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Understanding Catalysis of Phosphate-Transfer Reactions by the Large Ribozymes

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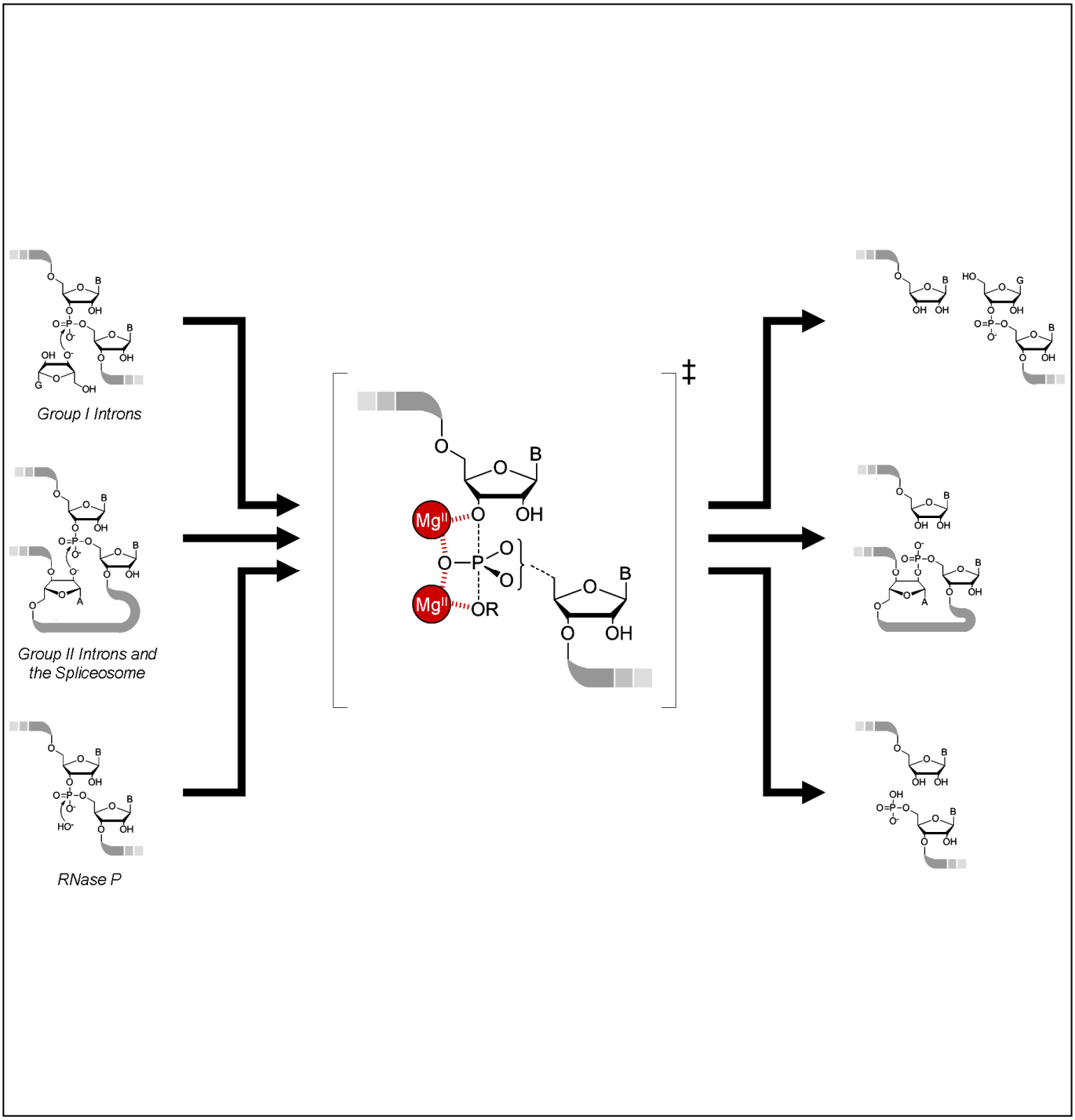

Abstract: The large ribozymes are unique among the catalytic RNA molecules in that their reactions involve intermolecular nucleophilic attack on an RNA phosphodiester linkage. Crystal structures of near atomic resolution are now available for the group I and group II self-splicing introns and the RNA subunit of RNase P. The structural data agrees well with the earlier models proposed on the basis of biochemical studies and the evidence at hand suggests that all of the large ribozymes utilize a mechanism where coordination of Mg^{II} ions reduces the negative charge on the scissile phosphodiester linkage, as well as assists both the nucleophilic attack and the departure of the leaving group.

Keywords: large ribozymes • catalysis • phosphate transfer • transesterification • hydrolysis

Introduction

Large ribozymes are a group of catalytic RNA molecules that are related to each other not only by their great size but also by their reaction mechanism.[1] They include the first three classes of catalytic RNA molecules discovered in the early 1980s, the group I[2, 3] and II[4, 5] introns and the RNA subunit of RNase P.[6, 7] The eukaryotic spliceosome catalyzes a reaction identical to the self-splicing of group II introns and its catalytic center is largely made up of RNA but recent studies have identified a protein subunit in the catalytic center as well so some controversy still remains as to whether this immense ribonucleoprotein complex is a true ribozyme or not.[8, 9] The ribosome, on the other hand, has been compellingly shown to be a ribozyme but as a peptidyl (rather than phosphoryl) transferase it falls beyond the scope of this review.[10]

In contrast to RNA cleavage catalyzed by the small ribozymes or protein enzymes, the phosphate-transfer reactions of the large ribozymes are initiated by the attack of an external nucleophile on the scissile RNA phosphodiester bond (Scheme 1A). This nucleophile is the 3´-OH of a guanosine nucleoside or nucleotide in the case of group I introns (Scheme 1B),[11] the 2´-OH of an intramolecular but distant adenosine residue in the case of group II introns and the spliceosome (Scheme 1C)[12] and a water molecule in the case of RNase P (and some group II introns) (Scheme 1D).[13, 14] The scissile phosphodiester linkage, in turn, is part of the ribozyme sequence itself in the case of the self-splicing group I and II introns, whereas the spliceosome and RNase P are true catalysts with turnover, promoting transesterification or hydrolysis of a substrate RNA strand *in trans*. On the other hand, also with the self-splicing introns the catalyst and the substrate parts may be separated, resulting in *trans*-acting large ribozymes readily tractable by established methods of enzymology.[15, 16] In many cases, the results discussed below were obtained by using *trans*-acting ribozymes, even when not explicitly mentioned in the text.

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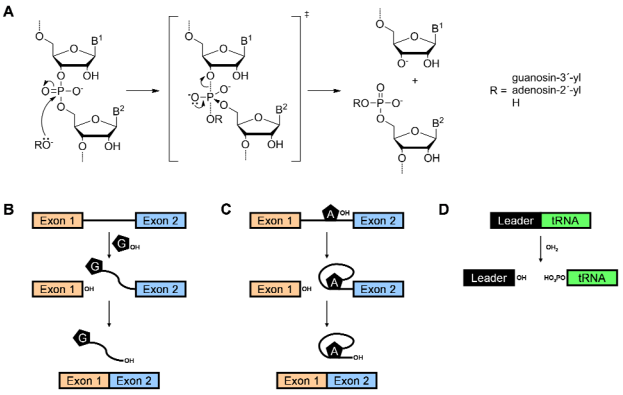


Scheme 1. (A) Transesterification and hydrolysis by the large ribozymes: self-splicing of group I (B) and II (C) introns and (D) pre-tRNA maturation by RNase P.

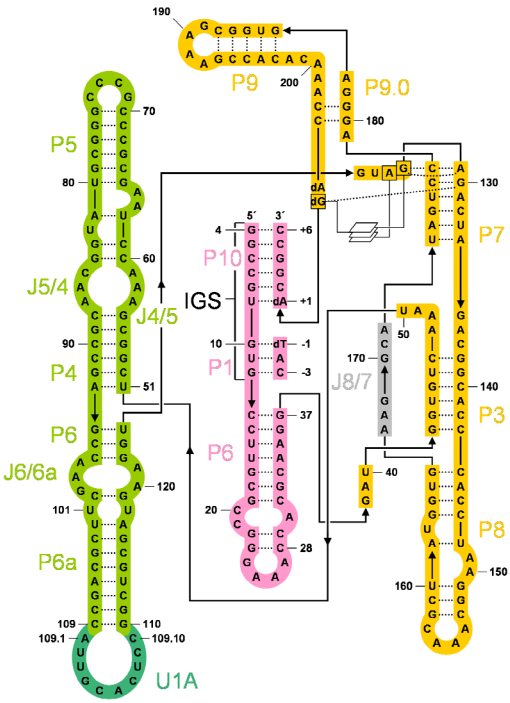


Figure 1. Secondary structure of the splicing intermediate of a group I intron from *Azoarcus sp. BH72* (adapted from ref. 41a). Hydrogen bonding interactions are presented as dotted lines and π-π stacking interactions as stacked rectangles connected to the respective nucleotides.

By the end of the decade following the discovery of RNA catalysis, conserved and critical domains as well as a number of tertiary interactions essential for the catalytic activity of large ribozymes had been identified by studying the digestion patterns of single- and double-strand-specific nucleases,[17] inhibition of the reactions by intercalators,[18] site-specific mutagenesis or modifications at the atomic level[12c, 19-21] and phylogenetic covariation of paired bases in the natural ribozyme sequences.[5, 22, 23] In general, the secondary structures of the large ribozymes are rather conserved even though the primary structures may differ considerably.[5a, 24-26] Representative secondary structures of group I and II introns and the RNA subunit of RNase P are depicted in Figures 1-3.

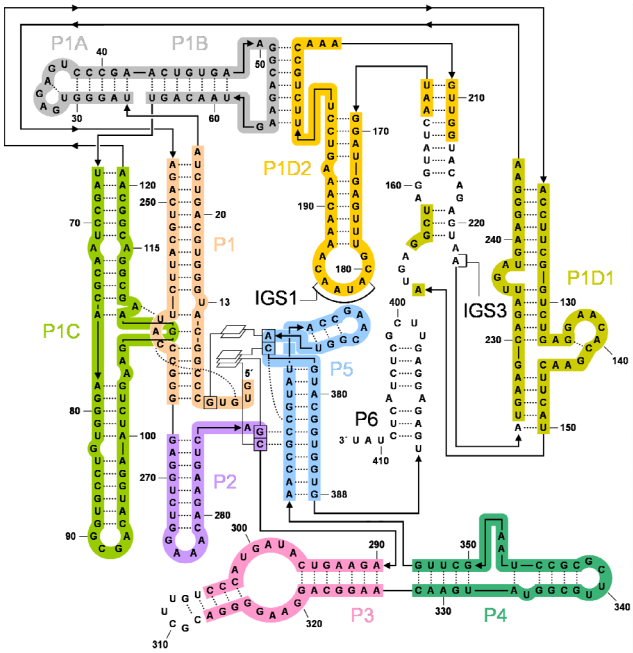


Figure 2. Secondary structure of a spliced group II intron from *Oceanobacillus iheyensis* (adapted from ref. 42a). Hydrogen bonding interactions are presented as dotted lines and π-π stacking interactions as stacked rectangles connected to the respective nucleotides.

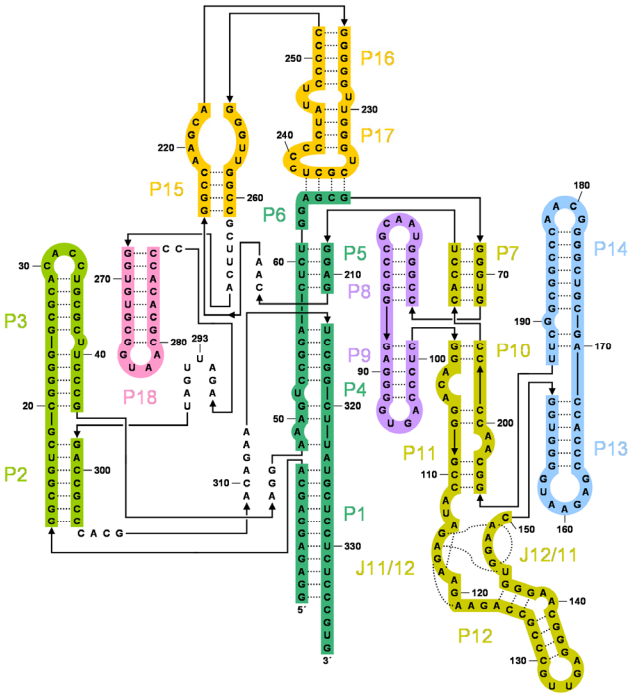
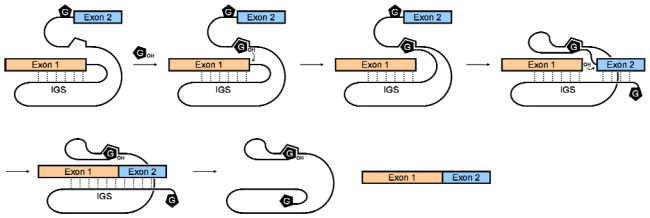


Figure 3. Secondary structure of a the RNA subunit of the RNase P from *Thermotoga maritima* (adapted from ref. 43b). Hydrogen bonding interactions are presented as dotted lines.

First steps towards elucidating the tertiary structure of large ribozymes were taken already in the late 1980s by indirect methods such as studying the cleavage patterns by RNA cleaving agents localized at a specific site,[27] accessibility of each nucleoside in the ribozyme sequence to various chemical and enzymatic probes[28-30] or the effect on catalytic activity of covalently linking together regions that are distant from each other in the secondary structure.[31-38] Since the mid-1990s these studies have been supplemented by direct visualization of the three-dimensional structure by electron microscopy and X-ray crystallography.[39, 40] During the past seven years, crystal structures of near-atomic resolution have become available for all of the large ribozymes except the huge spliceosome complex, providing a solid foundation for mechanistic studies. [41-43]

Substrate Recognition and Stereochemistry

For recognition of their substrate sequence, the self-splicing introns utilize a complementary strand known as the internal guide sequence (IGS, Schemes 2 and 3) [19, 20, 44, 45] The RNA subunit of RNase P, on the other hand, does not undergo extensive base-pairing with its target but the target itself must have a double-helical stem next to the cleavage site, analogously called the external guide sequence (EGS).[46] For precise positioning of the components of the catalytic core, the large ribozymes rely on additional hydrogen bonding and π-π stacking interactions, as well as metal ion coordination. Kinetic studies on large ribozymes are complicated by the fact that instead of the chemical reactions of interest, conformational changes of the ribozyme structure may be rate-limiting.[47-49] All of the transesterification and hydrolysis reactions catalyzed by the large ribozymes proceed with inversion at phosphorus, consistent with a direct in-line attack resulting in a pentacoordinate phosphorane-like transition state or a marginally stable intermediate.[47, 50-52]



Scheme 2. Interactions of the group I intron with the flanking exons and the guanosine cofactor during self-splicing.

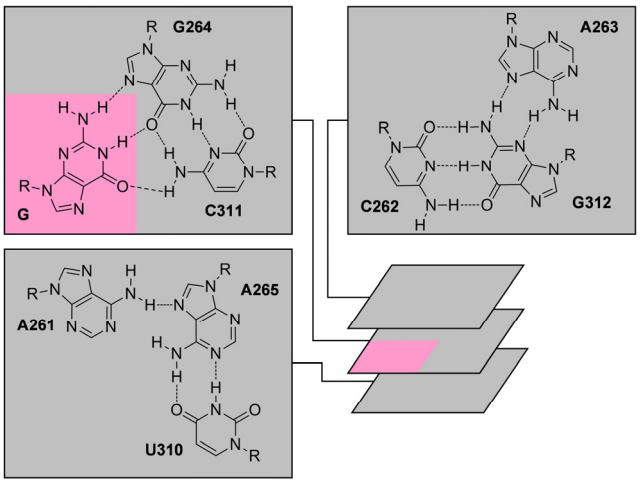
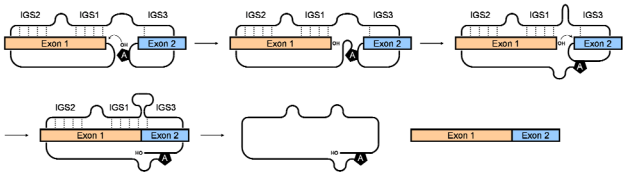


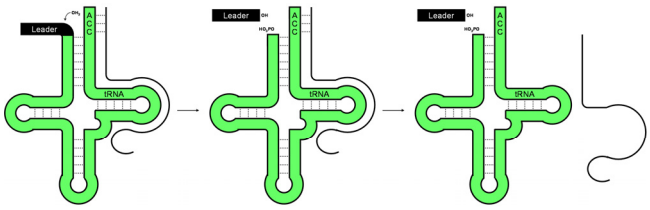
Figure 4. The guanosine binding site of group I introns composed of three stacked base triples.

During both steps of splicing of the group I introns, the 5′-exon is held in place by hybridization with the internal guide sequence (P1 in Fig. 1), as well as a number of tertiary contacts with the intron (Scheme 2).[41, 53-57] The 5′-splice site base-pair within this duplex is almost always a wobble U·G that is recognized by two mispaired adenine residues of a conserved loop motif (J4/J5-J5/J4) and required for the first but not the second step of splicing.[58] The external guanosine attacking in the first step and the 3′-terminal guanosine of the intron departing in the second step (ωG) occupy the same highly ordered binding site composed of three stacked base triples, the middle one including the splice site guanosine (Fig. 4). [32, 40b, 41, 59] For the second step of splicing (exon ligation), the 3′-exon is also hybridized with the internal guide sequence (P10 in Fig. 1) but, in contrast to 5′-splice site selection, few tertiary interactions beyond those of the leaving ωG with the guanosine binding site have been identified.[19e, 23, 41] Binding of the substrate sequence and the guanosine cofactor is not independent: with *trans*-acting group I intron ribozymes, the ternary ribozyme:substrate:guanosine complex is more stable than any of the binary complexes. Binding of the product oligonucleotide and guanosine, on the other hand, is slightly anticooperative, facilitating the successive steps of splicing.[49] The group I intron has been crystallized with both of the exons in place and the crystal structure largely corroborates the interactions inferred from biochemical studies.[41]



Scheme 3. Interactions of the group II intron with the flanking exons during self-splicing.

In the group II introns, the internal guide sequence complementary to the 3′-end of the 5′-exon is composed of two distinct parts that typically both form six base pairs with the exon (IGS1 and IGS2, Scheme 3).[20] The one flanking the 5′-splice site (IGS1) is needed in the second but not in the first step of self-splicing – evidently, the role of this guiding sequence is to position the 3′-end of the 5′-exon correctly for nucleophilic attack in the exon ligation.[9d, 60] The 3′-exon is hybridized by yet another internal guide sequence (IGS3), consisting of a single base-pair.[25, 61] Recently, crystal structure has been determined for a group II intron with an oligonucleotide representing the ligated exons. In this structure, IGS1 and IGS3 form a continuous base-pairing interface that simultaneously recognizes both exons and brings the reactive phosphodiester linkage to the catalytic center, consistent with the earlier results of biochemical studies on group II introns (the crystal structure was obtained for a group IIC intron lacking IGS2).[42b, 62] As discussed above, the nucleophile initiating the self-splicing of group II introns is typically the 2′-OH of a bulged adenosine residue.[12] However, neither the identity of the base nor its bulging out of the surrounding double helix determines the branch site.[63] Instead, the position of the attacking nucleoside within the P6 domain, as well as the identity of the base-pairs flanking it control the selection of the branch site in a systematic and predictable way.[63a] In the introns removed by the spliceosome, the splice sites as well as the branch point are surrounded by short consensus sequences that are recognized by the snRNA subunits of the spliceosome.[8, 64-66] While there are striking similarities between certain domains of these snRNAs and the group II introns,[9, 67, 68] spliceosomes are rather promiscuous in the selection of their branch point compared to the group II introns.[69]



Scheme 4. Interactions of the RNA component of RNase P with the pre-mRNA substrate during the maturation of tRNA by RNaseP.

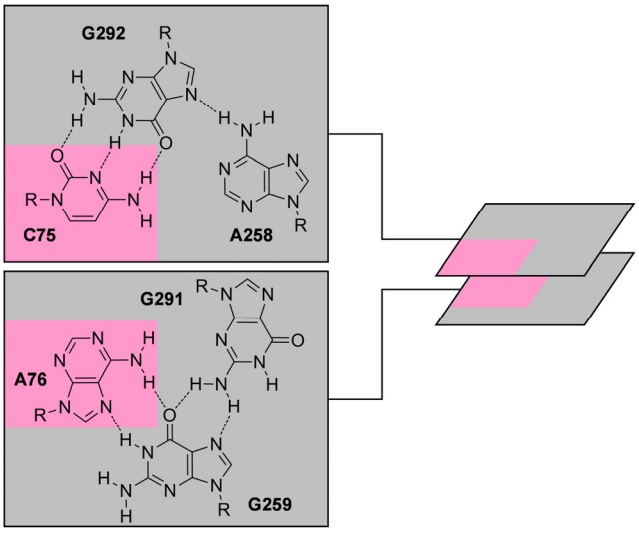


Figure 5. Recognition of the 3′-terminal CCA sequence of tRNA.

As the sequence containing the cleavage site is not conserved among the tRNA precursors processed by RNase P, substrate recognition cannot be based on hybridization of complementary regions in the ribozyme and its target.[70] As a result, RNase P tolerates a wide range of substrate sequences as long as they retain the acceptor stem and the T stem-loop motifs and the conserved 3′-terminal CCA sequence (Scheme 4).[46, 71] The former two are recognized based on their conformation and the interaction of specific 2′-OH groups in the T-loop with conserved adenosines in the P10/P11 helix of the RNase P RNA,[21a] whereas the first two bases of the CCA stretch are engaged in Watson-Crick base-pairing with a GG stretch of the ribozyme.[71b] Furthermore, the middle CG pair, as well as the following A, is part of higher-order structure comprised of two stacked base triples (Fig. 5).[72] The 5′-leader sequence mainly interacts with the protein subunit of RNase P[73] but the cleavage site nucleoside (usually U) appears to be base-paired with a conserved adenosine of the RNA subunit. [6g, 74] The RNA component of RNase P still remains to be crystallized with its pre-tRNA substrate. While modeling of the substrate on the crystal structure of the ribozyme has yielded a picture consistent with most of the experimental data, some conformational changes would be required to account for all of the proposed interactions. [6, 43]

Metal Ions in the Catalytic Core of Large Ribozymes

Because of their apparent lack of functional groups suitable for acid/base catalysis, all ribozymes were initially thought to be obligate metalloenzymes. While most small ribozymes are now known to rely on their nucleobases with perturbed pK_a values as general acid/base catalysts,[75] it still seems that all of the large ribozymes require divalent metal ions, primarily Mg^{II}, for catalytic activity.[13c, 14, 76-79] There are several roles that a metal ion might play in the phosphate transfer reactions of the large ribozymes, summarized in figure 6.[80] A metal ion may facilitate the deprotonation of the attacking oxygen, hence making it a better nucleophile, by A) directly coordinating to it or B) using a hydroxo ligand as a general base. Direct coordination of a metal ion to one or both of the non-bridging phosphoryl oxygens C) makes the phosphorus more electrophilic and/or stabilizes the negative charge developing upon nucleophilic attack on the phosphorus. A metal ion may also assist in the departure of the 3′-oxyanion by D) coordinating to it directly or E) through general acid catalysis by an aquo ligand. Finally F), a metal ion with no direct role in the catalysis could still be needed to stabilize the tertiary structure of the ribozyme.

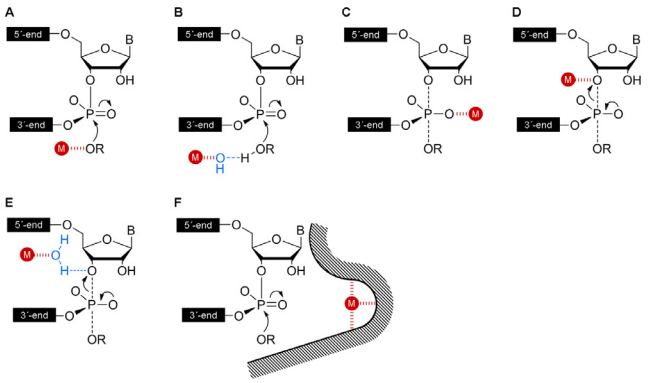


Figure 6. Possible roles for a metal ion in the phosphate transfer reactions of the large ribozymes.

Thio Effects in the Identification of Catalytic Metal Ions

Mapping thio effects for the substitution of oxygen atoms in the catalytic center for sulfur atoms has been a powerful method for identifying potential catalytically important metal ions.[81] According to Pearson's HSAB principle, oxygen, a hard Lewis base, will coordinate Mg^{II}, a hard Lewis acid, stronger than the relatively soft sulfur.[82] Loss of activity of a ribozyme upon thio substitution of one of its oxygen atoms may hence be attributed to coordination of a catalytically important Mg^{II} ion by that oxygen. The interpretation is further supported if replacing Mg^{II} with a softer metal ion, such as Mn^{II}, partially or completely restores activity (the so-called manganese rescue effect).

By studying the thio and manganese rescue effects, up to three metal ions have been identified to participate in the transesterification of group I introns (Fig 7A), coordinated to the 3′-oxygens of both the attacking and the departing nucleoside, as well as the *pro*-S_P oxygen of the scissile phosphate. [83-89] It should be noted that the in-line arrangement of the attacking and departing oxygens in a phosphorane-like transition state rules out the possibility that both of them would be coordinated to the same metal ion. In other words, at least two metal ions must be involved in the reaction. Like thio substitution, replacing a hydroxy group with an amino group also retards coordination of a Mg^{II} and this "amino effect" has been exploited to reveal a potential interaction between the 2′-OH of the guanosine nucleophile and a metal ion.[84, 88, 90-93] While all of these interactions could be accounted for by a model having two metal ions in the catalytic center, participation of a third one has been proposed on the basis of different affinities to Mn^{II} determined for the three sugar oxygens.[84] The second step of the splicing is essentially the reverse of the first step, with the guanosine binding site being occupied by the leaving group, the 3′-terminal guanosine residue of the intron (Fig. 7D). Accordingly, the metal ion requirements are the same for both steps of the splicing.

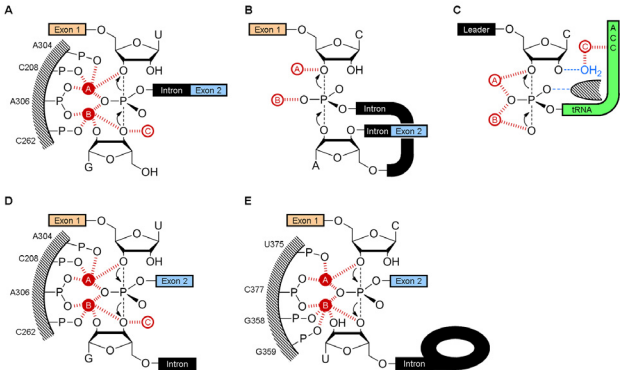


Figure 7. Metal ions in the (A) first and (D) second step of splicing of the group I introns, the (B) first and (E) second step of splicing of the group II introns and (C) hydrolysis catalyzed by RNase P. The participation of the metal ions presented as hollow spheres is suggested by biochemical studies whereas the participation of the metal ions presented as solid spheres has also been verified by crystallographic studies.

In the first step of the splicing of group II introns, thio and manganese rescue effects have revealed that the departing 3′-oxygen, the *pro*-R_P oxygen and, possibly, the *pro*-S_P oxygen interact with metal ions (Fig. 7B).[51, 94, 95] Coordination of a metal ion by the 3′-oxygen of the departing nucleoside is essential also in the second step of the splicing but instead of the *pro*-S_P oxygen of the scissile phosphate, which would be expected to interact with a metal ion if the reaction was simply a reverse of the first step, it is the *pro*-R_P oxygen that exhibits a strong thio effect (Fig. 7E) (note that an oxygen that is *pro*-R_P in the forward reaction would be *pro*-S_P in the reverse reaction).[95, 96] Evidently, considerable conformational rearrangement takes place between the two steps of the splicing.[97] A second metal ion, coordinated to the 3′-oxygen of the attacking nucleoside and, possibly, the *pro*-R_P oxygen, has also been identified in the second step of the splicing.[98] In all likelihood, this metal ion occupies the same binding site as the one stabilizing the leaving group in the first step of the splicing. 3′-Thio substitutions have the same effect in spliceosomal and group II introns, suggesting a similar role for catalytic metal ions in the reactions of these two classes of large ribozymes. [95, 99, 100]

The RNA hydrolysis catalyzed by the RNA component of *E. coli* RNase P is third-order in Mg^{II} concentration, indicating participation of three of these metal ions in the transition state (Fig. 7C). Upon 2′-deoxy substitution of the departing nucleoside, the reaction exhibits only a second-order dependence on Mg^{II}

concentration and is retarded 3400-fold, suggesting that the 2′-OH of the ribo substrate coordinates a catalytically important (but not absolutely necessary) Mg^{II} ion, either directly or through an aquo ligand.[101, 102] Thio substitution of either the *pro*-S_P or the *pro*-R_P oxygen of the scissile phosphodiester bond retards the reaction by at least three orders of magnitude[52, 103] but only in the latter case is reactivity restored by the more thiophilic Cd^{II} or Mn^{II}. Based on the observed second-order dependence of the Cd^{II} rescue on Cd^{II} concentration, coordination of two metal ions to the *pro*-R_P oxygen has been proposed.[103, 104] Similar rate retardation by thio substitution of the non-bridging phosphoryl oxygens is observed with the RNA component of *Bacillus subtilis* RNase P, representing the other main subtype of bacterial RNase P RNA. It should be noted, however, that in this case rescue by Cd^{II} alone is not achieved due to weak binding of the substrate but equimolar amounts of Ca^{II} or Mn^{II} are also needed for full activity.[105] Finally, the 3′-terminal CCA conserved among virtually all tRNAs lies close to the scissile phosphodiester bond and has been proposed to play a role in binding catalytically important metal ions. [102, 106, 107]

Active Site Metal Ions in the Crystal Structures of the Large Ribozymes

In the case of the group I introns, crystallographic data of sufficient quality to reveal metal ions in the catalytic core are available.[41, 108] In the crystal structure of the group I intron from *Azoarcus sp. BH72* trapped in the state preceding the second step of the splicing by appropriate 2′-deoxy substitutions, six phosphates converge to coordinate two metal ions, one of which assigned as Mg^{II} and the other one as K^{I}.[41a] The unexpected discovery of K^{I} in the catalytic core is probably an artifact resulting from the deletion of the 2′-OH of the departing guanosine, proposed to coordinate to one of the metal ions. Accordingly, studies with the corresponding structure including of all of the putative ligands for the catalytic metal ions have confirmed that both of the metal ions detected are in fact Mg^{II}.[41f] One of the Mg^{II} ions coordinates to the 3′-oxygen of the attacking nucleoside and one to the 3′-oxygen of the leaving nucleoside and both to the same non-bridging oxygen of the scissile phosphodiester linkage, in perfect agreement with the results obtained by kinetic studies with modified ribozymes. On the other hand, the direct participation of a third metal ion in the reaction center, also inferred by atomic mutation studies on group I intron ribozymes, has not been verified by any of the crystallographic studies. While the possibility that a third metal ion is disordered or missing in the crystal cannot be discarded, it should also be noted that the Mn^{II} affinities for the various sugar oxygens were determined for initial states having the guanosine binding site unoccupied whereas the crystal structures were obtained with all of the reaction components in place.

The group II intron has only been crystallized in the state following the second step of self-splicing. In this structure, two Mg^{II} ions reside in the catalytic core, one bound by two and the other one by three phosphate groups.[42] At least for the second step of the splicing, the crystallographic data confirms the results of biochemical studies. As discussed above, the stereochemical requirements of the two splicing steps are different, meaning that the results may not be directly applicable to the first step.

The exact number and location of metal ions in the catalytic center of the RNA subunit of RNase P still eludes crystallographic studies[109] but a pattern similar to the one observed with the self-splicing introns seems likely. In other words, one Mg^{II} ion would be bound to the attacking hydroxide ion, another one to the departing 3′-oxygen and both to the same non-bridging oxygen of the scissile phosphodiester linkage. Additionally, a third metal ion, suggested by the third-order dependence of the reaction rate on Mg^{II}, may act as a bridge between the 2′-oxygen of the leaving nucleoside and the 3′-terminal CCA of the ribozyme.

The Role of 2′-OH Groups Flanking the Reactive Phosphodiester Linkage

Even though the large ribozymes do not rely on the intramolecular nucleophilic attack of the vicinal 2′-OH group to initiate transesterification, they still generally exhibit a preference for RNA over DNA substrates. [46, 110-113] RNA substrates typically bind several orders of magnitude stronger than DNA substrates but the contribution of the 2′-OH group flanking the scissile phosphodiester linkage is predominantly to the transition state of the chemical reaction, not to ground state stability.[96, 114, 115] In the reactions of group I introns, rate retardation of 3 orders of magnitude is observed upon 2′-deoxy substitution at the departing nucleoside.[115] Furthermore, 2′-deoxyguanosine acts as a competitive inhibitor to the native guanosine substrate, indicating that the 2′-OH group is essential also in the attacking nucleoside.[11b] Group II introns tolerate DNA substrates better than the other classes of large ribozymes – replacing the departing 3′-linked nucleoside with its 2′-deoxy counterpart retards the rate of the hydrolytic cleavage catalyzed by a minimal group II intron ribozyme only by an order of magnitude.[116] In the second step of the splicing, the deleterious effect of 2′-deoxy substitution of the leaving group is more pronounced but still not as large as with group I introns.[96] With RNase P, the effect of 2′-deoxy substitution at the cleavage site on rate is comparable to that observed with group I introns. [101, 107]

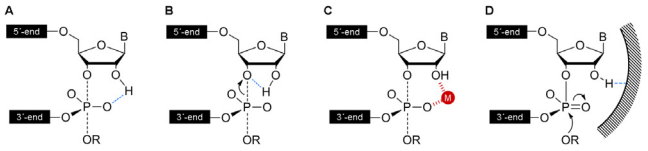
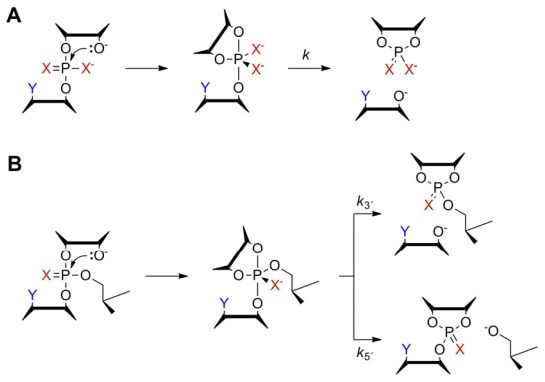


Figure 8. Possible roles for a reaction site 2′-OH in the phosphate transfer reactions of the large ribozymes.

Several explanations for the requirement of a ribonucleoside leaving group may be proposed (Fig. 8). The 2′-OH group may stabilize the developing negative charge on A) the non-bridging phosphoryl oxygens or B) the departing 3′-oxygen by donating a hydrogen bond. Alternatively, the 2′-oxygen may C) coordinate a catalytically important metal ion or D) help orient the substrate favorably by hydrogen bonding. As discussed above, coordination of metal ions by cleavage site 2′-oxygens has been documented with all three classes of large ribozymes. The different roles of the 2′-OH groups are not mutually exclusive, however, and distinguishing between them is a challenging task requiring various complementary approaches.

Studies with Small Molecular Models

With simple dinucleoside-3′,3′-monophosphate or phosphorodithioate models (Scheme 5A), as well as trinucleoside-3′,3′,5′-monophosphate or phosphorothioate models (Scheme 5B), the rate of cleavage initiated by an intramolecular attack of the 2′-oxygen of one of the 3′-linked nucleosides on the phosphorus is retarded 30-fold upon 2′-methoxy substitution of the departing 3′-linked nucleoside, whereas the respective 2′-ammonium substitution accelerates the reaction 50-fold (Tables 1 and 2).[117-121] In the case of the phosphotriester and the corresponding phosphorothioate models, P-O5′ fission competes with P-O3′ fission and the changes in product distribution upon methylation of the 2′-OH group may shed light on the origin of the observed rate retardation. If hydrogen bond stabilization of the departing 3′-oxyanion by the neighboring 2′-OH (Fig. 8B) played a key role, then cleavage of the P-O3′ bond would be expected to be specifically inhibited. Hydrogen bonding to a non-bridging phosphoryl oxygen of the phosphorane intermediate (Fig. 8A), in turn, would have equal effects on both reactions, so that the product ratio should remain largely unaffected by the 2′-methoxy substitution. With the phosphotriester models, P-O3′ cleavage accounts for 88% of the overall reaction under alkaline conditions regardless of whether the 2′-OH of the departing 3′-linked nucleoside is methylated or not (Table 2).[118] Only with the slightly accelerating 2′-trifluoroacetamido modification is complete suppression of the P-O5′ fission observed.[122] With the trinucleoside phosphorothioate models, the ratio of P-O3′ fission is 88% for the 2′-OH and, somewhat unexpectedly, 95% for the 2′-OMe analogue.[120] The fact that cleavage of the P-O3′ bond is not specifically retarded upon 2′-methoxy substitution suggests that it is the phosphorane intermediate (Fig. 8A) rather than the 3′-leaving group (Fig. 8B) that is stabilized by the 2′-OH group. This interpretation is further borne out by the observation that methylation of the 2′-oxygen also inhibits the 2′/3′-isomerization.[119-120] Theoretical studies on simple, unconstrained model compounds also identify the non-bridging phosphoryl oxygen, rather than the 3′-oxygen, as a hydrogen bond acceptor.[123] Finally, the 3′-OH of adenosine is only 0.7 pK_a units more acidic than the 3′-OH of 2′-*O*-methyladenosine, indicating that hydrogen bond stabilization of the departing 3′-oxyanion by the neighboring 2′-OH group could account for no more than a 5-fold rate acceleration.[124]



Scheme 5. Hydrolytic reactions of (A) dinucleoside monophosphate and phosphorodithioate and (B) trinucleoside monophosphate and phosphorothioate models for the catalysis by the large ribozymes.

Table 1. Relative second-order rate constants for the hydroxide ion catalyzed hydrolysis of the dinucleoside monophosphates and phosphorodithioates presented in Scheme 5A (T = 90 °C, I = 0.10 M).

Y[a] \ X[b]	O	S
OH	1	0.68
OMe	0.031	0.025
NH_3^+	50	N / A

[a] The 2′-substituent of the departing nucleoside (see Scheme 5). [b] The non-bridging ligands of the phosphorus atom (see Scheme 5).

Table 2. Relative second-order rate constants for the hydroxide ion catalyzed hydrolysis of the P-O3′ ($k_{3'}$) and P-O5′ bonds ($k_{5'}$) of the trinucleoside monophosphates and phosphorothioates presented in Scheme 5B (T = 25 °C, I = 1.0 M).

Y[a] \ X[b]	O		S	
	$k_{3'}$	$k_{5'}$	$k_{3'}$	$k_{5'}$
OH	0.89	0.11	0.046	0.0065
OMe	0.033	0.0045	0.0019	0.00010
NHTfa	1.9	-	N / A	N / A

[a] The 2′-substituent of the departing 3′-linked nucleoside (see Scheme 5). [b] The non-bridging ligand of the phosphorus atom (see Scheme 5).

Studies with Large Ribozymes Bearing Atomic Mutations

In contrast to the results obtained with small molecular models, studies on modified large ribozymes have yielded substantial evidence of hydrogen bonding between the vicinal 2′- and 3′-oxygens of the leaving (or, in the case of reverse reactions, the attacking) nucleoside in the reactions of group I and II introns.[85, 115, 125] Systematic replacement of the 2′-OH group with a methoxy, a methylamino and an amino group creates an atomic mutation cycle that allows breakdown of the overall impact of the modification on the rate of the reaction to steric, electrostatic and hydrogen bonding contributions.[126] A methoxy group is electrostatically similar to an OH group and has the same capacity to accept a hydrogen bond but is bulkier and cannot donate a hydrogen bond. A methylamino group can both donate and accept a hydrogen bond, like an OH group, but is electrostatically different and as bulky as a methoxy group. Finally, an amino group is isosteric with a hydroxy group and may both donate and accept a hydrogen bond but its electrostatic contribution is different.

Group I Introns

By studying the effects of atomic mutations, the 2′-OH groups of both the leaving and the attacking nucleosides have been identified as hydrogen bond donors in the first step of splicing of group I introns.[85, 127] When the departing 3′-oxygen is replaced with sulfur, a weaker hydrogen bond acceptor, the catalytic advantage of 2′-OH over 2′-deoxy substrates is lost, suggesting

that this oxygen acts as a hydrogen bond acceptor (Fig. 9A).[85] In contrast, a similar study on the reverse reaction (the second step of the splicing) revealed the same energetic penalty upon methylation of the 2′-OH of both 3′-oxygen and 3′-sulfur leaving groups, indicating that the 3′-oxygen of the attacking guanosine is not a hydrogen bond acceptor.[128] Some crystal structures suggest ribose zipper –type hydrogen bonding between the N7 and the 2′-OH of this guanosine and the 2′-OH of an active site adenosine but it is uncertain whether this interaction is shared by all group I introns.[41c, 127, 129]

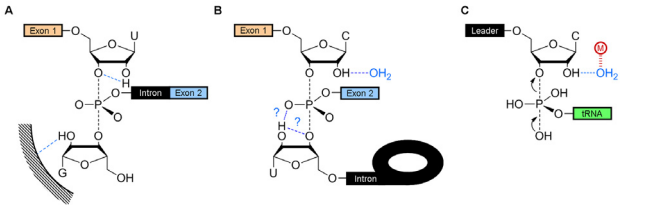


Figure 9. The role of the 2′-OH groups flanking the scissile phosphodiester linkage in the reactions of (A) group I and (B) group II introns and (C) RNase P.

Group II Introns and the Spliceosome

An atomic mutation cycle similar to the one described above for group I introns has also been applied to the spliced exons reopening reaction of group II introns.[130] The reaction is the reversal of the second step of self-splicing of group II introns but also related to the first step of self-splicing in the sense that in both reactions the leaving group is the 3′-terminal nucleoside of exon 1 (the sole nucleoside that occupies the same binding site in both steps of the splicing). As discussed above, 2′-deoxy substrates are tolerated relatively well in the first step of the splicing, ribo substrates being cleaved only an order of magnitude more rapidly.[116] In line with this observation, the atomic mutation studies have not clearly identified the 2′-OH group of the leaving group as a hydrogen bond acceptor, a hydrogen bond donor, a ligand for a catalytically important metal ion or a contributor to substrate binding. Based on the results of extensive quantitative structure – activity relationship studies, in particular the observation that 2′-hydroxyalkyl substrates are cleaved substantially faster than the respective 2′-alkyl substrates, interaction with a water molecule has been proposed instead (Fig. 9B).[130]

With ribozyme models mimicking the second step of the splicing of group II introns (exon ligation), replacing the 2′-OH of the departing nucleoside with an amino group is deleterious but considerably less so than the respective 2′-fluoro or 2′-deoxy substitutions, suggesting that this 2′-OH group may donate a catalytically important hydrogen bond.[96] Rate retardation upon 2′-deoxy substitution is similar with 3′-oxygen and 3′-sulfur leaving groups, indicating that the departing 3′-oxygen is not the acceptor for this hydrogen bond. Recent atomic mutation studies on the reverse reaction, on the other hand, suggest that the 2′-OH of the attacking nucleoside (*i.e.* the leaving group of the forward reaction) abstracts a proton from the 3′-oxygen, thus making it more nucleophilic.[125] When interpreted in terms of microscopic reversibility, this finding would mean that the 3′-leaving group accepts a proton from the neighboring 2′-OH, in apparent conflict with the earlier results on the exon ligation reaction. The *pro*-R_P oxygen is another viable candidate for the hydrogen bond acceptor based on the observed rate retardation by sulfur substitution at this position and the results on small molecular models discussed above.[96, 118, 120] However, due to the fact that no reaction is detected with the *pro*-R_P substrates regardless of the 2′-substituent, this proposed interaction remains to be confirmed (Fig. 9B). The ribo substrate requirements of the spliceosome parallel those of the group II introns: the 2′-OH of the departing nucleoside is not absolutely necessary in either splicing step but the second step proceeded approximately an order of magnitude faster with the natural ribo substrate relative to 2′-deoxy or 2′-methoxy substrates.[131]

RNase P

As discussed above, the 2′-OH group of the departing 3′-linked nucleoside in the hydrolysis catalyzed by RNase P has been proposed to interact with a divalent metal ion, possibly by donating a hydrogen bond to an inner-sphere aquo ligand (Fig. 9C).[101] This proposal receives further support from the finding that 2′-methoxy substitution, retaining the lone electron pairs on the oxygen but eliminating its capacity to serve as a hydrogen bond donor, is even more deleterious than the respective deoxy modification.[113] However, the possibility that the origin of the rate retardation observed with the 2′-methoxy analogue is steric crowding by the relatively bulky methyl group, rather than deletion of an important hydrogen bond, cannot be discarded based on the evidence at hand. More comprehensive atomic mutation studies, such as those performed on the group I and II ribozymes, would be required to establish the role of the cleavage site 2′-OH in the reactions of RNase P.

The 2′-OH Group as a Part of a Hydrogen Bonding Network

The apparent discrepancy between the results on stabilization of the 3′-leaving oxygen by the vicinal 2′-OH obtained using small molecular models or modified large ribozymes may be explained in terms of an intricate hydrogen bonding network in the catalytic center of the latter. [85, 127, 132, 133] Such a network could not only make the 2′-OH more acidic but also orient it favorably for hydrogen bonding with the neighboring 3′-oxygen.

Summary

The large ribozymes employ different methods for recognizing their substrates: the self-splicing introns achieve sequence-specificity through Watson-Crick base-pairing supplemented by tertiary contacts, whereas RNase P accepts a number of substrates having a compatible tertiary structure. The complex hydrogen bonding networks of the catalytic cores differ between the three classes of large ribozymes (and possibly even within a class) but fundamentally all utilize a remarkably similar catalytic strategy: coordination of one Mg^{II} ion by the attacking oxygen, another one by the departing oxygen and both by a non-bridging oxygen of the scissile phosphodiester linkage.[41, 42, 98, 100, 103] [134] At least in the case of RNaseP, additional metal ions may be required to bring the reaction components together. The two-metal-ion mechanism may be seen as an example of convergent evolution on the molecular level, as the large ribozymes are evolutionarily related neither to each other nor to the various protein enzymes, such as the 3',5'-exonuclease of *Escherichia coli* DNA polymerase I, that also use a similar mechanism.[135]

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Biographical Sketch

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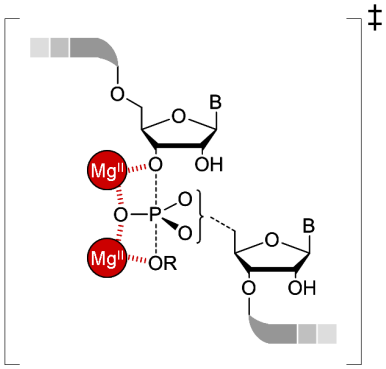
Entry for the Table of Contents (Please choose one layout only)

Layout 1:

Catch Phrase

Tuomas Lönnberg… Page – Page

Understanding Catalysis of Phosphate-Transfer Reactions by Large Ribozymes



The phosphate-transfer reactions catalyzed by the large ribozymes proceed by attack of an external nucleophile and departure of a 3′-linked nucleoside. The recently determined crystal structures as well as the results of various biochemical studies suggest that all of the large ribozymes utilize variations of a common two-metal-ion mechanism in their catalysis.