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Research Articles

Defining the Inside and Outside of a Catalytic RNA Molecule

JOHN A. LATHAM AND THOMAS R. CECH

Ribozymes are RNA molecules that catalyze biochemical reactions. Fe(II)-EDTA, a solvent-based reagent which cleaves both double- and single-stranded RNA, was used to investigate the structure of the *Tetrahymena* ribozyme. Regions of cleavage alternate with regions of substantial protection along the entire RNA molecule. In particular, most of the catalytic core shows greatly reduced cleavage. These data constitute experimental evidence that an RNA enzyme, like a protein enzyme, has an interior and an exterior. Determination of positions where the phosphodiester backbone of the RNA is on the inside or on the outside of the molecule provides major constraints for modeling the three-dimensional structure of the *Tetrahymena* ribozyme. This approach should be generally informative for structured RNA molecules.

RNA CATALYSIS, OBSERVED INITIALLY IN THE SELF-SPLICING of the precursor to the large ribosomal RNA (rRNA) of *Tetrahymena*, is not an isolated phenomenon (*1*). RNA self-splicing has been identified as a property of a number of other introns found in precursor RNA from both prokaryotes and eukaryotes, and ribonuclease P has been established as an enzyme with a catalytic subunit composed of RNA (*2*). In all cases, the structure of the RNA itself must form the catalytic center to perform precise RNA cleavage-ligation or hydrolysis reactions.

Recent work on a shortened form of the *Tetrahymena* intron, the L−21 Sca I RNA, has shown that this RNA is capable of mediating a variety of transesterification reactions (*3, 4*). The L−21 Sca I RNA has saturable binding sites for substrates and performs transesterification in a multiple turnover format identical to that of a classical enzyme.

There is, however, a striking difference between the L−21 Sca I RNA and classical protein enzymes. Proteins are assembled from amino acids whose side chains include both hydrophobic and hydrophilic functional groups. Nonpolar amino acids are usually in the interior of the catalyst where hydrophobic interactions are maximized, while the charged and polar amino acids are concentrated on the exterior and thus maximize interaction with the solvent. Catalytic RNA, like other RNA, consists of the four nucleotides. These do not have the structural diversity of amino acid side chains. The phosphates are anionic, and additional hydrophilicity comes from the sugar and base functional groups.

Like proteins, these ribozymes (RNA enzymes) require a specific structure for their biochemical activity. RNA secondary structure has been proposed by comparative sequence analysis of related group I introns, and confirmed by analysis of splicing defective mutations and second site suppressors that restore activity (*5, 6*). A

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model for the three-dimensional structure of the *Tetrahymena* intron was developed on the basis of conserved sequence elements found within the group I introns in combination with structure rules established with information derived from the crystal structure of phenylalanine transfer RNA ($tRNA^{Phe}$) (*7*). This model is only beginning to be tested experimentally.

One approach for investigating the three-dimensional structure of RNA involves the use of nucleic acid cleavage reagents. Dervan and co-workers (*8*) have developed affinity reagents that cause strand scission of the nucleic acid backbone. The metal-chelator EDTA is tethered to molecules capable of interacting with the nucleic acid through intercalation, hybridization, or groove binding (*9–11*). When mixed with the redox-active metal Fe(II), O_2, and reducing source, these EDTA-containing reagents generate a reactive oxygen species, either a hydroxyl radical or a ferryl-oxygen complex (*12*). Both are proposed intermediates on the Fe(II)-EDTA oxygen activation pathway, and either is capable of initiating oxidative degradation of the sugar in the backbone of the nucleic acid. Cleavage occurs close to where the redox-active metal complex is bound.

Tullius and co-workers have used this metal complex in a different form (*13*). In their studies, untethered Fe(II)-EDTA is used as a general cleavage reagent directed toward the nucleic acid backbone to probe for altered DNA structure, as in the case of kinetoplast DNA, or to localize protein-DNA contacts in the lambda repressor and Cro protein interaction with bacteriophage lambda DNA (*14*). They have argued that this untethered probe generates neutral hydroxyl radicals that can indiscriminately react with the sugar phosphate backbone to initiate strand scission as proposed by Hertzberg and Dervan (*9*).

We now describe experiments in which the *Tetrahymena* ribozyme is exposed to untethered Fe(II)-EDTA. This is an initial test to establish the higher order structure of this catalytic RNA in solution. In addition, experiments with yeast $tRNA^{Phe}$ lead us to conclude that this reagent should be generally applicable for studies of RNA structure.

Transfer RNA structure analysis. We hypothesized that tertiary structure of RNA would sterically exclude the solvent-based metal complex from any interior regions of the nucleic acid. This in turn would suppress strand scission in these areas, regardless of whether cleavage occurs by direct interaction of a ferryl-oxygen complex or by reaction of a diffusible but short-lived hydroxyl radical. To test this hypothesis, we assayed the cleavage technique on yeast $tRNA^{Phe}$, whose tertiary structure was established by x-ray crystal-

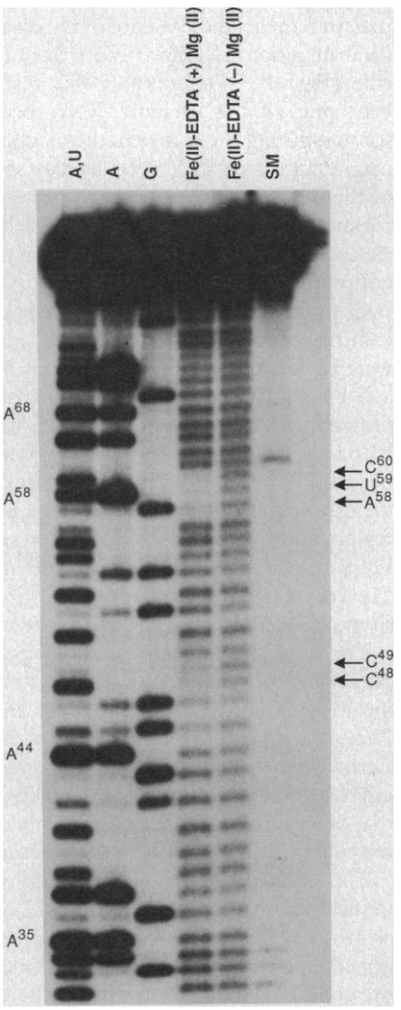

Fig. 1. Cleavage of synthetic $tRNA^{Phe}$ by Fe(II)-EDTA. Autoradiogram of a 10 percent polyacrylamide sequencing gel of synthetic yeast $tRNA^{Phe}$ labeled at the 5′ end with ^{32}P. A+U, A, and G are enzymatic sequencing lanes (*35*). Fe(II)-EDTA lanes contain cleavage reactions performed on 5′ end-labeled $tRNA^{Phe}$ in the presence and absence of Mg(II). Because of the different chemistries of Fe(II)-EDTA cleavage (which destroys the ribose) and nuclease cleavage (which leaves the nucleotide intact), the site where cleavage is mapped is offset one nucleotide in comparison to the sequencing ladder. The lane labeled SM is starting RNA incubated in buffer to control for background hydrolysis. Phage T7 RNA polymerase was used to synthesize tRNA (*36*). Although this synthetic tRNA molecule lacks the modified bases found in the natural tRNA, it is competent for charging by the cognate aminoacyl tRNA synthetase and has native structure as judged by Pb(II)-cleavage and ultraviolet (UV) crosslinking (*36, 37*). After purification, the tRNA was treated with calf intestinal phosphatase (NEN), extracted with phenol, and precipitated with ethanol. The dephosphorylated RNA was then 5′ end-labeled with [γ-^{32}P]ATP (ICN) and phage T4 polynucleotide kinase (U.S. Biochemical). Conditions for cleavage of $tRNA^{Phe}$ were as follows: 5′-[^{32}P]$tRNA^{Phe}$ (1×10^6 cpm) was incubated for 2 hours at 42°C in 50 m*M* tris, *p*H 7.5, 1 m*M* Fe(II), 2 m*M* EDTA, and 5 m*M* dithiothreitol, with and without 10 m*M* $MgCl_2$.

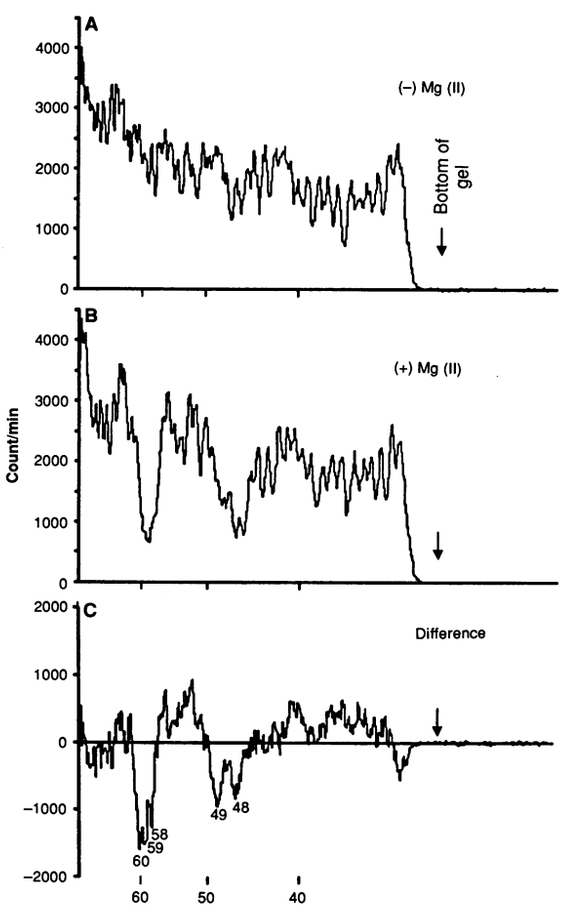

Fig. 2. Quantitation of Fe(II)-EDTA cleavage of 5′-[^{32}P]$tRNA^{Phe}$. Radioactivity of the polyacrylamide gel shown in Fig. 1 was quantitated with the use of the Ambis β particle detection system (Ambis System Inc., San Diego, CA). Each panel is corrected for background hydrolysis observed in the starting material and then normalized to adjust for loading differences. (**A**) Fe(II)-EDTA cleavage in the absence of $MgCl_2$. (**B**) Data for Fe(II)-EDTA cleavage in the presence of 10 m*M* $MgCl_2$. (**C**) The difference of panel B minus panel A. Negative peaks in this panel are regions of protection.

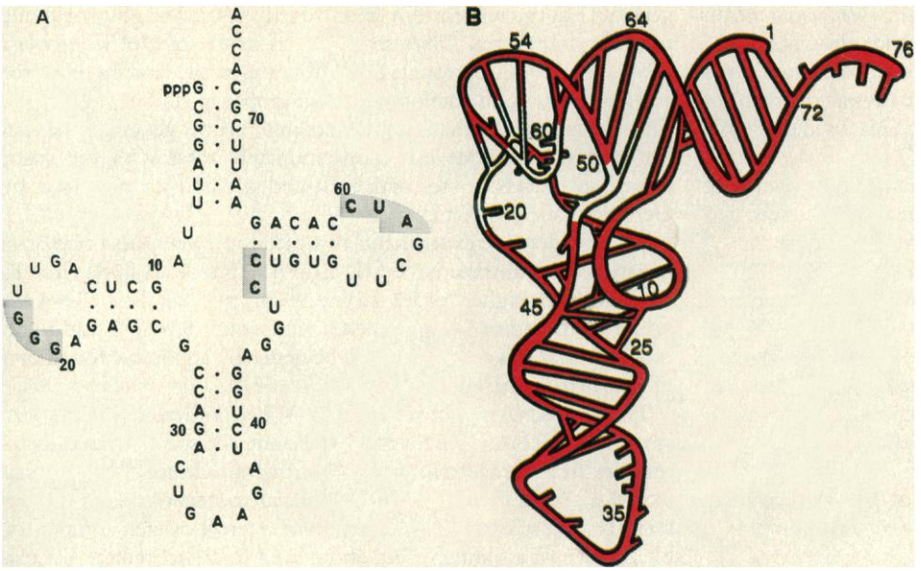

Fig. 3. tRNA^{Phe} cleavage data superimposed on the known secondary and tertiary structure of this molecule. (**A**) Shaded areas are regions of protection. The numbering system used is taken from Kim *et al.* (*15*). (**B**) Three-dimensional structure of tRNA^{Phe} (*15*). Colored backbone indicates cleavage, white backbone indicates protection. Cleavage was performed in the presence of Mg(II).

lography (*15*). Magnesium ion binds to loops and bends of the folded tRNA, thereby stabilizing the correct tertiary structure of the molecule (*16, 17*).

Yeast tRNA^{Phe} was subjected to cleavage with Fe(II)-EDTA in the presence and absence of magnesium ion (Fig. 1). In the absence of magnesium ion, cleavage occurs throughout the entire molecule. Addition of magnesium ion significantly suppresses cleavage in three specific areas. Quantification of the level of protection showed that cleavage is decreased four- to sixfold in these areas when compared to adjacent nucleotides (Fig. 2B). These regions have been identified as nucleotides 18 to 20, 48, 49, and 58 to 60 (Fig. 3) (*18*).

Earlier work on oxidative strand scission of nucleic acid has implicated the involvement of the 4′ hydrogen and possibly the 1′ hydrogen of the ribose sugar in the cleavage event (*19*). The accessible surface areas of the ribose 1′ and 4′ hydrogens of tRNA^{Phe} have been determined in order to establish the correlation with the observed Fe(II)-EDTA cleavage protection. We have found that residues with small 1′ and 4′ hydrogen surface areas are the residues where strong protection is observed. An earlier study by Romby *et al.* (*20*) on the chemical modification of tRNA^{Phe} with ethylnitrosourea corroborates our calculated and experimental results. Regions of greatest protection of phosphates from ethylnitrosourea are adjacent to the ribose sugars we find to be protected from Fe(II)-EDTA; in both cases, protection correlates with low backbone surface area.

These experiments indicate that lack of cleavage with Fe(II)-EDTA is due to inaccessibility caused by tertiary structure, which is only fully established and stabilized in the presence of magnesium. The results also establish the insensitivity of this reagent to secondary structure. Strand cleavage occurs in single- and double-stranded regions of RNA as evidenced by cleavage in the anticodon loop and both the acceptor and anticodon stems. Thus, this reagent requires only backbone accessibility for strand scission to occur and is not subject to the secondary structure constraints common to many other chemical probes of RNA structure (*21, 22*).

Catalytic RNA structure. Cleavage of the *Tetrahymena* ribozyme was studied with the same redox-active metal complex. The conditions in these experiments are identical to those used for catalysis by the RNA. Strand scission was assayed by high resolution polyacrylamide gel electrophoresis (Fig. 4). Distinct regions of strand cleavage alternate with extensive areas of protection.

Ribozyme activity has an absolute requirement for magnesium or manganese cation, implying that the correct three-dimensional structure requires metal for its formation and stabilization (*1–4, 23*). Like the tRNA data described above, the observed protection pattern requires the presence of the divalent cation. In the absence of divalent cation, cleavage occurs throughout the entire RNA molecule (Fig. 4). We interpret this result to mean that the entire backbone of the catalytic RNA becomes accessible for strand scission during the course of the incubation. Only in the presence of magnesium ion is the correct tertiary structure established, and areas of the RNA backbone are then protected. We have quantified the regions of protection with 5′ end-labeled RNA, and found that cleavage is suppressed up to tenfold in these areas relative to adjacent nonprotected areas (Fig. 5B).

Cleavage points in the L−21 Sca I RNA were mapped by comparison to standard sequencing ladders. The regions of cleavage were identified with the use of both 5′ end-labeled RNA (Fig. 4) and 3′ end-labeled RNA (*24*); extensive overlap in the data confirmed the cleavage locations. Points of strand scission are highlighted on the secondary structure diagram of Fig. 6. Four conserved helices, P3, P4, P6, and P7, act as core elements which help make up the catalytic center (*7, 25*). Cleavage is notably lacking in this core region, although P6 is subject to cleavage. P6a, P6b, and portions of P8, elements peripheral to this central core, exhibit strong cleavage. The central stem region composed of P4, P5, P5a, P5b, and P5c shows alternating cleavage and protection.

The strongest cleavage points mapped are restricted to a limited portion of the ribozyme (approximately 25 percent). These regions include P2.1; the 5′ portions of P5a and P6; P5b; the 3′ portion of P5c; P6a; P6b; P8; and the distal 3′ portion of P9.1 (Fig. 6). Certain duplex regions appear to be modified only on one side (for example, P5a, P5c, P9, P9.1, and P9.2); such asymmetry implies that one face of the helix contacts other portions of the intron while the other face is exposed to the exterior.

The cleavage points are mapped onto the three-dimensional model of Kim and Cech (*7*) in Fig. 7. This model includes the core elements envisioned to form the active site of the ribozyme but does not contain the peripheral structural elements shown in Fig. 6. P1 is the conserved helix generated by the base pairing of the substrate with the internal guide sequence of the intron; this duplex is used by the catalytic RNA to specify where phosphodiester cleavage occurs (*26*). The central regions proposed to form the site for P1 interaction are protected from cleavage, along with many of the helical components that make up this binding site. Cleavage is in large part

absent in the two extended helices formed by coaxial stacking of P4 with P6 and of P3 with P7. Clearly the structure shown in Fig. 7 could not by itself account for the extensive protection from cleavage. Structures not included in the model, such as P2, P2.1, and the central stem, must pack against the core to contribute to its protection.

Structural studies on a truncated ribozyme. Truncation of the *Tetrahymena* ribozyme at the Bgl II site (position 233, Fig. 6) instead of the Sca I site (position 409) gives an RNA that is catalytically inactive (*27, 28*). Both P3 and P6 are eliminated in this molecule because their 3′ sequence elements are not present. In addition, the phylogenetically conserved P7 and P9 helices are removed in their entirety.

This catalytically inactive RNA was studied to determine whether gross structure changes had taken place because of these deletions. Dramatic changes in the cleavage pattern relative to the L−21 Sca I RNA are observed (*24*). Regions proximal to and including P3, strongly protected in the L−21 Sca I RNA (Fig. 4), are now completely available for cleavage (*24*). The 5′ half of P3 along with the base portions of stems P2 and P2.1 are strongly cleaved. Little magnesium-dependent protection is detected in the 5′ portion of this truncated ribozyme. The structure of the central stem area (of which P4 is the base) retains much of the cleavage pattern it had in the L−21 Sca I RNA; the implication is that the structure present in this region is generated by the domain itself and that the core is not essential for its formation.

Thus using Fe(II)-EDTA, we have detected global structural changes in a truncated ribozyme that cannot be compensated by the remaining structural elements. In addition, we have identified a domain where structure is retained regardless of the status of the core.

Effect of binding site occupancy on Fe(II)-EDTA cleavage of the ribozyme. We have also used Fe(II)-EDTA to detect possible structural changes when known binding sites on the ribozyme are occupied. The oligonucleotide binding site, nucleotides 22 through 27 of the intron (Fig. 6), can be filled by the tight-binding inhibitor CCCUCU (*29*). Binding of the inhibitor forms the P1 helix (Fig. 7) (*5, 6, 26*). Cleavage experiments in the presence of CCCUCU show no detectable pattern changes (Fig. 8). Addition of guanosine, the required nucleoside substrate for this system, also leaves most of the cleavage pattern unchanged (Fig. 8). However, a change in cleavage is detected for nucleotide U^{75}. The level of cleavage of U^{75} increases when guanosine and the inhibitor are both added. The exact nature of this minor yet detectable structural change is not yet known.

Catalytic RNA has an inside and an outside. Protein enzymes, like other globular proteins, have tightly packed interiors. Nonpolar amino acid side chains are buried in the interior, minimizing contact with water, and form a hydrophobic core (*30*). The double helices in nucleic acid structure pack the planar bases into the middle of the molecule. On the outside exposed to solvent is the phosphodiester backbone. Because it is composed of anionic phosphates and polar sugars, the nucleic acid backbone seems poorly suited for higher order packing of the type found in proteins. Even the relatively compact tRNA has a much more extended structure than a typical

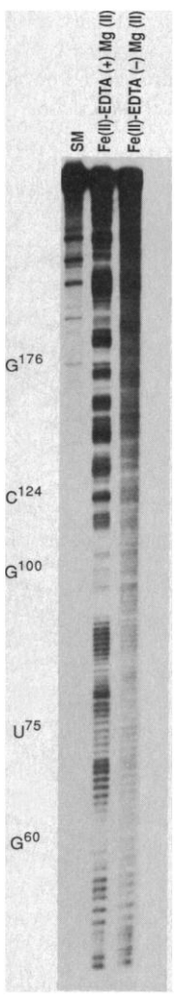

Fig. 4. Cleavage of the *Tetrahymena* ribozyme by Fe(II)-EDTA. Autoradiogram of a 6 percent polyacrylamide sequencing gel of L−21 Sca I RNA labeled at the 5′ end with ^{32}P. Fe(II)-EDTA lanes contain cleavage reactions in the presence and absence of $MgCl_2$ under the conditions of Fig. 1. The lane labeled SM is the starting 5′ end-labeled RNA incubated in buffer to control for background hydrolysis. L−21 Sca I RNA was prepared as described (*4*). Purified L−21 Sca I RNA was treated with calf intestinal phosphatase, extracted with phenol and precipitated with ethanol. This RNA was then 5′ end-labeled with [$\gamma^{32}P$]ATP and phage T4 polynucleotide kinase.

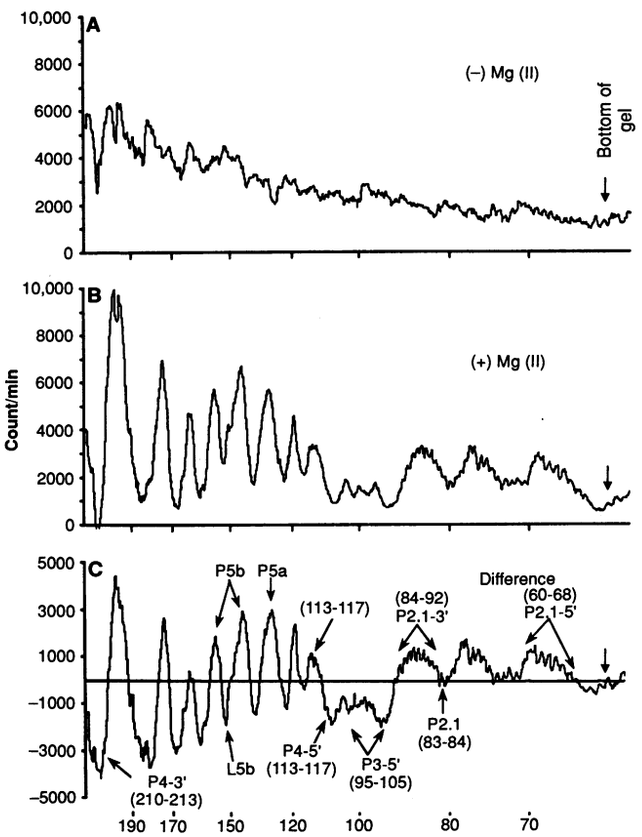

Fig. 5. Quantitation of Fe(II)-EDTA cleavage of L−21 Sca I RNA. Polyacrylamide gel shown in Fig. 4 was quantitated in the manner described in Fig. 2. (**A**) Fe(II)-EDTA cleavage of L−21 Sca I RNA in the absence of $MgCl_2$. (**B**) Fe(II)-EDTA cleavage in the presence of 10 m*M* $MgCl_2$. (**C**) The difference, panel B minus panel A, showing regions of protection (negative peaks) and regions of cleavage (positive peaks).

globular protein of the same molecular mass (*31*). In short, the polypeptide backbone can be on the inside or the outside of a protein, whereas one expects the backbone of a nucleic acid molecule to be on the outside accessible to its surroundings.

Given this background, it was not at all obvious that the catalytic RNA molecule studied here would have so much of its polynucleotide backbone protected from cleavage by a small solvent-based reagent. We interpret the protected regions as being inaccessible to Fe(II)-EDTA, or the activated oxygen species derived therefrom, because of the higher order folding of the RNA molecule (*32*). It is even possible that the protected regions are inaccessible to bulk solvent; however, Fe(II)-EDTA is substantially larger than a water molecule, and inaccessibility of the reagent should be sufficient to prevent cleavage independent of the entity performing strand scission (either a short-lived hydroxyl radical or a ferryl-oxygen complex). In either case, regional protection of the phosphodiester backbone from cleavage indicates a location on the interior of the molecule, as substantiated by the control experiments with tRNA^{Phe}. These data provide experimental evidence that a catalytic RNA molecule, like a protein, has an interior and an exterior.

The interactions that bury so much of the RNA backbone in the interior of the ribozyme are likely to be very different from the hydrophobic interactions that dominate protein folding. It has been argued that hydrophobic interactions do not contribute to the formation of RNA helices (*33*). Although we do not yet know the details of the interactions responsible for the formation of the interior structure of the ribozyme, there are several reasonable possibilities. Tertiary hydrogen-bonding interactions between bases, sugars, and phosphates of the sort found in tRNA could define much of the structure. Stacking interactions between the planar aromatic bases, essential for RNA helix formation, probably contribute to tertiary structure as well. Finally, magnesium ions, neutralizing the anionic phosphates and perhaps bridging helices, could allow the backbones of different helices to be packed close together.

Although the ribozyme and tRNA^{Phe} are both highly structured, we have observed a distinct difference in the overall level of cleavage protection between these two RNA forms. In the case of the catalytic RNA, the extent of protection in the presence of magnesium is higher than that observed for the tRNA under identical conditions (up to tenfold in the *Tetrahymena* case compared to sixfold with tRNA^{Phe}). In addition, the overall total number of nucleotides protected is significantly larger with the catalytic RNA. Approximately 40 percent of the total nucleotides in the *Tetrahymena* ribozyme are protected (Fig. 6) compared to 7 percent in the case of the tRNA (Fig. 3A). One possible explanation for these differences involves a distinctive format of helix packing in the catalytic RNA, which is not observed in the known tertiary structure of tRNA.

Structure of the *Tetrahymena* ribozyme. In addition to the general result that the *Tetrahymena* ribozyme has a definite interior

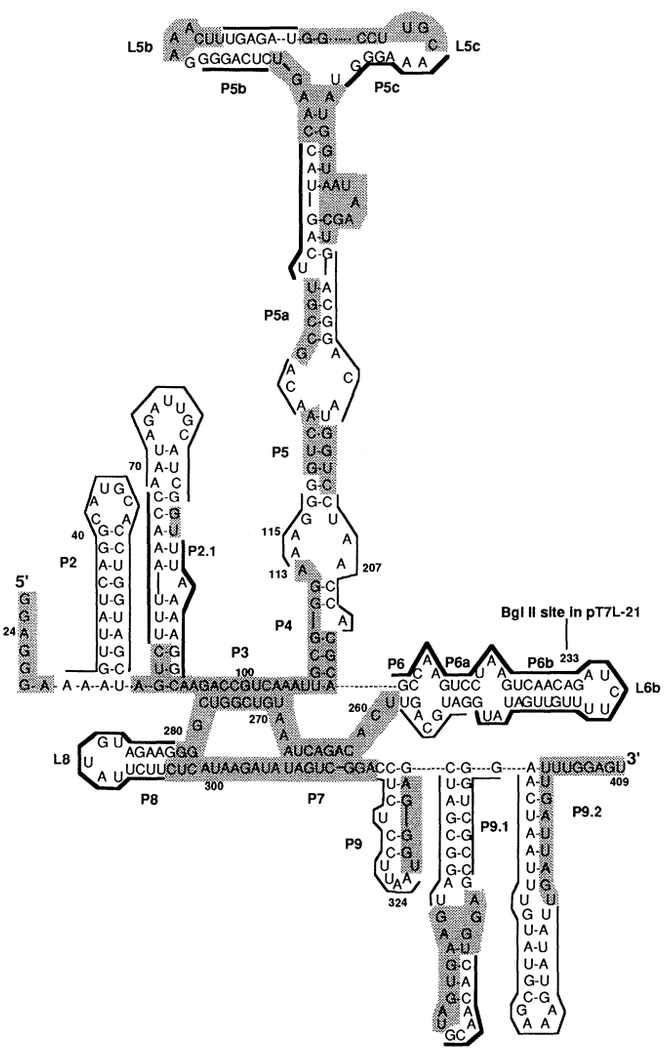

Fig. 6. Areas of protection of L−21 Sca I RNA from cleavage by Fe(II)-EDTA highlighted on the secondary structure diagram of the *Tetrahymena* group I intron (*5, 7, 25*). Shaded areas are those regions where strand scission is absent in the presence of magnesium ion. Solid lines indicate regions where cleavage is observed. Line intensity is proportional to cleavage intensity; thick line, heaviest cleavage; light line, moderate cleavage. Evaluation of the extent of cleavage is most uncertain for nucleotides at the very bottom of the gels (the first three nucleotides from the 5′ end and the ten nucleotides preceding the 3′ end).

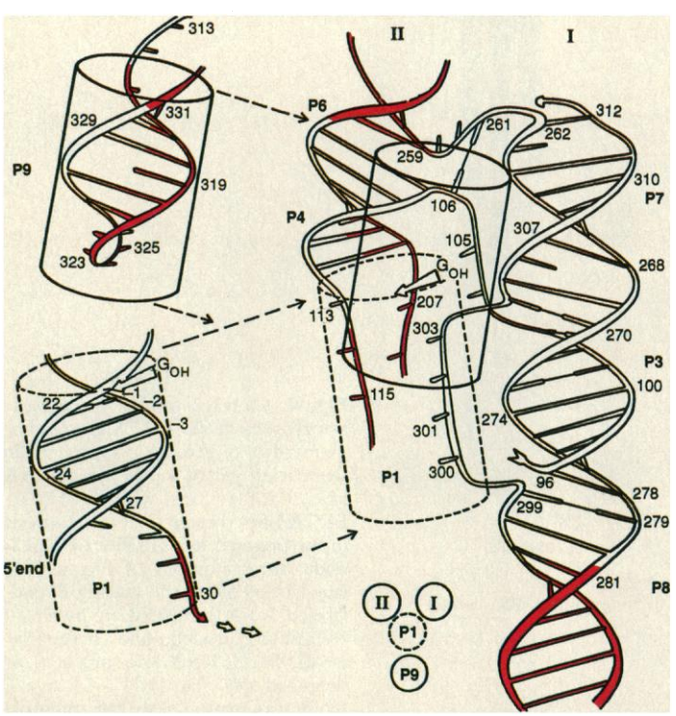

Fig. 7. Fe(II)-EDTA cleavage data for the *Tetrahymena* ribozyme superimposed on a provisional tertiary structure model of the catalytic core. Ribbon structure model is taken from Kim and Cech (7). White regions indicate areas of protection from cleavage by Fe(II)-EDTA in the presence of magnesium ion. Colored areas are regions where cleavage is observed.

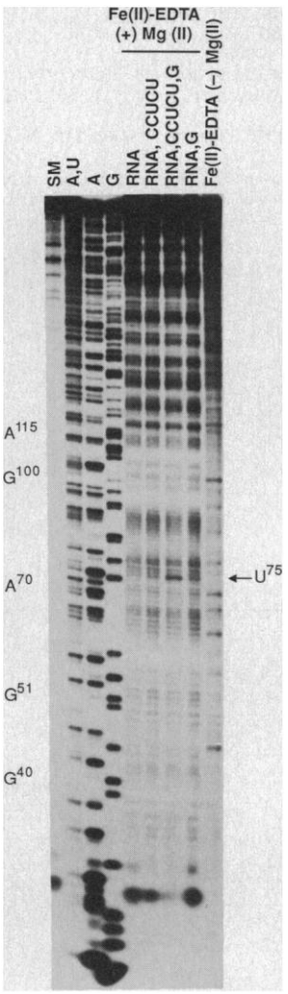

Fig. 8. Effect of binding site occupancy on Fe(II)-EDTA cleavage of L−21 Sca I RNA. Autoradiogram of a 6 percent polyacrylamide sequencing gel with 5′ end-labeled RNA as described in Fig. 4. Cleavage of L−21 Sca I RNA was performed as in Fig. 1 in the presence of 4 μ*M* pppGGCCCUCU (K_i = 4 n*M*) (*29*), pppGGCCCUCU plus guanosine (500 μ*M*), and guanosine alone. pppGGCCCUCU is abbreviated as CCCUCU, the portion of the oligonucleotide responsible for binding to the active site. All Fe(II)-EDTA cleavage reactions were performed in the presence of $MgCl_2$ except in the rightmost lane.

and exterior, the Fe(II)-EDTA cleavage pattern provides specific information about the structure of the molecule. The catalytic core composed of P3, P4, P6, and P7 is largely protected from cleavage. The protection observed is independent of the status of P1, whether it has formed by binding of the inhibitor CCCUCU or is absent when the experiment is performed without CCCUCU. Lack of cleavage in the core places these conserved elements in the interior of the ribozyme presumably surrounded by a close-packed layer of RNA structures. A strong candidate for a component of the outer layer is the central stem region composed of P4, P5, P5a, P5b, and P5c, which contains both protected and cleaved areas. Although this region of the ribozyme may appear to be an extended structural element from the secondary structure diagram (Fig. 6), it must be intimately involved in tertiary folding to account for the protected areas. In contrast, the uniform cleavage of P6a, P6b, and P8 indicates that they are extended into the solvent rather than being packed against the core.

Our cleavage data suggest an adjustment of the three-dimensional model. P9 needs to be rotated 90° from its position in the model of Fig. 7 to make its position consistent with observed strand scission. Rotation would place the modified face of this helix away from the central area, where catalysis is envisioned to take place, and redirect the modified portion of the helix toward the solvent, so that it is much more accessible for strand cleavage by Fe(II)-EDTA. Modification observed at the end of P4 and in the adjacent single-strand regions (nucleotides 114 to 116 and 206 to 209) is consistent with the current three-dimensional model. At these cleavage points, the sugar phosphate backbone is directed away from the cavity formed by the coaxially stacked helices and therefore could be accessible for cleavage, as has been observed.

The Fe(II)-EDTA data are in general agreement with previous experiments performed on the ribozyme with chemical and enzymatic structure probes (*22, 34*). Single- and double-strand specific nucleases are envisioned to pose two requirements in order to act on the RNA: (i) the correct recognition structure or sequence must be present, and (ii) the target structure or sequence must be physically accessible to the nuclease. The data from nuclease treatment (the sum of the single-strand and double-strand specific cleavage patterns) and Fe(II)-EDTA strand scission overlap well in both location and relative intensity. The agreement between these two independent methods argues strongly for the contribution of accessibility to strand scission observed with Fe(II)-EDTA. It strengthens our conclusion that this chemical probe is acting on regions of the RNA that are exposed and likely to be on the exterior surface. The increase in total cleavage in the case of Fe(II)-EDTA, when compared to the nucleases, can be attributed to the small size and low specificity of the former reagent. Fe(II)-EDTA is substantially smaller than the endonucleases, and therefore would be expected to penetrate further into interior areas of the RNA molecule. In addition, unlike the endonucleases, the operation of this chemical probe appears to be independent of RNA secondary structure.

Our data highlight the generality of Fe(II)-EDTA for studying the structure of large RNA molecules. This metal complex has been successfully applied for structure analysis of two RNA molecules, synthetic $tRNA^{Phe}$ and the *Tetrahymena* ribozyme, both of which have extensive tertiary structure. The reagent is straightforward to use and should be widely applicable for investigation of tertiary structure in all forms of RNA.

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32. An alternative interpretation might be that the cleavage reaction requires movement of the ribose sugar, and that the protected regions are the areas most rigidly held by the structure. Given the excellent correlation between accessible surface area and reactivity with chemical modification reagents in the case of $tRNA^{Phe}$ [S. R. Holbrook and S.-H. Kim, *Biopolymers* **12**, 1145 (1983)], we prefer the interpretation that accessibility rather than rigidity is the major determinant of the Fe(II)-EDTA reaction.
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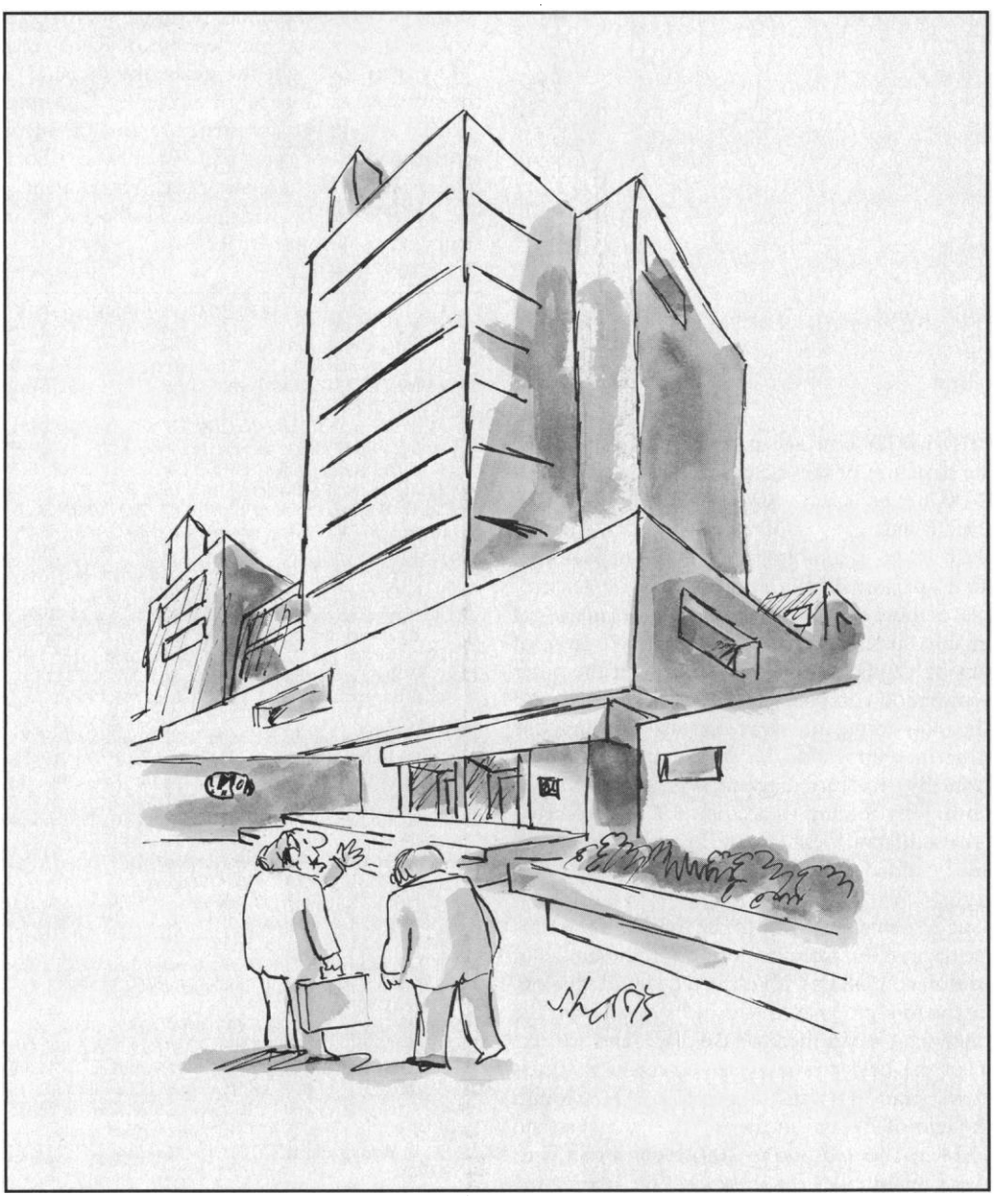

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