

Metal ion interaction with cosubstrate in self-splicing of group I introns

A.-S. Sjögren, E. Pettersson¹, B.-M. Sjöberg and R. Strömberg^{1,2,*}

Department of Molecular Biology and ¹Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-10691 Stockholm, Sweden and ²Laboratory of Organic and Bioorganic Chemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden

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ABSTRACT

The catalytic mechanism for self-splicing of the group I intron in the pre-mRNA from the *nrdB* gene in bacteriophage T4 has been investigated using 2'-amino-2'-deoxyguanosine or guanosine as cosubstrates in the presence of Mg²⁺, Mn²⁺ and Zn²⁺. The results show that a divalent metal ion interacts with the cosubstrate and thereby influences the efficiency of catalysis in the first step of splicing. This suggests the existence of a metal ion that catalyses the nucleophilic attack of the cosubstrate. Of particular significance is that the transesterification reactions of the first step of splicing with 2'-amino-2'-deoxyguanosine as cosubstrate are more efficient in mixtures containing either Mn²⁺ or Zn²⁺ together with Mg²⁺ than with only magnesium ions present. The experiments in metal ion mixtures show that two (or more) metal ions are crucial for the self-splicing of group I introns and suggest the possibility that more than one of these have a direct catalytic role. A working model for a two-metal-ion mechanism in the transesterification steps is suggested.

INTRODUCTION

Self-splicing of group I introns from precursors (pre-mRNAs) to messenger RNAs requires two consecutive transesterifications of internucleosidic phosphodiester linkages. The first step being cleavage of the exon 1–intron junction by an exogenous guanosine cosubstrate and the second being ligation of the exons (1,2). The pre-mRNA from the *nrdB* gene in bacteriophage T4 contains a self-splicing group I intron (Scheme 1a) (3,4). Our present work is focused on the first step of splicing, i.e., where the external guanosine cosubstrate, with the 3'-hydroxyl as the nucleophilic function, attacks the internucleosidic phosphodiester linkage at the exon 1–intron junction and becomes covalently attached to the 5'-end of the intron (Scheme 1b). It is well known that divalent metal ions are necessary for the function of group I introns (1,2,5). More recently, photo-crosslinking experiments in the L-21 ScaI system have shown that magnesium ion coordination near the splice site is crucial for folding into an active ribozyme (6). In addition, phosphorothioate substitution

interference experiments have identified several magnesium ion coordination sites close to where the cosubstrate binds (7).

Piccirilli *et al.* (8) have provided experimental support for the existence of a magnesium ion that promotes the reaction by coordinating to, and facilitating leaving of, the oxyanion of exon 1 (oxygen c in Scheme 1b). In an analogous model system (trimethoxyphosphorane dianion) calculations on the influence of metal ions suggest that the phosphorane intermediate would break down spontaneously in the presence of magnesium ions (9). Mechanisms involving two catalytic metal ions have been discussed (1,2,5,8–11). Steitz and Steitz suggested that splicing by group I introns utilises a two-metal-ion mechanism which may be general for many enzyme-catalysed phosphoryl transfers (10). Molecular dynamics simulation also shows the plausibility of a mechanistic model involving two catalytic magnesium ions (11). Furthermore, Streicher *et al.* (12) have reported indications of two defined metal ion binding pockets close to the cleavage site in the phage T4 *td* intron and suggested that these may be sites for catalytic ions. However, until now no experimental support has been reported for a metal ion that would be involved in promoting nucleophilic attack by the guanosine cosubstrate.

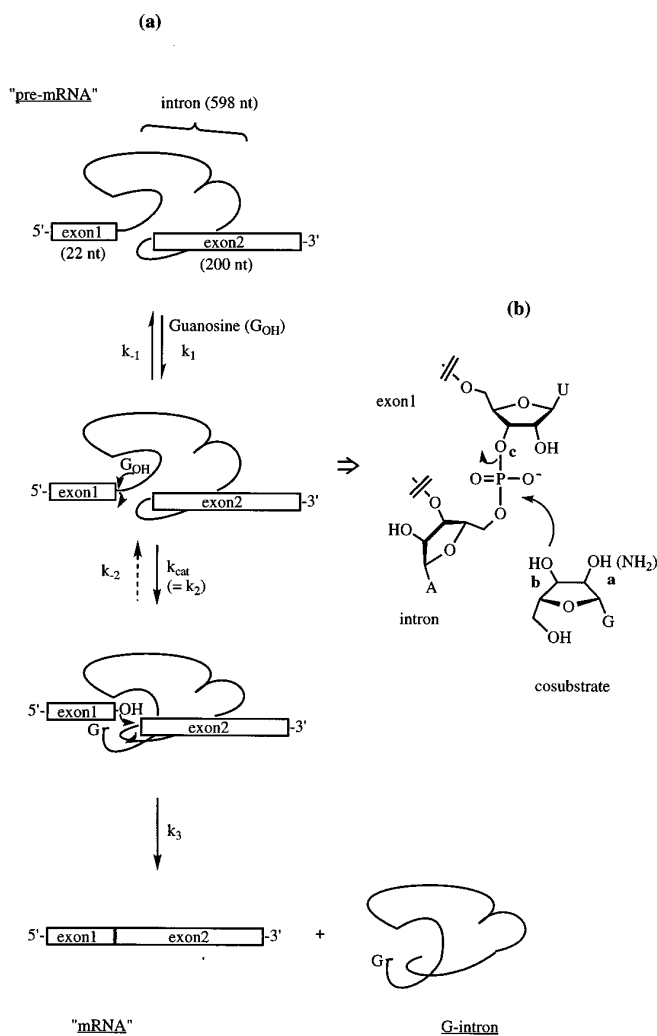
The 2'-hydroxyl of the cosubstrate is critical for the first catalytic step (13) but not as important for binding, since 2'-deoxyguanosine is a competitive inhibitor (14) and even binds slightly tighter than guanosine in the L-21 ScaI ribozyme (15). We have shown previously that 2'-amino-2'-deoxyguanosine can replace guanosine as cosubstrate in group I self-splicing, albeit with lower efficiency (16). In the present study we have used this amino analogue, in the presence of Mg²⁺ or Mn²⁺ as well as in mixtures of Mg²⁺ and Mn²⁺ or Zn²⁺, as a tool to further dissect the mechanism of catalysis.

MATERIALS AND METHODS

Materials

Deoxynucleotides, nucleotides, Nick-columns (G-50), RNA-guard and T7 RNA polymerase were from Pharmacia. Guanosine, spermidine, diethyl pyrocarbonate (DEPC) were from Sigma. Labelled [³⁵S]UTPαS was purchased from Amersham, *HpaI* from USB and DNaseI from Boehringer Mannheim. All solutions were DEPC-treated. 2'-Amino-2'-deoxyguanosine was

* To whom correspondence should be addressed at Karolinska Institutet. Tel: +46 8 728 7749; Fax: +46 8 311 052; Email: Roger.Stromberg@mbb.ki.se



Scheme 1. Schematic mechanism of self-splicing of the group I intron from the 'pre-mRNA' of the *nrdB* gene of bacteriophage T4 (a) with the first transesterification step shown in more detail (b). The length in nucleotides (nt) for exon 1, intron, and exon 2 is indicated in the upper graph of (a).

synthesized using published procedures (17,18) and purified as reported (16).

Methods

Preparation of RNA. The shortened version of the self-splicing pre-mRNA from the phage T4 *nrdB*-gene used in the kinetic experiments is a linearised plasmid, pBS5Δ1-650 (Sjögren, A-S, Strömberg, R. and Sjöberg, B-M., manuscript in preparation). It contains the T7 RNA polymerase promoter in front of 9 nucleotides (nt) of LacZ RNA sequence, 13 (intron proximal) nt of exon 1, the 598 nt intron and the 500 nt exon 2. *In vitro* transcription of pBS5Δ1-650, linearized with *HpaI*, produces a transcript of 820 nt; 22 nt of exon 1, 598 nt of intron and 200 nt of exon 2. Several (up to 10) transcription mixtures of 40 μl in 40 mM HEPES-KOH, pH 7.0, 1 mM each of ATP, CTP, GTP, 0.5 mM UTP, 1 μCi [³⁵S]UTPαS (400 Ci/mmol), 2 mM MgCl₂, 0.4 mM spermidine, 30 U RNA guard, 0.01 M dithiothreitol, 1 μg of linear plasmid and 120 U T7 RNA polymerase were incubated at 37°C for 50 min. The reaction was stopped by phenol:chloro-

form:isoamyl alcohol (25:24:1) treatment. The labelled transcript was DNaseI treated, extracted by phenol:chloroform:isoamyl alcohol (25:24:1), and desalted on Sephadex G-50 (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) to remove excess nucleotides. No detectable splicing of the pre-mRNA transcript occurred during transcription and purification. The purified pre-mRNA was precipitated in 4 M ammonium acetate and ethanol, dissolved in water and stored at -20°C.

Splicing conditions. Immediately prior to splicing reactions nanomolar concentrations of pre-mRNA were denatured at 95°C for 30 s, transferred to 4°C and left for 1.5 min and then brought to 32°C after which the folding buffer was added. The pre-mRNA was allowed to fold at 32°C for 4 min in 5/4 concentration of the splicing buffer. Splicing reactions were performed in 40 mM PIPES-KOH buffer, pH 7.2, 60 mM KCl and 4 mM for Mg²⁺ or 0.9 mM for Mn²⁺. Reactions were stopped by adding an equal volume of 10 M urea, 50 mM EDTA, 0.1% bromophenolblue and 0.25% xylene cyanol.

Splicing reactions in the presence of metal ion mixtures contained either 4.0 mM of total divalent metal ion concentration (Mg²⁺/Mn²⁺ ratios as given in Fig. 2) or 4 mM Mg²⁺ plus Zn²⁺ or Mn²⁺ (as indicated in Table 2). Folding of the RNA in the presence of only Mg²⁺ followed by addition of Mn²⁺ at the start of the splicing reaction gave similar cooperative effects as folding in mixtures of the metal ions.

Nucleoside concentrations were determined spectrophotometrically at pH 1.0. Extinction coefficients used were 12 200 M⁻¹ cm⁻¹ (256 nm) for guanosine and 12 500 M⁻¹ cm⁻¹ (255 nm) for 2'-amino-2'-deoxyguanosine (19).

Kinetic evaluation. Splicing products and intermediates were separated by electrophoresis on 3.4% polyacrylamide/8 M urea gels and quantified using the PhosphorImager system (Molecular Dynamics). The observed first order rate constants (*k*_{obs}) were determined by fitting a linear equation to the plot of the natural logarithm of the remaining precursor fraction [ln F(pre-mRNA)] versus time of incubation. The reactions were followed for up to 80% conversion and total RNA was calculated as the sum of remaining 'pre-mRNA' (221 uridine residues, Us), weighted intron (156 Us) and weighted AG- or G-intron-exon 2 (212 Us).

The kinetic analysis is based on the following observations. Since a difference in rate for the two cosubstrates is observed, a step preceding those involving the cosubstrate can not be rate-limiting. The disappearance of the pre-mRNA exhibits good first order kinetics throughout the experiments (over two half-lives), which suggests that *k*₋₁ must be substantially larger than *k*₂ and, since substrate saturation is obtained, we must have a real pre-equilibrium followed by a slower step. The concentration of the intermediate G-intron-exon 2 (or AG-intron-exon 2) is built up to later disappear during the course of the reaction, which suggests not too different *k*₂ and *k*₃ values (curve fitting using equations for consecutive reaction also suggest that *k*₃ is not dependent on substrate concentration). We quantify both this intermediate and the G-intron and treat the two splicing steps as simple consecutive reactions [i.e. first order kinetics of the disappearance of pre-mRNA to products (both G-intron-exon 2 and G-intron)]. The initial rate of formation of the intermediate G-intron-exon 2 appears to be identical to the rate of disappearance of the pre-mRNA, which is constant throughout the two half-lives through which we follow the reaction (while the rate of formation of the intermediate levels off). This shows that *k*₋₂ is small enough

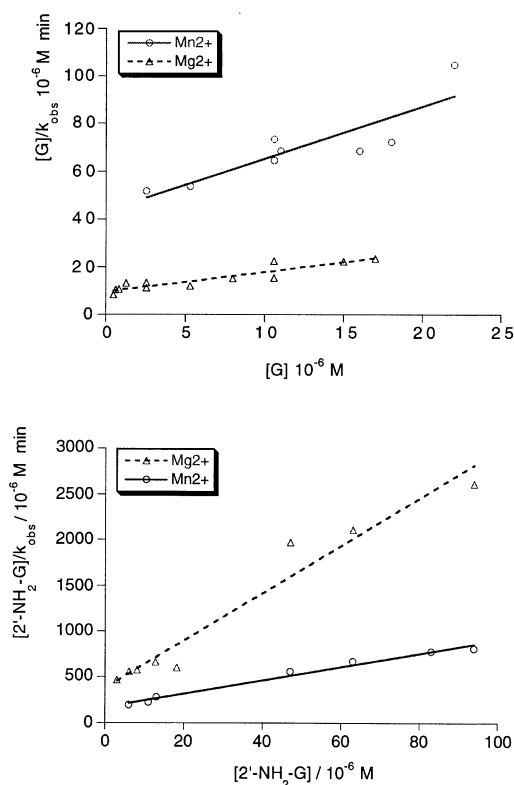


Figure 1. Graphical presentation of data in the form of Hanes–Woolf plots, $[\text{cosubstrate}]/k_{\text{obs}}$ versus $[\text{cosubstrate}]$, for guanosine (upper graph) or 2'-amino-2'-deoxyguanosine (lower graph) in the presence of either 4 mM Mg^{2+} or 0.9 mM Mn^{2+} . In the reactions with guanosine and Mg^{2+} the highest cosubstrate concentration was 17 μM , since the reaction rates at higher concentrations became too fast for accurate k_{obs} determination.

not to cause any significant equilibration back to pre-mRNA. These observations lead to the conclusion of limits to the ratios of rate constants that in practice means that an obtained k_{cat} value in essence is equal to k_2 . That we are monitoring the first catalytic step is also supported by the observations that $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_M)$ are linearly dependent on pH (Sjögren, A-S, Strömberg, R. and Sjöberg, B-M., manuscript in preparation) which is consistent with increased deprotonation of the 3'-hydroxyl at higher pH (as was also found in the L-21 Sca1 system by Herschlag and Khosla) (20).

RESULTS

Our experimental system consists of a shortened version of the self-splicing pre-mRNA (referred to as 'pre-mRNA') from the phage T4 *nrdB*-gene in which most of exon1 is deleted. The self-splicing of the 'pre-mRNA' can be treated kinetically as two consecutive reactions (the two transesterifications in Scheme 1) having rate constants of the same order of magnitude. Evaluations by curve fitting show that only k_2 and not k_3 varies with concentration of cosubstrate (data not shown). However, since the intermediate formed (G-intron–exon2 in Scheme 1) can be quantified the most accurate evaluation of the first splicing step (k_{cat}) is to base it on the disappearance of the 'pre-mRNA' (i.e., fraction of 'pre-mRNA' out of total RNA including both

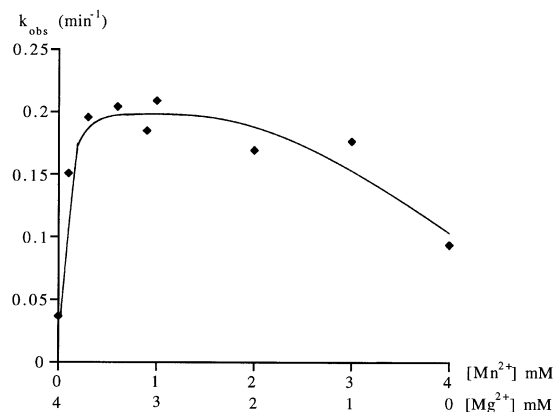


Figure 2. Observed first order rate constants (k_{obs}) for splicing with the cosubstrate 2'-amino-2'-deoxyguanosine at different ratios of magnesium and manganese ions. A cosubstrate concentration of 290 μM was chosen to ensure conditions where the intron is almost completely saturated with cosubstrate.

intermediate and product) which obeys first order kinetics. The kinetic behaviour of the system and the possibility of quantification of the intermediate ensures that the k_{cat} obtained reflects the first splicing step (see Materials and Methods).

The data obtained with 2'-amino-2'-deoxyguanosine and guanosine in the presence of either magnesium or manganese ions are presented in the Hanes–Woolf plots in Figure 1. The k_{cat} is 0.039 min⁻¹ and the K_M value is 15 μM when splicing is done with 2'-amino-2'-deoxyguanosine as cosubstrate in the presence of magnesium ions. The obtained k_{cat} is considerably lower than when guanosine is used as cosubstrate, whereas the K_M value for 2'-amino-2'-deoxyguanosine is similar to that of guanosine (Table 1). In the presence of manganese ions, the splicing with 2'-amino-2'-deoxyguanosine gives a four times higher rate constant for the first splicing step than in the presence of magnesium ions. In contrast, the reaction with guanosine in presence of manganese ions has a rate constant three times lower than that for the corresponding reaction with magnesium ions present (Table 1). The lower k_{cat} for guanosine with Mn^{2+} is probably not due to a lower inherent catalytic ability of the metal ion, since the Mn^{2+} aquo ion is reported to be a slightly better catalyst for this type of reaction (due to higher acidity and hence lower $\text{p}K_a$ value of coordinated water or hydroxyl function) in a diribonucleotide (21), but could be due to other effects such as imperfect positioning/folding. The K_M is indeed somewhat higher in the presence of Mn^{2+} with both cosubstrates.

Table 1. The kinetic parameters for splicing of the *nrdB* 'pre-mRNA' with guanosine or 2'-amino-2'-deoxyguanosine as cosubstrate in the presence of magnesium or manganese ions

Cosubstrate	k_{cat} (min ⁻¹)	K_M (μM)
Guanosine (with Mg^{2+})	1.2	12
Guanosine (with Mn^{2+})	0.46	20
2'-Amino-2'-deoxyguanosine (with Mg^{2+})	0.039	15
2'-Amino-2'-deoxyguanosine (with Mn^{2+})	0.14	24

Table 2. Splicing with the cosubstrate 2'-amino-2'-deoxyguanosine (168 μ M) in mixtures of magnesium and manganese or zinc ions

Metal ion	k_{obs} min ⁻¹	Relative k_{obs}	Metal ion with UpU ^d	Relative k with UpU ^d
4 mM Mg ²⁺	0.036 ^a	1	Mg ²⁺	1
0.9 mM Mn ²⁺ with 4 mM Mg ²⁺	0.22 ^b	6.1 (5.4–12) ^c	Mn ²⁺	6.2
0.1 mM Zn ²⁺ with 4 mM Mg ²⁺	1.3 ^b	35	Zn ²⁺	41

Relative first order rate constants (k_{obs}) are compared with the relative rates observed upon transesterification of uridine 3'-(uridine 5'-phosphate) when catalysed by the corresponding metal ions.

^aValue calculated from the equation of the curve fit to the Hanes-plot of Figure 1 for 168 μ M 2'-amino-2'-deoxyguanosine.

^bMean value of two different k_{obs} determinations.

^cAn average value for concentrations 0.3–1 mM Mn²⁺ taken from the graph in Figure 2 gives a relative rate of 5.4. If the negative influence of manganese ions, found with guanosine as cosubstrate, is taken into account then the relative rate for the reaction with 2'-amino-2'-deoxyguanosine can be as much as almost the double value.

^dData from Kuusela and Lsnberg (1993).

The observed reversed relative rates of the first splicing steps in presence of manganese or magnesium ions with the different cosubstrates (Table 1) indicate that a metal ion binds to the 2'-position of the cosubstrate (direct or via water) and that this clearly affects the rate of the transesterification reaction. With 2'-amino-2'-deoxyguanosine as cosubstrate the overall effect on the transesterification step may be about an order of magnitude higher with Mn²⁺ than with Mg²⁺ if taking into consideration the reversed situation with guanosine as cosubstrate. A probable catalytic role for the divalent ion interacting with the cosubstrate would be to promote the nucleophilic attack by the 3'-hydroxyl of the cosubstrate on the phosphodiester of the exon1-intron junction and to contribute to electrostatic stabilisation of the transition state. Apparently Mn²⁺ has a higher ability to catalyse the transesterification with an amino function at this site whereas an oxygen gives a higher rate with magnesium. A plausible explanation is a ligand effect on the metal ion. The more polarisable (softer) Mn²⁺ ions has a higher affinity to the softer amino group than the harder Mg²⁺ and the ligand can also affect the acidity of coordinated hydroxy functions. The acidity of metal ion complexes is known to be affected by the ligands to the metal ion and this is also reported to influence their ability to catalyse intramolecular transesterification in dinucleotides (21).

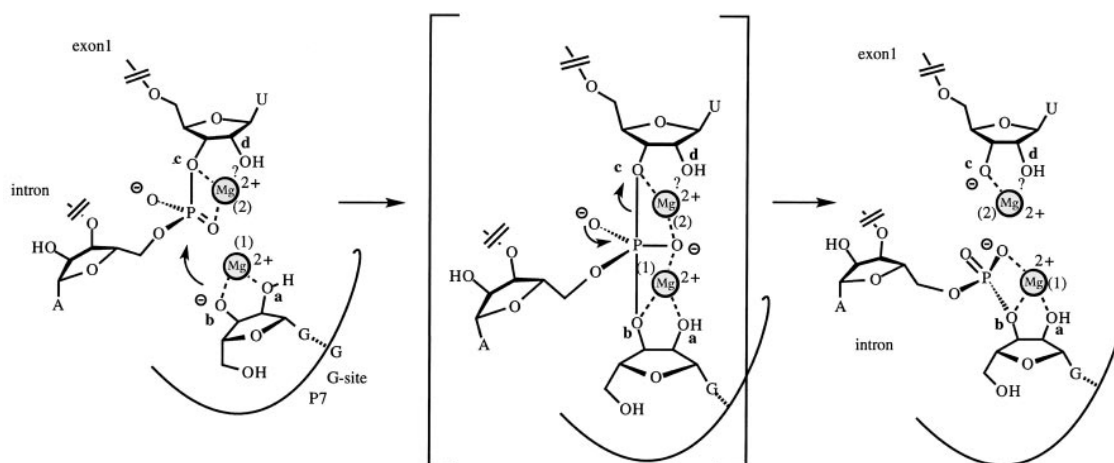
It is conceivable that a metal ion interacting with the cosubstrate and promoting the nucleophilic attack is non-identical with the magnesium ion that has been suggested to facilitate leaving of the oxyanion of exon1 (8) which means that two metal ions would be involved in catalysis of the transesterification. In order to investigate such a hypothesis we decided to carry out experiments in metal ion mixtures. The rate constants for splicing with 2'-amino-2'-deoxyguanosine are significantly higher in mixtures of Mn²⁺ and Mg²⁺ than with either ion alone, particularly in the range 0.3–1 mM Mn²⁺ where the magnesium ion concentration is in excess (Fig. 2). This cooperative effect is only observed with 2'-amino-2'-deoxyguanosine as cosubstrate. With guanosine as cosubstrate inclusion of manganese together with magnesium ions gives a gradual decrease in rate, approaching the rate with manganese ions alone for high Mn²⁺ content (data not shown). The observed relative rate constants for splicing with 2'-amino-2'-deoxyguanosine in Mn²⁺/Mg²⁺ mixtures are up to 5–12 times higher than with Mg²⁺ alone (Table 2). If this result reflects the higher ability of manganese ion to catalyse the transesterification one would expect an even more acidic metal ion like the zinc ion to increase the rate further and this is indeed

observed. With a mixture of Zn²⁺ and Mg²⁺ the rate enhancement is even more striking and the observed rate constant is 35 times higher than with Mg²⁺ alone (Table 2). With guanosine as cosubstrate the rate of splicing was unaffected by low concentration (<0.2 mM) of Zn²⁺ in a magnesium ion background, indicating that a low concentration of zinc ion does not influence folding of the pre-mRNA in a negative way.

DISCUSSION

In this study we provide evidence for a metal ion interaction with the 2'-position of the cosubstrate in self-splicing of a group I intron. The nature of this metal ion affects the rate of transesterification which lead us to assign it a catalytic role. Strong support for a catalytic role of the metal ion that interacts with the cosubstrate comes from the finding that the reaction rates in the different metal ion mixtures follow the acidity of the metal ions and correlate well with their corresponding effect on transesterification of phosphodiester linkages in diribonucleotides (Table 2) (21). In this model system the nucleophilic attack on the phosphodiester takes place with a hydroxylic function of similar pK_a value (~12–13) as the 3'-OH function of the cosubstrate. The effect of the metal ions in the model system was suggested to be largely due to enhancement of the nucleophilicity of the attacking hydroxyl (21). A probable catalytic role for the divalent ion interacting with the cosubstrate is to promote the nucleophilic attack by the 3'-hydroxyl of the cosubstrate on the phosphodiester of the exon1-intron junction and to contribute to stabilisation of the transition state.

Experimental support for a catalytic metal ion with the role to enhance the leaving ability of the exon1 alkoxide that is displaced by the cosubstrate has been reported (8). Would it be possible for a single metal ion to provide both these catalytic functions? If two (or more) metal ions are involved in catalysis, in metal ion mixtures with 2'-amino-2'-deoxyguanosine as cosubstrate one would expect a magnesium ion to coordinate the leaving exon 1-oxygen and a metal ion with higher nitrogen affinity to coordinate the cosubstrate. In metal ion mixtures of Mg²⁺ and Mn²⁺ or Zn²⁺ a relatively low concentration of any of the latter metal ions should be sufficient to compete for the amino ligand of 2'-amino-2'-deoxyguanosine since coordination of either a manganese or a zinc ion to a nitrogen ligand is considerably more favourable than coordination of magnesium ions (22,23). In contrast, low concentrations of Mn²⁺ or Zn²⁺ should not be able to compete



Scheme 2. A suggested working model for the mechanism of self-splicing of group I introns involving two catalytic magnesium ions (only the possible ligands to the magnesium ions discussed in this study are indicated).

efficiently with Mg^{2+} for binding neither to the 2'-OH of guanosine nor to the leaving 3'-oxyanion (due to relative affinities and higher concentration of Mg^{2+}). Thus, only with 2'-amino-2'-deoxyguanosine will such metal ion mixtures give a considerable portion of cosubstrate/pre-mRNA complex with the more acidic metal ion coordinating the cosubstrate, while the other sites are predominantly occupied with Mg^{2+} . Faster transesterification reactions are to be expected in such mixtures, since both manganese and zinc ions are known to be more efficient than magnesium ions in catalysing transesterification in diribonucleoside phosphodiester (UpU) (21). This is indeed observed also in our system.

The observed cooperative effects reported here are experimental indications of at least two simultaneously acting metal ions that influence the rate of reaction. A mechanism with one metal ion being catalytic and another influencing in a different way can not be completely excluded on a purely experimental basis. However, there are now a number of experimental observations that are consistent with a two-metal-ion mechanism (see discussion below) and a single-ion mechanism seems less likely from a theoretical point of view. If a single metal ion would promote catalysis on both sides of the phosphate linkage this would require a two-step transesterification with a conformational change at the stage of an intermediate phosphorane which, if a true intermediate, would be expected to have a very short life-time. We therefore suggest a working model involving two catalytic metal ions. It seems reasonable and consistent with our findings that if the role of one metal ion is to catalyse departure of the leaving group then the other metal ion that interacts with the 2'-position of the cosubstrate has the role of promoting the nucleophilic attack of the 3'-OH.

A plausible mechanism involving metal ion enhancement of both nucleophilicity and leaving ability as well as electrostatic stabilisation of the transition state is proposed in Scheme 2. The working model involves coordination of one magnesium ion ($\text{Mg}_{(2)}^{2+}$) to oxygen **c** to make the exon1 oxyanion a better leaving group. The other magnesium ion ($\text{Mg}_{(1)}^{2+}$) is suggested to coordinate the 3'-oxygen (**b**) in the cosubstrate (see discussion below) thereby promoting its deprotonation to the more nucleophilic anion. The transition state can be stabilised by the two metal

ions via neutralisation of emerging charges and counteraction of repulsion between the negatively charged nucleophile and the phosphodiester. Electrostatic stabilisation of the transition is suggested to be a most important factor in two-metal-ion catalysis, e.g., the rate acceleration with double Lewis-acid activation has been quantified to be 4×10^5 for cleaving a phosphodiester with a binuclear Co[III] complex (24) whereas comparable single Lewis-acid activation (Co[III]) gives rate accelerations that are three orders of magnitude lower (25). Coordination to the pro-Sp oxygen is inferred from phosphorothioate substitutions (26–29). Based on positions of metal ion dependent cleavage of the intron core and modelling, metal ion binding pockets relatively close to the 3'-O of the leaving oxyanion and to the cosubstrate have been suggested in the model of Streicher *et al.* (12) From these studies one can consider additional possible ligands to the magnesium ions. These ligands would be (using the numbering of the native *nrdB* intron) (30,31) the N-3 of the bulged C457 next to the G-site in P7 and the 5'-phosphodiester of A521 for $\text{Mg}_{(1)}^{2+}$ and the 5'-phosphodiester of U520 and A523 for $\text{Mg}_{(2)}^{2+}$ [these phosphates in P7 are those identified by Christian and Yarus (7) as most sensitive to phosphorothioate substitutions]. Although the distance of 8.6 between the magnesium ions in the model of Streicher *et al.* (12) is too large to suggest that both these metal ions would be involved simultaneously in catalysis the experimental data and the model in their study does not completely exclude this possibility (as also discussed by the authors).

The coordination to oxygen **d** is tentative (marked with a question mark symbol for the bonding interaction) and included on the basis of the following discussion. There is no direct experimental evidence for metal ion interaction with oxygen **d** (Scheme 2), but the nature of the group in this position influences the efficiency of cleavage (32). It was concluded that this is mostly via an inductive effect and the data for a hydroxy substituted derivative that deviated in a Brønsted plot (*vs* leaving group $\text{p}K_a$) was explained by possible internal hydrogen bonding to the 3'-hydroxyl (32), an alternative that also has been suggested to explain the role of the 2'-hydroxyl of the cosubstrate (16). Another explanation for the above mentioned deviation of the Brønsted relationship could be that oxygen **d** coordinates $\text{Mg}_{(2)}^{2+}$

(Scheme 2) and thus perturbs its efficiency in catalysis. It seems plausible that if the role of the 2'-OH of the cosubstrate is, at least partially, to enhance the effect of the catalysing $Mg_{(1)}^{2+}$ ion, the same kind of coordination also would improve the catalytic ability of the $Mg_{(2)}^{2+}$ ion. Examples where metal ions promote transesterification of internucleotidic phosphodiester linkages can be found both for dinucleotides (33–35) and oligonucleotides (19,36,37), and an example of metal ions lowering the pK_a of a dihydroxy system can be found in complexes with catechols(38). There is also precedence that two metal ions acting simultaneously provides catalysis that is considerably more efficient than with single metal ions acting in separate steps (24,33).

At this stage, we can also consider alternatives in the details of a two-metal-ion mechanism: interactions could involve water molecules between hydroxyl functions and metal ions. However, the mechanism presented in Scheme 2 follows the principle of microscopic reversibility irrespective of whether metal ions coordinate to the nucleoside alkoxy oxygens directly or via bound water/hydroxide. That catalysis is correlated to the acidity of the metal ions is consistent with coordination to the 3'-hydroxyl of the cosubstrate but the alternative involving deprotonation of the 3'-OH by metal-bound hydroxide (and protonation of the leaving group by metal-bound water) can not be excluded on the basis of current kinetic data. Models based on crystallographic data on protein enzymes (10) and the previously mentioned calculations (9,11) favours direct interaction. Conceivably, the strongest argument in favour of a direct interaction of the 3'-oxygen with the metal ion is that the differences in metal ion influence on catalysis obtained when comparing exon 1 oxanion and thioanion leaving groups (8) would be difficult to explain with an indirect interaction. Similarly our results with 2'-amino-2'-deoxyguanosine suggest direct interaction with the 2'-position of the co-substrate. On the basis of the arguments above we favour the mechanistic alternative involving direct coordination of the metal ion to the 2' and 3'-oxygens of both the nucleophile and the leaving group. The proposed mechanism has the additional attraction of generating a liberated exon 1 that is ready and activated for the subsequent ligation reaction (provided that the metal ion is not rapidly exchanged before the second splice site is properly aligned) in a fashion identical to that for the guanosine cosubstrate in the first step.

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