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Catalytic cleavage of *cis*- and *trans*-acting antigenomic delta ribozymes in the presence of various divalent metal ions

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ABSTRACT

Catalytic activity of four structural variants of the antigenomic delta ribozyme, two *cis*- and two *trans*-acting, has been compared in the presence of selected divalent metal ions that effectively support catalysis. The ribozymes differ in regions that are not directly involved in formation of the ribozyme active site: the region immediately preceding the catalytic cleavage site, the P4 stem and a stretch of the viral RNA sequence extending the minimal ribozyme sequence at its 3′-terminus. The variants show high cleavage activity in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+}, lower with Co^{2+} and Sr^{2+} and some variants are also active with Cd^{2+} and Zn^{2+} ions. In the presence of a particular metal ion the ribozymes cleave, however with different initial rates, according to pseudo-first or higher order kinetics and to different final cleavage extents. On the other hand, relatively small differences are observed in the reactions induced by various metal ions. The cleavage of *trans*-acting ribozymes induced by Mg^{2+} is partially inhibited in the presence of Na^{+}, spermidine and some other divalent metal ions. The inert $Co(NH_3)_6^{3+}$ complex is unable to support catalysis, as reported earlier for the genomic ribozyme. The results are discussed in terms of the influence of structural elements peripheral to the ribozyme active site on its cleavage rate and efficiency as well as the role of metal ions in the cleavage mechanism. Some implications concerning further studies and possible applications of delta ribozymes are also considered.

INTRODUCTION

Hepatitis delta virus RNA (HDV RNA) is a single-stranded circular RNA which replicates via the double rolling circle mechanism. In the genomic RNA strand as well as in its antigenomic counterpart generated during virus replication there are two sequences with ribozyme activities. These ribozymes, the genomic and antigenomic types, are required for self-cleavage of polymeric RNA transcripts into monomeric units (for selected reviews see 1–4). The spatial structure of the genomic ribozyme determined by X-ray analysis (5,6) and recent propositions on the mechanism of cleavage by delta ribozymes according to a general acid–base catalysis (7–9) has greatly increased our understanding of their functioning. However, one of the most important issues, concerning the role of divalent metal ions in folding or the mechanism of catalysis of delta ribozymes, remains unsolved.

Most evidence has consistently supported a requirement for divalent metal ions in HDV catalysis under physiological pH conditions. The delta ribozymes definitely require divalent ions, while other ribozymes, such as the hammerhead, hairpin and VS ribozymes, are also active in very high concentrations of monovalent ions (10). The presence of a 'general' metal ion-binding site in the genomic ribozyme has been suggested based on the results of metal ion-induced cleavage experiments. Specific cleavages are induced in the J4/2 region with Pb^{2+} (11) and also with Mg^{2+}, Ca^{2+} and Mn^{2+} (12). In the *trans*-acting antigenomic ribozyme, a specific Mg^{2+}-induced cleavage occurs at the bottom of the P2 stem (13). Moreover, although the 3′,5′-phosphodiester linkage at 'the functional cleavage site' is cleaved slightly faster in Ca^{2+} than in Mg^{2+}, the 2′,5′-linkage is cleaved in Mg^{2+} (or Mn^{2+}) but not Ca^{2+} (14). This dramatic difference is strongly suggestive of a crucial metal ion interaction at the active site. Recently, it has been shown that imidazole buffer rescues the activity of a mutant ribozyme with a C76→U substitution (7). These data are consistent with imidazole-enhanced cleavage by a general base mechanism, but another possibility is that imidazole could coordinate a catalytic metal ion, presumably replacing a ligand lost with mutation of C76 (7). In another mechanistic proposition (8), C75 acts as the general acid with the pK_a of the ring nitrogen N3 shifted upwards to ~7 as a consequence of interaction with a phosphate residue and the presence of a tight metal ion-binding site in its vicinity. An ionized metal ion hydrate acts as the general base in the proposed mechanism. All the above observations suggest the presence of essential divalent metal ion-binding sites in the delta ribozymes, although the crystal structure of the 3′ cleavage product of a genomic ribozyme (5,6) does not reveal a metal ion in the catalytic pocket.

Catalytic activity of delta ribozymes in the presence of various divalent metal ions has been compared for the genomic variant (15). For the antigenomic ribozyme, several authors describe experiments for testing the activity of different variants with divalent ions (14,16–18). In most cases, however, these data cannot be directly compared since it is not possible to separate the two effects on catalysis: the kind of catalytic divalent

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metal ion and the structure of a particular ribozyme variant. Precise kinetic data are needed in order to compare the activity of the same ribozyme in the presence of a broader spectrum of divalent metal ions. In order to gain more information on the role of divalent metal ions in catalysis we compared the activity of closely related variants of the antigenomic ribozyme in the presence of various divalent ions. The variants differed in regions that were not directly involved in formation of the ribozyme catalytic core. Thus the role of these peripheral elements in modulating ribozyme activity could also be assessed.

MATERIALS AND METHODS

Materials

The materials used in this study were from the following sources. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham and all the chemicals were from Serva or Fluka. Polynucleotide kinase, T7 RNA polymerase, RNase inhibitor and T4 DNA ligase were purchased from MBI Fermentas. AmpliTaq polymerase was from Perkin Elmer. Chemically synthesized oligoribonucleotides *S1* (5′-GGGCGGGUCGG-3′), *S2* (5′-CUUCGGGUCGG-3′) and *S3* (5′-CUUUCCUCUUCGGGUCGGCA-3′) were purchased from Xeragon AG.

DNA template constructs

All oligodeoxyribonucleotides were synthesized at the 0.2 µmol scale, deprotected after synthesis and purified by electrophoresis on denaturing 8% (w/v) polyacrylamide gels. DNA bands were excised, eluted with 0.3 M sodium acetate, pH 5.2, 1 mM EDTA and precipitated with ethanol. The DNA was recovered by centrifugation and dissolved in TE buffer.

The DNA templates for *in vitro* transcription of the *cis(W)* ribozyme and the *R* oligomer, the ribozyme component of *trans(S1)* and *trans(S2)*, were prepared as follows (12,19). For *cis(W)*, two DNA oligomers were synthesized, A1 (5′-CTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCG-GATGCCCAGGTCGGACCGCGAGGAGGTGGAGATG-CCATGCCGACCC-3′) and B1 (5′-*TAATACGACTCACTATA*-GGGTCCTTCTTTCCTCTTCGGGTCGGCATGGCA-3′) (letters in italic mark the T7 RNA polymerase promoter; complementary sequences are underlined). For the *R* oligomer, two other oligomers were synthesized, A2 (5′-GAAAAGTGGC-TCTCCCTTAGCCATCCGAGTGCTCGGATGCCCAGGT-CGGACCGCGAGGAGGTGGAGATGCCC-3′) and B2 (5′-*TAATACGACTCACTATA*GGGCATCTCCACC-3′). Equimolar amounts of both oligomers (A1, B1 or A2, B2) were annealed and double-stranded DNA templates were generated by PCR. The reaction mixtures contained 1 µM both DNA oligomers, 10 mM Tris–HCl, pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 200 µM each dNTP and 25 U/ml AmpliTaq polymerase. The reactions were performed on a Biometra UNO II thermocycler for five cycles of 30 s at 94°C, 30 s at 46°C and 2 min at 72°C. The mixtures were extracted with phenol/chloroform (1:1) and the reaction products precipitated with ethanol, dissolved in TE buffer and used in transcription reactions.

RNA preparation

The *in vitro* transcription reactions contained 0.4 µM DNA template, 40 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM each NTP, 750 U/ml RNase inhibitor and 2000 U/ml T7 RNA polymerase (MBI Fermentas). To obtain the *R* oligomer with a monophosphate group at its 5′-end for subsequent preparation of *cis(L)*, 5 mM 5′-GMP was used in the transcription reaction. Following incubation of the mixtures at 37°C for 4 h, the RNA transcripts were purified on 8% denaturing polyacrylamide gels, localized by UV shadowing, eluted with 0.3 M sodium acetate, pH 5.5, 1 mM EDTA, precipitated with ethanol and dissolved in sterile water containing 0.1 mM EDTA.

The *cis(W)* ribozyme was internally labeled with ^{32}P during transcription by including [γ-^{32}P]ATP in the reaction mixture. To minimize ribozyme self-cleavage the mixture was incubated at 4°C for 48 h, the RNA purified and, following autoradiography, recovered as described above. 5′-^{32}P-labeled *cis(L)* was prepared by ligation of 5′-^{32}P-labeled oligomer *S3* and oligomer *R* with T4 DNA ligase in the presence of a splint oligodeoxynucleotide spanning 10 nt on each side of the ligation junction (14,20). Equimolar amounts of the three oligomers were heated at 95°C for 2 min, cooled to 25°C for 10 min in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl buffer. Subsequently, the ligation reaction (total volume 15 µl) was carried out in 50 mM Tris–HCl, pH 7.5, 16.7 mM NaCl, 11 mM $MgCl_2$, 10 mM DTT, 0.3 mM EDTA, 1 mM ATP buffer with 1000 U/ml T4 DNA ligase for 4 h at room temperature. The ligated RNA was purified on a denaturing 8% polyacrylamide gel. The oligoribonucleotide substrates *S1*, *S2* and *S3* were labeled at their 5′-ends using [γ-^{32}P]ATP and T4 polynucleotide kinase under standard conditions.

Cleavage reaction

Prior to self-cleavage the *cis*-acting ribozymes, internally ^{32}P-labeled *cis(W)* (100 000 c.p.m., 0.5–1 pmol RNA in 50 µl reaction volume) or 5′-^{32}P-labeled *cis(L)* (~50 000 c.p.m., 0.1 pmol RNA in 50 µl reaction volume), were subjected to a denaturation–renaturation procedure in the standard reaction buffer, 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA by incubating at 100°C for 2 min, 0°C for 10 min and finally at 37°C for 10 min. The reactions of *cis(L)* were performed in the presence of 100 mM NaCl which was added during the denaturation–renaturation procedure, after incubation of the samples at 0°C (17). The *trans*-acting ribozymes, *trans(S1)* or *trans(S2)*, were prepared by mixing the 5′-^{32}P-labeled substrates *S1* or *S2* (~50 000 c.p.m., 0.1 pmol RNA in 50 µl reaction volume) with the *R* oligomer in the standard reaction buffer to obtain the final RNA concentrations of <2 nM and 700 nM for the substrate and ribozyme components, respectively. The mixtures were subjected to the same denaturation–renaturation procedure as described above and under these conditions all the substrate molecules were bound in substrate–ribozyme complexes (16,21). The cleavage reactions were initiated by adding an appropriate divalent metal chloride solution and the reactions proceeded at 37°C. In some experiments (see Figs 5 and 6) additional cofactors (NaCl, spermidine or other divalent metal ions) were added during the denaturation–renaturation procedure after incubation at 0°C and the samples were incubated at 37°C for 10 min prior to initiation of the reaction. Aliquots of the reaction mixtures (5 µl) were removed at specified time points and quenched with equal volumes of 20 mM EDTA, 7 M urea mixture. The digestion products were analyzed by electrophoresis on 12% polyacrylamide, 0.75%

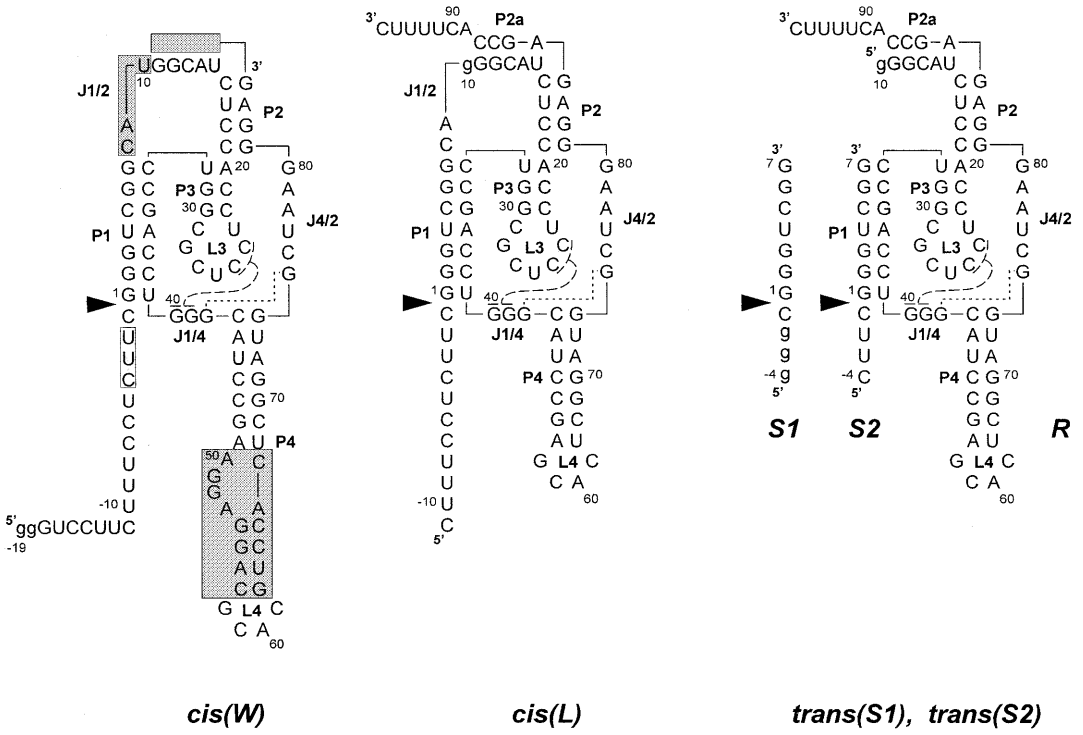

Figure 1. Secondary structure models of *cis*- and *trans*-acting antigenomic delta ribozymes. Numbering of nucleotides corresponds to the wild-type ribozyme sequence and nucleotide changes are shown in lower case letters. Base paired segments are denoted P1–P4 and single-stranded regions as J1/2, J1/4 and J4/2. Nucleotides connected with broken and dotted lines indicate the 2 bp helix P1.1 and non-standard G-G interactions analogous to those found in the crystal structure of the genomic variant. Catalytic cleavage sites are marked by filled triangles. The shaded segments in *cis(W)* denote regions changed in the ribozyme variants.

bis-acrylamide, 8 M urea gels. Autoradiography was performed at –70°C with an intensifying screen. For quantitative analysis radioactive bands were cut off and counted in a Beckman LS5000TA counter. Some gels were quantified using phosphorimaging screens, a Typhoon 8600 Imager with ImageQuant software (Molecular Dynamics). The fraction of substrate cleaved was plotted versus time and fitted to single or double exponential equations. First order rate constants (k_{obs}) were calculated from data fitted to a single exponential equation: $[P]_t = [EP](1 - e^{-kobs \times t})$, where k_{obs} is the first order rate constant and $[P]_t$ and $[EP]$ are the fractions cleaved at time t and the reaction end point, respectively. The double exponential equation was $[P]_t = [ep'](1 - e^{-k' \times t}) + [ep''](1 - e^{-k'' \times t})$, where ep' and ep'' represent the amplitudes of the biphasic time course and k' and k'' the corresponding rate constants. These parameters were estimated by non-linear regression analysis using the Levenberg–Marquardt algorithm and Microcal Origin 5.0 software. Reactions of the same ribozyme variant in the presence of various divalent metal ions were usually performed on a gel side by side. At least two independent data sets were collected and variation between experiments was not greater than 20%.

RESULTS

Cleavage in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} ions

We synthesized four variants of the antigenomic delta ribozyme, two *cis*- and two *trans*-acting, which are shown in Figure 1. The sequence of variant *cis(W)* matches the minimal ribozyme sequence of the antigenomic RNA strand of the HDV virus. The transcript contains 19 nt upstream of the catalytic cleavage site, 17 nt of the wild-type sequence and two extra G residues at its 5′-end, facilitating *in vitro* transcription by T7 RNA polymerase. In the second variant, *cis(L)*, the 5′-leader sequence has 11 nt, a single mutation U10→G is present in the J1/2 region, the P4 stem is shortened and an additional 11 nt extend the minimal ribozyme sequence at its 3′-end. Both the *trans*-acting ribozymes, *trans(S1)* and *trans(S2)*, are derivatives of *cis(L)* in which the substrate and ribozyme strands are separated in the J1/2 region between A9 and G10 and two nucleotides, C8 and A9, are deleted from the oligonucleotide substrates. The substrates differ in the regions preceding the cleavage site; the wild-type sequence $_{-1}$CUUC$_{-4}$ in *S2* is replaced by $_{-1}$CGGG$_{-4}$ in the *S1* oligomer.

Catalytic activity of the four ribozyme variants was determined at 1 mM concentrations of Mg^{2+}, Ca^{2+} and Mn^{2+} ions (Fig. 2 and Table 1). The *cis*-acting ribozymes, *cis(W)* and *cis(L)*, are cleaved in the presence of all three metal ions in a multi-phasic mode and the data fit well to curves expressed by the sum of two exponentials. It suggests that each of these ribozymes contains a mixture of fast and slow cleaving molecules (17,22). In the case of *cis(W)* the percentages of fast cleaving fractions are low, ~10–30%, depending on the metal ion used in catalysis, and the corresponding rate constants k' range between 2 and 9 min^{-1}. The rate constants k'' for slow cleaving fractions are lower by a factor of 30–50 and, finally, after

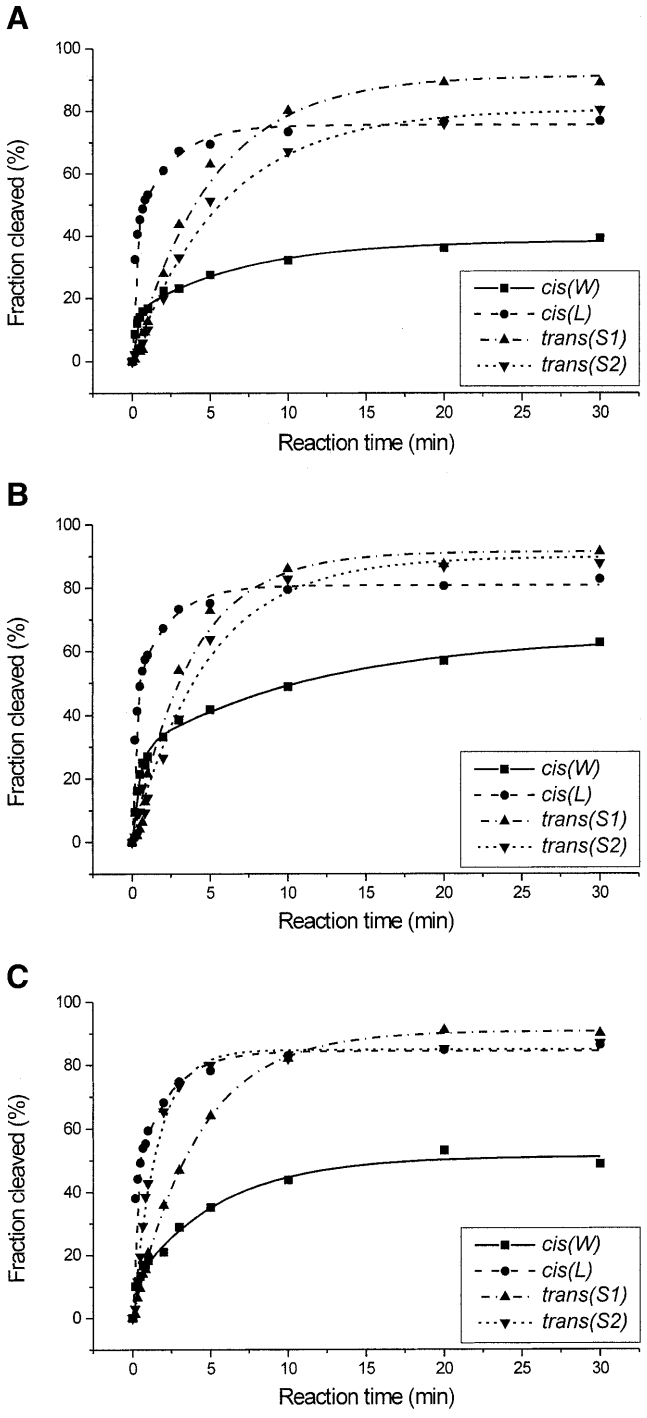

Figure 2. Cleavage kinetics of the *cis(W)*, *cis(L)*, *trans(S1)* and *trans(S2)* ribozymes in the presence of Mg^{2+} (**A**), Ca^{2+} (**B**) and Mn^{2+} (**C**) ions at 1 mM ion concentration.

30 min only 40–60% of the ribozyme molecules are cleaved. The *cis(L)* ribozyme self-cleaves with the k' constant ranging between 6 and 10 min^{-1}, has an almost identical k'' value of 0.4–0.5 min^{-1} and the final cleavage extent reached 70% for Mg^{2+}- and 80% for Ca^{2+}- and Mn^{2+}-induced reactions. The *trans*-acting ribozymes, with either the *S1* or *S2* substrates, are

Table 1. Summary of kinetic data

Ribozyme	Metal ion	1 mM	
		k' (*ep'*); (min^{-1}) (%)	k'' (*ep''*); (min^{-1}) (%)
cis(W)	Mg	3.95 ± 0.75 (15)	0.14 ± 0.02 (23)
	Ca	2.16 ± 0.25 (29)	0.08 ± 0.02 (36)
	Mn	8.8 ± 3.9 (11)	0.18 ± 0.02 (40)
cis(L)	Mg	7.65 ± 0.92 (40)	0.44 ± 0.05 (36)
	Ca	5.61 ± 0.69 (45)	0.48 ± 0.07 (36)
	Mn	10.36 ± 1.61 (41)	0.49 ± 0.05 (44)
		k_{obs} (*EP*); (min^{-1}) (%)	
		1 mM	10 mM
trans(S1)	Mg	0.19 ± 0.01 (92)	0.92 ± 0.07 (87)
	Ca	0.26 ± 0.02 (92)	
	Mn	0.24 ± 0.02 (92)	
trans(S2)	Mg	0.17 ± 0.01 (81)	1.32 ± 0.16 (86)
	Ca	0.20 ± 0.02 (90)	2.31 ± 0.32 (86)
	Mn	0.57 ± 0.04 (86)	1.87 ± 0.25 (86)

cleaved under single turnover conditions much slower than the *cis* variants. The reactions are, however, well approximated by pseudo-first order kinetics and the cleavage extent reached 80–90% in the presence of all three metal ions. The calculated rate constants k_{obs} for Mg^{2+}-, Ca^{2+}- and Mn^{2+}-induced reactions are 0.19, 0.26 and 0.24 min^{-1} for *trans(S1)* and 0.17, 0.20 and 0.57 min^{-1} for *trans(S2)*, respectively. Cleavage rate constants for the *trans*-acting ribozymes at 10 mM concentration of the metal ions were also determined (Table 1). The *S1* substrate is cleaved at 10 mM Mg^{2+} with a rate constant k_{obs} of 0.92 min^{-1}. The k_{obs} values with the *S2* oligonucleotide are 1.32, 2.31 and 1.87 for Mg^{2+}-, Ca^{2+}- and Mn^{2+}-induced reactions.

It has been shown that a *trans*-acting antigenomic ribozyme with a long L4 stem is cleaved in the presence of 10 mM Mg^{2+} with a k_{obs} of 0.91 min^{-1} (21), which is essentially identical to the value found for the *trans(S1)* variant. Another ribozyme, with stem L4 shortened but stem P2 extended by two additional GC base pairs, was cleaved with a lower, but comparable to our data, rate constant, ranging between 0.22 and 0.32 min^{-1} (16,23). A more rigid structure of stem P2 could explain the lower activity in that case (24). On the other hand, there are only very limited data on cleavage rates of antigenomic delta ribozymes in the presence of Ca^{2+} and Mn^{2+}. For two *trans*-acting ribozymes that differed in stems P4 and P2, in the presence of 10 mM Ca^{2+} the k_{obs} values ranged from 0.4 to 0.9 min^{-1} (13,14,16). The reported rate constant of 0.06 min^{-1} in 10 mM Mn^{2+} (13), much lower than in Mg^{2+} and Ca^{2+}, seems to be unexpectedly low. Comparable cleavage extents in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} were shown on an autoradiogram by other authors (14). However, for the Mn^{2+}-induced reaction the cleavage rate constant has not been determined.

The dependency of the observed k_{obs} values for *trans(S1)* on concentration of Mg^{2+}, Ca^{2+} and Mn^{2+} is shown in Figure 3A. The rates increase almost linearly up to ~5 mM concentration of divalent ions and reaches a plateau at ~10 mM. At that 'saturating concentration' the cleavage reaction is fastest with

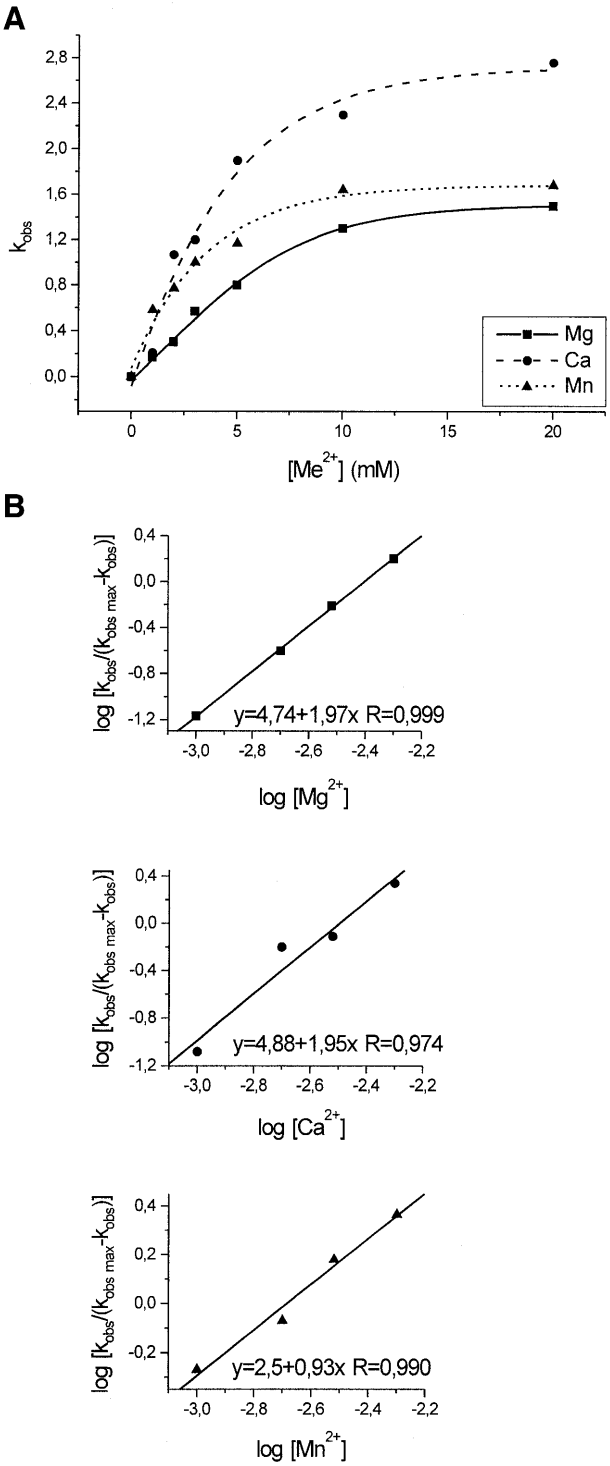

Figure 3. Dependence of the cleavage rate constants k_{obs} for the *trans(S2)* ribozyme on the concentration of Mg^{2+}, Ca^{2+} and Mn^{2+} ions (**A**) and Hill analysis of the data (**B**). The correlation coefficients *R* were calculated during the least squares regression analysis using the Microcal Origin 5.0 computer program.

Ca^{2+}, then with Mn^{2+}, and slowest with Mg^{2+}. It should be noted that this order of relative activity is essentially observed over the entire range of metal ion concentrations. The concentrations of divalent ions at half maximal cleavage rates, $K_{½}$, are ~4.5 for Mg^{2+}, 3.5 for Ca^{2+} and 2.5 for Mn^{2+}. The Hill coefficients calculated from the linear parts of curves are 1.97, 1.95 and 0.93 for the Mg^{2+}-, Ca^{2+}- and Mn^{2+}-induced reactions. These values suggest the involvement of two active Mg^{2+} or Ca^{2+} or one Mn^{2+} ion in catalysis.

Other divalent metal ions active in catalysis

Some other divalent metal ions are also active in catalysis (Fig. 4). The *trans(S2)* ribozyme is cleaved in the presence of 1 mM Co^{2+}, Sr^{2+}, Zn^{2+} and Cd^{2+} with the following first order rate constants and reaction end points: 0.016 min^{-1} and 67%, 0.003 min^{-1} and 25%, 0.009 min^{-1} and 16% and 0.006 min^{-1} and 49%. These values were, however, determined at 1 mM, likely a sub-saturating concentration of these ions. Since the ions might differ in metal ion–RNA binding affinities the rate constants do not necessarily reflect the relative effectiveness of these metals in catalysis. This effect may be responsible for differences in the cleavage of *trans(S1)*; after 60 min the cleavage extents at 1 mM divalent ions were Co^{2+}, 45% and Sr^{2+}, 5% (Fig. 4), but at 5 mM Co^{2+} it was 45%, whereas at 10 mM Sr^{2+} it was 25% (data not shown), suggesting a higher binding affinity of Co^{2+} than Sr^{2+} for the RNA. On the other hand, it is known that at higher concentration some of these metal ions may form polyhydroxy species, insoluble precipitates, perturb the RNA structure or even cause its degradation. To avoid misleading interpretations we therefore consider cleavage data obtained at low, 'safe' concentrations.

An interesting observation concerns a high cleavage activity of *cis(L)* in the presence of 1 mM Co^{2+} and Sr^{2+}, with corresponding rate constants k' of 4.67 and 0.65 min^{-1} and reaction end points of 76 and 66% for the Co^{2+}- and Sr^{2+}-induced reactions, respectively (Fig. 4B). These values are comparable to those obtained with the ions most active in catalysis, i.e. Mg^{2+}, Ca^{2+} and Mn^{2+} (Table 1). Clearly, cleavage rates depend strongly on the structure of the particular ribozyme variant and *cis(L)* turns out to be exceptionally active. Figure 4C shows that *cis(W)* is also active in the presence of 5 mM Co^{2+}, although the cleavage extent does not exceed ~10%. On the other hand, the inert complex $Co(NH_3)_6^{3+}$ at the same 5 mM concentration does not induce the reaction. The other ribozyme variants, *cis(L)* and *trans(S2)*, also remained inactive under these conditions (data not shown). This suggests either direct coordination of the active metal ion to the RNA chain or that ionization of a ligand coordinated to the ion is required in catalysis (discussed later).

Influence of Na^+, spermidine and other divalent metal ions on the Mg^{2+}-induced cleavage reaction

The cleavage of *trans(S1)* induced by Mg^{2+} is inhibited by sodium ions present at 200 mM concentration (Fig. 5A). This inhibition is almost complete at concentrations of Mg^{2+} <1 mM, at 2 mM the effect becomes weaker and it almost disappears at >10 mM. This influence of Na^+ on the cleavage of *cis*-acting ribozyme variants has also been observed by other authors (17,22). In that case, the effect depended strongly on ribozyme structure, on the ability of the 3′-terminus extension of the minimal ribozyme sequence to pair with the J1/2 region, forming an additional helix P2a. With a variant unable to form P2a the cleavage rate at 10 mM Mg^{2+} decreased in 100 mM NaCl from 0.61 to 0.38 min^{-1}, while the presence of P2a stimulated

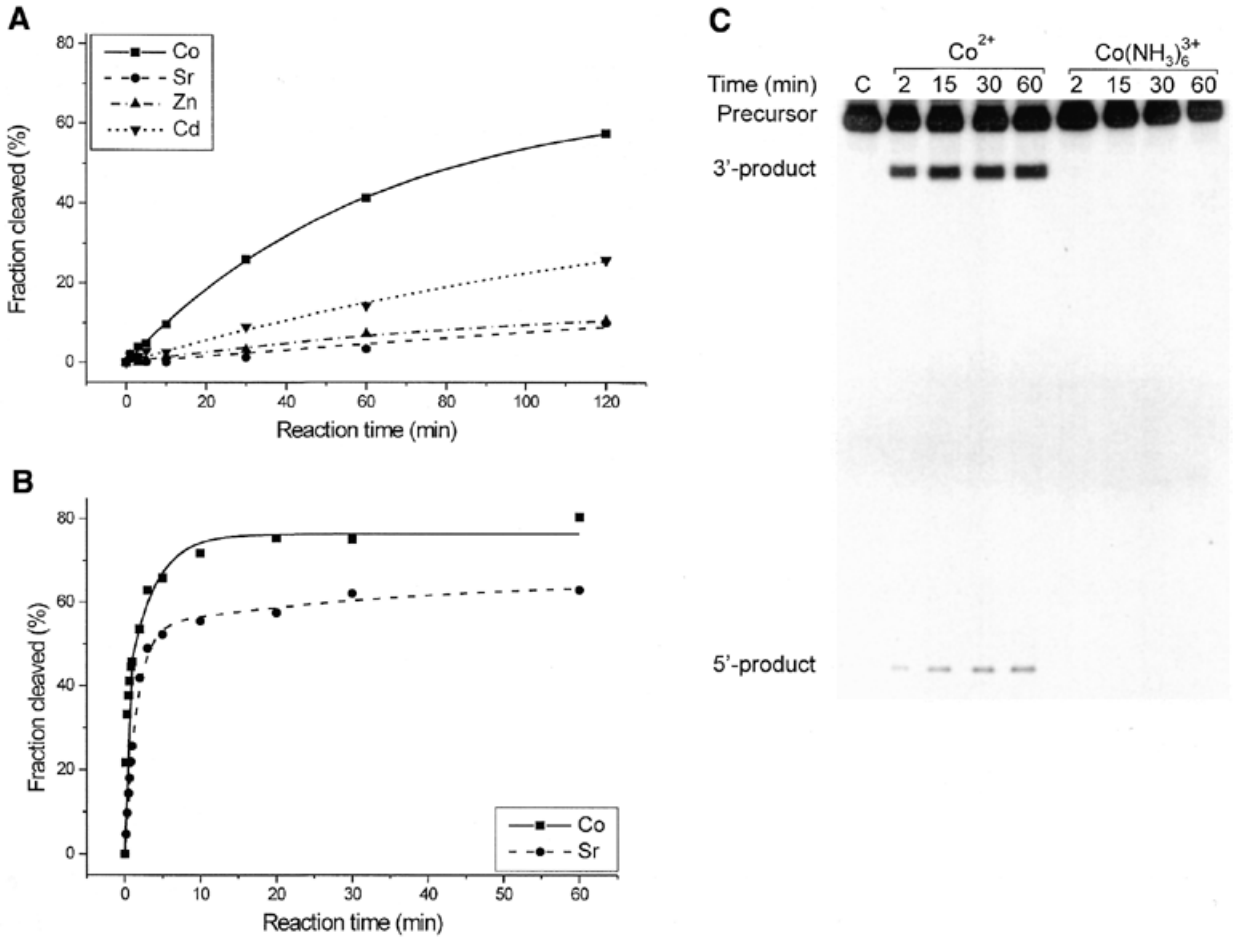

Figure 4. Cleavage reaction of the antigenomic ribozyme variants in the presence of Co^{2+}, Sr^{2+} Zn^{2+}, Cd^{2+} and $Co(NH_3)_6^{3+}$. (**A**) Cleavage reaction of the *trans(S2)* ribozyme induced by Co^{2+}, Sr^{2+}, Zn^{2+} and Cd^{2+} at 1 mM ion concentration. The corresponding pseudo-first order rate constants k_{obs} and the reaction end points were 0.016 min^{-1} and 67%, 0.003 min^{-1} and 25%, 0.009 min^{-1} and 16% and 0.006 min^{-1} and 49%, respectively. (**B**) Cleavage reaction of the *cis(L)* ribozyme in the presence of 1 mM Co^{2+} or Sr^{2+}. The data were fitted to an expression describing the sum of two exponentials (see Materials and Methods) and the following rate constants k' and k'' were obtained: 4.67 and 0.29 for Co^{2+} and 0.65 and 0.02 min^{-1} for the Sr^{2+}-induced reaction. The reaction end points were 76 and 66% for Co^{2+}- and Sr^{2+}-induced cleavage. (**C**) Autoradiogram of cleavage of the *cis(W)* ribozyme in the presence of 5 mM Co^{2+} and 5 mM $Co(NH_3)_6^{3+}$.

self-cleavage by a factor of 1500 in 100 mM NaCl compared to low Na^+/Mg^{2+} conditions (17,22).

The influence of spermidine on the activity of *trans*-acting ribozymes in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} has also been tested (Fig. 5B and C). Two concentrations of metal ions were used, 1 and 10 mM. Spermidine was at concentrations ranging between 0 and 5 mM, the cleavage extents were determined after 10 min reaction and the values were normalized to those obtained in control reactions with no spermidine added. The first concentration of Mg^{2+} is close to its physiological concentration (25), while the second minimizes simple competition for binding to the RNA chain between the positively charged polyamine and metal ions due to electrostatic interactions. It turns out that spermidine inhibits the cleavage of *trans(S1)* and *trans(S2)* under all the conditions tested. The effect is stronger at 1 mM concentration of divalent ions and the Mg^{2+}-induced reaction seems to be the most affected. We determined the concentration of spermidine that inhibited by 50% (K_i) the cleavage of *trans(S2)* induced in the presence of 10 mM Mg^{2+} (Fig. 5D). The fraction inhibition was plotted as a function of spermidine concentration and the data gave a K_i of 2.6 mM. A similar experiment performed in the presence of 200 mM NaCl gave a higher K_i of ~16 mM (data not shown). The inhibitory effect of spermidine is, therefore, relatively weak compared to, for example, that observed for the delta ribozymes and some antibiotics (K_i values in the micromolar range) (11).

Catalytic activity of the *trans(S1)* ribozyme induced by Mg^{2+} is inhibited in the presence of a 0.5 mM concentration of some other divalent metal ions (Fig. 6). This effect may result from binding of inactive metal ions or ones showing low catalytic activity to the same sites that bind Mg^{2+}. Alternatively, these ions may interact with the ribozyme interfering with formation of its active structure. The *trans(S1)* ribozyme is active in the presence of various metal ions, including those that are not considered to be particularly efficient in promoting the folding of functional RNA molecules (see the preceding section). This suggests that the observed inhibition of the Mg^{2+}-induced reaction by other metals is rather a consequence of competition with the catalytically active ion than a result of interfering with

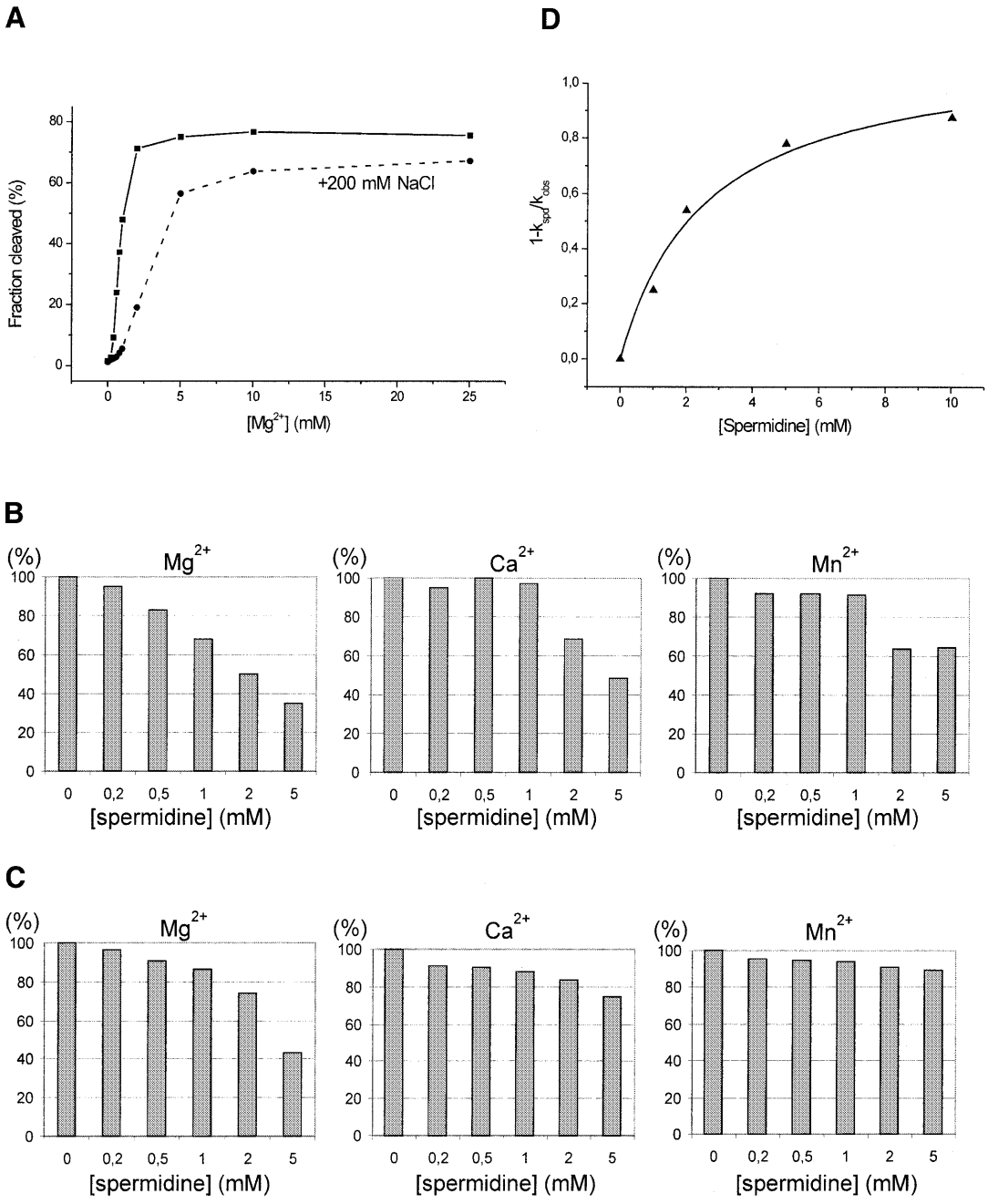

Figure 5. Effect of sodium ions and spermidine on cleavage of *trans*-acting antigenomic ribozymes induced by Mg^{2+}, Ca^{2+} and Mn^{2+} ions. (**A**) Cleavage reaction of the *trans(S1)* ribozyme as a function of Mg^{2+} ion concentration. The reaction was carried out in the presence or absence of 200 mM NaCl at 37°C for 5 min. (**B**) Dependence of the extent of cleavage of the *trans(S1)* ribozyme induced by 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} ions at 37°C for 10 min on the presence of spermidine at concentrations in the range 0–5 mM. The extents of cleavage were normalized to those obtained in control reactions with no spermidine added and the relative values are expressed as percentages. (**C**) Dependence of the extent of cleavage of the *trans(S2)* ribozyme induced by 10 mM Mg^{2+}, Ca^{2+} and Mn^{2+} ions at 37°C for 10 min on the presence of spermidine at concentrations in the range 0–5 mM. As above, the relative extents of cleavage are shown as percentages. (**D**) Concentration dependence of spermidine inhibition of the cleavage reaction of the *trans(S2)* ribozyme induced by 10 mM Mg^{2+}. The data were fitted to a hyperbolic, bimolecular binding equation to give K_i of 2.6 ± 0.7 mM. The symbols k_{spd} and k_{obs} are first order cleavage rate constants determined in the presence and absence of spermidine, respectively.

folding of the global ribozyme structure. Since the observed concentration of Mg^{2+} at half-maximal cleavage rate ($K_{½}$) is ~4.5 mM (Fig. 3A), such competition by other divalent ions should be particularly evident in reactions occurring at 2 and 5 mM Mg^{2+} (Fig. 6). The effect is more pronounced with Cd^{2+}, Co^{2+} and Ni^{2+} than with Sr^{2+}, Ba^{2+} and Zn^{2+}. Such a classification of these ions into two groups, according to their higher and lower ability to compete with Mg^{2+}, is consistent with the observation that at high concentrations of Mg^{2+} (5, 10 and 25 mM) the extents of cleavage are very similar in the presence

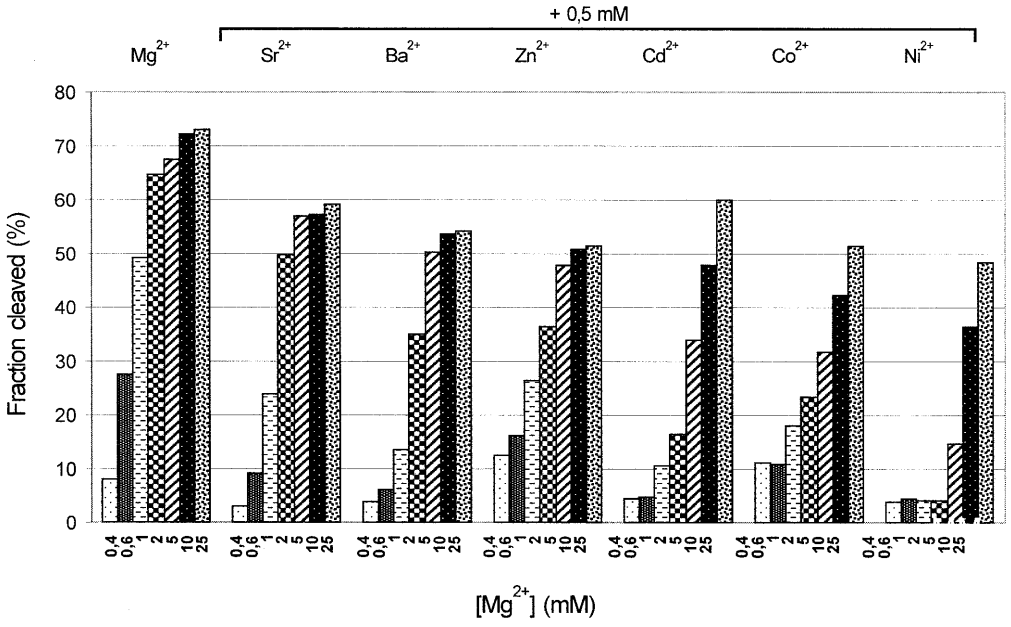

Figure 6. Inhibition of Mg^{2+}-induced cleavage of the *trans(S1)* ribozyme caused by a 0.5 mM concentration of selected divalent metal ions. The reactions were carried out in 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA buffer at 37°C for 5 min (for details see Materials and Methods).

of Sr^{2+}, Ba^{2+} and Zn^{2+}, but differentiated in the case of Cd^{2+}, Co^{2+} and Ni^{2+}.

DISCUSSION

Structural elements peripheral to the ribozyme active site influence cleavage rate and efficiency

Four variants of the antigenomic ribozyme (Fig. 1) show high cleavage activity in the presence of 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} (Fig. 2 and Table 1). The reactions induced by a particular metal ion occur, however, according to pseudo-first or higher order kinetics, with different rates, and result in different final cleavage extents. These differences are a consequence of structural changes introduced in the ribozyme regions not directly involved in formation of its catalytic core.

The P4 stem is present in its wild-type, long form only in the *cis(W)* variant. The variant undergoes fast self-cleavage in the presence of all three metal ions and the experimental data fit well to biphasic kinetics. However, after 30 min a relatively high percentage of molecules remains uncleaved. Several ribozymes closely related to the wild-type sequence behave similarly, showing a high tendency for conformational heterogeneity (2,4). Such behavior is not as evident in variants in which the P4 stem is shortened. We show that although cleavage of *cis(L)* also deviates from first order kinetics, the final cleavage extent reaches on average 80% in the presence of all three metals. This observation is consistent with some earlier data showing that other *cis*-acting ribozymes with short P4 stems were cleaved to a high percentage (26). On the other hand, some variants with the native or a slightly shortened P4 were cleaved well, but they were acting *in trans* (14,21,27). Therefore, we conclude that the native P4 stem contributes to incomplete cleavage of antigenomic ribozymes but only in ribozyme variants acting in *cis*. Possibly, in misfolded molecules the polypurine stretch $_{51}GGAG_{54}$ of P4 interacts with region $_{19}CCUC_{16}$, as we suggested earlier on the basis of structural studies of a 3′-truncated ribozyme (19).

It has been shown that separation of *cis*-acting delta ribozymes in the J1/2 region into the substrate and ribozyme strands results in a several-fold decrease in catalytic activity of *trans*-acting variants (4,21). In the case of *cis(L)*, on the one hand, and *trans(S1)* or *trans(S2)*, on the other, the corresponding rate constants k' and k_{obs} differ by a factor of 10–40 depending on the ribozyme variant and catalytic metal ion (Fig. 2 and Table 1). What is responsible for these differences is not clear. Interestingly, the catalytic activity of hammerhead ribozymes acting in *cis* or in *trans* does not differ substantially unless the substrate–ribozyme association step limits the reaction rate (28). The substrate–ribozyme association step is definitely not responsible for slower cleavage of *trans*-acting delta ribozymes since reactions are usually performed under single turnover conditions, ensuring binding of all substrate molecules in substrate–ribozyme complexes (16,21). It has been suggested that the active site in *trans*-acting ribozymes is less rigidly structured relative to that of *cis*-acting ribozymes, thus slowing the reaction (21). If this is the case, the observed rate constants might reflect an additional conformational change preceding the chemistry step of the cleavage reaction. One may speculate that conformational changes would be more difficult in the presence of Mg^{2+} than Ca^{2+} or Mn^{2+}, taking into account the different abilities of these ions to stabilize folded RNA structures. Indeed, the k_{obs} constants for the *trans(S1)* and *trans(S2)* ribozymes are lowest in the Mg^{2+}-induced reactions (Table 1).

An additional short duplex, in which a 3 nt sequence $_{11}GGC_{13}$ of J1/2 pairs with a sequence just outside the 3′-boundary of the ribozyme, may be formed in the *cis(L)*,

trans(S1) and *trans(S2)* ribozymes. It has been shown that an analogous duplex extended by a U10–A89 interaction, P2a, can both inhibit and enhance ribozyme activity depending on cleavage conditions (17,22). The *cis(L)* variant was tested in 100 mM NaCl, i.e. under conditions known to greatly enhance the activity of an antigenomic ribozyme with the P2a element present. Indeed, this variant shows exceptionally high activity not only with Mg^{2+}, Ca^{2+} and Mn^{2+} but also with other divalent ions, Co^{2+} and Sr^{2+} (Fig. 4B). At low Na^+ concentrations, as expected, cleavage activity was marginal (data not shown). The P2a element can also be formed in the *trans*-acting ribozymes, *trans(S1)* and *trans(S2)*. However, the presence of 200 mM NaCl did not stimulate cleavage of *trans(S1)*. On the contrary, it inhibited the reaction and the effect was particularly strong at low Mg^{2+} concentrations (Fig. 5A). Thus the P2a element plays a modulatory role only in *cis*-acting ribozyme variants. It has been suggested that P2a and P2 form a coaxial stacked helix, most likely roughly parallel to P1. The main effect of additional stabilization of this arrangement appears to be consistent with stabilization of both the inactive and active conformations and a slowing down of the conversion from misfolded to active forms (22).

It has been shown that sequences immediately upstream of the ribozyme cleavage site influence its catalytic activity. Closer examination (23) of the role of nucleotides –1 to –4 in efficient cleavage with a collection of small substrates that possessed single and multiple mutations in this region showed the optimal sequence to be $_{-1}$HRHY$_{-4}$ (H = U, C or A; R = purine; Y = pyrimidine). The best substrate contained the sequence $_{-1}$AAUC$_{-4}$ and was cleaved with a rate constant of 0.69 min^{-1} at 10 mM $MgCl_2$. Moreover, two consecutive pyrimidines at positions –1 and –2 were suggested to be responsible for the poor cleavage of substrates with the sequences $_{-1}$UCGG$_{-4}$ and $_{-1}$UUGG$_{-4}$ (23). Oligomers *S1* and *S2* used in our studies have the sequences $_{-1}$CGGG$_{-4}$ and $_{-1}$CUUC$_{-4}$ (Fig. 1). Thus both sequences differ from the optimal one, *S1* at positions –3 and –4 and *S2* at position –2, and *S2* contains two consecutive pyrimidines immediately adjacent to the cleavage site, which were suggested earlier to be detrimental to cleavage in other substrates. The oligomers are, however, cleaved well with k_{obs} of 0.92 for *S1* and 1.32 min^{-1} for *S2*, at 10 mM Mg^{2+} (Table 1). These values are higher than the rate constant of 0.69 min^{-1} observed by Perreault and co-workers (23) with the optimal substrate. In another *trans*-acting ribozyme the sequence $_{-1}$CUU$_{-3}$, with two pyrimidines at positions –1 and –2 preceding the cleavage site, the rate constant was in the range 0.7–0.9 min^{-1} (14,21). It seems, therefore, that sequential preferences for nucleotides –1 to –4 depend on the particular ribozyme variant used in the *trans*-acting system.

The role of divalent metal ions in the cleavage mechanism

It has been shown that the maximum rate constant for RNA cleavage of 0.022 min^{-1} can be achieved under strong alkaline conditions. Therefore, natural RNA-cleaving enzymes must make use of alternative or additional catalytic strategies that allow them to exceed the maximum rate constant permitted by general base catalysis alone (29). In two recently proposed mechanisms of self-cleavage the ability of the delta ribozymes to carry out a general acid–base catalysis has been assumed (7–9). The authors postulated an important role of a divalent metal ion in the reaction mechanism. The ion may act either directly as an ionized hydrate or indirectly by binding in the vicinity of the cleavage site and modulating the properties of its close environment.

Although the ribozyme variants used in our studies differ in their catalytic activities, with given variants acting either *in cis* or *in trans*, relatively small differences in the reactions induced by several metal ions have been observed. Despite the fact that the ions differ substantially in the ability of their hydrates to ionize, the pK_a values of metal hydrates are 11.4, 12.7 and 10.6 for Mg^{2+}, Ca^{2+} and Mn^{2+}, respectively (30). Strikingly, the cleavage rate constants k_{obs} determined for *trans(S2)* at a 10 mM concentration of divalent ions differ by not more than 2-fold and increase in the order $Mg^{2+} < Mn^{2+} < Ca^{2+}$ (Table 1 and Fig. 3A). In the case of both *cis*-acting variants the faster rate constants k' increase as follows: $Ca^{2+} < Mg^{2+} < Mn^{2+}$, consistent with an increasing concentration of ionized metal ion hydrates. The differences in cleavage rates are, however, far below expectation assuming a direct involvement of an ionized hydrate in catalysis. This is in contrast to hammerhead ribozyme cleavage in 10 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, for which the relative rates were ~1/16, 1 and 10, supporting the proposed role of the hydrate (31). Thus, in the antigenomic delta ribozymes the availability of an ionized metal ion hydrate, which has been proposed to act as the general base in the reaction mechanism (8), does not seem to be the rate-limiting step in ribozyme self-cleavage. Recently, it has been suggested that the overall rate-limiting step appears to be cleavage of the bond between the phosphorus and the 5′-leaving oxygen (32).

The metal ions used in our studies differ not only in pK_a values of their hydrates but also in binding preferences for nucleic acid ligands, phosphate, ribose and nucleic acid base residues. Similar cleavage rates in the presence of Mg^{2+} and Ca^{2+} and also Mn^{2+}, and for some ribozyme variants even with Co^{2+} and Sr^{2+}, suggest rather low specificity interactions in the metal ion-binding pockets. Presumably, the ions bind to phosphate or ribose residues and not to nucleic acid bases. Support for this conclusion comes from a well-characterized metal ion-binding pocket in the D-TΨC region of yeast $tRNA^{Phe}$ in which a Pb^{2+} ion binds directly to the nucleic acid bases U59 and C60 (33–35). Specific cleavage of the D loop at D16 is induced by an ionized Pb^{2+} hydrate and a transesterification mechanism. Some other ions, Mg^{2+}, Mn^{2+} and Eu^{3+}, also induce cleavage in this region. However, the number of cleavages, their relative intensities and positions are substantially changed, most likely because of different coordination properties of these metal ions (36–39).

Finally, we show that the inert $Co(NH_3)_6^{3+}$ complex is unable to support catalysis in the antigenomic delta ribozymes, acting either *in cis* or *in trans*, consistent with earlier reported data concerning a *cis*-acting genomic variant (8). These observations suggest inner sphere coordination of the active metal ion to the polynucleotide chain or that ionization of a ligand coordinated to the ion is required in catalysis. Plots of cleavage rates of the *trans(S2)* ribozyme on concentration of Mg^{2+}, Ca^{2+} and Mn^{2+} and Hill analysis of the data suggest that there are two active ions in the reactions induced by Mg^{2+} and Ca^{2+} but one ion in the case of Mn^{2+}. Similar analysis performed for a *cis*-acting genomic ribozyme (8) suggested only one Mg^{2+} ion involved in catalysis, but the significance of this difference is not clear.

Implications for further studies and possible application of hepatitis delta ribozymes

Catalytic activity of antigenomic delta ribozymes is strongly influenced by changes in the regions located outside the ribozyme catalytic core. Thus in detailed studies, particularly on the cleavage mechanism or the role of metal ions in catalysis, only data obtained with the same ribozyme variant can be directly compared. Since several ribozyme variants have been studied in different laboratories this point has to be stressed. On the other hand, certain ribozyme variants may be particularly well suited to solving specific problems. For example, the *cis(L)* ribozyme, which can be readily synthesized and is very active with a variety of metal ions, is currently being used in our laboratory in NAIM experiments designed to unravel the specific localization of the metal ions active in catalysis.

The delta ribozymes, as the only ribozymes naturally active in human cells, are potentially attractive tools in the strategy of directed RNA degradation. Cleavage of an mRNA in *trans* by an antigenomic delta ribozyme has been demonstrated, for the first time, with mRNA encoding the only protein of HDV virus, the delta antigen (40). The studies showed, however, that further characterization of the effects influencing ribozyme performance is needed before successful application of the delta ribozymes as therapeutic agents or useful biochemical tools. In the *trans*-acting mode, their cleavage activity is comparable to that of the hammerhead ribozyme but at low Mg^{2+} ion concentrations the delta ribozymes show the highest cleavage rates among all known ribozymes. We show, however, that at low concentrations of Mg^{2+} considerable inhibition of cleavage occurs in the presence of monovalent ions. Since most activity assays are usually performed in low salt conditions, ribozymes designed for practical applications should be tested at concentrations of divalent and monovalent ions relevant to physiological conditions. Moreover, stimulation of ribozyme activity by polyamines cannot be expected, in contrast to that observed with the hammerhead or hairpin ribozymes (41–44). Although the addition of extra 3′-terminal nucleotides may be detrimental for some ribozyme variants the effect occurs only with ribozymes acting *in cis*. Similarly, the native form of the P4 stem increases the fraction of misfolded ribozyme molecules that are resistant to cleavage only in *cis*-acting ribozyme variants. In ribozymes acting *in trans* the P4 stem could be used as an engineered element that would enable modulation of the activity or performance of the ribozyme. For example, in applicable variants it could govern their intracellular transport or interactions with protein cofactors.

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