Research Article

Visualizing the Higher Order Folding of a Catalytic RNA Molecule

DANIEL W. CELANDER AND THOMAS R. CECH

The higher order folding process of the catalytic RNA derived from the self-splicing intron of *Tetrahymena thermophila* was monitored with the use of Fe(II)-EDTA–induced free radical chemistry. The overall tertiary structure of the RNA molecule forms cooperatively with the uptake of at least three magnesium ions. Local folding transitions display different metal ion dependencies, suggesting that the RNA tertiary structure assembles through a specific folding intermediate before the catalytic core is formed. Enzymatic activity, assayed with an RNA substrate that is complementary to the catalytic RNA active site, coincides with the cooperative structural transition. The higher order RNA foldings produced by Mg(II), Ca(II), and Sr(II) are similar; however, only the Mg(II)-stabilized RNA is catalytically active. Thus, these results directly demonstrate that divalent metal ions participate in general folding of the ribozyme tertiary structure, and further indicate a more specific involvement of Mg(II) in catalysis.

CATALYTIC RNA'S (RIBOZYMES) COMPRISE A CLASS OF RNA molecules that accelerate phosphodiester cleavage and ligation reactions (*1*). For example, the L-21 Sca I RNA derived from the self-splicing intron of *Tetrahymena thermophila* has activity as a sequence-specific endonuclease (*2, 3*). The function of ribozymes, like that of proteins, relies upon formation of their correctly folded structures. Thus, there is an "RNA folding problem" akin to the much-studied "protein folding problem" (*4*): to identify the interactions that stabilize the final folded structure and to characterize the pathway or pathways of folding.

Unlike protein folding, the stable folding of nucleic acid structures is accompanied by juxtaposition of negatively charged phosphodiester groups and therefore requires positively charged counterions. Thus, it is not surprising that cations are necessary for ribozyme activity (*5, 6*) and also affect their specificity (*3, 7, 8*). More surprising is the limited repertoire of cations that can serve as cofactors for some ribozymes. In the case of the endoribonuclease activity of the L-21 Sca I RNA, only Mg(II) or Mn(II) can serve as the sole catalytic cofactor (*9*). However, the group IIa divalent metal ions Ca(II), Ba(II), and Sr(II) can reduce the Mg(II) or Mn(II) requirement. On the basis of such metal cation supplementation experiments, Grosshans and Cech (*9*) postulated that the *Tetrahymena* ribozyme has two distinct classes of metal ion binding sites: structural sites and those more directly involved in catalysis. Similar ideas had been described earlier for the catalytic RNA subunit of ribonuclease P (*6*).

In previous work, the involvement of counterions in ribozyme structure was not assessed from structural studies, but was inferred from studies of ribozyme activity. The reductive chemistry of Fe(II)-EDTA provides a method to directly evaluate tertiary structure formation at every position in a large RNA such as the L-21 Sca I ribozyme (*10, 11*). The combination of a dioxygen species and Fe(II)-EDTA initiates the production of free radicals, often considered to be hydroxyl radicals (*12, 13*), that cleave the sugar-phosphate backbone of DNA and RNA (*10, 12, 13*). Cleavage by this solvent-based reagent under our reaction conditions has little specificity for nucleic acid sequence or secondary structure (*14*), making it ideal as a probe for tertiary structure in RNA. In the case of the L-21 Sca I RNA, formation of a structure with a core resistant to Fe(II)-EDTA depends on Mg(II) ions (Fig. 1) (*10*). This cleavage-resistant core, including the contiguous regions of P3, P4, and P7 (*15*), corresponds to the catalytic center of the molecule (*1*).

As described in this article, the Fe(II)-EDTA cleavage reagent can be used to visualize the RNA folding process. By modulating the folding process with divalent metal ions, we gain new information about how metal ions participate in folding. Structure-function relations are derived by monitoring ribozyme-catalyzed cleavage of RNA substrates at the same concentrations of Mg(II). In the case of a "matched" RNA substrate [one complementary to the active site of the ribozyme (*3*)], cleavage is coincident with folding. In the cleavage of a "mismatched" RNA substrate, however, additional Mg(II) is required beyond that necessary for formation of the tertiary structure.

Visualization of RNA folding. Iron(II)-EDTA–catalyzed strand scission was performed on L-21 Sca I RNA incubated at 42°C in solutions containing different concentrations of divalent cation. The intensity of the cleavage pattern undergoes a sharp transition at about 0.75 mM $MgCl_2$ (Fig. 2). Below 0.75 mM $MgCl_2$, the pattern is similar to that observed with no added Mg(II), while at 0.75 mM and above, the pattern stabilizes into one of alternating regions of cleavage and protection like that observed previously at 10 mM $MgCl_2$ (*10*). Calcium(II) also promotes RNA tertiary structure formation (Fig. 2). As observed for the Mg(II) form of the RNA molecule, a sharp transition is evident in the Fe(II)-EDTA cleavage pattern as the $CaCl_2$ concentration is varied from 0 to 2.5 mM; however, the transition occurs at slightly higher cation concentrations (about 1 mM) for the Ca(II)-stabilized structure.

Mg(II) and Ca(II) are not the only counterions that permit tertiary structure formation. The ribozyme's tertiary structure also

The authors are with the Howard Hughes Medical Institute in the Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309–0215.

folds in the presence of 10 mM Sr(II) or in the presence of 0.5 mM Mg(II) in combination with either 1.0 mM Ca(II), 1.0 mM Sr(II), or 0.20 mM spermidine (*16*). The folded tertiary structure did not form under any of these conditions when 0.30 M NaCl was present (*16*). Neither the Na(I) ion (0.05 to 1.6 M) nor spermidine (0.02 to 0.20 mM) is sufficient to stabilize the tertiary structure (*16*), again suggesting a special role for a divalent cation.

Substantial tertiary structure is evident at −10°C through 22°C when the L-21 Sca I RNA is equilibrated in either 0.75 mM or 1.5 mM $MgCl_2$ buffers (Fig. 3). Above 22°C, a transition in RNA tertiary structure toward the unfolded form begins to occur for the RNA equilibrated in 0.75 mM $MgCl_2$ buffer; only the tertiary structure of the RNA equilibrated in 1.5 mM $MgCl_2$ buffer persists at 45°C. The metal ion requirement for formation of the folded tertiary structure of L-21 Sca I RNA is therefore temperature dependent, higher Mg(II) concentrations being required at higher temperatures.

Quantitation of the RNA folding process. As an indicator of tertiary structure formation, the extent of strand scission was determined at various positions (*17*) within the region bounded by structural elements P2.1 and P8. The cleavage pattern of L-21 Sca I RNA equilibrated at a given $MgCl_2$ concentration was determined relative to the cleavage observed at 0 mM $MgCl_2$ and then normalized to the amount of cleavage at 2.0 mM $MgCl_2$. No further change in the cleavage pattern occurred above 2.0 mM $MgCl_2$, and therefore the cleavage at 2.0 mM was taken to define the completely folded tertiary structure at 42°C.

Different folding trends were observed at different positions (Fig. 4). The vast majority of the single-position folding trends behave as would be expected for a two-state transition. That is, the "fraction folded" for a given position can be interpreted as the fraction of molecules that are in the final folded state. The folding properties of a small number of positions defy simple description of belonging to either the unfolded or folded state during the folding process; during the initial stages (generally between 0 and 0.1 mM $MgCl_2$), these positions attain negative values for "fraction folded." Negative values can arise when a position becomes initially accessible to cleavage before arriving at its final protected state, or when a position first becomes more protected before attaining a final accessible state.

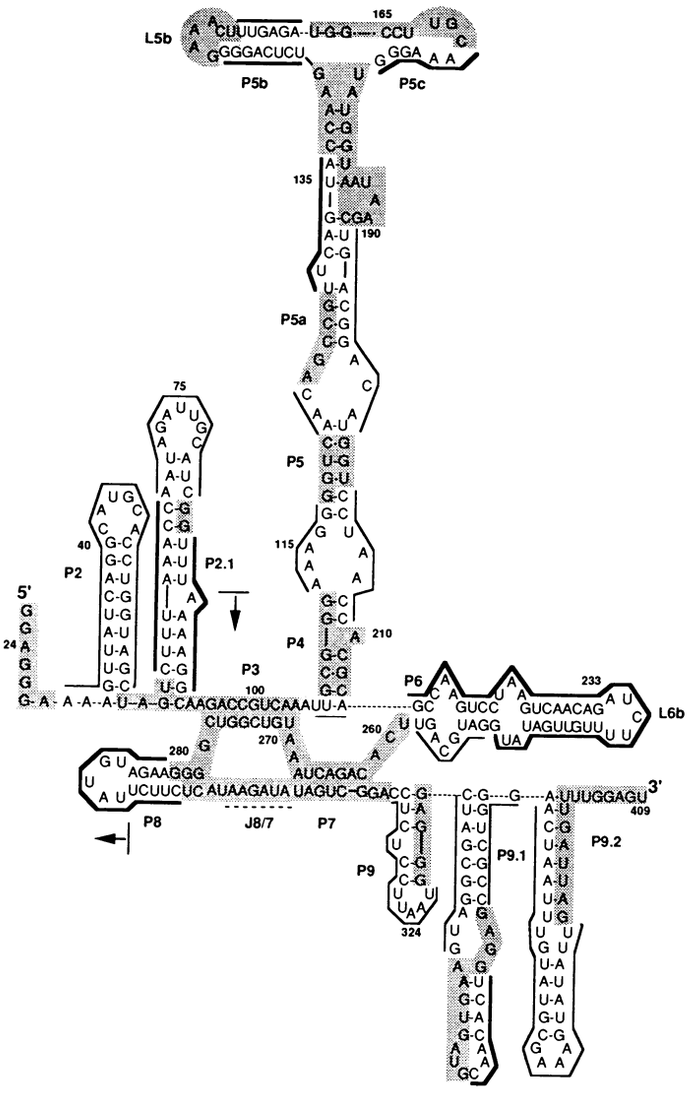

Fig. 1. The interior-exterior surface map determined by Fe(II)-EDTA cleavage superimposed onto the secondary structure of the L-21 Sca I RNA. The nucleotide positions of cleavage enhancement (overlined in bold) and protection (shaded) are indicated for RNA equilibrated in 10 mM $MgCl_2$ relative to no $MgCl_2$. The regions of RNA folding that were quantitated in the present study are bounded by the arrows indicated in P2.1 and P8 [P denotes a paired region (*15*)]. The dashed line shown under a portion of J8/7 [joining region between P8 and P7; nomenclature described in (*15*)] indicates the region exposed to Fe(II)-EDTA cleavage for RNA equilibrated in $CaCl_2$ or $SrCl_2$ (*16*). [Adapted from (*10*) with permission; the secondary structure from (*15*)]

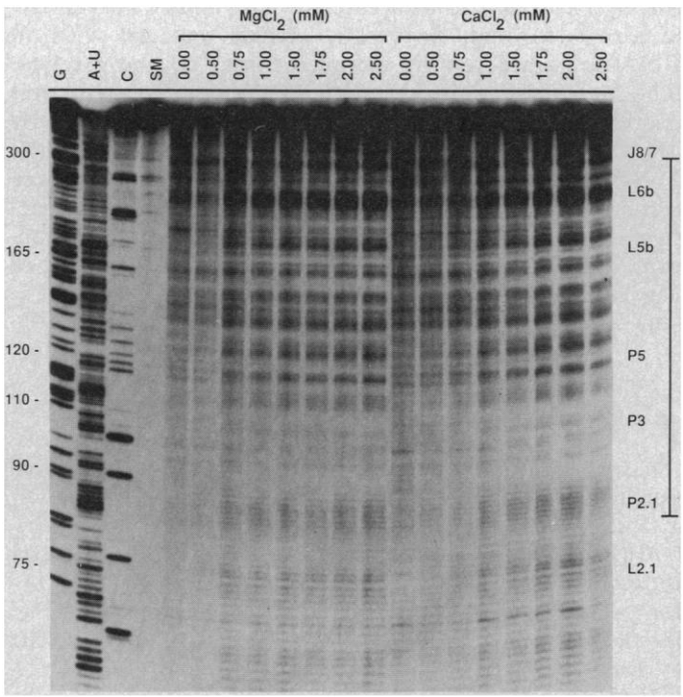

Fig. 2. Extent of L-21 Sca I RNA folding in increasing concentrations of $MgCl_2$ and $CaCl_2$. All cleavage reactions contained 5′ end-labeled [^{32}P]-RNA (10 to 20 nM) in 20 mM tris-HCl (pH 7.5), 1.1 mM Na_2EDTA, 1.0 mM $NH_4Fe(II)SO_4$, 5 mM dithiothreitol and were performed for 80 minutes at 42°C. The reactions were processed as described (*36*). Lane SM contained end-labeled RNA starting material incubated in 20 mM tris-HCl (pH 7.5) for 80 minutes at 42°C without Fe(II)-EDTA treatment. Control experiments revealed that very little breakdown of the starting material occurred when the RNA was incubated in buffers containing any of the metal ions at the concentrations indicated (*16*). The lanes denoted as G, A+U, and C contained sequencing standards generated by partial, base-specific cleavage of the RNA by the endonucleases T1, PhyM (*37*), and CL3 (*38*), respectively. Nucleotide positions, determined from these sequencing markers, are indicated on the left. The locations of some structural elements within the L-21 Sca I RNA molecule are indicated on the right; the vertical line delineates the boundaries of the quantitation reported for the Fe(II)-EDTA cleavage assays.

Table 1. Effect of ionic environment on L-21 Sca I RNA tertiary structure formation and cleavage of matched and mismatched RNA substrates.

Ionic environment*	Fraction folded†	k_{cat}/K_m (min^{-1} M^{-1})‡	
		$G_2CCCUCUCUA_5$	$G_2CCCGCUA_5$
1.5 mM $MgCl_2$	0.91 ± 0.17	$1.2 \times 10^7 \pm 0.2 \times 10^7$	$1.2 \times 10^6 \pm 0.02 \times 10^6$
0.5 mM $MgCl_2$	0.15 ± 0.03	$3.5 \times 10^6 \pm 0.9 \times 10^6$	$5.4 \times 10^4 \pm 0.1 \times 10^4$
0.5 mM $MgCl_2$ + 1.0 mM $CaCl_2$	0.88 ± 0.16	$1.1 \times 10^7 \pm 0.4 \times 10^7$	$3.2 \times 10^4 \pm 0.3 \times 10^4$
0.5 mM $MgCl_2$ + 1.0 mM $SrCl_2$	0.94 ± 0.18	$6.0 \times 10^6 \pm 0.2 \times 10^6$	$7.5 \times 10^4 \pm 1.2 \times 10^4$
0.5 mM $MgCl_2$ + 0.2 mM spermidine	0.85 ± 0.16	$2.4 \times 10^7 \pm 0.4 \times 10^7$	$6.4 \times 10^5 \pm 0.7 \times 10^4$
1.5 mM $MgCl_2$ + 300 mM NaCl	0.17 ± 0.03	≤6.7×10^3§	≤1.1×10^2§
2 mM $CaCl_2$	0.84 ± 0.16	≤6.7×10^3§	≤1.1×10^2§
10 mM $SrCl_2$	0.86 ± 0.16	≤6.7×10^3§	≤1.1×10^2§

*In addition to the defined buffer components, each assay contained 20 mM tris-HCl (pH 7.5) and was performed at 42°C. †Average fraction folded is as defined in the legend to Fig. 5. The L-21 Sca I RNA structure equilibrated in 2 mM $MgCl_2$ had a fraction folded value equal to 1.00. ‡The kinetic parameter k_{cat}/K_m was determined as described in the legend to Fig. 6. The L-21 Sca I RNA concentration used in cleavage assays performed on the matched RNA substrate, $G_2CCCUCUCUA_5$, was 5.0×10^{-9} M; in the case of the mismatched RNA substrate, $G_2CCCGCUA_5$, the ribozyme concentration was 3.0×10^{-7} M. §Upper limit based upon ≤1 percent total reaction in a 5-hour incubation.

Cooperativity of folding. The narrow structural transition observed with Mg(II) and Ca(II) illustrates qualitatively that the RNA folding process occurs as a steep function of divalent cation concentration. The overall extent of tertiary structure formation at any given $MgCl_2$ concentration can be described quantitatively as the fraction folded for each assessed position averaged for all positions. The overall folding process of the L-21 Sca I RNA follows a well-defined Mg(II) dependence over four independent Fe(II)-EDTA cleavage experiments (Fig. 5A). To determine the cooperativity of the folding transition, we performed a Hill analysis. The slope of the line in the region of mid-transition is described as the Hill coefficient and provides a measure of cooperativity, as well as a lower limit of the number of ions involved in the folding process. The Hill coefficient is 3.1 ($[Mg^{2+}]_{1/2} \approx 0.8$ mM) and indicates that at least three additional Mg(II) ions (beyond those already present in the unfolded state) are binding to promote the cooperative folding of the tertiary structure of the ribozyme (Fig. 5B).

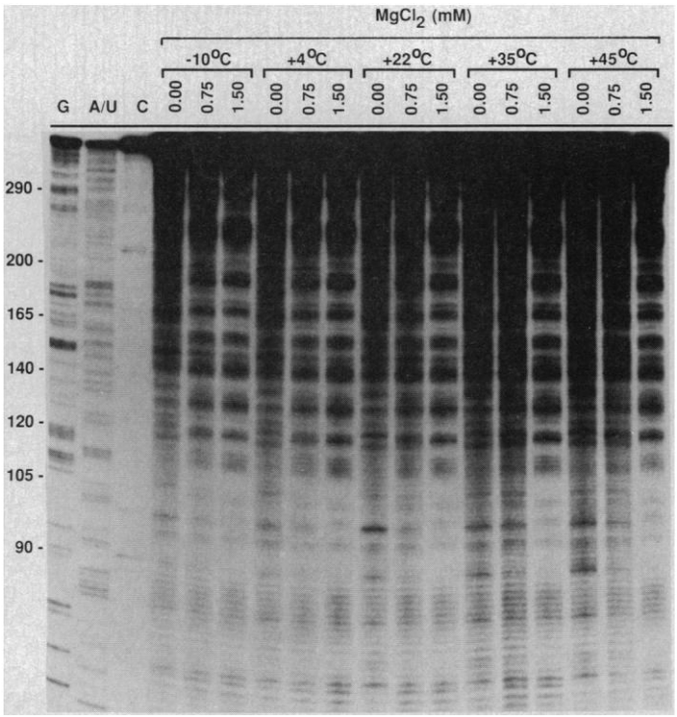

Fig. 3. Effect of temperature on $MgCl_2$-induced folding of the L-21 Sca I RNA. The reactions were performed as described (*39*).

The Hill analysis provides insight about the overall folding characteristics for the L-21 Sca I RNA. Examination of single-position folding characteristics, however, gives more specific information about the pathway of folding. The P5abc region of the molecule, represented here by nucleotide positions 145 to 168 (Fig. 5C), generally folds at lower Mg(II) concentrations than those observed for P3, P4, and P7 regions that comprise the catalytic core, as indicated by positions 100 to 115 and 212 to 280. On the basis of these folding characteristics, we suggest that the RNA tertiary structure assembles through the ordered folding of at least two structural domains. Confirmation and additional characterization of this proposed intermediate might be obtained with an independent structural probe.

The dependence of RNA folding on divalent cation concentration was compared for Mg(II), Ca(II), and Sr(II) (Fig. 5D). As evaluated from the midpoints of the transitions, Mg(II) and Ca(II) are more effective than Sr(II) in forming the RNA tertiary structure. The lower apparent plateau levels observed for folding the tertiary structures with Ca(II) and Sr(II) reflect the fact that these stabilized forms do not quite achieve the final extent of Fe(II)-EDTA cleavage and protection of the Mg(II)-stabilized form at the highest cation concentration tested.

RNA folding correlates with acquisition of catalytic activity. To determine the relation of the structural transition observed with

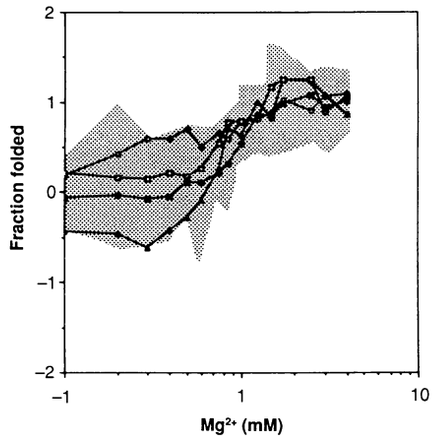

Fig. 4. The relative extent of RNA folding at individual quantitated positions as a function of $MgCl_2$ concentration. A value of fraction folded refers to the fraction of molecules (assuming a two-state model) for a given position that has achieved the final cleavage pattern observed when the RNA is equilibrated in 2.0 mM $MgCl_2$. More than 95 percent of the data fall into the diagrammed gray zone, indicating the range of data obtained for all positions at the assessed $MgCl_2$ concentrations, less the highest and lowest values obtained at each assessed $MgCl_2$ concentration. Indicated within the gray zone are some representative folding trends observed for several positions as a function of $MgCl_2$ concentration. Quantitation was achieved as described (*40*).

the Fe(II)-EDTA cleavage reagent to ribozyme function, the kinetic parameter k_{cat}/K_m was assessed for two different RNA substrates over the same range of $MgCl_2$ concentrations. This parameter was chosen because it represents the second-order rate constant for reaction of free enzyme and free substrate, and therefore includes the component (free enzyme) on which the structural studies were performed. Under k_{cat}/K_m conditions ([E] » [S];[E] « K_m), the measured value of k_{cat}/K_m reflects the concentration of correctly folded enzyme molecules that are competent to catalyze the RNA cleavage reaction.

The first RNA substrate, GGCCCUCUAAAAA, has perfect base-pairing complementarity with the internal guide sequence (*15, 18*) portion of the ribozyme active site (Fig. 6). In the presence of guanosine, this "matched" substrate is cleaved into the products, GGCCCUCU and GAAAAA. The ratio k_{cat}/K_m follows a dependence on Mg(II) concentration similar to that observed with the Fe(II)-EDTA structure probe (Fig. 6). The Hill coefficient of 2.7 (at $[Mg^{2+}]_{1/2} \approx 1$ mM) for the ribozyme activity is similar to that observed for the folding transition as assessed with the structure probe. The Mg(II)-stabilized tertiary structure of the L-21 Sca I RNA corresponds to a catalytically active conformation of the ribozyme.

Other conditions that permit tertiary structure formation of the L-21 Sca I RNA molecule also generate a catalytically active structure. Combinations of $MgCl_2$ with $CaCl_2$, $SrCl_2$, or spermidine permit both tertiary structure formation and catalytic function (Table 1). Calcium chloride is equivalent to a corresponding amount of $MgCl_2$ when supplementing reactions containing $MgCl_2$, whereas $SrCl_2$ appears less effective. Consistent with previous work with a different form of the *Tetrahymena* ribozyme (*8*), spermidine serves as an effective supplementary counterion in promoting catalytic activity. As was expected from the structural study, catalytic activity is diminished for the L-21 Sca I RNA incubated in the presence of 1.5 mM $MgCl_2$ and 0.3 M NaCl. In contrast to those structures that contain some Mg(II), the tertiary structures of the ribozyme stabilized by only Ca(II) or Sr(II) do not have measurable catalytic activity.

The ribozyme's ability to catalyze cleavage of another RNA substrate had a Mg(II) requirement different from that observed for ribozyme tertiary structure formation. The second substrate, GGCCCGCUAAAAA, forms a duplex with the internal guide sequence that contains a single-base mismatch at the position underlined (see Fig. 6). The reaction of the mismatched RNA substrate with the ribozyme in the presence of guanosine yields the products GGCCCGCU and GAAAAA. Ribozyme activity was tested with this RNA substrate as a function of $MgCl_2$ concentration under k_{cat}/K_m conditions (Fig. 6). Optimal ribozyme activity with the mismatched RNA substrate occurs at concentrations of $MgCl_2$ approximately ten times higher than that observed for ribozyme tertiary structure formation or for catalysis of an RNA substrate complementary to the ribozyme active site. A Hill analysis of the additional cofactor requirement indicates that at least one additional magnesium ion is required for catalysis of the mismatched RNA substrate (Hill coefficient of 1.2 at $[Mg^{2+}]_{1/2} \approx 8$ mM; the analysis excluded data with $[Mg^{2+}] < 1.75$ mM, where the ribozyme is still folding). Not all conditions that permit catalysis with the matched substrate are equally effective with the mismatched substrate; only spermidine can serve as a supplementary counterion with 0.50 mM $MgCl_2$ for

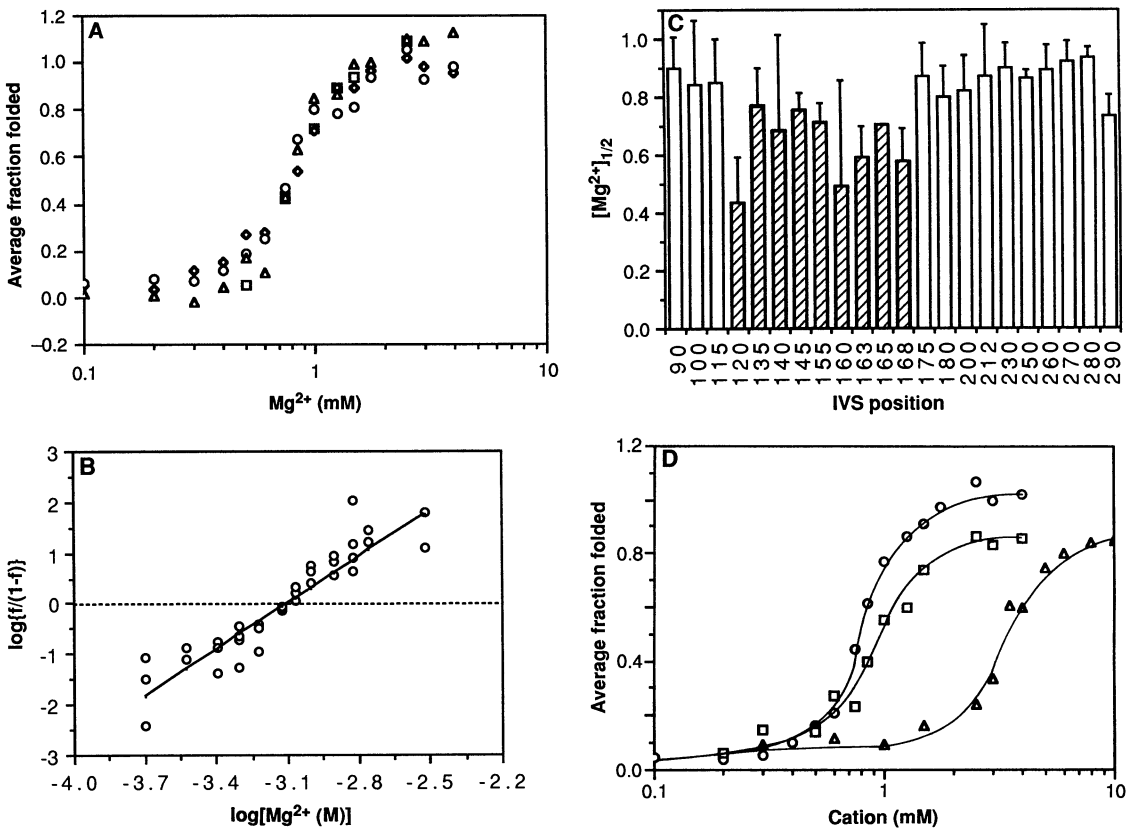

Fig. 5. The L-21 Sca I RNA folds in a cooperative manner. (**A**) The extent to which all assessed positions achieve the final folded state (expressed as the average fraction folded) as a function of $MgCl_2$ concentration is represented for four independent experiments (denoted by the different symbols). (**B**) Hill analysis of the data in (A). The plot describes the linear relation, $\log\{f/(1 - f)\} = n_H \log[Mg^{2+}] - \log K_d$ where the Hill coefficient (n_H) is the slope of the line at mid-transition; f (fraction folded averaged over all assessed positions) $= \Sigma F_{i,x}/\Sigma i$ where $F_{i,x}$ and i are as described (*40*); and K_d describes the equilibrium constant for the reaction: RNA·n$Mg^{2+} \rightleftharpoons$ RNA + nMg^{2+}. It is assumed that $[Mg^{2+}]_{free} \approx [Mg^{2+}]_{total}$. The uncertainty in measuring f was 19 percent. The line shown in (B) was calculated from a linear least-squares subroutine and has a slope of 3.1 ± 0.3 and an intercept of 9.5 ± 1.1. (**C**) The transition midpoint value of folding each assessed position and the associated standard variation (denoted by error bars) are indicated. The positions shown correspond to portions of the P5abc region (shaded bars) and the P3, P4, P6, P7, and P8 regions (open bars) as depicted in Fig. 1. (**D**) The dependence on the average fraction folded of RNA equilibrated in different concentrations of Mg(II) (circles), Ca(II) (squares), and Sr(II) (triangles). The data represent an average of three Ca(II) and Sr(II) and four Mg(II) independent Fe(II)-EDTA cleavage experiments. The average fraction folded is expressed relative to the 2.0 mM $MgCl_2$-stabilized structure in (A–D).

cleavage of the latter RNA substrate (Table 1).

Structural basis of ribozyme function. The Fe(II)-EDTA probe revealed that Mg(II), Ca(II), and Sr(II) could stabilize similarly folded RNA structures. In contrast, Na(I) cannot stabilize ribozyme tertiary structure, even at concentrations that provide equal stabilization of RNA secondary structure, as judged by thermal denaturation analysis (*19*). When Mg(II) is present, Ca(II) or Sr(II) can facilitate generation of an active conformation; however, ribozymes stabilized only by Ca(II) or Sr(II) are catalytically defective. Evidently, Mg(II) provides structural or catalytic roles (or both) that are not satisfied by Ca(II) and Sr(II). Subtle differences in tertiary structure of the L-21 Sca I RNA stabilized by the different divalent cations may account for the catalytic deficiency of the Ca(II) and Sr(II) forms of the RNA. In this regard, the 3′ portion of the joining region, J8/7, whose sequence is highly conserved among group I introns (*15*), is protected in the Mg(II) form of the RNA, whereas it is exposed in the RNA stabilized by Ca(II) or Sr(II) (Fig. 1) (*16*). In addition, the arrangement of catalytic center components within the interior could be affected by the type of divalent cation used to stabilize the global RNA conformation. Since the solvent-based cleavage reagent cannot gain access to the interior of the catalytic RNA (*10*), the probe could not detect such internal variations in RNA structure. In terms of a catalytic role in ribozyme function, Mg(II) might, for example, act as a Lewis acid in stabilizing negative charges that develop as the transesterification reaction proceeds through the transition state. Because of their lower charge densities compared to Mg(II), the divalent cations Ca(II) and Sr(II) might not stabilize the transition state as well.

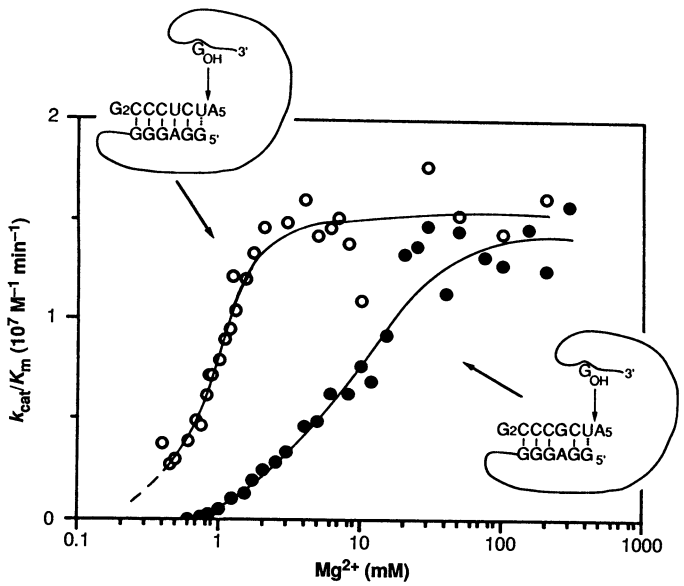

Fig. 6. Dependence of k_{cat}/K_m on $MgCl_2$ concentration for L-21 Sca I RNA catalyzed cleavage of a matched RNA substrate (open circles) and a mismatched RNA substrate (closed circles). The kinetic analysis was performed as described (*41*). Most data points represent an average of 2 to 4 independent determinations of k_{cat}/K_m at a given $MgCl_2$ concentration. The greatest range of variation in determination of k_{cat}/K_m was twofold and was observed for experiments done on separate days; the variation was less than 20% when k_{cat}/K_m was determined in duplicate on the same day with identical preparations of reagents. A model of L-21 Sca I RNA–substrate-guanosine ternary complex with either a perfect match RNA substrate or a single-base mismatch RNA substrate is shown adjacent to each activity relation. Each RNA substrate is shown base-paired with the internal guide sequence (5′-GGAGGG-3′) of the active site that is located within the interior of the ribozyme (*10*). The guanosine binding site in P7 (*46*) is also located within the interior of the ribozyme (*10*). Position of attack of the 3′-hydroxyl oxygen of the bound guanosine on each RNA substrate occurs at the phosphodiester bond indicated. The line connected to the internal guide sequence represents the remaining portions of the tertiary structure of the L-21 Sca I RNA.

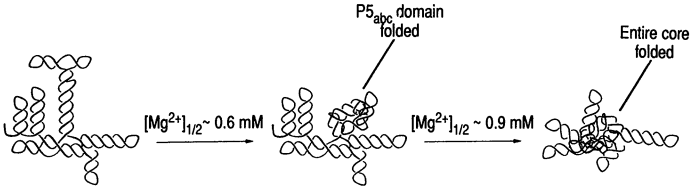

Fig. 7. A model describing the higher order folding of the L-21 Sca I ribozyme.

The role of specific ions in the stabilization of RNA tertiary structure is well established in the case of transfer RNA. Divalent and polyvalent cations bind to regions of the tRNA molecule where two or more portions of the phosphodiester backbone come into close proximity in the tertiary structure (*20*). The effect of these ion-phosphate interactions is to neutralize the repulsive negative charge density that develops in these regions, which in turn stabilizes the tertiary structure of the molecule. Divalent cations protect the tRNA tertiary structure from thermal denaturation (*21*), presumably by serving as the specific counterions in these regions of local, highly negative charge density. Similar functions may also arise in the L-21 Sca I RNA; however, in tRNA, tertiary structure can form in the absence of Mg(II) (*22*), whereas we observe that the native structure of the catalytic RNA, as judged by our assays, requires Mg(II). Therefore, Mg(II) may have a novel role in the ribozyme's folded structure. For example, an array of Mg(II) ions could be positioned to bridge phosphates on separate secondary structure elements, thereby producing a tightly packed catalytic center that may be essential for activity and also confer protection from Fe(II)-EDTA cleavage.

The L-21 Sca I RNA catalytic center is composed of a set of phylogenetically conserved secondary structures and specific sequences characteristic of the group I intron family (*15, 23*). Mutation of a number of sites in these introns impairs catalytic activity; however, activity can often be restored by increased $MgCl_2$ concentrations (*24, 25*). Metal ion complementation of such mutations suggests that most of these variants are defective in higher order RNA folding. This hypothesis can now be tested with the approach described here for the wild-type RNA.

Metal ions as determinants of ribozyme specificity. In contrast to the cleavage of the matched substrate, optimal ribozyme cleavage of the mismatched substrate is achieved at $MgCl_2$ concentrations ten times greater than required for RNA tertiary structure formation. Further, Mg(II) was the only divalent cation tested that could fulfill the additional metal ion requirement for optimal cleavage of the mismatched substrate. There is a potential practical application of these findings in the area of sequence-specific cleavage of RNA by *Tetrahymena* ribozymes: 2 mM $MgCl_2$, or even lower concentrations of $MgCl_2$ in combination with $CaCl_2$ or $SrCl_2$, should provide more specificity than is possible with 10 mM or 20 mM $MgCl_2$ alone.

The direct measurement of substrate RNA binding to the L-21 Sca I RNA has revealed that divalent cations influence substrate binding events at concentrations beyond that necessary for tertiary structure formation (*26*). A greater Mg(II) ion concentration was required for ribozyme binding to the mismatched substrate than to the matched substrate (*26*). The additional Mg(II) requirement in mismatched substrate cleavage described above may reflect the greater metal ion requirement for the binding of this substrate in the active site. The apparent selectivity of Mg(II) in serving as the additional cofactor in mismatch substrate cleavage may be ascribed to a substrate binding mode uniquely suited for an ion with the

radius and coordination geometry of Mg(II), but not Ca(II) nor Sr(II).

Cooperativity and a folding intermediate. The functional assays with the perfect-match substrate demonstrate that the catalytically active structure arises through cooperative interactions of several Mg(II) with RNA. Because a similar Mg(II) dependence was observed when ribozyme folding was monitored by Fe(II)-EDTA cleavage chemistry, we infer that several Mg(II) interact cooperatively with the RNA to promote the correct folding of the catalytic structure. Similar conclusions were derived earlier for one specific tertiary interaction that could be monitored by formation of a photochemical cross-link (*27*); the Fe(II)-EDTA reagent allows the entire molecule to be monitored. The overall folding transition can be adequately described by a two-state model in which an equilibrium exists between unfolded and folded structures.

In the case of protein folding, the success of the two-state approximation turned out to be compatible with intermediates, the existence of which are easily obscured by the high cooperativity of the folding transition (*28*). The two-state approximation for higher order folding likewise appears to be an oversimplification for the *Tetrahymena* ribozyme. Some local structures fold at metal ion concentrations different from that observed on average, an indication that the entire tertiary structure does not fold simultaneously. From consideration of the transition midpoint values for the assessed positions (Fig. 5C), we suggest that one dominant Mg(II)-induced folding pathway exists for the L-21 Sca I RNA, as illustrated in Fig. 7. At low Mg(II) ion concentrations (0.1 mM), we consider that much of the secondary structure is established with very little higher order structure evident (*29*). As the Mg(II) concentration is raised, the higher order structure of the P5abc portion of the central stem begins to fold prior to the bulk of the P3, P4, and P7 regions of the catalytic core, which folds at higher Mg(II) concentrations (Fig. 7) (*30*).

Other observations support the idea that the P5 central stem behaves as a distinct structural domain. Iron(II)-EDTA cleavage analysis indicates that this central stem can fold to some extent even when isolated from the remainder of the L-21 Sca I ribozyme (*31*). Furthermore, when added in trans, the central stem can functionally complement defective *Tetrahymena* ribozyme molecules from which this region has been deleted (*32*). Interactions of A183 with the third base pair of P4 may be involved in folding of this domain (*25*).

In addition to this postulated folding intermediate (at 0.6 mM $MgCl_2$), there is some indication that additional intermediates arise much earlier in the folding process. The evidence is the occurrence of negative values of F (fraction folded) measured for some positions at 0.10 mM Mg(II), relative to the initial reference state of 0 mM Mg(II).

Considering the phylogenetic conservation of group I intron structure and function, we anticipate that members of this family share not only similar tertiary structures (*33*) but also similar higher order RNA folding processes. Structural studies of higher order folding with other members of this family may therefore provide some additional insights about the nature of the folding process observed for the *Tetrahymena* ribozyme. The Fe(II)-EDTA cleavage reaction also promises to enable analysis of the higher order folding not only of other catalytic RNA's (*34*), but of structured RNA's in general (*35*).

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29. In Fig. 7, the secondary structure is shown as formed, even at the lowest Mg(II) ion concentration studied. While this postulate seems reasonable, given that monovalent cations contributed by the buffer should facilitate secondary structure formation, we have not tested it [see (*19*)].
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blue, 0.05 percent xylene cyanol containing either 40 mM D-glucose or 40 mM thiourea]. The products were fractionated on 10 percent polyacrylamide (acrylamide:bis-acrylamide, 19:1 or 29:1), 50 percent (w/v) urea gels.

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40. The relative extent of cleavage was determined by densitometric quantitation of films similar to that illustrated in Fig. 2 with an LKB enhanced laser densitometer. Total area integrations were obtained for 20 to 30 RNA positions for RNA cleavage reactions containing different concentrations of $MgCl_2$. Since analytical treatment of the data required reliable estimates of the unfolded state (defined by RNA equilibrated in 0 mM $MgCl_2$), an averaged area integral was obtained for each position with at least three independent cleavage reactions of RNA equilibrated in 0 mM $MgCl_2$. The relative extent of cleavage (E) at a position i for $MgCl_2$ concentration x is defined by the relation: $E_{i,x} = (I_{i,x})(\Sigma I_{i,0}/\Sigma I_{i,x}) - I_{i,0}$ where I is the area integral and $\Sigma I_{i,0}$ is the sum of area integrals for all assessed positions in a given reaction (in this case, 0 mM $MgCl_2$). This treatment corrects for differences in measured area integrations that may arise due to differences in lane shape or sample loading. Although this treatment does not correct for any differences in the total extent of cleavage, direct measurement of the extents of reactions with the use of an Ambis radioanalytic scanner indicated that reaction extents varied less than 5 to 10 percent among different lanes. The fraction folded (F) at position i for $MgCl_2$ concentration x is defined by the relation: $F_{i,x} = E_{i,x}/E_{i,2.0}$ where $E_{i,2.0}$ is the relative extent of cleavage observed at position i at 2.0 mM $MgCl_2$. Note that $E_{i,2.0}$ can be either positive or negative and, depending on the sign of $E_{i,x}$ relative to that of $E_{i,2.0}$, the value $F_{i,x}$ can be negative in some instances. The physical interpretation of a negative value for the fraction folded is described in the text.
41. Kinetic assays were performed under k_{cat}/K_m conditions after the initial equilibration of the L-21 Sca I RNA in 20 mM tris-HCl (pH 7.5) buffers containing the indicated concentration of $MgCl_2$ at 42°C. The guanosine concentration was 6×10^{-4} M, and the final substrate RNA concentrations were estimated to be 1×10^{-10} to 5×10^{-10} M. The concentration of L-21 Sca I RNA was 5×10^{-9} M in reactions with the matched RNA substrate and 3×10^{-8} M in reactions with the mismatched RNA substrate. These substrate and ribozyme concentrations are expected to be below the K_m for each RNA substrate under all assessed reaction conditions (*42*). The reaction was initiated by addition of 5′ end-labeled [^{32}P]RNA substrate (that had been incubated at 42°C) to the buffer solution containing L-21 Sca I RNA and guanosine. Samples (1 μl) were removed from the reactions at various times and quenched in 5 μl of 10 M urea, 0.5 × TBE, 20 to 75 mM Na_2EDTA. The labeled substrate and product RNA's were resolved on 20 percent polyacrylamide [acrylamide:bis-acrylamide, 29:1] gels. The gels were dried under vacuum, and the positions corresponding to labeled substrate and product RNA's were quantified with either a radioanalytic scanner (Ambis) or a phosphor-imager (Molecular Dynamics). The disappearance of substrate RNA was generally monitored for more than three half-lives.
42. At 50°C and 10 mM $MgCl_2$, the K_m's for the matched substrate and mismatched substrate under single-turnover conditions are 3.9×10^{-6} M and 1.25×10^{-5} M, respectively (*43*). K_m can be defined by the relation: $K_m = (k_{-1} + k_c)/k_1$ where k_{-1} is the rate constant for $E{\cdot}S \rightarrow E + S$, k_1 is the rate constant for $E + S \rightarrow E{\cdot}S$, and k_c is the rate constant for $E{\cdot}S \rightarrow E{\cdot}P$. From our present knowledge of how these rate constants are influenced by variations in temperature and magnesium ion concentration (*26, 43–45*), we expect that under our experimental conditions relative to those used previously (*43*), no greater than 3-fold and 30-fold variations in the K_m would occur for the matched and mismatched RNA substrates, respectively. Thus, we expect that the substrate concentrations used in the experiments of Fig. 6 are 100-fold below K_m for the matched substrate and 10- to 80-fold below K_m for the mismatched substrate. These parameters refer to the active population of ribozyme molecules in solution, and the analysis is based on the assumption that the unfolded fraction does not significantly alter catalysis by the active fraction.

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" You simply associate each number with a word, such as 'lipoprotein' and 3,467,009."