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A general two-metal-ion mechanism for catalytic RNA

(phosphoryl transfer mechanism/ribozyme/group I splicing/spliceosome/group II splicing)

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ABSTRACT **A mechanism is proposed for the RNA-catalyzed reactions involved in RNA splicing and RNase P hydrolysis of precursor tRNA. The mechanism postulates that chemical catalysis is facilitated by two divalent metal ions 3.9 Å apart, as in phosphoryl transfer reactions catalyzed by protein enzymes, such as the 3′,5′-exonuclease of *Escherichia coli* DNA polymerase I. One metal ion activates the attacking water or sugar hydroxyl, while the other coordinates and stabilizes the oxyanion leaving group. Both ions act as Lewis acids and stabilize the expected pentacovalent transition state. The symmetry of a two-metal-ion catalytic site fits well with the known reaction pathway of group I self-splicing introns and can also be reconciled with emerging data on group II self-splicing introns, the spliceosome, and RNase P. The role of the RNA is to position the two catalytic metal ions and properly orient the substrates via three specific binding sites.**

The finding that RNA can catalyze a chemical reaction (1, 2) was unexpected, in large part because RNA does not contain functional groups that have pKa values and chemical properties similar to those considered important in protein-based enzymes. How then can an RNA enzyme be an effective catalyst? Studies of protein enzymes have shown that high rates of catalytic activity result from the contributions of several factors: (*i*) the specific orientation of substrates relative to each other and to the catalytic groups of the enzyme, (*ii*) design of the active site of the enzyme to be complementary to the structure of the reactants in the transition state of the reaction, (*iii*) use of acid–base catalysis, and (*iv*) formation of a covalent enzyme–substrate intermediate (3–6). As RNA molecules show complex and specific three-dimensional structures (7–10), one can imagine how an RNA enzyme could achieve specific binding and orientation of substrates. But what chemical groups would assist in the chemistry of catalysis?

Both a general mechanism involving the expected requirement for an acid and a base (11) and specific mechanisms using one, two, or three Mg^{2+} ions have been suggested for RNA enzymes (12–14, 59). Although these proposals are consistent with available data, none has a precedent in the more extensively studied protein enzymes. Structural and biochemical studies have shown that the number of different mechanisms by which proteins achieve catalysis is much smaller than the number of enzymes catalyzing a particular category of reaction. Thus, it is reasonable to expect that one of the mechanisms of phosphoryl transfer used by protein enzymes might also be used by RNA enzymes if that mechanism does not depend on the chemical properties of protein side chains (13, 15, 16).

We now expand in detail an earlier suggestion (15, 16) regarding how a two-metal-ion mechanism might function in RNA splicing and in hydrolysis of precursor tRNA by RNase P. This mechanism is used by several protein phosphoryl-transfer enzymes and does not require that protein side chains of the enzyme participate directly in the chemistry of the reaction. Thus, the mechanism can be easily accommodated by an enzyme composed of RNA. Moreover, evidence supporting the existence of one of the two metals has been obtained for a group I intron by Cech and coworkers (17). Finally, this two-metal-ion mechanism predicts the existence of three critical substrate-binding sites on the ribozyme and is, therefore, useful in organizing the rapidly accumulating genetic and biochemical data on the several RNA splicing systems and RNase P.

Mechanisms of Nucleic Acid Hydrolysis

Three general categories of mechanisms for RNA or DNA hydrolysis have emerged from structural, mutagenic, and biochemical studies of protein enzymes. RNA hydrolysis that results in a 3′-PO_4 and 5′-OH and proceeds through a 2′,3′ cyclic phosphoryl intermediate was first established for pancreatic ribonuclease A (18), which uses one lysine and two histidines to facilitate the reaction. The same 2′,3′ cyclic phosphate is formed by the "hammerhead" self-cleaving ribozyme, although how the RNA accelerates this reaction is unknown. Bovine pancreatic DNase I represents a second category of nucleases and uses a divalent cation to interact with the scissile phosphate and a histidine residue as a general base to activate an attacking water molecule (19).

A third mechanism uses two metal ions and applies to those RNA or DNA phosphoryl-transfer reactions that yield 5′-PO_4s and 3′-OHs. For example, in the 3′,5′-exonuclease of *Escherichia coli* DNA polymerase I (pol I), the hydrolytic phosphoryl-transfer reaction is facilitated by two divalent metal ions spaced 3.9 Å apart (15, 16). The same mechanism (described below) has been established for both steps of the alkaline phosphatase reaction (20), is likely for RNase H (21), and is possible for P1 nuclease (22) and phospholipase C (23). In all five enzymes, there are two divalent metal ions 3.8–4 Å apart capable of interacting with the scissile phosphate.

Two-Metal-Ion Phosphoryl-Transfer Mechanism

A two-metal-ion-catalyzed phosphoryl-transfer mechanism (Fig. 1) has been derived from the crystal structure of the DNA polymerase I 3′,5′-exonuclease domain complexed with single-stranded DNA or product (15, 16) and is consistent with mutagenic and kinetic studies (24, 25). Neither the structure nor mutagenesis implicates any protein side chain in the chemistry of catalysis. Rather, protein residues bind and correctly orient for catalysis two divalent metal ions, the 3′ terminal and penultimate residue of the DNA substrate, and the attacking water molecule. In the crystal structure the two metal ions form inner-sphere complexes with the scissile phosphate and water and, thus, facilitate formation of the attacking hydroxide ion and stabilize the transition state. Specifically, metal ion A forms a metal OH^- ion that is

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Abbreviation: snRNA, small nuclear RNA.

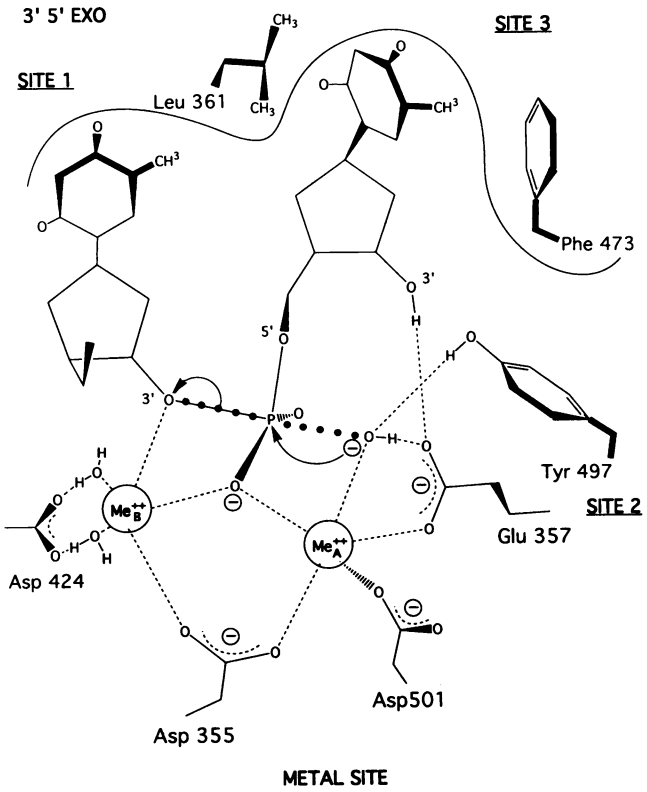

FIG. 1. The proposed transition state (or intermediate) in the mechanism of the 3',5'-exonuclease reaction of *E. coli* DNA polymerase I (16). The 3',5'-exonuclease activity can be supported by Mg^{2+}, Mn^{2+}, or Zn^{2+}, but the native enzyme is likely to contain Zn^{2+} in site A and Mg^{2+} in site B (15, 23). Metal ion A is proposed to facilitate formation of an attacking OH^- that has its lone pair of electrons oriented toward the phosphorus by interactions with the metal ion, Tyr-497, and Glu-357. Metal ion B is hypothesized to facilitate leaving of the 3'-OH group and to stabilize the 90° O—P—O bond angle between apical and equatorial oxygen atoms. The bases on either side of the scissile phosphate are positioned by interactions with the side chains of Phe-473 and Leu-361. Positions of all atoms in this figure were established by x-ray crystal structures, except for the positions of the phosphorous and the three equatorial oxygens, which have been moved by a few tenths of an Angstrom to construct the transition state.

properly oriented with the help of Tyr-497 and Glu-357 for in-line attack on the phosphorus of the scissile phosphate. Metal ion B acts as a Lewis acid in facilitating the leaving of the 3' oxyanion (26); it also serves to stabilize the pentacovalent transition state (or intermediate) by interacting with both the nonbridging and 3' bridging oxygen atoms in a manner analogous to what has been observed with certain organophosphates, such as methylethylene phosphate (27). The bond angle between the O—P—O bridged by the ethylene group in methylethylene phosphate is distorted to 99° from the tetrahedral bond angle of 109° (28), closer to the 90° that occurs in the transition state. Westheimer (27) proposed that strain induced by the ethylene bridge accounted for the enhanced hydrolysis rate of methylethylene phosphate (some 10^6 times faster than trimethyl phosphate); a similar role is suggested for the bridging metal ions in Fig. 1.

Alkaline phosphatase provides an example of how the same active-site configuration can carry out a two-step reaction. Alkaline phosphatase contains binding sites for three closely positioned divalent metals, two of which bind phosphate as described above (20). The mechanism proposed for each stage of the reaction (Fig. 2) is comparable to that of the 3',5'-exonuclease. In the first step, the phosphate is transferred to the OH group of Ser-102; reversal with OH^-,

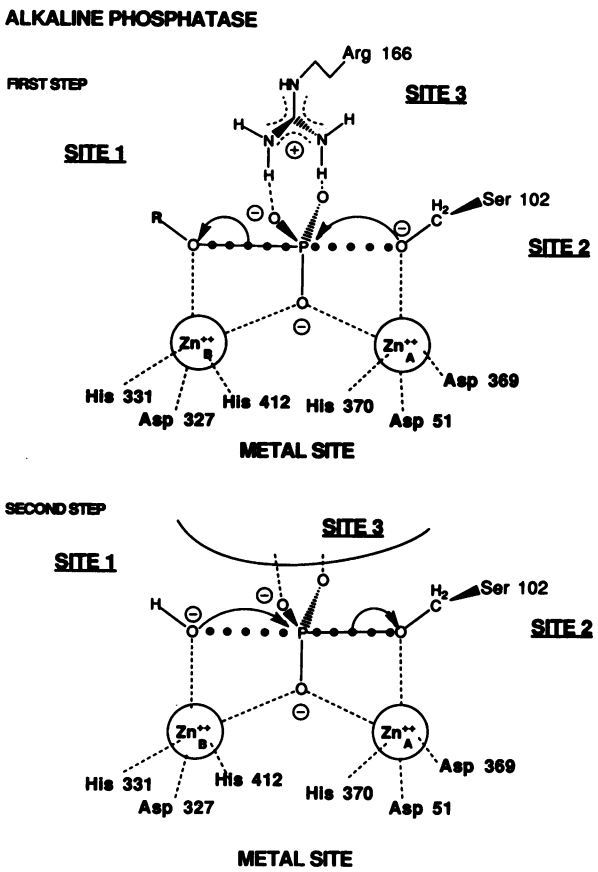

FIG. 2. The two-step mechanism of *E. coli* alkaline phosphatase. Transition states for each of the two steps are stabilized by two Zn^{2+} ions (20).

now occupying substrate binding site 1, achieves phosphate release. The RNase H domain of human immunodeficiency virus reverse transcriptase likewise binds two divalent metal ions 4 Å apart in the active site, implying a similar mechanism (21). The single-stranded P1 nuclease has three bound zinc ions, two of which are observed to coordinate to the 5' phosphate of a bound nucleotide (22). Finally, phospholipase exhibits the same overall protein structure as P1 nuclease, including three Zn^{2+} atoms at the active site (23).

Although all of these five enzymes may use a two-metal-ion mechanism of phosphoryl transfer, only two (P1 nuclease and phospholipase) have obviously homologous structures. This observation implies enormous variability in the structural context capable of positioning two metal ions precisely relative to the phosphoryl group. That the intermetal ion distance is 3.9 Å in four enzymes of separate evolutionary origin implies an important chemical role for this positioning. This distance may be related to the distance (in the transition state) between the apical attacking and leaving oxygen atoms directly bonded to the metal ions. Could RNA molecules also position two Mg^{2+} ions 4 Å apart (perhaps using the phosphates of the RNA backbone rather than carboxylates) to achieve relevant interactions with a substrate phosphoryl group? Can ribozyme groups be identified that position the attacking water or sugar, the leaving ribose oxyanion, and its adjacent nucleotide?

Group I Splicing Conforms to the Two-Mg^{2+}-Ion Mechanism. The group I self-splicing intron catalyzes two phosphoryl-transfer reactions that result in intron removal and ligation of flanking exons (Fig. 3) (29). In each step of the splicing reaction, the 3' OH of a ribose attacks the backbone phosphate of an RNA chain. The chemistry of this transesterification reaction is identical to the hydrolysis reactions

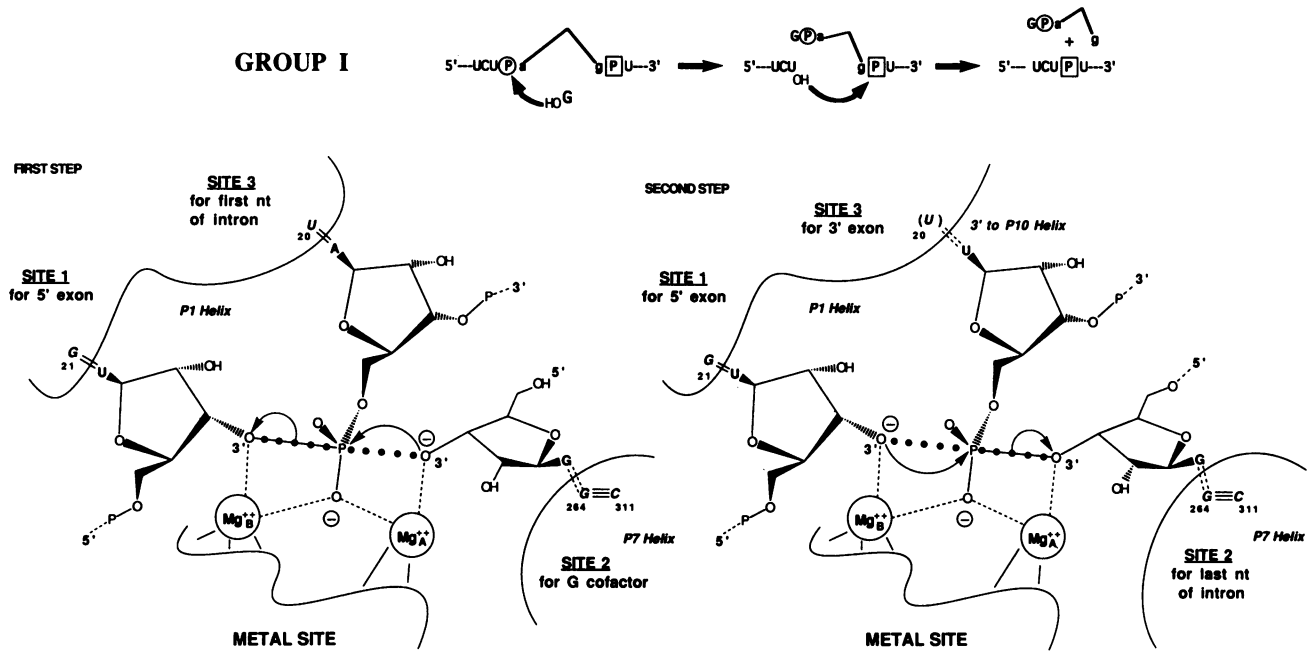

FIG. 3. Mechanism for group I self-splicing. References for assignment of *Tetrahymena thermophila* intron nucleotides and helices to substrate-binding sites 1–3 during the first and second steps of the reaction (shown above) are given in text. Intron nt 18–20 are included in helix P1 for step 1, whereas nt 13–19 pair with 3′ exon sequences to form P10 for the second step.

considered above, except that, instead of water, the 3′-OH of a nucleotide (guanosine in the first step and uridine in the second step) is activated for nucleophilic attack on the phosphoryl group. In Fig. 3, the 3′-OH of the guanosine that initiates the reaction is activated by Mg^{2+} ion A in exactly the same fashion as the attacking water in the 3′,5′-exonuclease reaction (Fig. 1). Also, as with the exonuclease, a second Mg^{2+} ion B is postulated to act as a Lewis acid to facilitate (26) leaving of the 3′-oxyanion of uridine and stabilize the pentacovalent intermediate (or transition state). Evidence for this metal ion comes from the observation that, although it is unknown whether phosphates and/or bases bind the metals, replacing Mg^{2+} by Mn^{2+} or Zn^{2+} rescues cleavage of a phosphorothioester at the 5′-splice site (17), as anticipated from the known preference of Mg^{2+} versus Mn^{2+} for coordination by oxygen versus sulfur. After step 1, the chain containing the newly added guanosine must dissociate from sites 2 and 3, allowing a new substrate, the 3′-splice site, to take its place. The newly created 3′-OH of uridine at the end of the 5′ exon remains in the same position, whereas the guanosine residue at the 3′ end of the intron occupies the same guanosine-binding site as the guanosine cofactor (site 2) and the first nucleotide of the 3′ exon now occupies site 3. Hence, in the second step, the Mg^{2+} ions reverse roles and Mg^{2+} B activates the attacking 3′-OH of uridine, whereas Mg^{2+} A facilitates the leaving oxyanion of guanosine. The mirror symmetry of the two metal ions in the catalytic center reflects the identical chemistry of the two steps of the reaction.

Not only is the chemistry of the group I intron the best understood among ribozymes, but assignment of RNA moieties that form binding sites for the substrates is most advanced. The uridine at the end of the 5′ exon (as well as several preceding residues) is base-paired to the so-called internal guide sequence and remains bound in this configuration (called helix P1) during both steps of the reaction (29) (site 1 in Fig. 3). The location of the binding site for the guanosine cofactor for the first step of the reaction (site 2) is also known (30): critical interactions are made with the G^{264}-C^{311} pair in the core of the ribozyme (helix P7). The same binding site orients the guanosine at the 3′ end of the intron for the second step of the reaction (30, 31) and also serves to bind water for ribozyme-catalyzed hydrolysis at the 5′ site (32). The third substrate-binding site, which holds the 5′ end of the intron during the first step and the 3′ exon during the second step of the reaction, has likewise been identified for the group I intron (29). A sequence adjacent to the internal guide sequence initially pairs with bases at the 5′ end of the intron (P1) but then exchanges partners to form several pairs with the 3′ exon (called P10) for the second step of the reaction. Because all three substrate-binding sites are identical for the two steps of group I splicing, inhibition of only one (the second) reaction step by an R_p phosphorothioate diasteriomer at the splice site is observed (33, 34).

Finally, critical metal-ion-binding sites have been located near position 207 (in P5) and 306 (near P7) in the catalytic core of the group I intron (35).

A Model for the Active Sites of Group II Introns and the Spliceosome. Intron removal by group II self-splicing introns (36) and by the spliceosome (37), which assembles from five small nuclear RNAs (snRNAs) (U1, U2, U4, U5, and U6) and many proteins, follows a different two-step pathway (Fig. 4, see top). In the first phosphoryl-transfer reaction, the 2′-OH group of an adenosine residue located upstream of the 3′-splice site attacks the backbone phosphate at the 5′-splice site. Magnesium ion A is proposed to activate this OH group (Fig. 4, first step) as in Figs. 1 and 3 (first step), whereas Mg^{2+} ion B again stabilizes the leaving oxyanion and pentacovalent intermediate. After step 1, the lariat structure must be replaced by the 3′-splice site in sites 2 and 3 (Fig. 4, second step). Meanwhile, the excised 5′ exon is held fixed, so that in the second step of the reaction its newly created 3′-OH can attack the phosphoryl group between the conserved residue at the 3′ end of the intron and the 3′ exon. This relationship is pictured as a reversal of the first reaction.

In contrast to group I splicing, where all three substrate-binding sites remain unchanged for the two reaction steps, our fragmentary understanding of group II introns and the spliceosome argues that two of these binding sites (2 and 3) change. Yet, in the simplest formulation of the model, site 1 (which holds the 5′ exon) and the positions of the two metal ions could remain unaltered. How can current knowledge of

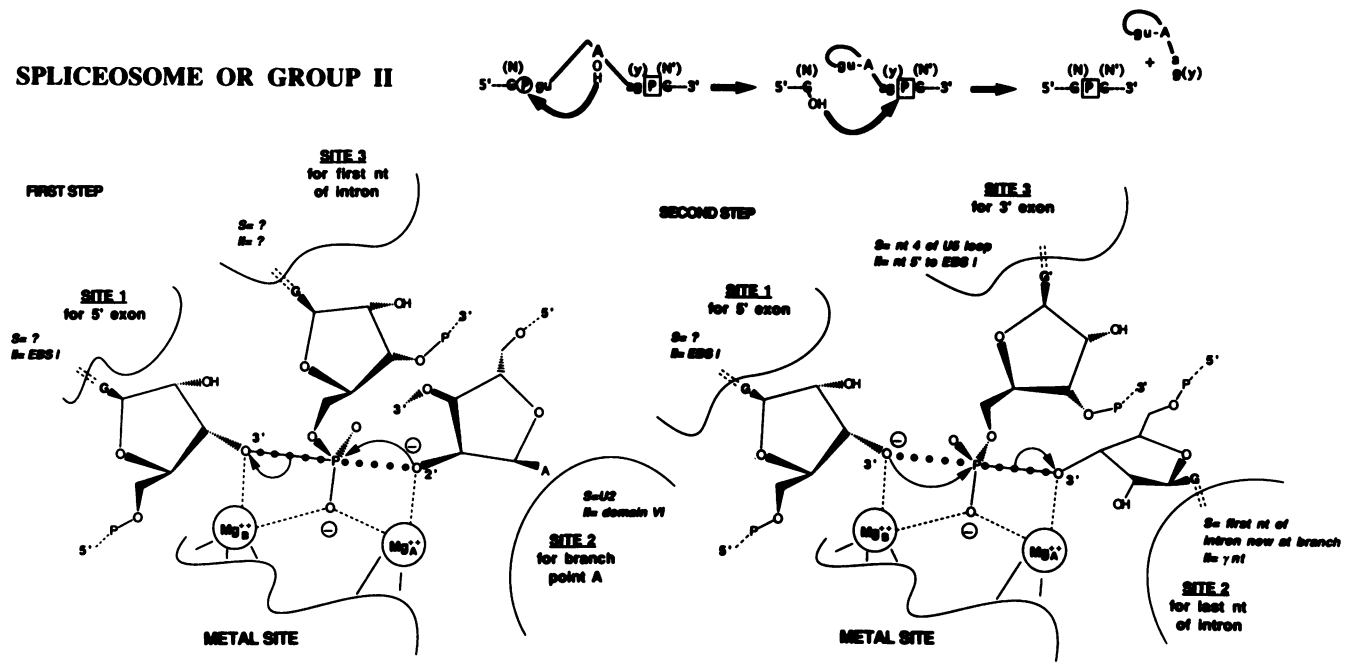

FIG. 4. Mechanism for spliceosome and group II splicing. Above is shown the two-step reaction with consensus nucleotides indicated for the spliceosome-catalyzed reaction and, in parentheses, consensus nucleotides that differ in group II self-splicing introns. In both subgroup IIa and IIb, N and the adjacent 5 nt of the 5′ exon covary with EBS1; N′ in subgroup IIa covaries with the intron nucleotide 5′ to EBS1; y indicates a pyrimidine. The two steps shown below use the spliceosome consensus sequences. Experimental evidence supporting the assignment of particular group II intron (II) or snRNA (S) nucleotides to binding sites 1–3 is cited in text. A rotation of binding site 3 by 120° away from the reader between the two reaction steps, whereas sites 1 and 2 are approximately fixed, is proposed.

functional or conserved residues in the group II intron and in the snRNAs of the spliceosome be reconciled with the model (Fig. 4)?

In group II introns, the 5′ exon is held by base-pairing to two nonadjacent sequences in domain I of the intron, termed EBS1 and EBS2 (36). The 5′-splice site is determined by its position opposite the 5′-most nucleotide in EBS1 (38, 39). Because the 5′ exon–EBS1 interaction appears to be maintained through both steps of splicing (36), it precisely fits the requirements of site 1 in Fig. 4. Domain VI of the intron (36), a double helix from which the branchpoint A residue is bulged, comprises binding-site 2 for the first step of the reaction, whereas for the second step the pairing of the last nucleotide of the intron with an intron nucleotide (located between domains II and III) termed γ is critical (36, 40). Yet, only a small displacement of domain VI between steps may be required because its structure is known to influence 3′-splice-site recognition (41). The third substrate-binding site likewise cannot be identical for the two steps of the reaction. No nucleotide has yet been identified as interacting with the first base of the intron during the first step (36, 42, 43), but intron nt 3 and 4 (and perhaps adjacent residues) base-pair with 2 nt called ϵ in intron domain I (36, 40); this pairing is maintained for the second step of the reaction (40). For step 2, introns of subgroup IIA pair the intron nucleotide 5′ to EBS1 with the first nucleotide of the 3′ exon; this interaction is important for 3′-splice-site specificity (36, 38) and, therefore, probably contributes to site 3 for the second step, but this same intron nucleotide does not appear to pair with the first base of the intron during step 1 (36). Finally, domain V is an excellent candidate for binding the two metal ions: it is the most highly conserved of the six intron domains and, when added *in trans* to domains I–III, it stimulates 5′-splice-site hydrolysis (44).

In the spliceosome, only three snRNAs (U2, U5, and U6) are believed to contribute functionally once catalysis begins (37). No RNA has yet been identified in contact with the last nucleotide of the 5′ exon during either step of the reaction, although an earlier interaction of the penultimate nucleotide of the exon with an absolutely conserved 9-nt loop sequence in U5 has been documented both genetically and biochemically (45, 46). In striking analogy to group II introns, the branch site A residue is bulged from a helical interaction with U2 snRNA, identifying binding-site 2 as containing U2 snRNA (37) for the first reaction step. Before the second step, the model (Fig. 4, second step) proposes that the invariant guanosine at the 3′ end of pre-mRNA introns replaces the branch-point A residue in site 2. A slight shift of the U2 snRNA within the spliceosome might achieve such a realignment: a non-Watson–Crick interaction between the first and last bases of pre-mRNA introns has been proposed based on genetic suppression data (47), and the first intron nucleotide is now attached to the branch-point A residue. The third substrate-binding site again would require significant remodeling between steps. The results of chemical crosslinking have suggested that intron nucleotides downstream of the 5′ splice site contact the conserved loop sequence in U5 snRNA during the first step (48), whereas genetic results argue that the first two positions of the 3′ exon interact with adjacent nucleotides in the same U5 conserved loop for the second step of the reaction (49). Thus, two different portions of the U5 conserved loop could comprise site 3 for the two reaction steps, suggesting movements that might be facilitated by known U6 contacts (48, 50) (that perhaps mimic ϵ in the group II intron) with sequences near the 5′ end of the intron. Specifically, a 120° shift in the position of site 3 (a rotation about the axial oxygens in the pentacovalent intermediate) between the two steps of pre-mRNA splicing (Fig. 4) would place the other nonbridging oxygen between the two metals. This geometry would rationalize the results of Moore and Sharp (51), who recently observed that both steps of the spliceosome reaction are inhibited by the same phosphorothioate diasteriomer, whereas neither step is affected by the opposite diasteriomer. Finally, an interaction between conserved sequences in U2 and U6 snRNAs recently identified by genetic analyses (52) in yeast exhibits uncanny

structural resemblance to domain V of group II introns. This snRNA–snRNA duplex is, therefore, a most attractive candidate to provide metal-binding sites in the active spliceosome.

Ribonuclease P

Ribonuclease P catalyzes the simple hydrolysis of a specific phosphodiester bond in pre-tRNAs to create the 5′-PO_4 terminus of all mature tRNA molecules (53, 54). Although the native enzyme contains both RNA and protein, the RNA alone is active in the presence of Mg^{2+} under nonphysiological salt conditions (2). The two-metal-ion model for the RNase P active site again includes three substrate-binding sites (see Fig. 1): site 1 for the last nucleotide of the 5′ leader sequence, site 2 for the attacking OH^-, and site 3 to hold the first nucleotide of the mature tRNA. Biochemical experiments have suggested candidate residues in the *E. coli* RNase P RNA that may serve as sites 1 and 3 for substrate [C^{92} (55) and A^{246} (56)] and metal [positions 257 and 295 (57)] binding.

Perspectives

We have described how a mechanism of phosphoryl transfer documented for several protein enzymes could be used by RNA enzymes involved in splicing and RNA hydrolysis. In the protein enzymes, no side chains are directly involved in the chemistry of catalysis; rather, they serve to bind two catalytically essential metal ions and orient the substrates. These functions can equally well be assumed by RNA. The same mechanism could also be used by RNA enzymes, past or present, to catalyze other, but related, reactions, including RNA or DNA polymerization reactions (16, 58).

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