A Labile Phosphodiester Bond at the Ligation Junction in a Circular Intervening Sequence RNA

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Abstract. *The excised intervening sequence of the* Tetrahymena *ribosomal RNA precursor mediates its own covalent cyclization in the absence of any protein. The circular molecule undergoes slow reopening at a single phosphodiester bond, the one that was formed during cyclization. The resulting linear molecule has 5′-phosphate and 3′-hydroxyl termini; these are unusual products for RNA hydrolysis but are typical of the other reactions mediated by this molecule. The reopened circle retains cleavage-ligation activity, as evidenced by its ability to undergo another round of cyclization and reopening. The finding that an RNA molecule can be folded so that a specific phosphate can be strained or activated helps to explain how the activation energy is lowered for RNA self-splicing. The proposed mechanisms may be relevant to several other RNA cleavage reactions that are RNA-mediated.*

In some species of the ciliated protozoan *Tetrahymena*, an intervening sequence (IVS) of about 400 base pairs (bp) interrupts the gene for the large ribosomal RNA (*1*). An early step in maturation of the ribosomal RNA is RNA splicing, which involves cleavage of the RNA at the ends of the intervening sequence and ligation of the adjacent coding sequences (*2*). Splicing requires a free guanosine molecule that is linked to the 5′ end of the IVS RNA during excision (*3, 4*). The IVS RNA is subsequently converted into a covalently closed circular form (*5*). Cyclization is a cleavage-ligation reaction. It involves joining the 3′-hydroxyl terminus of the linear form to the phosphate at position 16 in the chain, with concomitant release of an oligonucleotide containing the first 15 nucleotides of the molecule (*6*).

These RNA splicing and cyclization reactions occur in vitro in the absence of any protein (*6, 7*). The folded RNA molecule provides both the cleavage-ligation activity and the RNA substrates (the internucleotide linkages that are cleaved and joined). Each of these reactions appears to occur by a transesterification (phosphoester transfer) mechanism (*6, 7*). Having bond formation always linked to bond breakage explains the lack of a requirement for an external energy source such as adenosine triphosphate (ATP).

Understanding the thermodynamics of the reaction does not tell us how the activation energy is lowered to facilitate the reaction at a specific site. Binding of the guanosine substrate to a specific site on the RNA, presumably with its 3′-hydroxyl group in proximity to the phosphate at the splice site, provides at least a partial solution to the activation energy problem for the initial splicing reaction (*8*). We now find that the structure of the circular IVS RNA molecule makes one of its 399 phosphodiester bonds particularly susceptible to hydrolysis. The hydrolysis reaction resembles a reverse cyclization reaction. On the basis of these results, we suggest that enhancement of the reactivity of the phosphate to be attacked is another way in which the activation energy is lowered for transesterification.

Circular IVS RNA reopens at the ligation junction. When purified linear IVS RNA is incubated under cyclization conditions for a time longer than that required to achieve cyclization, an RNA molecule about 15 nucleotides (nt) shorter than the original L IVS (*9*) begins to accumulate (*6*) (Fig. 1A). We have proposed that this "L–15" RNA species might be nicked C IVS RNA or it may be an abortive cyclization product in which the 15-nt fragment was released without cyclization occurring (*6*). A version of the first possibility is correct. As is described below, the L–15 RNA is derived from the C IVS. However, the specificity of the reaction is such that it is more accurately termed "circle reopening" than nicking.

When purified C IVS RNA was incubated at 42°C in a solution buffered at *p*H 7.5, it was converted to L–15 RNA (Fig. 1A) with a half-life ($t_{1/2}$) of 450 minutes. The reaction required Mg^{2+}. Dideoxynucleotide sequence analysis (Fig. 1B) indicated that the L–15 had a specific 5′ end—namely, nucleotide 16 in the sequence of the L IVS. Therefore, the L–15 RNA was a product of precise reopening of the C IVS at the phosphodiester bond that was formed during cyclization. The specific 5′ end of the L–15 RNA formed under these conditions contrasts with the random ends of the nicked circles produced by limited hydrolysis of C IVS RNA at 95°C at either *p*H 9.0 (data not shown) or *p*H 7.5 (discussed below).

At *p*H 9.0, the C IVS RNA was converted to a linear form at a much faster rate, $t_{1/2} \approx 8$ minutes (Fig. 1C). Dideoxynucleotide sequence analysis gave an unexpected result in that the final product had a 5′ end at nucleotide 20 (Fig. 1D) and was therefore an L–19 species. The L–15 species was seen early in the reaction. The kinetics of its formation and disappearance (Fig. 1E) are consistent with its being an intermediate in the production of L–19 RNA. The reaction proceeds to give the L–19 RNA at *p*H 7.5 as well, but the reactions are so slow that the L–15 RNA is still the predominant linear species after 1 hour.

The structure of the 5′ and 3′ ends of the L–15 and L–19 RNA's was analyzed by digestion with ribonuclease T1 followed by two-dimensional (chromatography and electrophoresis) mapping of the oligonucleotides (fingerprinting). The L–15 RNA oligonucleotide maps (data not shown) contained two spots not found in the pattern of the C IVS RNA. One of these was identical to the normal 3′-terminal oligonucleotide of the L IVS RNA, UACUCG-OH (*4*) (U, uridine; A, adenine; C, cytidine; G, guanosine). The other, which migrated faster than spot 41 (*4*) in the electrophoretic dimension, was identified as pACCUUUGp (nucleotides 16 to 22 of the IVS). With RNA labeled with [α-^{32}P]ATP, secondary digestion of this spot with ribonuclease T2 yielded pAp as the only labeled product. Thus, the L–15 RNA has 5′-phosphate and 3′-

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hydroxyl termini. The L–19 RNA oligonucleotide maps also contained the 3′-terminal UACUCG-OH (Fig. 2). In addition, a new spot 40A appeared on the nucleotide maps of L–19 RNA labeled with [α-^{32}P]uridine triphosphate (UTP) (Fig. 2) or [α-^{32}P]guanosine triphosphate (GTP), but was absent in the nucleotide patterns of RNA labeled with ATP or CTP (cytidine triphosphate) (data not shown). Ribonuclease T2 digestion of UTP-labeled spot 40A produced only labeled pUp; digestion of GTP-labeled spot 40A produced equal amounts of labeled Up and Gp. Thus, the spot 40A oligonucleotide has the sequence pUpUpGp (nucleotides 20 to 22 of the IVS) and is followed by a G. The L–19 RNA has 5′-phosphate and 3′-hydroxyl termini.

A minor site of cyclization of linear IVS RNA. The major L IVS RNA cyclization reaction involves attack at A^{16} and release of a 15-nt fragment (*6*). Recently we have found that a minor portion of the cyclization takes place at U^{20} with release of a 19-nt fragment (*10*). Thus the nucleotide that forms the 5′ end of the L–19 RNA is also a minor site of cyclization of the L IVS RNA (Fig. 4). The relative amount of 15-nt and 19-nt fragments depends on the cyclization conditions in vitro. With reactions carried out at 42°C in 10 m*M* $MgCl_2$, the proportion of 19-nt fragment (calculated as the ratio of the 19-nt fragment to the sum of the 19-nt plus the 15-nt fragments) is 0.04 at *p*H 7.5, and is 0.20 at *p*H 9.0 (*10*).

The reopened circle undergoes another round of ligation and reopening. The L–19 RNA contains four fewer nucleotides than the C IVS from which it is produced. In what form are these nucleotides released? The C IVS RNA was incubated at *p*H 9.0, and the reaction products were analyzed by 20 percent polyacrylamide, 8*M* urea gel electrophoresis. The formation of L–19 RNA was accompanied by the release of a single 4-nt fragment (Fig. 3B). This oligonucleotide was purified, subjected to secondary digestion, and identified as pApCpCpU-OH (*11*). Thus, nucleotides 16 to 19 are released in a single endonucleolytic step.

At what stage in the reaction is the tetranucleotide released? Since the L–15 RNA appeared to be an intermediate in the production of L–19 RNA and the phosphate between nucleotides 19 and 20 was a minor site of cyclization of the L IVS RNA, it seemed likely that the tetranucleotide was released during cyclization of the L–15 RNA at the nucleotides 19–20 linkage. Reopening of this

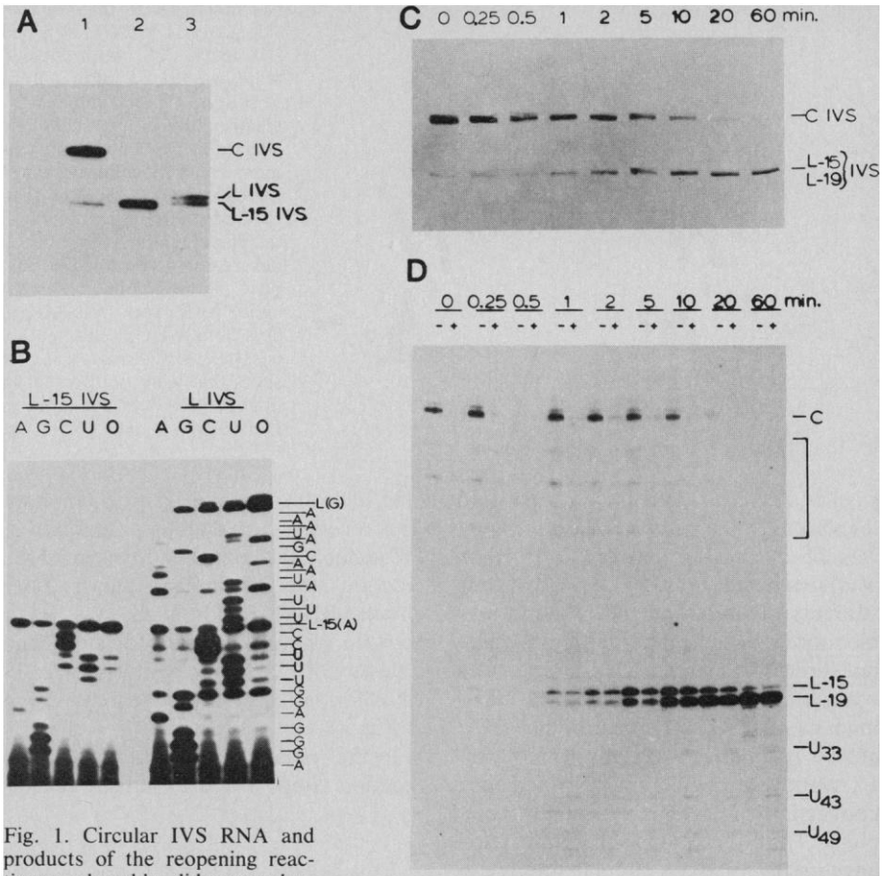


Fig. 1. Circular IVS RNA and products of the reopening reactions analyzed by dideoxynucleotide sequencing (*24*) and primer extension (*25*). (A) Autoradiogram of ^{32}P-labeled IVS RNA after electrophoresis in a 4 percent polyacrylamide, 8*M* urea gel (*3*). (Lane 1) Circular IVS RNA, containing a small amount of reopened circle (L–15). [The C and L–15 species contain the same number of nucleotides, but the conformation of the circular form causes it to be retarded during electrophoresis in a denaturing gel (*5*).] (Lane 2) L–15 RNA; (lane 3) a mixture of linear IVS RNA and L–15 RNA. (B) Dideoxynucleotide sequence analysis (*24*) of (left-half) L–15 RNA and (right-half) L IVS RNA. A 5′ end-labeled, 14-base deoxyoligonucleotide, complementary to nucleotides 31 to 45 of the IVS (*26*), was hybridized to the RNA. AMV reverse transcriptase was then used to extend the primer for 15 minutes at 37°C in the presence of all four dNTP's (deoxynucleoside triphosphates; 400 μ*M* each) and a single ddNTP (dideoxynucleoside triphosphate; 200 μ*M*). A, G, C, and U indicate the nucleotide in the RNA, that is, the nucleotide complementary to the ddNTP added. (O) No ddNTP added. Electrophoresis was in a 12 percent polyacrylamide, 8*M* urea sequencing gel. (C) C IVS RNA, uniformly labeled with [^{3}H]UTP during transcription, was incubated in 10 m*M* $MgCl_2$, 50 m*M* CHES, *p*H 9.0 at 42°C. At the indicated time, the reaction was stopped on ice by the addition of EDTA (25 m*M* final concentration). The conversion of circular to linear RNA was assayed by electrophoresis in a 4 percent polyacrylamide, 8*M* urea gel followed by tritium fluorography. (D) Primer extension analysis of the same samples shown in (C). A 17-base deoxyoligonucleotide, complementary to nucleotides 96 to 112 of the IVS, was hybridized to the RNA and extended by AMV reverse transcriptase for 30 minutes at 37°C. In alternate (+) lanes, the reactions contained 80 μ*M* ddATP in addition to 400 μ*M* of each dNTP to allow reading of the U's in the sequence (clearly visible on longer exposure). (E) Quantitation of the gel shown in (D). Individual bands of radioactivity corresponding to the L–19 end, the L–15 end, and the high molecular products resulting from primer extension through the cyclization junction (the prominent band "C" and the bracketed portion of the gel below that band) were cut from the (−) lanes of the gel (no ddNTP included in reaction) and analyzed by liquid scintillation counting. Results are presented as the fraction of radioactivity in each RNA species, with the radioactivity in the bands resulting from the C IVS multiplied by a correction factor (2.51) to account for the less efficient extension of primers into the high molecular weight region. (○) C IVS; (□) L–15; (△) L-19.

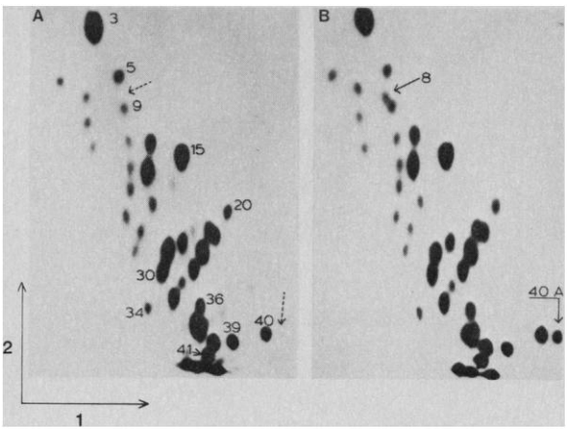


Fig. 2. Ribonuclease T1 two-dimensional oligonucleotide analysis of (A) C IVS and (B) L–19 IVS, both labeled with [α-^{32}P]UTP during transcription. First dimension, electrophoresis on cellulose acetate at *p*H 3.5. Second dimension, PEI cellulose chromatography at *p*H 3.5. The methods of identification of all the oligonucleotides have been described (*4*, *6*). For the purpose of orientation, note that spot 3 is Gp, spot 15 is AUGp, spot 20 is UUGp, and spot 40 is UUUUGp. Arrows indicate spots that were not present in C IVS RNA and appeared in L–19 RNA.

smaller circle (C′ IVS in Fig. 4) would then produce the L–19 RNA. This model was directly tested. Purified L–15 RNA was incubated at *p*H 9.0 for various amounts of time, and the reaction was monitored by gel electrophoresis (data not shown). A circular RNA species was observed to form; it reached its maximum amount (≃ 10 percent of the RNA) at 5 to 10 minutes and disappeared after 60 minutes of incubation, at which time conversion to L–19 is essentially complete. Thus the L–15 → L–19 reaction appears to involve a circular intermediate. Analysis of the same reaction products on a 20 percent sequencing gel revealed a progressive increase in the amount of tetranucleotide throughout the reaction.

A model for the cyclization and reopening reactions. Our data suggest that the L–19 RNA is derived from C IVS RNA mainly by reactions 3, 4, and 5 of Fig. 4. When the circle reopens to form the L–15 RNA, it regenerates the 3′ terminal nucleophile G-OH. The G-OH attacks at position 20, forming the circle C′ and releasing the tetranucleotide pACCU-OH. This cyclization reaction, like the original L → C reaction, occurs by transesterification and, therefore, has no external energy requirement. In reaction 5, the circle C′ undergoes site-specific hydrolysis at the ligation junction. This reaction is analogous to reaction 3. Presumably the conformation around the reaction sites is similar; that is, both circular RNA's fold in such a way as to activate whatever phosphodiester bond follows G^{414}. A minor pathway of L–19 RNA formation involves reactions 2 and 5 (Fig. 4).

In the initial L IVS RNA cyclization reaction (steps 1 and 2), attack at U^{15}–A^{16} is preferred over U^{19}–U^{20}. This could reflect the relative probability of two alternative structures of the RNA, with UpA positioned in the active site in one structure and UpU in the site in the other structure. Otherwise there could be a single structure in which the UpA and the UpU have different reactivity because of their position in the molecule or the base composition of the dinucleotide. In the L–15 species, only the U^{19}–U^{20} site remains, and it is to that site that cyclization occurs.

Reopening requires the native structure of the RNA. When C IVS RNA was incubated at 95°C in Mg^{2+}-containing buffer at *p*H 7.5, there was progressive conversion of circular to linear forms (Fig. 5A). Primer extension analysis, however, showed that the resulting linear forms had random ends with no preferential cleavage at either A^{16} or U^{20} (Fig. 5B). There is no indication that the reopening reaction (hydrolysis at G^{414}–A^{16} leaving the phosphate at the 5′ end) occurs under these denaturing conditions, even though the conditions accelerate the random hydrolysis of RNA. In a separate experiment, C IVS RNA was incubated at 42°C in a 10 m*M* $MgCl_2$, *p*H 9 buffer containing 8*M* urea; neither specific reopening nor random nicking occurred to a significant extent (data not shown).

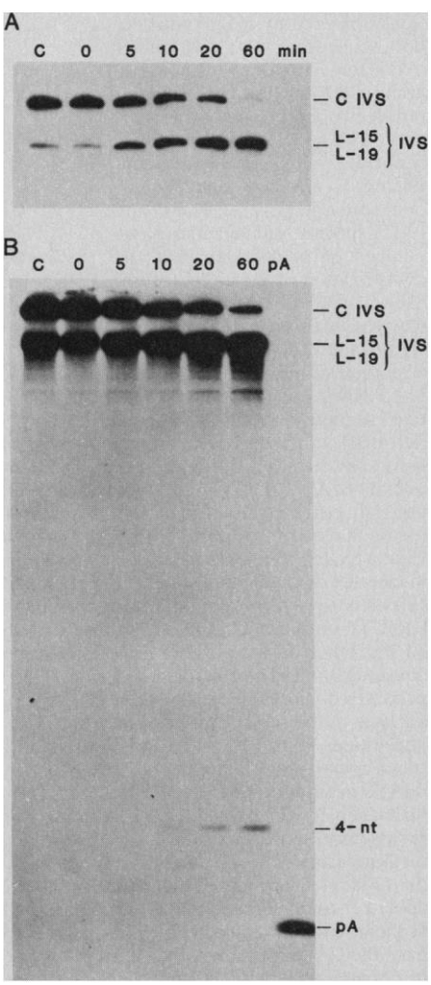


Fig. 3. A tetranucleotide released during incubation of C IVS RNA at *p*H 9.0. Uniformly labeled C IVS RNA was incubated for the times indicated with 10 m*M* $MgCl_2$, 50 m*M* CHES, *p*H 9.0. The reactions were stopped by addition of EDTA and analyzed by electrophoresis (A) in a 4 percent polyacrylamide, 8*M* urea gel, or (B) in a 20 percent polyacrylamide, 8*M* urea sequencing gel. The autoradiogram of the sequencing gel was intentionally overexposed to show the low molecular weight region; this exaggerated the apparent amount of L–15 RNA in the C IVS preparation. (Lane C) Untreated C IVS; (lane pA) ^{32}P-labeled adenosine 5′-monophosphate is the marker.

Implications for the Mechanism of Self-Splicing RNA

The cleavage-ligation activity that is intrinsic to the IVS RNA is maintained through multiple reactions. We had previously described three such reactions: guanosine addition, exon ligation, and IVS cyclization (*3*–*7*). We have now found that the IVS RNA can catalyze three additional reactions: circle reopening, recyclization at a second site, and reopening of the smaller circle. One of these is a transesterification reaction like those found previously. The other two are hydrolysis reactions in which the number of phosphodiester bonds decreases. In each round of cyclization and circle reopening, the IVS RNA acts as a nuclease on its 5′-proximal region. The first 19 nucleotides of the molecule are acting as substrate for the cleavage-ligation activity. The final form of the molecule, the "L–19" RNA (Fig. 4), fails to undergo further reaction. We interpret this to mean that there is no more available substrate on which it can act, while the activity remains intact. This interpretation is strengthened by the recent finding that the IVS RNA can undergo a

seemingly limitless number of forward and reverse cyclization reactions when it is supplied with a high enough concentration of an oligonucleotide substrate (*12*).

The circle reopening reaction is similar to the other reactions catalyzed by the IVS RNA in that it produces 5′-phosphate and 3′-hydroxyl termini. (This differs from standard alkaline hydrolysis of RNA, which produces first a 2′,3′-cyclic phosphate and subsequently a mixture of 2′- and 3′-monophosphates.) More specifically, the autoreopening reaction resembles a reverse cyclization reaction. A true reverse reaction (Fig. 4) would involve the 15-nt fragment attacking the circle at the G^{414}–A^{16} bond, joining to A^{16} and leaving a 3′-hydroxyl at G^{414}. Instead, the reopening reaction involves attack by water at the G^{414}–A^{16} bond, leaving the phosphate at A^{16} and a 3′-hydroxyl at G^{414}.

Circle reopening occurs 30 to 60 times faster at *p*H 9.0 than at *p*H 7.5. A more detailed analysis of the *p*H dependence (*10*) has shown that the reaction is first order in hydroxide ion concentration. Such a *p*H dependence is consistent with a mechanism in which a hydroxide ion undergoes direct nucleophilic attack on the phosphate. Thus, there is no need to invoke titratable groups on the RNA bases (*13*) to explain the faster rate at *p*H 9.0, although the data do not allow us to rule out such a possibility.

The circle reopening reaction has important implications for the activation energy problem for self-splicing RNA. Prior to this finding, we had evidence that precise positioning of the 3′-hydroxyl group of an attacking nucleotide was one factor contributing to catalysis (*8*). Splicing requires the 3′-hydroxyl of a free guanosine nucleoside or nucleotide (*3*). Cyclization involves the 3′-hydroxyl at the 3′ end of the linear IVS RNA (*6*). We now find that in the absence of any 3′-hydroxyl group, external or internal, the circular IVS RNA is able to cleave itself at the ligation junction.

The unspliced ribosomal RNA precursor also undergoes site-specific hydrolysis at the 3′ splice site (*12*). We therefore infer that increasing the reactivity of the phosphate that is to undergo nucleophilic attack is another way by which the RNA structure lowers the activation energy for self-splicing.

We envision the overall secondary and tertiary structure of the RNA molecule producing an unusual local conformation that facilitates cleavage at a particular site. For example, the phosphate could be distorted from tetrahedral toward a trigonal bipyramidal transition state, with one apical position occupied by the 3′-O of G^{414} and the other apical position unoccupied, available for the attacking nucleophile (*14*). In addition, the phosphate between G^{414} and A^{16} could be made more electrophilic by specific coordination of Mg^{2+} ion or by hydrogen binding of a 2′-OH group such as that of G^{414} (*15*).

Comparison to Other Systems

Nonenzymatic RNA cleavage reactions have been described previously. It is well known that folded transfer RNA molecules can bind metal ions to facilitate specific cleavage (*16, 17*). These cleavage reactions probably have no role in normal RNA metabolism, although they may be important in lead toxicity (*17*). There is one reported case in which RNA self-cleavage occurs at a site that is used in RNA processing in vivo, the case of the precursor to species-1 RNA of bacteriophage T4 (*18*). This reaction appears to have no requirement for a metal ion, as it is not inhibited by EDTA. In all of these previously described nonenzymatic reactions, cleavage leaves the phosphate on the 3′ end of the chain, opposite to the specificity observed with the *Tetrahymena* IVS RNA.

Ribonuclease P, the enzyme responsible for the maturation of the 5′ ends of transfer RNA's in both prokaryotes and eukaryotes, has both an RNA and a protein subunit (*19*). Altman, Pace, and their co-workers have found that the RNA moiety of ribonuclease P catalyzes the accurate processing of transfer RNA precursors in the absence of the protein

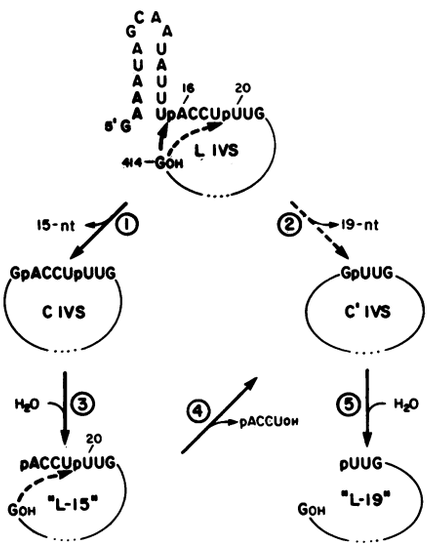
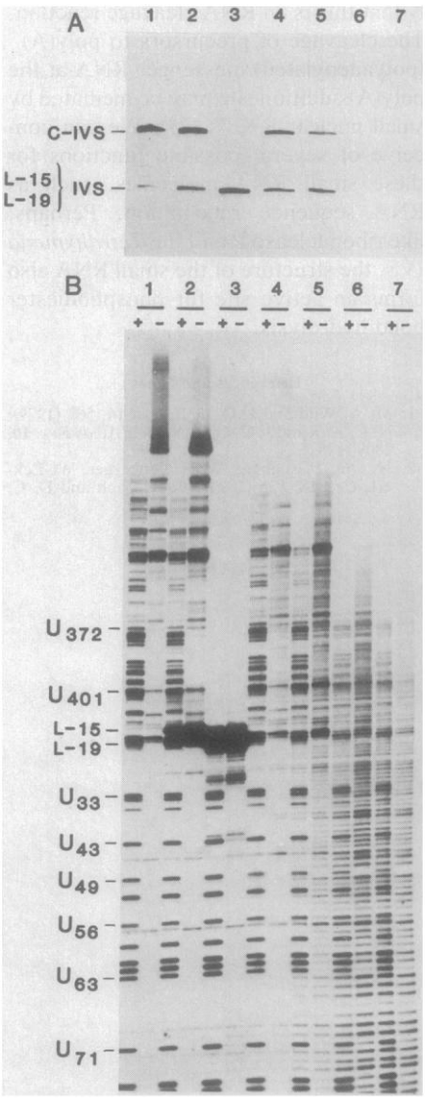


Fig. 4 (left). Model for the IVS RNA cyclization and reopening reactions. (**1**) Predominant cyclization reaction (*6*). (**2**) Minor cyclization reaction (*10*). (**3**) Reopening of the C IVS, which reestablishes the 3′ terminal nucleophile (G-OH). (**4**) Recyclization of the L–15 RNA to the secondary cyclization site. (**5**) Reopening of C′ IVS, the smaller circle. Evidence for reactions 3 to 5 is presented in the text. Reactions 1, 2, and 4 are transesterification reactions and are therefore expected to be reversible, although the forward reactions predominate at the low RNA concentrations used in this study. Reactions 3 and 5 are hydrolysis reactions and should therefore be irreversible in aqueous solution. Fig. 5 (right). Circle reopening requires the native structure of the RNA. (A) C IVS RNA, labeled with [^{3}H]UTP during transcription, was (lane 1) untreated, or was incubated for 1 hour at 42°C (lane 2) in 10 m*M* $MgCl_2$, 50 m*M* HEPPS, *p*H 7.5 or (lane 3) in 10 m*M* $MgCl_2$, 50 m*M* CHES, *p*H 9.0. Other samples were incubated at 95°C in 10 m*M* $MgCl_2$, 50 m*M* HEPPS, *p*H 7.5 for (lane 4) 1 minute, (lane 5) 2 minutes, (lane 6) 5 minutes, or (lane 7) 10 minutes. For the high temperature points, the RNA was equilibrated at 95°C in buffer; the reaction was then initiated by the addition of the $MgCl_2$ and stopped by the addition of EDTA, all at 95°C. (B) The same RNA analyzed by primer extension as in Fig. 1D, with the alternate (+) lanes containing a 1:5 ratio of ddATP to dATP.



component in vitro in solutions containing 60 m*M* Mg^{2+} (*20*). Ribonuclease P and the *Tetrahymena* IVS therefore provide two examples where RNA lowers the activation energy for very specific RNA cleavage; in the first case the catalytic agent acts on other molecules, while in the second it acts on itself. It seems possible that these two RNA molecules operate with similar mechanisms. One possibility is that the ribonuclease P RNA binds the pre–transfer RNA in such a way as to make the phosphate at the 5′ end of the mature transfer RNA portion more susceptible to hydrolysis by one of the mechanisms proposed above for the IVS RNA circle reopening reaction (*21*).

The generation of the mature 3′ end of histone H3 messenger RNA in *Xenopus* oocytes requires a small RNA molecule, perhaps in the form of a small nuclear ribonuclear protein (RNP) (*22*). It is likely that this is an RNA cleavage reaction. The cleavage of precursors to poly(A)$^+$ (polyadenylated) messenger RNA at the poly(A) addition site may be mediated by small nuclear RNP's (*23*). We can conceive of several possible functions for these small RNA molecules, such as RNA sequence recognition. Perhaps, like ribonuclease P and the *Tetrahymena* IVS, the structure of the small RNA also forms an active site for phosphodiester bond hydrolysis.

References and Notes

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9. Abbreviations: L IVS RNA, the linear intervening sequence (414 nt) that is the direct product of pre–ribosomal RNA splicing; C IVS RNA, the major circular form (399 nt); L–15 RNA (read "L minus 15"), a 399-nt linear form missing the first 15 nt of the L IVS; and L–19 RNA, a 395-nt linear form missing the first 19 nt of the L IVS.
10. A. J. Zaug, J. R. Kent, T. R. Cech, in preparation.
11. RNA was labeled with [α-^{32}P]CTP and UTP, so digestion of the tetranucleotide with nuclease P_1 produced only labeled pC and pU. Digestion with ribonuclease T2 produced only labeled pAp and Cp in a ratio of 1 to 2.2, determined by liquid scintillation counting. The lack of labeled Up was indicative of a 3′-hydroxyl, since the next nucleotide in the sequence is also a U and would be labeled. Digestion of the tetranucleotide with ribonuclease A produced labeled Cp and a larger labeled product. The latter was isolated, cleaved with ribonuclease T2, and found to give equal amounts of label in pAp and Cp. This confirmed the sequence of the tetranucleotide as pApCpCpU-OH.
12. F. X. Sullivan, T. Inoue, T. R. Cech, in preparation.
13. Uridine and guanosine have pK_a's in the range of 9.2 to 9.4 [P. O. P. Ts'o, *Basic Principles in Nucleic Acid Chemistry* (Academic Press, New York, 1974), p. 453]. Their pK_a's in an RNA molecule will depend on the proximity of phosphates and on the ionic strength. Thus, some non-base-paired U and G residues could be partially deprotonated at *p*H 9.0.
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21. Another conceivable mechanism would involve the 3′ terminus of ribonuclease P undergoing transesterification with the phosphodiester that links the precursor-specific sequence with the mature transfer RNA. Hydrolysis of this covalent intermediate would then regenerate the 3′ end of the ribonuclease P RNA. Such a mechanism would be analogous to that of the IVS RNA circularization-reopening cycle. The recent observation that ribonuclease P does not require a 3′-hydroxyl group for activity rules out such a mechanism for this reaction (T. Marsh and N. Pace, in preparation).
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