Group II Intron Ribozymes and Metal Ions – A Delicate Relationship

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Group II introns are naturally occurring ribozymes in plants, fungi, bacteria, and lower eukaryotes that undergo a fascinating array of reactions. These large molecular machines with a size ranging between 600 and 2500 nucleotides are self-splicing introns also capable of reinserting themselves into RNA or DNA, thus making them mobile genetic elements. The structural information available on group II intron ribozymes is very scarce. So far, only one crystal structure and one NMR solution structure of two domains located in the catalytic core are available. For proper folding and function, each intron requires specific concentrations of monovalent and divalent metal ions. Although most of these metal ions are used for charge screening, some are bound to distinct sites as has been shown by hydrolytic cleavage experiments. These specifically bound ions are crucial for tertiary contact formation and catalysis. This review will discuss the different metal-ion requirements of self-splicing group II introns, the available structural data and information on the binding location and affinity of metal ions, as well as the methods applied to investigate the metal-ion binding properties of these large RNAs. Due to the size of these introns, the richness of local structures, the catalytic versatility and the involvement of metal ions in all of the above-mentioned aspects, group II introns are an ideal target to be studied by combined means from the fields of Biochemistry, Molecular Biology, Analytical, and (Bio)Inorganic Chemistry.

1. Introduction

In contrast to the typical misconception that RNA is a stringy, single-stranded molecule without defined architecture, most functional RNA molecules are compact, stable, and tightly folded into a unique conformation.[1–4] Even mRNAs, which are normally thought of as linear "tapes" encoding for protein sequences, contain highly structured regions at their termini that serve as signals for regulating gene expression.[5,6] In the early 1980s, the research in RNA biochemistry "exploded" after the exciting discovery by Altman, Cech, and Pace that RNA is able to catalyze various reactions in living cells without the aid of amino acids.[7,8] Thus, the old dogma of only proteins being enzymes was overthrown and ribozymes were "born".

In the past twenty years, many more catalytic and noncatalytic functions of RNA in Nature have been and are still being discovered.[9] To name just a few: Aside from its diverse roles in protein synthesis (e.g., tRNA, rRNA), RNA is also involved in protein transport (signal recognition par-

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MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

ticle, SRP).[10] Furthermore, in the last couple of years, small RNAs composed of about 21–23 nucleotides in lengths were discovered to occur in periodic cycles within the cell. Such small interfering RNAs (siRNAs) play a key role in cell development (RNA interference, RNAi).[11,12] Only very recently, so-called coRNAs (coregulatory RNA) or riboswitches[13–19] were found to be important for gene regulation. Last, but not least, RNA molecules also fulfil diverse functions regarding RNA editing as well as RNA processing, i.e., they carry out post-transcriptional modifications. Catalytic RNAs are found among several of these groups; they are involved in protein synthesis, gene regulation, RNA editing, as well as RNA processing.[9] In all of these RNAs, catalytic or noncatalytic, metal ions play an essential role in their architectural assemblies and modes of action that are central to RNA biology.

Today, three different families of naturally occurring ribozymes are known: (i) The large phosphoryl transfer ribozymes (group I and group II introns, ribonuclease P), (ii) the small phosphoryl transfer ribozymes [hammerhead, hairpin, hepatitis delta, and Varkud satellite (VS) ribozyme], and (iii) the aminoacylesterase ribozymes (ribosome).[20] All these ribozymes either catalyze the cleavage and/or joining of RNA and DNA molecules through reactions at phosphate centers[20] or catalyze peptidyl transfers during protein synthesis, which involve the transfer of an activated ester at a carbon center.[21]

Very recently two new ribozymes have been discovered. First, the *glms* ribozyme, which belongs to the small phosphoryl transfer ribozymes [family (ii) mentioned above].[18] This ribozyme consists of a RNA motif found within a mRNA that self-cleaves upon binding to the reaction product of its encoded protein, therefore providing self-regulation in gene expression. Second, the co-transcriptional cleavage (CoTC) RNA motif that occurs in the 3′-flanking regions of primate β-globin genes and promotes transcription termination.[22] There is also strong evidence that another large ribonucleoprotein complex within the cell is a ribozyme – the so-called spliceosome.[23,24] This molecular machine, composed of five different snRNA molecules (small nuclear RNA) and numerous proteins, is found in all higher eukaryotes and is responsible for the correct splicing of freshly transcribed RNAs (thus belonging to the first mentioned family).[25,26]

As indicated in the above paragraphs, natural ribozymes encompass a surprisingly large chemical and functional repertoire in living organisms. The structures and reaction mechanisms of some of these ribozymes, like the Hammerhead, the Hairpin, or group I introns have been resolved to some detail in the past few years.[27–33] This short review focuses on group II introns, their occurrence, the reactions they catalyze, their structure, as well as on what is known about their delicate and complicated interaction with metal ions. For example, the Mg^{2+} requirements of different group II introns originating from the same gene within one organism can differ by a factor of more than ten. In addition, these large RNAs have one very remarkable quality: A group II intron broken into individual domains will reassemble into the catalytically active ribozyme! It is this modular behavior of the components, together with the anticipated richness of structural motifs and the strict requirement of metal ions for folding and catalysis that makes group II introns excellent systems to be studied by a combination of biochemical, analytical, and (bio)inorganic methods.

2. What are Group II Intron Ribozymes?

Group II introns are a very interesting class of naturally occurring ribozymes. These large molecular machines consist of about 600 to 2500 nucleotides and are second in size only to ribosomal RNAs. More than 750 gene locations of group II introns are known.[34] They are found primarily in organellar genes of plants, fungi, and lower eukaryotes, but also in many bacteria.[35] These RNAs are best known for their ability to undergo self-splicing, but they also perform a variety of other reactions including RNA and DNA hydrolysis as well as intron mobility into RNA and DNA substrates.[35,36]

RNA splicing is defined as follows: Most genes are transcribed into mRNA precursors that are not ready to undergo translation into proteins. Before translation, these mRNAs (as well as most tRNAs, rRNAs, and snRNAs) must undergo the process of splicing, in which noncoding introns (*in*tervening sequences) are removed from the coding exons (*ex*pressed regions). RNA splicing can be achieved by either autocatalytic introns (group I and group II introns), the eukaryotic spliceosome, or proteins (tRNA splicing).[37] The spliceosome releases the excised introns from the mRNA precursor as branched lariat molecules. This formation of lariat RNA resembles very much the splicing pathway of group II introns, with the only difference that the catalytic components responsible for splicing reside within the intron itself (Figure 1).[38–40]

In group II introns, the first step of the splicing reaction consists of a nucleophilic attack of the 2′-OH of a highly conserved adenosine located in a bulge within domain 6 (D6) (see below). In the second step, the 5′ and 3′ exons are ligated and the lariat intron is simultaneously released (Figure 1). Both steps of this branching reaction are highly reversible, explaining the fact that free lariat group II introns can catalytically insert themselves into RNA and DNA.[41–43] This ability makes group II introns infectious genetic elements that are capable of migrating into new hosts or new positions within a host genome.[44–50] Unlike other systems of genetic transposition, in group II introns, this process depends on the reactivity of the intron itself, which catalyzes its own reverse splicing into new genomic locations. Studies of intron mobility have shown that group II introns can be designed to target and insert themselves at any desired location, thereby either knocking out or transforming a gene.[45,48,51]

The transesterification reaction in group II intron splicing is in constant competition with an alternative hydrolytic pathway: A water molecule can readily react as nucleophile

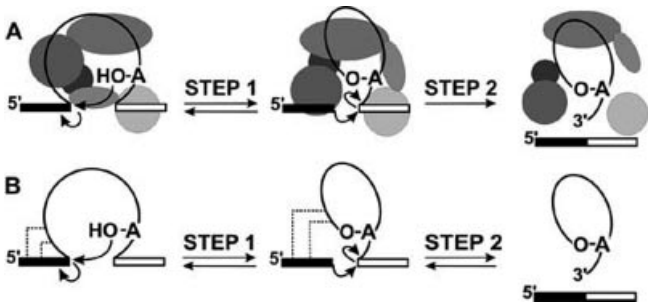

Figure 1. Comparison of the similar splicing pathways of the spliceosome and group II introns. **(A)** In higher eukaryotes the spliceosome removes introns and joins exons together. Five RNAs and several proteins assemble around the splice site and remove the intron as a lariat. An intronic 2′-OH of an adenosine serves as the nucleophile. After removal of the intron and joining of the exons, the RNAs and the proteins disassemble and then reassemble at the next splice site. **(B)** Group II introns fold into the active structure around the splice site. Correct cleavage site recognition by the EBS-IBS base pairings is indicated by dashed lines. In a first step, an internal 2′-OH of a conserved adenosine attacks the 5′-splice site to form a lariat. In a second step, the two exon pieces are joined and the intron removed as a lariat. Both steps are reversible, enabling group II introns to reinsert themselves into RNA or DNA.

during the first step of splicing (both in vitro and in vivo) making the two steps irreversible.[52–54] Although autocatalytic in vivo, group II introns often employ proteins to stabilize the intron structure or to enhance their functionality (e.g., reverse transcriptase activity during intron mobility).[36,55–57] Many group II introns have the innate capability to function in vitro, but only a few have been characterized in more detail. Among those best studied regarding folding, tertiary structure, and catalytic activity is ai5γ, which is located in the cytochrome oxidase 1 gene of mitochondria in baker's yeast *Saccharomyces cerevisiae*.[36,58]

Despite a relative lack of sequence conservation among group II introns they all possess a conserved set of six domains that contain well-defined secondary structural elements (Figure 2).[36,58] Each domain has a specific function: Domain 1 (D1) is an independent folding unit and a molecular scaffold for docking of the other domains.[59] In addition, this domain recognizes the 5′-exon through two EBS-IBS (exon/intron binding site) base pairing interactions, which are not conserved but always co-vary.[60] D1 contains components important for catalysis, including motifs involved in the λ[61] and ε[62] tertiary interactions, and forms together with domain 5 (D5) the minimal structure capable of supporting catalytic activity.[63,64] D2 lacks phylogenetic conservation but may aid to stabilize the tertiary structure of the folded intron.[64,65] D3 is a catalytic effector that increases the rate of catalysis by D1 and D5.[66–68] D4 does not contribute to self-splicing activity but often contains an open reading frame (ORF) encoding a maturase protein that aids in splicing and intron mobility in vivo.[69,70] Unlike the other domains of the intron, D5 is highly conserved and represents the most critical active-site component. It forms a very stable hairpin and binds through the ζ-ζ′[71] and the κ-κ′[72] tertiary interactions to the central region of D1 (Figure 2). Combination with the λ-λ′[61] (and ε-ε′)[62] interaction places the 5′ splice site right next to the "chemical face" of D5, i.e., to functional groups which have been identified to play a direct role in catalysis.[73,74] D6 contains the conserved bulged adenosine branch-point that serves as the nucleophile during the first step of branching.[38] The branch-point region of D6 contains conserved functionalities that position the bulged adenosine relative to the active-site components in D1 and D5.[72,75]

Group II introns have the interesting ability that their domains are independent folding units.[59] Thus the individual domains can be added together *in trans* (i.e. not covalently linked) and they will reassemble (at the proper metal-ion concentration) to the active three dimensional fold.[61,68,76] It should be added that the individual domains or combinations thereof can be easily synthesized by in vitro transcription from double-stranded DNA (dsDNA) templates, e.g. linearized plasmids or synthetic dsDNA, and purified by polyacrylamide gel electrophoresis (PAGE).[77,78] All these points make group II introns ideal targets to study with respect to either structure-function and/or metal-ion binding-function relationship.

3. The Need of Metal Ions in RNA Folding and Catalysis

Due to the polyanionic nature of the phosphate-sugar backbone metal ions are inextricably involved in the process of RNA folding and function. A simplified two-step folding pathway of large RNAs starts with the transition from a random coil to the secondary structure followed by the second step of compaction to the tertiary structure, in which specific long-range interactions orient all the structural elements in space (Figure 3).[79,80] Monovalent ions tend to play an important role in charge-screening, thereby allowing the secondary structure to form (Figure 3). Only recently one has begun to decipher the repertoire of tertiary interaction motifs that stabilize folded RNA molecules.[71,81] Tertiary structural elements are stabilized by interactions with divalent metal ions, preferably Mg^{2+}, but also the monovalent potassium appears to have an additional role in binding to high affinity sites.[82] The stabilization of nucleobase quartets by alkaline metal ions,[83,84] e.g. in telomeres,[85,86] is just one prominent example. In general, the formation of RNA tertiary structure is dependent on four parameters: (i) RNA sequence, (ii) metal-ion identity, (iii) metal-ion concentration, and (iv) the presence of RNA binding proteins and polyamines.[20] However, in addition to employing metal ions within structural elements, ribozymes also use them directly for catalysis.

One of the major differences between nucleic acids and proteins is that in DNA or RNA, due to the existence of only four nucleobase moieties (compared to 21 amino acids) a much smaller diversity of functional groups is present, none of which having a pK_a value near the physiological pH. This lack in diversity can be overcome by several factors: Stabilization of a protonated spezies by hydrogen

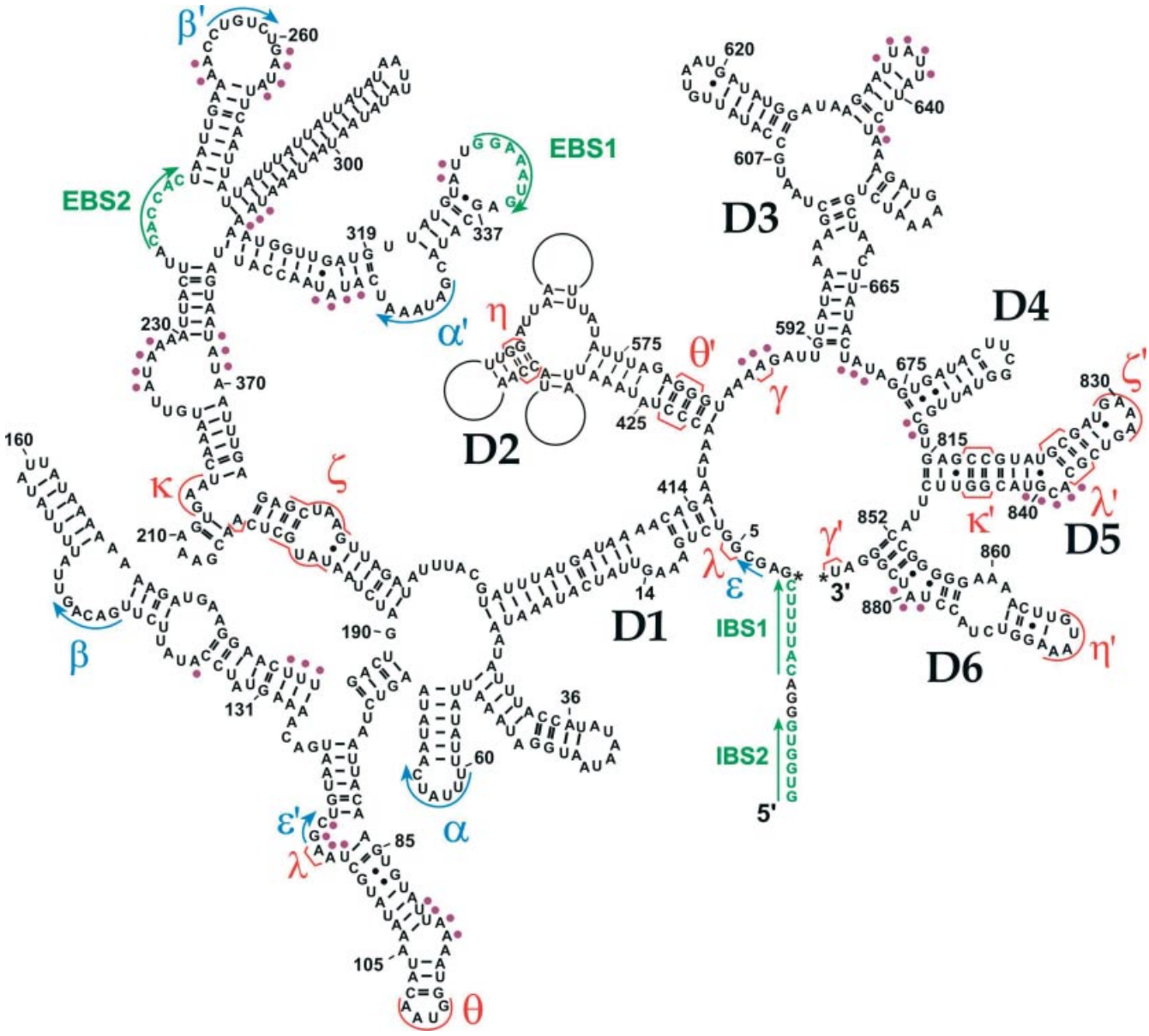

Figure 2. Secondary structure of a ribozyme derived from the group II intron ai5γ from *S. cerevisiae*. Domain 2 (D2) is only partly drawn; further regions making no known contacts to the other intronic domains are indicated as circles. Domain 4 (D4), which in some introns contains an open reading frame (ORF) is truncated and reduced to a hairpin, although the numbering of the nucleotides corresponds to that of the full lengths intron. The last sixteen nucleotides of the 5′ exon are also shown and separated from the intronic nucleotides by the splice site. Both, the 5′ and the 3′ splice sites are indicated by asterisks. Long-range tertiary contacts are labeled with pairs of Greek letters: Of the latter ones, intra-domain contacts are colored in blue, whereas inter-domain contacts are shown in red. Intron-exon interactions (IBS1-EBS1 & IBS2-EBS2) are shown in green. Sites of intense Tb^{3+} cleavage are marked by filled circles in magenta (see also ref.[78]).

bonding like in A·C$^+$ base pairs,[23] formation of a special fold with distinct electrostatic properties, and/or by the aid of metal ions.[87–89] Coordination of a metal ion to a nucleobase can lead to large pK_a shifts at amino and/or imino groups.[90–95] As a consequence, hydrogen bonding might be strengthened,[92,94,96,97] and/or rare tautomers stabilized[98] leading to new base pairing patterns.[96] In addition, such acidified protons could now participate directly in catalysis at a pH close to neutral. In fact, in most ribozymes structurally and/or biochemically investigated today, divalent metal ions are found within the catalytic core. There they are used, e.g., to activate the attacking nucleophile for phospho-diester cleavage, to stabilize the transition state, or the leaving group.[20,99,100] It follows that in nucleic acids not only kinetically inert metal ions, e.g. Pt^{2+},[101,102] but also labile metal ions[103] can coordinate in close neighborhood to each other,[104–106] sometimes even to the same nucleobase.[107] In a few cases, divalent metal ions can be replaced by high, i.e. molar, concentrations of monovalent ions, suggesting a mechanism where metal ions "only" have an electrostatic role and are thus probably not coordinated at the active site. However, it is unclear if under such conditions

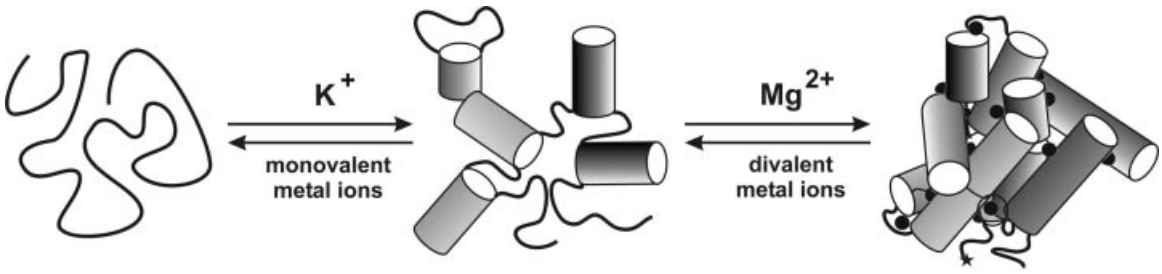

Figure 3. Schematic view of group II intron folding. Monovalent metal ions are needed for charge-screening and enable formation of the secondary structure. Tertiary structure formation to the catalytically active architecture only takes place in the presence of divalent metal ions like Mg^{2+}. Several Mg^{2+} ions (in some instances also K^+ ions) occupy specific binding sites stabilizing local structural motifs (adapted from ref.[79,80]).

of nonnatural high concentrations of M^+ ions, the mechanism of catalysis corresponds to the one in the presence of divalent ions.

To summarize, both Mg^{2+} and K^+ play an indispensable role in the world of RNA metabolism (see Figure 3), but in limited cases, also Na^+ and Li^+ (and NH_4^+) can contribute to correct folding of certain RNAs. Ca^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} can sometimes substitute for Mg^{2+}, but they are rarely the natural cofactors.[108,109]

4. Metal Ions and Group II Introns

Large ribozymes like group II introns are particularly dependent on metal ions for function and are therefore ideal "targets" for study by bioinorganic chemists. It is interesting to note, although so far not understood, that different group II introns have rather different requirements for divalent and monovalent ions in vitro (see Table 1).[110–112] This specificity does not only relate to ribozymes from different organisms, but also to those originating from within one gene.[110] Considering the highly conserved secondary structure of these molecules, the range of Mg^{2+} concentrations varying between 0.1 and 100 mM is astonishing – even more so because there seems to be no correlation with the GC content of the nucleic acid sequence. Today, only one catalytic Mg^{2+} ion is known, which binds to the 3′-oxygen of the scissile phospho diester bond in both steps of splicing thereby stabilizing the 3′-oxoanion leaving group.[24] This Mg^{2+} coordination is observed in group II introns and the spliceosome, thus extending the parallels between these two splicing machineries.[24,113]

Binding studies of metal ions to folded RNA molecules are hampered because Mg^{2+} and K^+ are spectroscopically silent and are further complicated by the fact that these ions serve multiple roles (structural and catalytical) within the same molecule; hence, it is difficult to focus on one particular binding site. In addition, high Mg^{2+} concentrations are often required as Mg^{2+} binding is predominantly weak. Therefore, in experimental studies Mg^{2+} is often partly replaced by other metal ions. Besides d-transition metal ions, as well as Zn^{2+}, Cd^{2+}, and Pb^{2+}, lanthanides have lately become important mimics of Mg^{2+} to probe RNA structures and functions.[114,115] Like Mg^{2+}, lanthanide ions are mostly redox inactive and coordinate preferably to hard ligands such as phosphate oxygen atoms. Their +3 charge enables them to interact stronger with RNA than Mg^{2+}, but also leads to lower pK_a values for coordinated water molecules compared to the situation encountered with Mg^{2+}. The lanthanide hydroxide species can either directly attack the phospho diester bond, or alternatively depro-

Table 1. Concentrations of K^+ (or NH_4^+) and Mg^{2+} needed for achieving the best splicing activity in vitro with group II introns from different organisms. In column 1, the organism is given, in which the group II intron (column 2) belonging to one of the two subgroups (column 3) is found. The gene location is given in column 4. Optimal concentrations of K^+/NH_4^+ (column 5) and Mg^{2+} (column 6) are listed together with the tested concentration range, in which the ribozyme is active.

Organism	Group II intron	Subgroup	Location[a]	[K^+][M]	[Mg^{2+}][mM]	Ref.
Azotobacter vinelandii	Av.hsp60	IIB	hsp60	0.5	100	[111]
Chlamydomonas sp.	Chs.psbA1	IIB	psbA	1 (0.5–1.5)	25 (12–100)	[148]
Lactococcus lactis	Ll.ltrB	IIA	ltrB	0.5–1.5[b]	50	[51,149]
Podospora anserina	Pa.coI I1	IIA	cox1 su1	0.5–1.25[b]	60	[134]
Pylaiella littoralis	Pl.lsu/1	IIB	lsu rRNA	1	100	[110]
	Pl.lsu/2	IIB	lsu rRNA	1 (0.1–1)	5 (0.1–100)	[110]
Saccharomyces cerevisiae	aI1	IIA	cox1	1	100	[150]
	aI2	IIA	cox1	2[b]	100	[57]
	ai5γ	IIB	cox1	0.5	50–100	[112,132]
	bI1	IIB	cob	1.25[b]	10	[122]
Scenedesmus obliquus	So.lsu/1	IIB	lsu rRNA	1.25[b]	60[c]	[151]

[a] Hsp60, heat shock protein 60kD; psbA, photosystem II thylakoid protein domain 1; ltrB, relaxase gene of conjugative element pRS01; cox1, cytochrome oxidase 1; su1, subunit 1; lsu rRNA, large subunit of ribosomal RNA; cob, apocytochrome B. [b] NH_4Cl. [c] In the presence of 2 mM spermidine.

tonate a 2′-hydroxy group, which itself can then act as a nucleophile to break the scissile phospho diester linkage. This reaction mechanism has two consequences: (i) only those metal-ion binding sites are detected, where the Ln^{III} ion is in close neighborhood to either the phospho diester or the 2′-OH, and (ii) under physiological conditions Ln^{3+} ions cleave RNA more efficiently at their coordination site than Mg^{2+}.[77,116,117]

Taking advantage of the above-mentioned properties of Ln^{3+} ions, we have applied them to locate the metal-ion binding sites in domains 1, 3, 5 and 6 of the group II intron ai5γ (Figure 4).[77] The ribozyme was labeled at either the 5′ or the 3′ end with ^{32}P and subsequently incubated with sufficient K^+ and Mg^{2+} at pH 7 to achieve the correct three-dimensional fold. Micromolar amounts of Tb^{3+} (or other lanthanides) were then added and the reaction mixture put on ice to protect the ribozyme from additional unspecific cleavage. As the natural Mg^{2+} ions are bound kinetically labile at their coordination sites, these ions are now in equilibrium with the higher charged Ln^{3+} ions. Binding of the lanthanide ions within the binding pocket subsequently leads to cleavage of the phospho diester backbone close to their binding site. The resulting RNA pieces of different length can then be separated by denaturing polyacrylamide gel electrophoresis (PAGE) and thus the multiple metal-ion binding sites within the RNA can be located.

Analysis of the PAGE gels shows that some sites are cleaved with high specificity whereas other regions are completely protected from hydrolysis.[77] When plotting the determined metal-ion binding sites onto the secondary structure of the RNA, it is striking to see that the metals are spread out all over the intron and are located mostly at single-stranded regions, suggesting unspecific cleavage (Figure 2). However, comparison with biochemical data from footprinting experiments[118] shows that almost all binding sites are located in the inner core of the active three dimensional architecture. Furthermore, the cleaved nucleotides are mostly known to be either crucial for long-range or tertiary contact formation via diverse hydrogen bonding patterns, or to take part in catalysis itself. Additional evidence that Ln^{3+} ions bind to the same sites as Mg^{2+} ions in the active structure is given by the fact that only the corresponding hydrolytic cleavage experiments with Mg^{2+} yield the same sites, though the cleavage intensities are much lower than with, e.g., Tb^{3+} and the gels therefore more difficult to interpret.[77] Hence, it is reasonable to assume that indeed $Ln^{3+}$and Mg^{2+} ions bind to the same sites in group II introns and RNA in general.

Divalent d-transition metal ions, as well as Zn^{2+} and Pb^{2+}, have also been applied in metal-mediated cleavage reactions of the RNA backbone.[119–122] These metal ions usually have a well defined coordination geometry but often prefer the softer nitrogen donor atoms instead of the harder oxygen atoms of the phosphate and carbonyl groups, which are the predominant binding partners of Mg^{2+}. In contrast, Ln^{3+} ions have the distinct advantage of preferentially binding to oxygen atoms as well. Although their preferred coordination geometry does not fit the one of Mg^{2+}, it is very

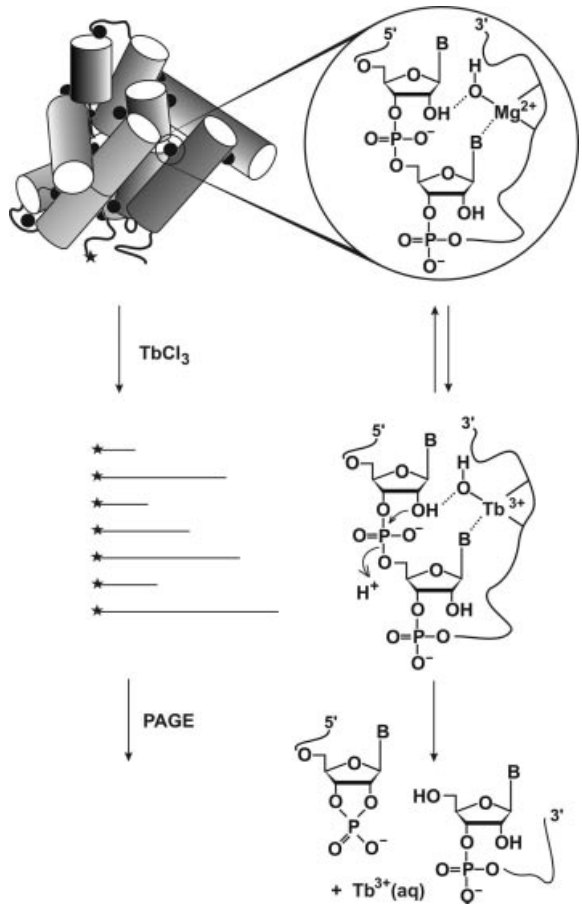

Figure 4. Lanthanide(III)-mediated cleavage of the phosphate-sugar backbone of large RNA molecules. The ribozyme, which is labeled with ^{32}P either at its 5′ or 3′ end (indicated by an asterisk), is folded to its active three-dimensional structure in the presence of K^+ and Mg^{2+}. Subsequently added Tb^{3+} competes with Mg^{2+} at its binding sites and cleaves the phosphate-sugar backbone by hydrolysis (B indicates the nucleobase moiety). The resulting RNA fragments of different lengths are separated by denaturing polyacrylamide gel electrophoresis (PAGE) and visualized with a phosphoimager. The cleavage sites, and thus coordination sites, of the Tb^{3+} ions appear as dark bands and these can be correlated with their position in the intron by a nuclease T1 digest run in parallel.[77]

flexible and can thus adopt easily to different environments. Thus, lanthanide ions seem to preserve the prefolded tertiary structure and not to disturb the local geometry within the binding pocket. The relative rigidity of the coordination sphere of Pb^{2+} is reflected by the fact that in case of the group II intron ai5γ, backbone cleavage induced by this metal ion leads to fewer detectable sites but these being identical with those induced by Tb^{3+} or Mg^{2+}.[77]

With another group II intron, bI1 from the cob gene in yeast, Mn^{2+} and Zn^{2+} were used to test for metal-ion binding sites.[122] In this case not the primary cleavage pattern was investigated, but a primer extension analysis was chosen. Although primer extension is not as sensitive as the direct observation, this method has the advantage that it can be applied to very large RNAs.[116,122] In the case of bI1, Mn^{2+} and Zn^{2+} induced cleavage revealed the same sites. However, some sites detected with Tb^{3+} did not show

up, like the one in the bulge of domain 5, which is part of the catalytic center (see also below).[77]

Tb^{3+} has another striking feature: When applied at millimolar concentrations, Tb^{3+} nonspecifically cleaved also intronic regions with a high AU content. Most of these regions are not crucial for catalysis; they are believed to lie on the surface of the ribozyme and are therefore likely to be dynamic or disordered in secondary structure. Under such conditions of high concentration, Tb^{3+} coordinates unspecifically to the surface nucleotides of the intron, leading to an enhanced hydrolysis of the phospho diester bridges at these positions. Thus, depending on the concentration applied, Tb^{3+} can also be readily used as a probe for unstructured regions within a large folded RNA.

In addition to their use to determine metal-ion binding sites in large RNA molecules like group II introns, hydrolytic cleavage experiments with Ln^{3+} ions also yield information about the affinity of these ions to the RNA at each specific site.[114] Variation of the Tb^{3+} concentration shows that at some sites cleavage already occurs at low micromolar concentrations, whereas at other sites high micromolar or even millimolar amounts of this lanthanide(III) ion are needed to cleave the backbone.[114] Assuming a 1:1 binding behavior, the dependence of cleavage intensity on the Tb^{3+} concentration can be fitted with a nonlinear least-squares fit and affinity constants for each site determined. It is interesting to see that the intensity of the cleavage does not coincide with the affinities of Tb^{3+} at the single sites. This simply means that at some sites the Tb^{3+} ion is geometrically ideally positioned to promote cleavage but that this positioning is independent from its affinity at the site.[114] To conclude, in such experiments it is always important to perform Tb^{3+} titrations over a large concentration range, as the cleavage picture/intensity at just a single concentration can easily be misleading regarding the affinity of the metal ion to a given site.

5. Structure and Metal Ion-Binding Properties of the Catalytic Domain 5

5.1. The Role of Magnesium Ions

Domain 5 (D5) (see Section 2 and Figure 2) is the phylogenetically most conserved region of group II introns and is an essential active-site component for any reaction catalyzed by these ribozymes. D5 forms a hairpin-loop structure with usually about 34 nucleotides in length.[36,75] This hairpin always consists of two helical stems separated by a two-nucleotide bulge and is closed on one side by a GNRA tetraloop (N = any nucleotide, R = purine). Almost every nucleotide within D5 has at least one functional group, which is important for either catalysis itself or for binding of D5 to other intronic domains. Close to the 5′ end of D5, the so-called AGC, or catalytic, triad is located. These three nucleotides are almost invariable and their major groove functional groups have been shown to be crucial for the catalytic step.[74] D5 has a nanomolar affinity for the other intronic components,[123] of which advantage is taken in so-called *trans*-splicing experiments, where D5 acts as the "enzyme" in Michaelis–Menten-type kinetics.[76] The major interaction between D5 and the central region within D1 is a tetraloop-tetraloop receptor interaction, which is highly dependent on the presence of divalent metal ions.[124] In addition, further contacts between these two domains are known.[61,72] There are no phylogenetic covariations of Watson–Crick base pairing between nucleotides in D5 and other intronic or exonic regions, thus, these interactions are made through conserved hydrogen bonding patterns, which can be rather stable,[102] involving nucleobase and sugar moieties thus building higher order structures like base triples.[61,62,71,72]

Only few of these inter domain contacts are structurally characterized, mainly based on nucleotide analog interference mapping studies (NAIM).[72] Indeed, any structural information on group II introns is very scarce.[78,125] Regarding D5, phylogenetic alignment clearly shows a two-helix structure. However, the secondary structure around the bulge is still a matter of debate (Figure 5). On the one hand a GU wobble pair leaving the adjacent A and C unpaired, and on the other hand an AU Watson–Crick pair with flipped out C and G nucleobases is proposed. In favor of the first base pairing scheme with the GU wobble is a crystal structure of a permuted D5D6 construct.[125] The first helix is closed by the wobble pair between U823 and G840 and the second helix is stacked on top thus forming one straight long helix with two flipped out nucleotides A and C in the middle. The two flipped out nucleotides form crystal lattice contacts with neighboring molecules, possibly mimicking similar contacts within the whole ribozyme (Figure 5B).[125] In the NMR solution structure of D5 in the presence of Mg^{2+} the bulge adopts a hitherto unknown structure.[78] The two bulged AC nucleotides are stacked within the hairpin and the downstream G840 does not form a wobble pair but instead is in a *syn* conformation and thus flipped down into the major groove of stem 1 (Figure 5C). As a consequence, the two helixes are slightly twisted against each other, exposing the AU base pair below to the solvent providing a possible platform for stacking interactions with incoming nucleotides of the above λ-λ′ contact. In support of the flipped down *syn*-G840 is the biochemical observation that the NH_2 group of G840 is known to be crucial for catalysis.[72,126] In the NMR structure the exocyclic amino group is very close to the AGC triad[78] and thus, to the reaction center.[61] In addition, Tb^{3+} cleavage experiments of D5 alone and in the presence of D1 both revealed a metal-ion binding site in the bulge, suggesting that D5 adopts a similar structure in solution as in the ribozyme.[77]

Several metal-ion binding sites have been detected in D5. Thio rescue experiments revealed a Mg^{2+} coordinated to the S_p-oxygen atom of the A816 phosphate group within the AGC triad, thus being important for binding of D5 to the other domains.[127] Titration studies of D5 with $MgCl_2$ by NMR revealed further binding sites of Mg^{2+} within D5.[78,128] In fact, such sites may closely neighbor each other.[107] The recording of 2D $^1H,^1H$-NOESY spectra al-

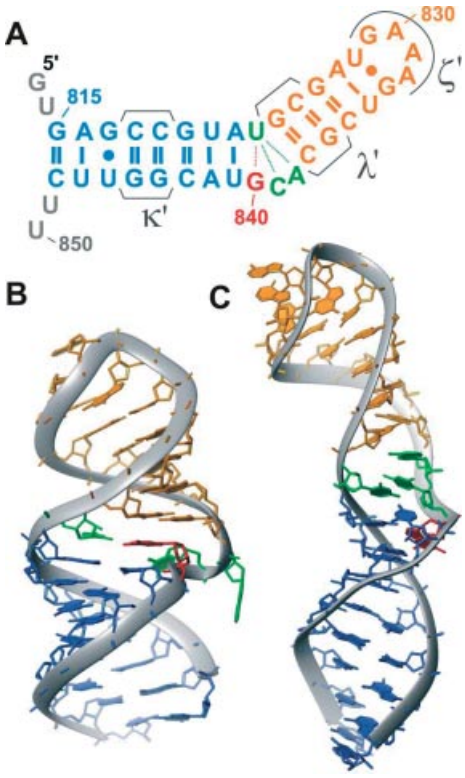

Figure 5. Solution and crystal structures of D5 together with its secondary structure. (**A**) Secondary structure of D5 from ai5γ together with its numbering scheme and tertiary contacts with other domains indicated by Greek letters (see also Figure 2). Helix one is colored in blue, helix two in orange, G840 in red, and the other bulge nucleotides in green. Nucleotides shown in grey, flanking D5, are not included in the two structures. (**B**) In the crystal structure (pdb entry 1KXK),[125] D5 adopts a straight helix with the two bulge nucleotides A838 and C839 flipped out making lattice contacts with neighboring molecules. (**C**) The NMR structure (pdb entry 1R2P)[78] reveals a more flexible structure with the two helices slightly kinked as all bulge-nucleotides are found within the helix. The syn-G840 is flipped down into the major groove. Both structures were drawn with the program MOLMOL,[147] based on the pdb entries given above.

lowed us to follow the chemical shift changes of the aromatic nucleobase, as well as the aliphatic sugar protons and yielded therefore a very detailed picture not only about the location, but also about the strength of the binding.[78,128] Almost all protons in D5 are affected by the addition of Mg^{2+} implying that in general Mg^{2+} binds unspecifically to the phosphate groups of this oligonucleotide. However, some chemical shifts show a much larger dependence on Mg^{2+} concentration, e.g., resonances belonging to nucleotides around the bulge, the tetraloop and the tandem GC pairs in helix 1. In line with the above-mentioned results from the Tb^{3+} cleavage experiments[77] and the structural orientation of the bulge, is the strong metal-ion binding in this region (log $K = 2.52 \pm 0.12$ M^{-1})[77] for Mg^{2+}, K being the stability constant), which is high compared to e.g., $Mg(pUpU)^-$ (log $K = 1.84 \pm 0.04$ M^{-1}).[129] Indeed, several carbonyl oxygen atoms are positioned in the minor groove of the bulge, providing possible multiple coordination sites for the hard Mg^{2+} ion. The large chemical shift changes of about 0.3 ppm may be indicative of a structural change in the bulge upon Mg^{2+} binding, although the NOESY pattern did not change up to 7.5 mM $MgCl_2$ added. Metal-ion coordination within the bulge region together with a hydrophobic stacking interaction of incoming nucleotides with the below lying AU platform, might facilitate the formation of the λ-λ′ tertiary contact by providing charge compensation for the anionic backbone.

5.2. The Effect of Other Metal Ions on Group II Intron Reactivity

Monovalent cations are primarily used for charge screening, but in a few cases also very specific binding sites for, e.g., K^+ ions, are known.[130] In living cells, the concentration of Na^+ and K^+ is (depending on the organism and kind of cell) usually between 2 and 150 mM. In addition, polyamines and RNA binding proteins are abundant in the cell to stabilize large three dimensional RNA structures such as group II introns. For in vitro splicing, much higher salt concentrations are needed; usually between 500 and 1500 mM (see Table 1 and refs. therein). However, also for in vitro splicing, usually 100 mM Mg^{2+} (Table 1) can be partly replaced (e.g. 10 mM $MgCl_2$ total) by either polyamines like spermidine (2 mM)[38] or protamine (5 nM),[53] or RNA binding proteins: Experiments with the Ll.ltrB intron have shown that the self-encoded maturase protein LtrA binds to intronic sequences in D4 and enables splicing also under reduced salt conditions (450 mM NaCl, 5 mM $MgCl_2$).[131]

KCl can be replaced in most instances by ammonium salts like NH_4Cl or $(NH_4)_2SO_4$ without much loss of overall activity, but having an effect on the chosen splicing pathway: Ammonium sulfate favors transesterification (branching), KCl the hydrolytic pathway, whereas NH_4Cl yields the products of both pathways in similar amounts.[53,132–134] Thus, for many biochemical experiments, like NAIM (nucleotide analog interference mapping), NAIS (nucleotide analog interference suppression), as well as for reverse-splicing reactions ammonium salts are preferred.[72,131] In intron insertion reactions into DNA, i.e. in the presence of a self-encoded maturase protein, Na^+ is readily used (at low Mg^{2+} concentrations). However, there are only few examples of in vitro splicing reactions where Na^+ is used instead of K^+ (or NH_4^+),[131] as the presence of high amounts of Na^+ can lead to a loss in activity.[110] Li^+ can promote folding of some RNAs,[135] but it is only rarely employed. However, one should be aware that, although not specifically reported, sodium (and lithium) ions are almost always present to some extent: Many lithium and sodium salts of nucleotide triphosphates and of buffers are used during transcription and purification of the RNA as well as in the splicing reaction itself (MOPS, Edta, to name just two).

All group II introns investigated up to now need Mg^{2+} to perform splicing or branching. However, other divalent metal ions are often added in low millimolar amounts, e.g., in thio-rescue, hydrolytic cleavage experiments, and NAIS/

NAIM studies. In thio-rescue experiments, mainly $CdCl_2$ and $MnCl_2$ are used in concentrations between 2 and 10 mM in the presence of 100 mM Mg^{2+}.[72,127,136] Under such conditions, both branching and splicing still take place. It should be added here that it is a common misconception that Mn^{2+} is significantly more thiophilic than Mg^{2+}. Mn^{2+} does not show an increased affinity towards thiophosphate groups compared to phosphate groups.[137] However, due to its position in the Irving–Williams series, its affinity to (thio)phosphate groups is slightly higher than that of Mg^{2+}, explaining the few observed "rescue" effects (related results are given in refs.[137–140]).

5.3. The Special Effects of Calcium Ions

Above, the need of group II introns for K^+/NH_4^+ and Mg^{2+} as well as the use and effect of various mimicking metal ions has been summarized. But what about other alkaline earth metal ions? For example, Ca^{2+} is abundant in Nature, although in living cells the free calcium concentration is normally kept low. Ca^{2+} is slightly larger than Mg^{2+}, but it also prefers oxygen donors as ligands and therefore effectively binds carbonyl groups.[141] Complexes of Ca^{2+} with monophosphate groups[142] are known to be weaker by a factor of about 0.75 compared to those with Mg^{2+}. For the simultaneous coordination to several phosphate groups like in a nucleoside triphosphate, the factor between Mg^{2+} and Ca^{2+} is further reduced to about 0.4.[143] One would therefore assume that small amounts of Ca^{2+} have only little effect on the reactivity of group II introns. Surprisingly, the opposite is observed. We have performed *trans* cleavage assays, in which the 5′-exon is cleaved from the intronic domains 1, 2, and 3 upon addition of D5 (Figure 6).[144,145] Such a setup has been shown to be an excellent mimic for the first step of splicing obeying Michaelis–Menten kinetics, when D5 is added in excess.[76]

Under saturating conditions of D5 (500 mM KCl, 100 mM $MgCl_2$) the observed rate constant $k_{obsd.}$ equals 0.044 ± 0.002 min^{-1}.[76,144,145] In the presence of only 1 mM Ca^{2+} (and, 99 mM Mg^{2+}), the rate of the reaction decreases significantly (Figure 6).[144,145] The slightly decreased concentration of Mg^{2+} cannot be the reason for reduced catalysis, especially as at concentrations down to 50 mM Mg^{2+} the ribozyme still remains folded and active.[112] The mode of inhibition by Ca^{2+} is unclear. The displacement of Mg^{2+} from the active site is a first possibility, although a less likely one. Nevertheless, it is evident that, despite having normally a lower affinity towards the constituents of nucleic acids, Ca^{2+} must somehow alter the three dimensional fold of a local metal-ion binding motif or even of the whole ribozyme, for example by binding to (additional) carbonyl groups due to its larger size and higher coordination number. This observation proves that group II intron ribozymes indeed are a very delicate molecular machinery being extremely sensitive to small changes in its environment, and despite its size show a high specificity for certain metal ions.

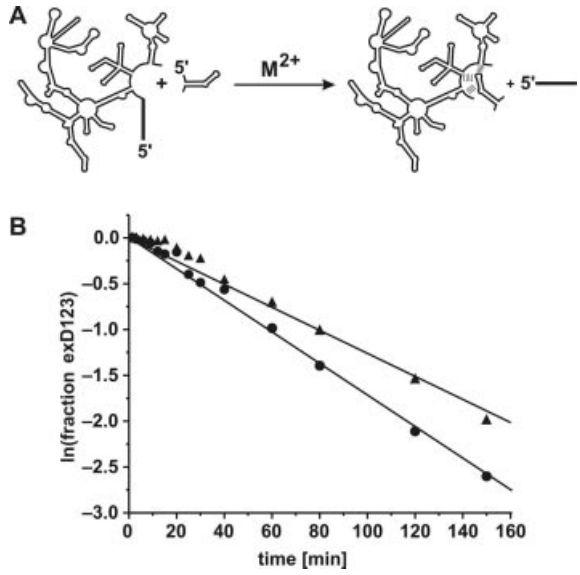

Figure 6. Effect of Ca^{2+} on the splicing reaction of the group II intron ai5γ. (**A**) Reaction scheme of the *trans* splicing reaction of exD123 upon addition of D5 in the presence of divalent metal ions. (**B**) Comparison of the observed rate of the above reaction in the presence of only Mg^{2+} (100 mM, ●) and a ratio of Ca^{2+}:Mg^{2+} = 1 mM:99 mM (▲). The fraction of precursor is drawn vs. time. The reaction is clearly slower in the presence of Ca^{2+}.[144,145]

6. Conclusions and Outlook

In this review, I have tried to show why group II introns are fascinating molecules and worthwhile targets for studies by Bioinorganic Chemists. After a short summary of the diverse array of reactions catalyzed by group II intron ribozymes, the role of metal ions in folding and activity of these catalytic RNAs was discussed. In living cells only concentrations in the lower millimolar range of monovalent and divalent metal ions are needed, as polyamines and RNA binding proteins support the folding of these large RNAs to the active three dimensional structure. Such stabilizing effects by biomolecules other than RNA are usually replaced in vitro by high concentrations of metal ions, also supporting catalysis. However, it is reasonable to assume that both in vivo and in vitro, metal ions are occupying the same key sites within the catalytic core or regions of tertiary interactions.

In the light of the highly conserved secondary structure of group II introns it is not understood why introns from different organisms and gene loci exhibit such a diverse need regarding the concentration of Mg^{2+}. A set of minor groove binding sites for metal ions has been determined by hydrolytic cleavage experiments for the ai5γ and bI1 introns from yeast.[77,122] Not all binding sites appear to correspond to each other. However, as different metal ions have been used for the promoted backbone hydrolysis as well as different methods to visualize the cleavage sites, a thorough interpretation is difficult.

It will be interesting to see if group II introns from different organisms have high affinity binding sites for metal ions at different locations within the intronic domains. One

can speculate if group II introns do not manifest one particular fold and reaction pathway, but rather that there might be subtle differences in the structure depending on the subgroup and the origin of a particular group II intron. Such a scenario is quite well possible, as two existing models of the catalytic centers of ai5γ and Pl.lsu/2 cannot be superimposed.[118,146] The two structures of single domains of the group II intron ai5γ solved so far,[78,125] together with the two models of the catalytic core,[118,146] are first important steps in the direction of building a structural basis for these molecular machines. Indeed, more structural information is highly needed for the interpretation of metal-ion binding necessary for folding and catalysis. It is evident that much work needs to be done because at present hardly any exact binding site is known; for example, if a divalent metal ion binds to a G unit, where is the metal ion exactly located? At N1, O6, N7 or even at N3? Is the phosphate group also involved? Only if we are able to answer these questions down to the atomic level we shall be able to characterize and understand the reaction processes in detail.

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[1] Abbreviations and Definitions: For names and locations of group II introns see Table 1; CoTC RNA, co-transcriptional cleavage RNA; dsDNA, double-stranded DNA; Edta, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NAIM, nucleotide analogue interference mapping, NAIS, nucleotide analogue interference suppression; ORF, open-reading frame; PAGE, polyacrylamide gel electrophoresis; RNA, ribonucleic acid; RNAi, RNA interference; coRNA, co-regulatory RNA; mRNA, messenger RNA; rRNA, ribosomal RNA; siRNA, small interfering RNA; snRNA, small nuclear RNA; tRNA, transfer RNA.

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