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Lead cleavage sites in the core structure of group I intron-RNA

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ABSTRACT

Self-splicing of group I introns requires divalent metal ions to promote catalysis as well as for the correct folding of the RNA. Lead cleavage has been used to probe the intron RNA for divalent metal ion binding sites. In the conserved core of the intron, only two sites of Pb^{2+} cleavage have been detected, which are located close to the substrate binding sites in the junction J8/7 and at the bulged nucleotide in the P7 stem. Both lead cleavages can be inhibited by high concentrations of Mg^{2+} and Mn^{2+} ions, suggesting that they displace Pb^{2+} ions from the binding sites. The RNA is protected from lead cleavage by 2'-deoxyGTP, a competitive inhibitor of splicing. The two major lead induced cleavages are both located in the conserved core of the intron and at phosphates, which had independently been demonstrated to interact with magnesium ions and to be essential for splicing. Thus, we suggest that the conditions required for lead cleavage occur mainly at those sites, where divalent ions bind that are functionally involved in catalysis. We propose lead cleavage analysis of functional RNA to be a useful tool for mapping functional magnesium ion binding sites.

INTRODUCTION

Ribozymes have an absolute requirement for divalent cations to promote catalysis. For the cleavage reaction of the ribozyme derived from the group I intron of *Tetrahymena thermophila*, two types of divalent cation binding sites have been postulated: class 1 sites bind cations functionally involved in catalysis. Only Mg^{2+} and Mn^{2+} are active at these sites, whereas Ca^{2+}, Zn^{2+}, Co^{2+} or Pb^{2+} do not promote the reaction. Class 2 sites bind cations involved in structure-stabilizing interactions, with Ca^{2+} and Mg^{2+} being the preferred ions, less well substituted by Mn^{2+}, Sr^{2+} and Ba^{2+}. Ions at these sites have been proposed to promote the global folding of the RNA (1).

Some divalent cations, like Pb^{2+} and Zn^{2+}, have long been known to catalyse site-specific cleavage of the sugar-phosphate backbone in tRNA molecules (2,3). In the proposed cleavage mechanism a lead-bound hydroxyl group abstracts the proton from a 2'-OH group, thus facilitating nucleophilic attack of the adjacent phosphate group. The cleavage generates a 5'-hydroxyl group and a 2',3'-cyclic phosphate (4,5).

The best studied example of lead cleavage is $tRNA^{Phe}$. X-ray analysis of uncleaved and cleaved $tRNA^{Phe}$-Pb^{2+} complexes as well as mutational studies enabled the elucidation of the configurational requirements that have to be met for lead cleavage to occur. Three lead binding sites could be identified in the $tRNA^{Phe}$ molecule, but only at one binding site lead cleavage of the sugar-phosphate backbone was detected. Pb^{2+} ions bind to complexely folded single-stranded regions of RNA, preferentially cleaving domains where rigorous stereochemical requirements are met. In the X-ray diffraction studies of $tRNA^{Phe}$ it was shown that the Pb^{2+} ion that cleaves the RNA displaces a Mg^{2+} ion, which is bound nearby in the native structure (4, 6). At the other two lead binding sites observed in $tRNA^{Phe}$, the lead ions displace a spermine and a water molecule; cleavage does not occur. Analysis of yeast $tRNA^{Phe}$ mutants showed that only two nucleotides in a single-stranded domain are directly involved in the cleavage mechanism by coordinating the active lead ion (7).

Pan and Uhlenbeck developed an *in vitro* selection procedure to isolate RNA molecules that are cleaved by lead in the presence of magnesium. A small oligoribonucleotide was selected, which was not only cleaved by lead, but which also hydrolysed the terminal 2',3'-cyclic phosphate, generating a 3' phosphomonoester. This metalloribozyme depends on Pb^{2+} for cleavage, with Mg^{2+} being required, but not sufficient, for the reaction (8, 9). The role of Mg^{2+} ions in group I introns was studied by Sugimoto et al. (10), using the circle reopening reaction of the *Tetrahymena* ribozyme as a model. The covalently closed circular RNA molecule can be opened by the addition of oligoribonucleotides (11) or by hydrolysis (12). The reaction requires a weakly bound Mg^{2+} ion, and both the ribozyme as well as the substrate (a diribonucleotide) are involved in the formation of a Mg^{2+} binding pocket. Additionally, it was shown that the 2' OH group of the 3' sugar of the substrate is required for Mg^{2+} binding (10). The chemistry of the reaction was studied by Mc Swiggen & Cech by introducing a single phosphorothioate into the substrate RNA at the 5'splice site.

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These studies revealed a stereochemical order for the reaction leading to the hypothetical model that Mg^{2+} could stabilize the transition state (13).

We analysed the Pb^{2+} cleavage sites in self-splicing group I introns and detected two major sites of cleavage in the catalytic core of the intron, which is conserved both in sequence and structure. The sites of lead cleavage coincide with sites where phosphorothioate substitutions lead to splicing deficiency in the presence of Mg^{2+}, but not in the presence of Mn^{2+}, suggesting that the phosphates are involved in magnesium binding (14; E.Christian and M.Yarus, personal communication). Additionally, we showed that the intron RNA can be protected from lead cleavage by 2'-deoxyGTP, an inhibitor of the first step of splicing.

MATERIAL AND METHODS

Plasmids

NdC, a truncated version of the T4 phage derived *td* gene, contains 79 nt of exon I, 265 nt of the intron (delta P6-2) and 21 nt of exon II cloned into the vector pTZ18U (15, 16). In the mutant NdU (C263U, numbering according to the *Tetrahymena* intron) the bulged nucleotide in P7 is mutated to a U (16). The plasmid containing the *Tetrahymena thermophila* intron was pBGST7 (17). The plasmid containing the *sunY* intron was SYC1.3 (kindly provided by F.Michel).

Preparation of precursor RNA transcripts

Plasmid-DNA was prepared by the alkaline lysis procedure and linearized with Hind III for the *td* and *Tetrahymena* introns, and with EcoR1 for the *sunY* intron. For the experiments trimming the 3' end of the *td* precursor RNA, the plasmid was linearized with Xba I. *In vitro* transcription and purification of precursor RNA were performed as described previously (18).

Lead cleavage procedure

20.000 cpm (about 50 ng) of precursor RNA were preincubated without buffer for 1' at 90°C, prewarmed buffer (50 mM Tris−HCl pH 7.3, 0.4 mM spermidine, 5 mM magnesium chloride, otherwise as indicated in the figures) was added, and renaturation was performed for 10' at 37°C (19). For the *Tetrahymena* intron and the *sunY* intron, 200 mM NaCl or 50 mM NH_4Cl were included, respectively. The cleavage reaction was initiated by the addition of freshly prepared lead acetate solution and performed for 3 minutes at room temperature. The reaction was stopped by addition of EDTA and tRNA to a final concentration of 2 mM and 80 μg/ml, respectively. Samples were ethanol precipitated, resuspended in loading buffer (0.1% bromophenol blue, 0.1% xylene xyanol, 10 mM Tris-Cl pH 7.6, 80% formamide), and run on 5% acrylamide/7M urea gels. Products were visualized by autoradiography.

Mapping of the lead cleavage sites

Lead cleavage was performed as described above. Following ethanol precipitation, the RNA was resuspended in water, annealed to appropriate ^{32}P-labeled oligodeoxyribonucleotides, and a primer extension reaction was performed in buffer containing 50 mM Tris−HCl pH 8.3, 74 mM KCl, 3 mM magnesium chloride, 0.5 mM dNTP, 10 mM DTT with 1 unit reverse transcriptase (USB) for 30' at 37°C. Reactions were stopped by addition of loading buffer and run on 8% acrylamide/7M urea gels. Sequencing reactions were performed with Pharmacia T7 sequencing kit according to the protocol with the same ^{32}P-labeled oligodeoxyribonucleotides. The following oligodeoxyribonucleotides were used for primer extension analyses: MB14: 5' TGT CAC CAT GCA GAG CAG AC 3' for the *td* intron, CGM 1289: 5' GAA ACA ATG TAA, TTT, GGA, AGC, TTT TTC, TTA ATT TCC 3' for the *sunY* intron (kindly donated by F.Michel) and TET1: 5' TCA TCC GCT A-GC TCC CAT TAA 3' for the *Tetrahymena* intron.

RESULTS

Lead cleavage of group I introns

Incubation of bodylabeled precursor RNA of the T4 phage derived *td* intron (mutant C263U) with lead acetate resulted in four major cleavage products (labeled with * and $^+$ in Fig. 1A). Based on lead cleavage analyses of yeast $tRNA^{Phe}$ it is assumed that lead ions displace magnesium ions from their binding sites. Thus, by increasing the concentration of Mg^{2+}, competition between both metal ions for the binding site should lead to inhibition of lead cleavage. As expected, addition of increasing amounts of magnesium (Fig. 1A, lanes 4−8) or manganese (Fig. 1A, lanes 13−16) to the cleavage reaction resulted in inhibition of cleavage. At concentrations of Mg^{2+} or Mn^{2+} higher than 30 mM, lead cleavage is repressed, suggesting that the cleavages observed in the precursor RNA result from lead ions that can be displaced by Mg^{2+} or Mn^{2+}.

To show that addition of high amounts of Mg^{2+} or Mn^{2+} does not alter the overall intron structure, which could prevent lead cleavage, the splicing reaction was started by addition of GTP after incubation with lead and high amounts of magnesium or manganese. Splicing does take place under these conditions as can be seen in figure 1A (lanes 9 and 17), indicating that high concentrations of Mg^{2+} (or Mn^{2+}) do not alter the intron structure.

Sizes of the cleavage products were estimated in comparison to known sizes of splicing products and marker. As the added sizes of each pair of products (Fig. 1A, * and $^+$) correspond to the length of the precursor RNA, it is likely that the four main cleavage products are generated by two cuts of the intron RNA. To identify the cleavage products generated by distinct cleavages, precursor RNAs differing in the length of the 3' exons were prepared. This was achieved by linearising the plasmid for template preparation with different restriction endonucleases. In Figure 1B, precursor RNA of the *td* intron (mutant C263U) was transcribed with Hind III linearized template (lanes 1−6, left panel) and with Xba I linearized template (lanes 7−11, right panel). Lead cleavage of these precursor RNAs generated the equally sized products derived from the 5' part of the molecule and the 3' products, which differ in size (Figure 1B). Each cleavage event of the bodylabeled precursor RNA results in two products (marked with $^+$ and * and 5' or 3', respectively; Fig. 1B). According to their sizes, we expected the cleavages to occur in the core structure of the intron, probably in P7 and in J8/7. As no distinct band of the size of the distance between P7 and J8/7 (70 nucleotides) was detectable, we assume that most RNA molecules are cut only once. Some minor cleavage products are detectable, but they are less pronounced, less well competed by Mg^{2+}, and not located in the core.

To investigate if the cleavages are conserved among group I introns, cleavage was performed with three different introns, namely the T4 phage derived *td* (wildtype and C263U mutant) and *sunY* introns, both belonging to group IA2, and the

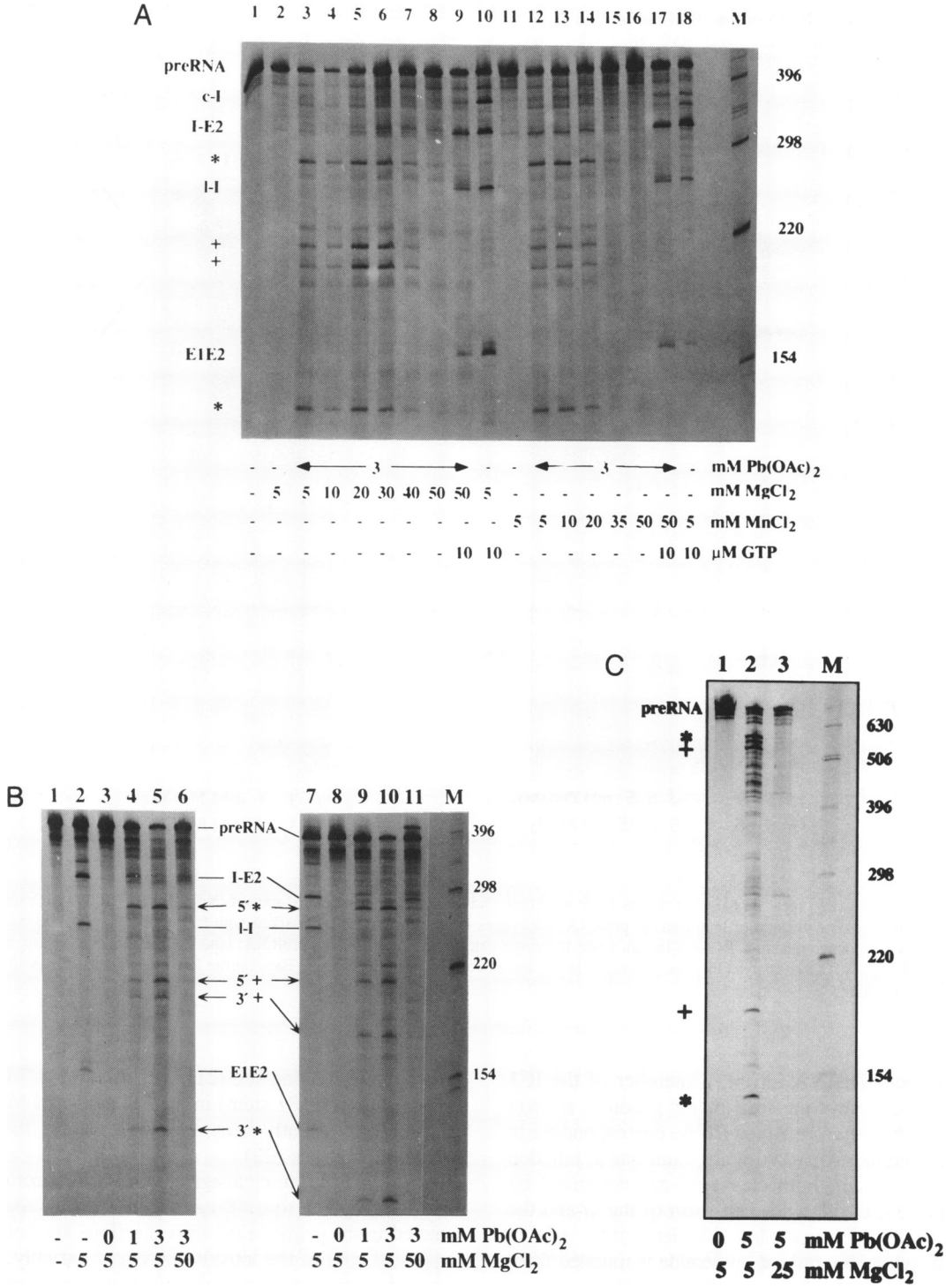

Figure 1. A. Lead cleavage of the precursor RNA (preRNA) of the T4 phage derived *td* intron. Bodylabeled precursor RNA of the C263U mutant (numbering of residues is according to the respective sequence in the *Tetrahymena* intron) was treated with lead. Lane 1: preRNA; lane 2, 11: preRNA incubated without lead; lanes 3−8: preRNA incubated with lead and increasing amounts of Mg^{2+}; lanes 12−16: preRNA incubated with lead and increasing amounts of Mn^{2+}; lane 9,17: preRNA incubated with lead and 50 mM $MgCl_2$ or $MnCl_2$ with subsequent addition of 10 μM GTP; lane 10,18: preRNA splicing with 10 μM GTP. Bands marked with $^+$ are products of cleavage at the bulged nucleotide in P7 with sizes 288 and 133, bands marked with * are products of cleavage in J8/7 with sizes 218 and 203. Splicing products are marked I-E2 for intron-exon 2, E1E2 for ligated exons, l-I for linear intron and c-I for circular intron. **B.** Lead cleavage of precursor RNAs of the T4 phage derived *td* intron, linearized with Hind III (left panel, lanes 1−6) and with Xba I (right panel, lanes 7−11). Lanes 2 and 7 show products of the splicing reaction after addition of 70 μM GTP. Lanes 4,5 and 9,10 show lead cleavage products labeled with * or + and with 5′ or 3′ according to their position to the cleavage site. In reactions shown in lanes 6 and 11, 50 mM $MgCl_2$ was added. Other products are as labeled in Fig. 1A. **C.** Lead cleavage of the precursor RNA (preRNA) of the *Tetrahymena thermophila* rRNA intron. Lane 1: preRNA incubated without lead; lanes 2 and 3: preRNA incubated with lead and 5 or 25 mM $MgCl_2$, respectively. Bands marked with + are products of cleavage at the bulged nucleotide in P7 (A263) with sizes of 536 and 180 nucleotides, bands marked with * are products of cleavage in J8/7 (U305) with calculated sizes of 579 and 137 nucleotides.

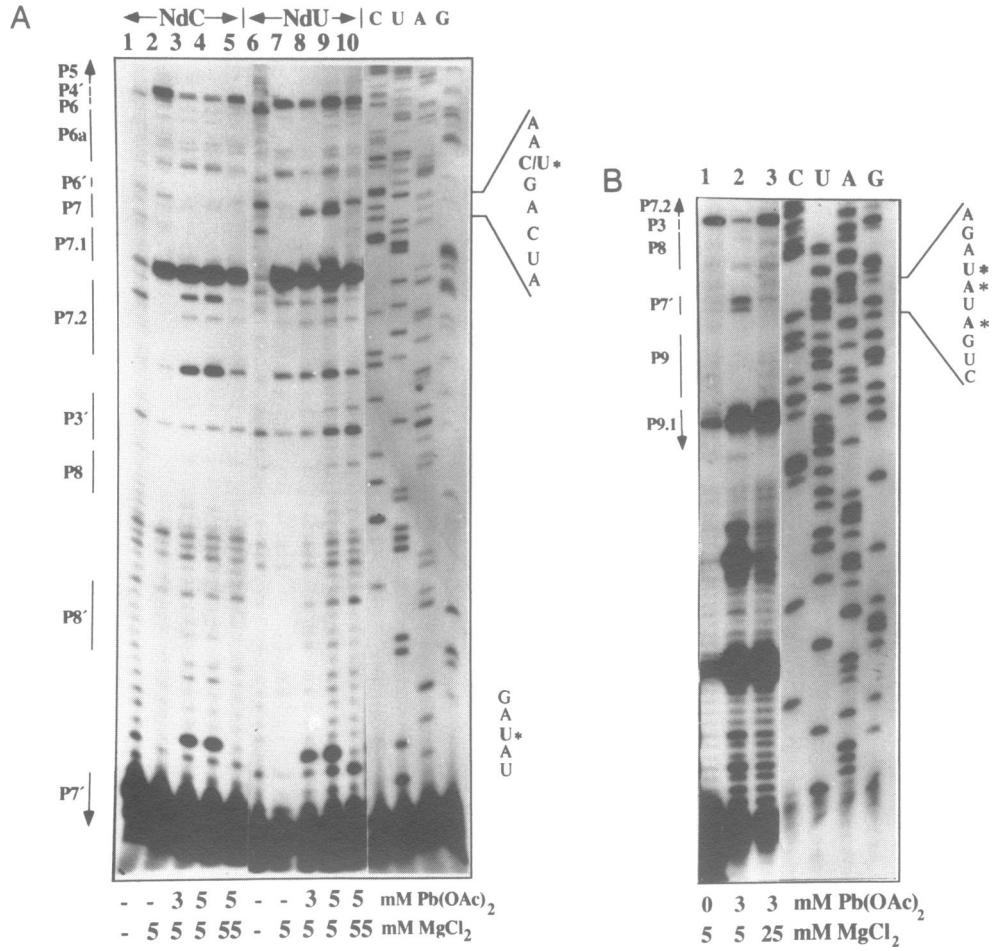

Figure 2. Mapping of the lead cleavage sites. **A:** *td* intron. Lanes 1–5: wild type (NdC). Lanes 6–10: C263U mutant (NdU) Lanes 1,6: Primer extension of untreated preRNA; lanes 2–5, 7–10: Primer extension of lead cleaved preRNA. Cleavages in the intron core are marked with *. Bars on the left side indicate the positions of the stems. CAUG shows a sequence reaction. **B:** *sunY* intron. Lane 1: Primer extension of non-cleaved preRNA; lane 2–3: Primer extension of preRNA cleaved with lead and 5 or 25 mM $MgCl_2$, respectively. Only the cleavages in the intron core are marked with *. Bars on the left side indicate the positions of the stems. CAUG shows a sequence reaction.

Tetrahymena thermophila rRNA intron, a member of the IC1 group (20). Figure 1C shows the four major products of lead cleavage of the *Tetrahymena* precursor RNA, corresponding in length to two cuts, one in P7 marked with $^+$ and one in junction J8/7 marked with *. Again, both cleavages are competed by Mg^{2+} (Fig 1C, lane 3). In the wild-type form of the *td* and the *sunY* introns, cleavage at P7 was almost undetectable; only in the mutant C263U, where the bulged nucleotide is mutated from C to U, the cleavage is readily observed (Fig. 2B).

The major lead cleavage sites are located in the catalytic core of the intron

For the exact localization of the cleavage sites, a primer extension analysis was performed (Fig. 2A, B) and cleavage positions were determined by comparison with a sequencing reaction performed with the same primer. Bands corresponding to the expected cleavage sites were obtained. One of the cleavage sites is located 5′ to the bulged nucleotide (A263) in the P7 helix, the other is 5′ to U305 in J8/7 (all numbers referring to the *Tetrahymena* intron, Fig. 5). Both cleavages are located in the catalytic core of the intron and are at functionally important sites. The bulged nucleotide in P7 is next to G264, which has been shown to be part of the G-binding site (21). The junction 8/7 is located near the 5′ splice-site (P1 stem) in the 3D model of Michel-Westhof (20) and has recently been shown to contact the P1 helix via 2′-OH interactions (22).

In the *sunY* intron, cleavage at U305 was accompanied by two minor cleavages 5′ to A306 and A308 (Fig. 2B). Additional bands detected in the primer extension reactions are not located in the conserved core of the introns and occur variably. One example of such a minor cleavage in the *td* intron was mapped at the basis of stem P7.2. Some of these additional minor bands are also not readily competed by Mg^{2+} and may not be due to lead cleavage, but to conformationally induced stops of the reverse transcriptase.

Magnesium dependence of lead cleavage

As already shown in figures 1A and 1B, Mg^{2+} ions interfere with lead cleavage in a way that high amounts of Mg^{2+} prevent cleavage. Incubation of the precursor RNA of the *td* intron (C263U mutant) with lead in the absence of and with increasing amounts of Mg^{2+} clearly demonstrates that the concentration of Mg^{2+} is a crucial parameter for lead cleavage to occur (Figure 3). In the absence of Mg^{2+} (Fig. 3; lane 2), no cleavage is detected. The concentration of Mg^{2+}, where the highest

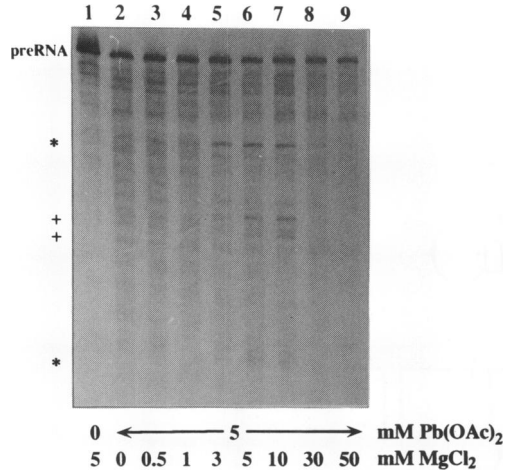

Figure 3. Magnesium dependence of lead cleavage. Bodylabeled precursor RNA (preRNA) of the *td* intron (mutant C263U) was incubated with Pb^{2+} in the absence of and with increasing amounts of Mg^{2+}. Lane 1: preRNA incubated without lead; lane 2: preRNA incubated with lead in the absence of Mg^{2+}; lanes 3–9: preRNA incubated with lead and increasing amounts of Mg^{2+}. Bands are labeled as in figure 1A.

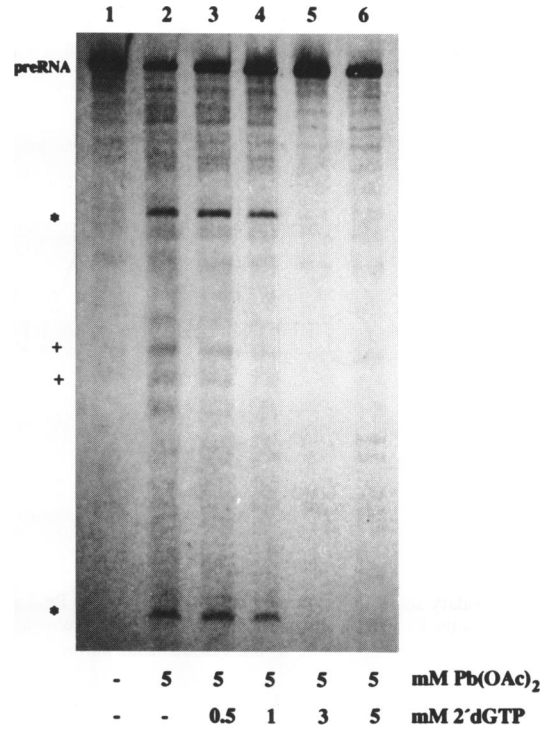

Figure 4. Inhibition of lead cleavage by 2′-deoxyGTP. Bodylabeled precursor RNA (preRNA) of the *td* intron (mutant C263U) was treated with lead. Lane 1: preRNA incubated without lead; lane 2: preRNA incubated with lead; lane 3–6: preRNA incubated with lead in the presence of increasing amounts of 2′dGTP. Bands are labeled as in Fig. 1A

efficiency of cleavage is observed, is approximately 10 mM (Fig. 3; lane 3–9). At low Mg^{2+} concentrations, the RNA is probably not correctly folded; at too high Mg^{2+} concentrations, lead is displaced. Pb^{2+} has long been known to unspecifically degrade RNA molecules (23), preferentially in unstructured or single-stranded regions. Pb^{2+}-induced degradation of RNA can also be observed for the group I intron RNA (compare Fig. 3, line 1 with line 2). The unspecific degradation of RNA by Pb^{2+} is not influenced by the Mg^{2+} concentration (Fig. 3). However, the two observed distinct cleavages in the core of the group I intron are strongly influenced by the concentration of Mg^{2+}, and cleavage is much more efficient than unspecific degradation.

2′-deoxyGTP, an inhibitor of splicing, protects the intron RNA against lead cleavage

Self-splicing of group I introns is inhibited by 2′dGTP via competition with the substrate guanosine (24). Lead cleavage experiments performed in the presence of 2′dGTP resulted in reduced and, at high concentrations, in inhibition of cleavage (Fig. 4). The two cleavages are not equally blocked by 2′dGTP: for the mutant *td* intron, cleavage at the bulged nucleotide in P7 was inhibited upon addition of 1 mM 2′dGTP, whereas cleavage at U305 was inhibited by addition of 3 mM 2′dGTP. The self-splicing reaction of the *Tetrahymena* intron is inhibited by 2′dGTP with a Ki of 1.1 mM (24), in the same concentration range at which lead cleavage is inhibited. Inhibition of lead cleavage by 2′dGTP was not altered by raising the Mg^{2+} concentration from 5 to 10 mM (data not shown), indicating that the effect of 2′dGTP is not simply due to unspecific binding of divalent ions to nucleotides.

DISCUSSION

Lead cleavage analysis of self-splicing group I introns was performed, leading to the detection of two major cleavages in the core structure of three tested group I introns. We suggest that the cleavages result from Pb^{2+} ions located at functional Mg^{2+} binding sites. Our interpretation is supported by the following observations:

The two lead cleavage sites are proximal to the substrate binding sites

In the three dimensional model of Michel & Westhof (20), the two major lead cleavage sites are proximal to the substrate binding sites. The cleavage site in J8/7 is close to a region that contacts the P1 stem, which is formed by the pairing of the 5′ splice-site with the internal guide sequence. The 5′ splice-site can be considered the substrate in a ribozyme reaction. The positioning of the P1 helix, which contains the 5′ cleavage site, has recently been experimentally demonstrated by Pyle et al. (22), who showed that the 2′ OH group of the U-3 residue interacts with A302 in J8/7 of the *Tetrahymena* ribozyme. The cleavage site 5′ to the bulged nucleotide in P7 (A263) is close to the G-binding site (binding site for the substrate guanosine), which has been partially localized at the conserved G (G264) in P7 (21) (Fig. 5). Both cleavage sites are located where the transesterification reaction during splicing is expected to take place. In a recent review by T. Cech et al. (25), where a model for the ribozyme-substrate complex in the transition state was proposed, the Mg^{2+} ions, which stabilize the transition state, were modeled in proximity to the positions where Pb^{2+} cleavage is observed.

Substitution of the phosphates by phosphorothioates at the lead cleavage sites results in splicing deficiency

The role of the phosphate groups in the *Tetrahymena* intron has been investigated by substitution of phosphates by phosphorothioates with subsequent analysis of their effect on splicing. Magnesium can only coordinate the oxygen atom of a phosphate, but not the sulfur atom of a phosphorothioate, whereas

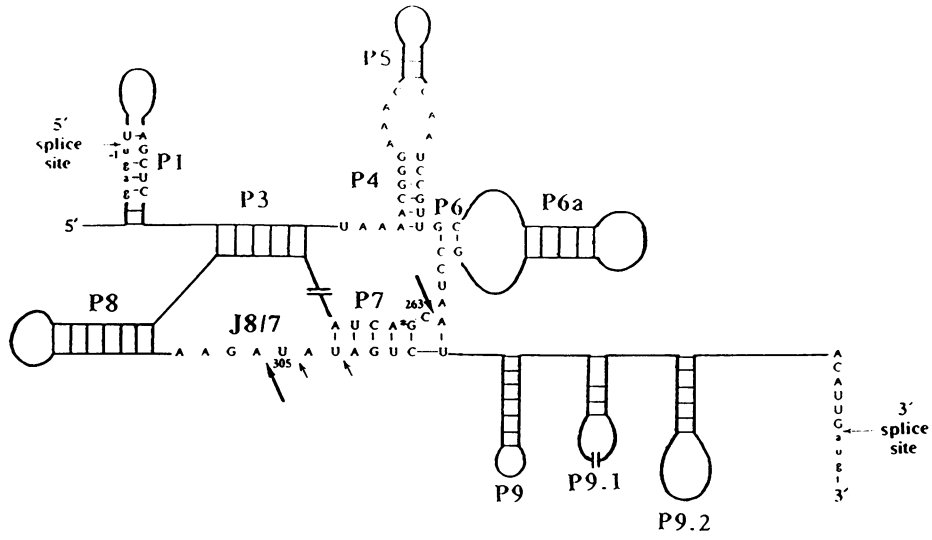

Figure 5. Secondary structure of group I introns. Stems P1 to P9.2 are shown, conserved sequence elements are given in sequence for the T4 phage derived *sunY* intron. *G marks the G-binding site; large arrows indicate the two conserved lead cleavage sites, small arrows indicate additional minor cleavages in the *sunY* intron. Numbering of residues is according to the *Tetrahymena* intron.

manganese is able to coordinate both oxygen and sulfur (25). Substitution of phosphates by phosphorothioates showed that mainly the thiophosphates in J8/7 are sensitive positions, with no splicing in the presence of magnesium, but with restored splicing activity in the presence of manganese (14, E.Christian and M.Yarus, personal communication). This implies these phosphates to be involved in Mg^{2+} binding. Interestingly, substitution of the phosphates by phosphorothioates 5′ to either U305, A306 or A308, but not at U307, resulted in splicing inhibition (14), corresponding to the series of three cleavages by lead observed at these positions in the *sunY* intron (Figure 2B, Fig.5). In the Michel-Westhof model (20), these nucleotides surround the phosphate group 5′ to residue U-1, possibly creating a lead/magnesium binding pocket.

The cleavage 5′ to the bulged nucleotide in P7 is very weak and almost undetectable in the wild-type. In *td*, only after we mutated this nucleotide from C to U, lead cleavage was readily observed. Mutation of the bulged nucleotide results in an increased magnesium requirement for splicing as well as in an increased Km for GTP. Furthermore, the mutant intron is resistant against site specific hydrolysis at pH 9 (16). We think that the increased lead cleavage in the mutant at this position is due to a destabilization of the bulged nucleotide, making the metal ion binding site more flexible. Chemical modification experiments showed that this nucleotide is deprotected in the mutant when compared to the wild-type (U.v.A. & R.S.; unpublished results).

In the X-ray diffraction studies of yeast $tRNA^{Phe}$ it was shown that the cleaving Pb^{2+} ion binds close to a binding site that is normally occupied by Mg^{2+} (4). As both ions cannot bind simultaneously, this means that the lead ion is able to displace the magnesium ion from its binding site. The fact that the two lead cleavages in the intron RNA can be competed by Mg^{2+} and Mn^{2+} suggests that the cleaving lead ion, as in the tRNA, occupies magnesium/manganese binding pockets.

Sugimoto et al. (10) studied the role of Mg^{2+} ions in group I intron catalysis. They proposed that the 2′ OH of U-1 (last nucleotide of the 5′ exon) participates in binding Mg^{2+} in conjunction with some yet unidentified group on the ribozyme. The phosphate group 5′ to U305 is a good candidate for such an interaction as it has been modeled in proximity to the 5′ exon and as it is cleaved by lead.

A splicing inhibitor inhibits lead cleavage

2′-deoxyGTP has been shown to inhibit the first step of the self-splicing reaction by competition with the substrate guanosine via binding to the G-binding site (24, 21). We showed that 2′dGTP also protects the intron RNA against lead cleavage. Cleavage at the bulged nucleotide in P7 occurs close to the G-binding site, so that binding of 2′dGTP to the G-binding site could prevent lead from entering an adjacent lead binding pocket. When using increasing amounts of 2′dGTP to prevent lead cleavage, the cleavage at the bulged nucleotide is inhibited first, indicating that this position has a higher affinity to the inhibitor. As shown by Sugimoto et al. (10), binding of the substrate altered the affinity of the ribozyme to Mg^{2+}. In analogy, binding of 2′dGTP to the intron RNA may alter the affinity for Mg^{2+}, so that Pb^{2+} cannot displace a bound Mg^{2+} ion. Alternatively, a conformational change induced by 2′dGTP binding could disrupt the stereochemical structure required for lead cleavage. This may also be true for the cleavage in J8/7, as conformational changes may affect even more distant regions of the catalytic core of the intron in a way that prevents lead binding or cleavage.

Pb^{2+} cleavage as a tool for probing functional Mg^{2+} binding sites?

We observed unstructured RNA molecules to be very sensitive to degradation in the presence of lead (data not shown). On the other hand, complexly structured RNAs, like group I introns, are only rarely cleaved. The extent and specificity of cleavage depends on reaction conditions, suggesting that binding sites with different affinities to lead exist in the molecule. As the two major cleavages observed occur in the catalytic core near the substrate binding sites, where the splicing reaction takes place, and as they are at phosphates that interact with magnesium ions, it is likely

that these magnesium ions are involved in catalysis or substrate binding, therefore occupying class 1 binding sites. Thus, we suggest that under the chosen conditions only class 1 magnesium binding sites are occupied and cleaved by lead, and that class 2 magnesium binding sites are either not occupied by lead, or lead cleavage requirements are not met.

Pb^{2+} has long been known to be very efficient in depolymerizing RNA (23). Additionally, Pb^{2+} has been used to probe the structure of large RNA molecules (26). Gornicki et al. reported a preferential affinity of Pb^{2+} for interhelical and loop regions. They suggested that specially flexible and dynamic regions of the RNA molecule are highly sensitive to Pb^{2+}. Our results are in well agreement with these observations, however, the rareness of cleavage is rather astonishing. We observe two major cleavage sites in the core of the intron and only a few minor cleavages in a molecule of 421 bases (*td* intron). The tRNA molecule shows a single lead cleavage site. Lead cleavage analysis of the RNase P RNAs from *E.coli*, *B.subtilis* and *Chromatium vinosum* also showed a limited number of cleavage sites, which occur in regions of complex tertiary structure (K.Zito, A.Hüttenhofer and N.R.Pace, personal communication). Taken together, we conclude that strong and efficient lead cleavages in RNA molecules are rare and distinct from the lead-induced depolymerization. Two types of lead cleavages have already been postulated by Gornicki et al. (26), who analysed the susceptibility of the 3′ end of the *E.coli* 16S rRNA towards lead.

We propose that the specific lead cleavages observed in structured RNA molecules reflect a catalytic function of the RNA molecule itself. This would imply that the yeast $tRNA^{Phe}$ also harbours catalytic properties. These have indeed been recently proposed for fragments of tRNA molecules (27, 28). As only two major sites of lead cleavage could be detected in the three tested group I introns, and as these sites seem to be relevant for catalysis, we suggest the analysis of a functional RNA molecule by lead cleavage to be a potentially useful tool for mapping functional magnesium binding sites.

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