Ca^{2+} to the splicing reaction rises, the splicing rate appears to rise only slightly. At 1 mM Ca^{2+} the splicing rate increased by 10% compared to that in the absence of Ca^{2+}. In *Tetrahymena thermophila* large rRNA precursor Ca^{2+} acts to reduce a portion of Mg^{2+} requirement for splicing activity (8).

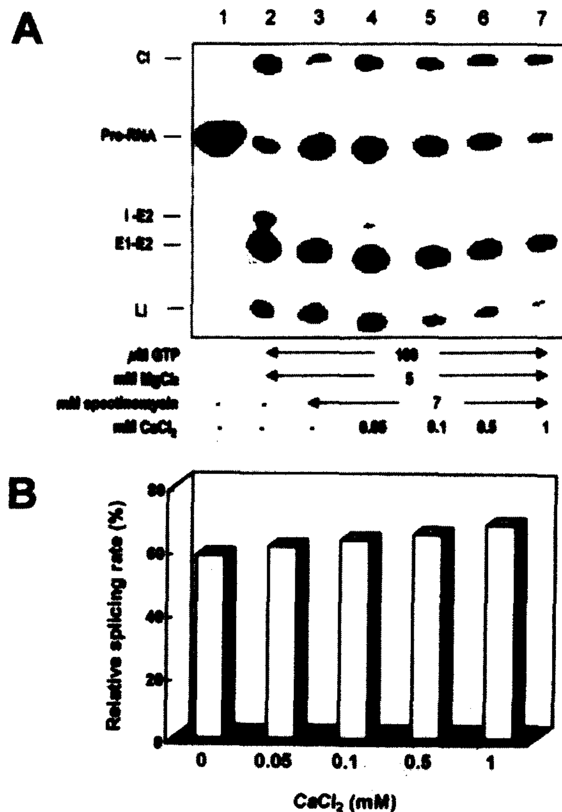

Fig. 2. Effects of Ca^{2+} on self-splicing inhibition by spectinomycin. A. Autoradiogram showing the effect of varying concentrations of Ca^{2+} (0.05~1 mM) on self-splicing. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; E1-E2, exon 1-exon 2; LI, linear intron; CI, circular intron. B. Splicing rates of the intron RNA as a function of Ca^{2+} concentration. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity.

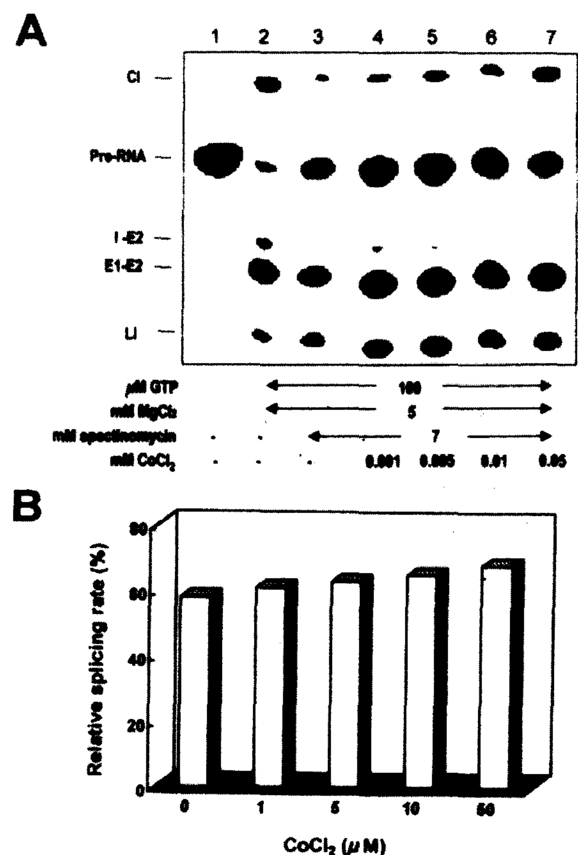

Fig. 3. Effects of Co^{2+} on the self-splicing inhibition by spectinomycin. A. Autoradiogram showing the effect of varying concentrations of Co^{2+} (1~50 μM) on self-splicing. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; E1-E2, exon 1-exon 2; LI, linear intron; CI, circular intron. B. Splicing rates of intron RNA as a function of Co^{2+} concentration. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity.

They 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 RNA.

The effect of Co^{2+} on self-splicing inhibition by spectinomycin is shown in Fig. 3. The inhibition of the splicing rate by spectimomycin was recovered slightly by adding Co^{2+} whose concentration ranges from 1 to 50 μM.

At 50 μM Co^{2+} the recovered splicing rate was about 10%, demonstrating the enhancement of the formation of the circular intron and exon 1-exon 2 with the corresponding reduction of pre-RNA.

The effect of $MgCl_2$ on the self-splicing inhibition of the *td* intron RNA by spectinomycin is shown in Fig. 4. As shown in lane 2, normal splicing products such as the circular intron, intron-exon 2, exon 1-exon 2 and the linear intron were detected in the absence of spectinomycin. Upon the addition of 7 mM spectinomycin to the normal splicing reaction, the formation of the circular intron and intron-exon intermediate was almost completely abolished with the corresponding increase of pre-RNA (lane 3). As illustrated in Fig. 4A the formation of splicing products such as the circular intron, intron-exon 2, exon 1-exon 2, and linear intron were progressively reduced as the concentration of spectinomycin was gradually increased. In particular, the formation of the I-E2 intermediate was appreciably reduced relative to the formation of the E1-E2 ligation product and the linear intron, indicating the inhibition of the first step of self-splicing. It is of a great interest to note that splicing inhibition was reversed by increasing the concentration of Mg^{2+} ions, suggesting the competitive nature of the Mg^{2+} ion with spectinomycin in the splicing reaction. Furthermore, increasing the Mg^{2+} concentration up to 8 mM fully restored the splicing activity to the normal splicing level. This strongly suggests that inhibition by spectinomycin was specifically

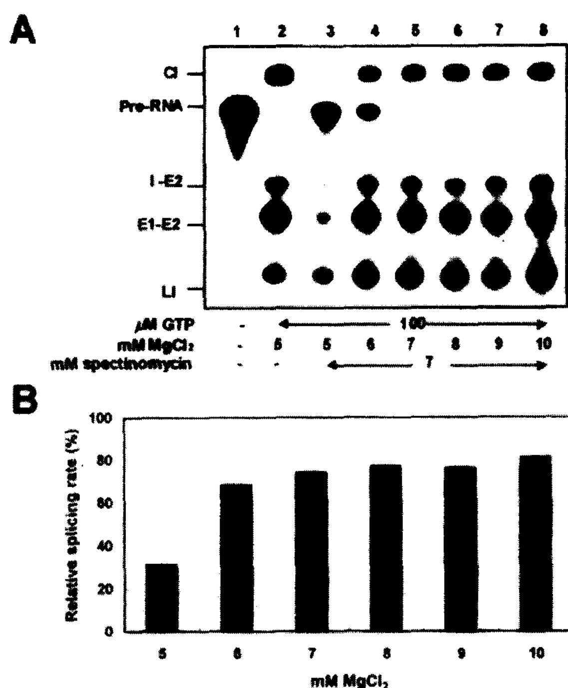

Fig. 4. Effects of Mg^{2+} on self-splicing inhibition by spectinomycin. A. Autoradiogram showing the effect of varying concentrations of Mg^{2+} (5~10 mM) on self-splicing. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; E1-E2, exon 1-exon 2; LI, linear intron; CI, circular intron. B. Splicing rates of intron RNA as a function of Mg^{2+} concentration. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity.

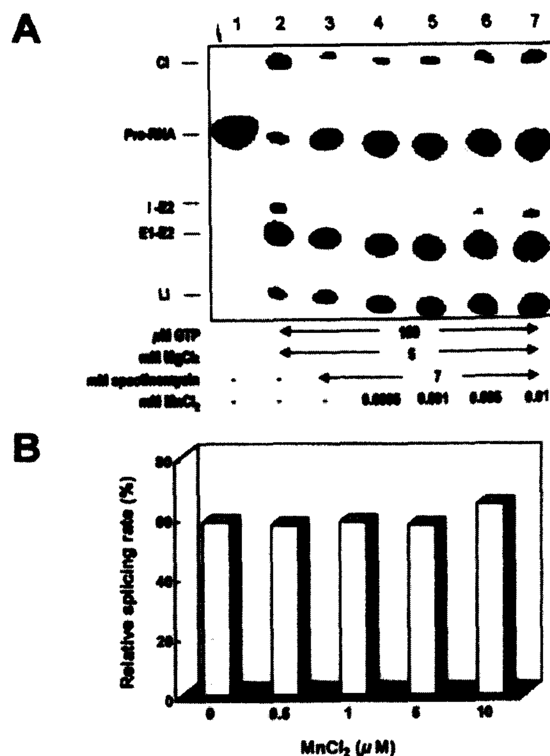

Fig. 5. Effects of Mn^{2+} on self-splicing inhibition by spectinomycin. A. Autoradiogram showing the effect of varying concentrations of Mn^{2+} (0.5~10 μM) on self-splicing. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; E1-E2, exon 1-exon 2; LI, linear intron; CI, circular intron. B. Splicing rates of intron RNA as a function of Mn^{2+} concentration. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity.

Mg^{2+} dependent, probably interfering with the catalytic function of Mg^{2+} in the splicing reaction. If the interaction of spectinomycin with intron RNA is primarily ionic, increasing concentrations of Mg^{2+} should alleviate spectinomycin inhibition of the splicing activity by acting as a competing counterion. This appears to be what happened in the present study. One possibility is that Mg^{2+} ions probably saturate the hydroxyl residue of spectinomycin, neutralizing its charges and possibly protecting the splicing activity from inhibition by spectinomycin. Besides its role as a catalytic cofactor, Mg^{2+} is thought to be involved in proper folding and general electrostatic interaction to shield the phosphodiester backbone (8). Similarly, the splicing inhibition by viomycin and tuberactinomycin was fully reversed by increasing the Mg^{2+} concentration (17). It has been suggested that the tuberactinomycin antibiotic family have more than one contact site with the intron RNA via the G-binding site and via additional contacts with the RNA backbone (17). This implies that spectinomycin may interact with other specific sites within the intron RNA structure which could be Mg^{2+}-binding sites although its inhibitory mechanism is not fully understood. Recently, the antibiotic neomycin B has been shown to inhibit self-splicing of the *td* intron RNA by binding to the internal loop between the stems P4 and P5 and displacing Mg^{2+} ions in the catalytic core (9). The splicing inhibition by neomycin was strongly dependent on pH and Mg^{2+}concentration, suggesting electrostatic interactions and competition with Mg^{2+}.

The effect of Mn^{2+} on self-splicing inhibition by spectinomycin is shown in Fig. 5. With the concentration ranging from 0.5 to 5 μM Mn^{2+}, the splicing rate was not affected at all but at 10 μM Mn^{2+} the splicing rate was recovered about 6%. For the splicing reaction of the human hepatitis delta virus ribozyme, Mn^{2+} was the most effective divalent cation at concentrations below 1 mM (14). Above 1 mM Mn^{2+}, however, nonspecific degradation occurred. In contrast, Mn^{2+} behaves very similarly to Mg^{2+} in the splicing activity of *Tetrahymena* ribozyme (8). This suggests that the roles of the Mn^{2+} vary with the type of ribozymes.

The effect of Zn^{2+} on self-splicing inhibition by spectinomycin is shown in Fig. 6. The inhibition of the

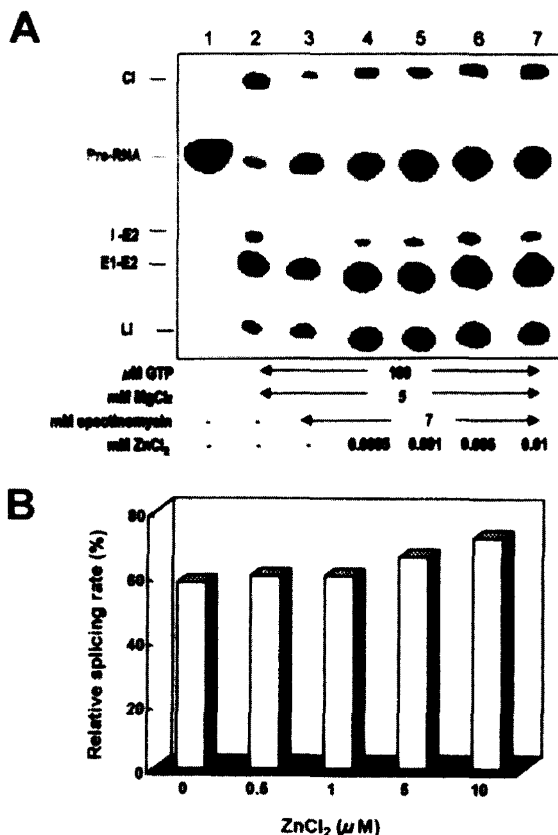

Fig. 6. Effects of Zn^{2+} on self-splicing inhibition by spectinomycin. A. Autoradiogram showing the effect of varying concentrations of Zn^{2+} (0.5~10 μM) on self-splicing. Lane 1, unspliced pre-RNA; I-E2, intron-exon 2; E1-E2, exon 1-exon 2; LI, linear intron; CI, circular intron. B. Splicing rates of intron RNA as a function of Zn^{2+} concentration. The ratio of E1-E2 ligation produced in each splicing reaction to that of the normal splicing reaction was expressed as relative splicing activity.

splicing rate by spectinomycin was not affected until the concentration of Zn^{2+} added reached 5 μM. At 10 μM Zn^{2+}, the splicing rate was recovered by 15%. It has been shown that Zn^{2+} can partailly fulfill the catalytic ion requirement only in the presence of spermidine (6).

Of all divalent cations, Mg^{2+} was the most effective at suppressing splicing inhibition by spectinomycin. Although the mechanism by which Mg^{2+} ions actually interact with spectinomycin in the splicing reaction of the *td* intron RNA has not been clearly established yet, we propose that Mg^{2+} ions subsequently bind to the hydroxyl groups on spectinomycin, either neutralizing the charges or interfering with its binding to some specific sites in the RNA structure.

Acknowledgments

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