entails having an unspecified translation as the starting point of an inductive property (*30*).

Since conjoint structures are weakly ordered cartesian products, they are not ordered relational structures as defined above; however, they can be recast in that form. So the concepts of homogeneity and uniqueness apply to them. Further, because they can be recoded in a natural way in terms of operations closely related to PCSs, their study is greatly simplified (*23, 28*). We cannot go into the details here.

Concluding Remarks

Because of the differences in their respective phenomena, physical and behavioral data require different mathematical representing structures and therefore different procedures of measurement. Processes that may allow behavioral attributes to have strong forms of measurement have been developed, and measurements of such attributes, if they exist, will act in much the same way as physical units. Moreover, it is mathematically feasible for them to be combined among themselves and with physical units in just the same way as physical units combine. We have also described the mathematical possibilities (scale types) for those strong forms of measurement involving homogeneous structures and have shown that although they are greatly limited in number they are far more general than the usual models used in physical measurement. Their inherent limitations naturally suggest strategies for scientific experimentation and discovery, since much of their description can be captured by qualitative axioms.

The results reported here do not cover some important situations in which there are distinguished elements (for example, upper or lower bounds, as in probability and relativistic velocity). It is not yet clear how best to classify them.

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The Chemistry of Self-Splicing RNA and RNA Enzymes

THOMAS R. CECH

Proteins are not the only catalysts of cellular reactions; there is a growing list of RNA molecules that catalyze RNA cleavage and joining reactions. The chemical mechanisms of RNA-catalyzed reactions are discussed with emphasis on the self-splicing ribosomal RNA precursor of *Tetrahymena* and the enzymatic activities of its intervening sequence RNA. Wherever appropriate, catalysis by RNA is compared to catalysis by protein enzymes.

THE ABILITY OF RNA TO ACT AS A BIOLOGICAL CATALYST has become well established in the last few years. The examples of such ribozymes fall into two categories. Self-splicing (*1–3*) and self-cleaving (*4–8*) RNAs exemplify intramolecular catalysis (*9*) in which the folded structure of the RNA mediates a reaction on another part of itself. In addition, RNA also acts as a

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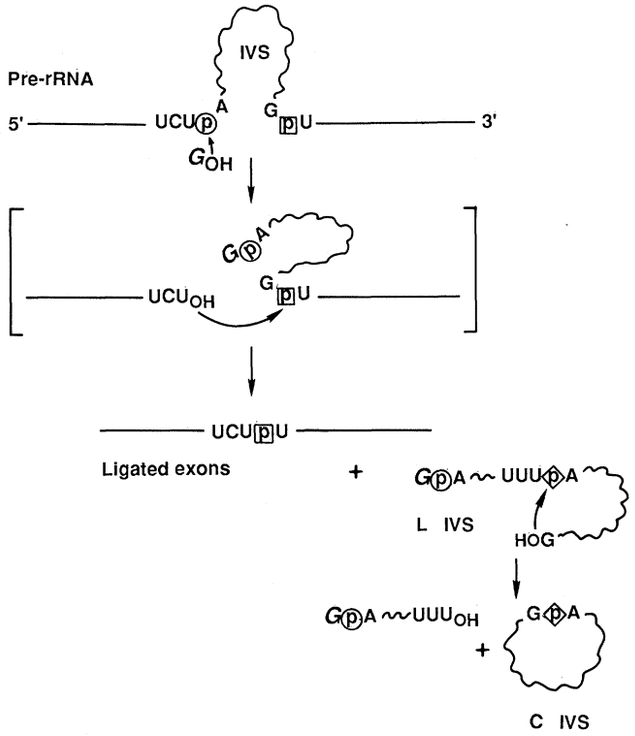

Fig. 1. Self-splicing of the *Tetrahymena* pre-rRNA and cyclization of the excised IVS by consecutive transesterification reactions (*32*). ___, Exons; ~~~, IVS. Square brackets designate a transient intermediate that has been isolated and characterized (*34*). C IVS, covalently closed circular form. The 5′ slice site phosphate is shown in a circle. The 3′ splice site, which becomes the splice-junction phosphate, is shown in a square. The cyclization site phosphate is shown in a diamond.

catalyst on other molecules (substrates), cutting and joining them without itself being changed in the process (*10–14*).

The finding of catalytic RNA has several implications. First, biologists can no longer assume that all biochemical reactions are catalyzed by proteins. For those cellular reactions that involve RNA, such as the small nuclear RNAs required for many RNA processing reactions (*15, 16*), the small RNAs involved in the priming of mitochondrial DNA replication (*17, 18*), and ribosomal RNA (rRNA) in protein synthesis (*19*), the possibility of RNA catalysis must be considered. Second, there are implications for prebiotic evolution. The ability of RNA, an informational molecule, to act as a catalyst has been widely interpreted (perhaps overinterpreted) as answering the chicken-and-the-egg problem of the origin of life—which came first, the protein or the nucleic acid?—in favor of RNA (*20*). Finally, there is the hope that the study of RNA enzymes will reveal some new principles of catalysis that are either unique to RNA enzymes or are general to all enzymes, and will expand our understanding of the fundamentals of phosphate chemistry (*21*).

An RNA catalyst, like its protein counterpart, greatly accelerates the rate of a biochemical reaction and shows extraordinary specificity with respect to the substrates it acts upon and the products it generates (*16*). For me, a satisfying understanding of RNA catalysis would require knowing what happens in the reaction—the chemical mechanism—and identifying the structures in the RNA that are responsible for mediating the chemistry and providing the substrate specificity. In the case of the self-splicing pre-rRNA of *Tetrahymena*, one of the RNA structures that is responsible for substrate specificity has been established (*22–27*). That information has been covered by recent reviews (*16, 28*). In this article I will concentrate on the more chemical questions of how the RNA-catalyzed reactions occur, and what catalytic mechanisms might be involved. My approach focuses on the self-splicing pre-rRNA of *Tetrahymena* and the enzymatic activity of its excised intervening sequence RNA. Near the end of this article I will extend the discussion to other examples of RNA catalysis.

Transesterification

In the genes of eukaryotes, coding sequences (exons) are often interrupted by stretches of noncoding DNA termed intervening sequences (IVS) or introns. Transcription of such "split genes" by RNA polymerase yields a mosaic precursor RNA. The precursor must undergo specific cleavage-ligation reactions (RNA splicing) to produce a functional RNA molecule, which can be a messenger RNA (mRNA), transfer RNA (tRNA), or rRNA (*15, 29, 30*).

The pre-rRNA of *Tetrahymena* contains an IVS of 413 nucleotides. Accurate and efficient splicing of this pre-rRNA occurs in vitro in the absence of protein; the RNA is self-splicing (*1*). Splicing requires a divalent cation (Mg^{2+} or Mn^{2+}). It also requires guanosine or a guanosine nucleotide, which becomes covalently joined to the 5′ end of the IVS RNA during its excision (*1*). Thus, the excised IVS contains 414 nucleotides. Splicing does not involve the hydrolysis of GTP or of any other nucleotide cofactor, distinguishing it from a number of enzyme-catalyzed nucleic acid ligation reactions that require hydrolysis of ATP or of diphosphopyridine nucleotide (*31*). On the basis of these observations, *Tetrahymena* pre-rRNA self-splicing was proposed to occur by two consecutive phosphoester transfer (transesterification) reactions, as shown in Fig. 1.

The proposed transesterification reactions are similar to those discussed in every undergraduate organic chemistry text,

except that the esters are of phosphate rather than carbon, and some of the R groups are very large (R′, the 5′ exon, contains about 5000 nucleotides and has a molecular weight exceeding 1 million daltons; R″, the IVS plus 3′ exon, contains about 1000 nucleotides; R is guanosine or GTP).

Confirmation of the reaction scheme of Fig. 1 came from several sources. First, the transesterification model was found to have substantial predictive value. A variety of self-catalyzed, splicing-related reactions, including cyclization of the excised IVS RNA (*32*), oligomerization of IVS RNA (*33*), and intermolecular exon-ligation (*24, 34*) were found to take place by concomitant cleavage and rejoining of RNA. These were readily explained by the transes-

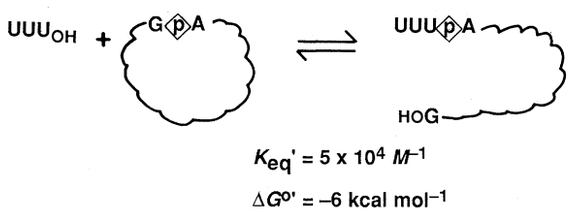

Fig. 2. Reversibility of IVS RNA cyclization (*36*). Equilibrium constant was determined from measurements of the equilibrium concentrations of circular and linear forms as a function of concentration of the trinucleotide UUU, and from measurements of the rate constants for the forward and reverse reactions. Reaction was at 42°C in 100 m*M* $(NH_4)_2SO_4$, 10 m*M* $MgCl_2$, 30 m*M* tris-HCl, *p*H 7.5.

terification model. Second, the postulated splicing intermediate (square brackets in Fig. 1) was subsequently detected, purified, and found to undergo the second step of splicing when incubated with an oligonucleotide analog of the 5′ exon (*34*). Third, the splice-junction phosphate (square in Fig. 1) was found to be derived from the 3′ splice site, as predicted (*35*). Finally, a transesterification reaction would be expected to be readily reversible. While reversibility of splicing has not yet been demonstrated, the cyclization of the excised IVS RNA has been shown to be reversible (*36*) (Fig. 2).

If RNA self-splicing occurs by two consecutive transesterification reactions, each of which is approximately isoergonic (constant free energy), then why does the reaction appear to proceed to completion instead of coming to equilibrium at some intermediate ratio of products to precursor? The reaction sequence shown in Fig. 1 is driven in the downward direction because the concentration of guanosine or GTP is high relative to that of the RNA (*37*). Stated in thermodynamic terms even if $\Delta G^{\circ\prime} = 0$, ΔG will still be negative at high ratios of GTP/pre-rRNA. Furthermore, cyclization of the excised IVS RNA, which proceeds essentially to completion at low RNA concentrations, helps drive splicing to completion by removing one of the reaction products. Thus, while RNA conformational changes occur during the reaction and could contribute to the free energy change, such changes do not need to be invoked to explain the thermodynamics. Additional factors that are expected to help drive the reaction to completion in vivo are the rapid degradation of the excised IVS RNA (*38*) and the sequestration of the spliced exons by export to the cytoplasm.

How, in detail, might self-splicing proceed? There is ample precedent from enzyme studies of phosphotransferases and phosphatases for reaction with inversion of configuration at phosphorus (*39*), probably by an S_N2 (P) or "in-line associative" pathway. Although there is no such detailed information yet available about the self-splicing reaction, a reasonable S_N2 (P) mechanism can be drawn (Fig. 3). The reaction could proceed via a trigonal bipyramidal transition state, as shown in Fig. 3. Alternatively, the trigonal bipyramidal species might be a true pentacovalent intermediate (*40*).

According to the reaction scheme of Fig. 1, it might appear that three distinct transesterification reactions are required to accomplish RNA splicing and cyclization of the excised IVS RNA. As noted by Inoue (*34, 41*), however, all the reactions can be described as the forward or reverse direction of the single reaction:

$$\text{YYUpN + G-OH} \leftrightarrows \text{YYU-OH + GpN}$$

(where Y is a pyrimidine and N is any nucleotide), if free guanosine nucleotide and the guanosine at the 3′ end of the IVS are equivalent (can occupy the same binding site within the IVS RNA). That is, the first step of splicing is

$$\text{...UCUpA... + pppG-OH} \rightarrow \text{...UCU-OH + pppGpA...,}$$

the second step of splicing is essentially its reverse

$$\text{...UCU-OH + ...GpU...} \rightarrow \text{...UCUpU... + ...G-OH}$$

and cyclization is once again a forward reaction

$$\text{...UUUpA... + ...G-OH} \rightarrow \text{...UUU-OH + ...GpA...}$$

Thus, the IVS RNA may perform the entire series of transformations by repetition of a single transesterification reaction, with conformational rearrangements serving to bring the different guanosines and the different YYU sequences into the active site.

Approximately 30 IVSs have been categorized along with the *Tetrahymena* pre-rRNA IVS as belonging to group I, on the basis of conserved sequence elements and RNA secondary and tertiary structure (*22, 23*). These IVSs come from far-flung corners of biology—protists, fungi, plants and bacteriophage T4—and include mitochrondrial and chloroplast as well as nuclear examples. In addition to the *Tetrahymena* IVS, eight other group I IVSs have been shown to be self-splicing in vitro (*2*). In all cases splicing is initiated by attack of a guanosine nucleotide, and transesterification can explain both splicing and IVS cyclization. Although the uridine adjacent to the 5′ splice site is universal, the preceding nucleotides need not be pyrimidines. The guanosine residue preceding the 3′ splice site is universal. Thus, the Inoue formalism can be generalized for all group I IVSs as

$$\text{XXUpN + G-OH} \leftrightarrows \text{XXU-OH + GpN}$$

where each X can represent any of the four nucleotides, and each IVS has a binding site for its own particular XXU sequence (*34, 41*).

All Phosphodiester Bonds Are Not Equal

The 3′-hydroxyl groups and phosphodiester bonds of RNA are normally exceedingly unreactive chemical species (*42*). What, then, enhances the reactivity of certain nucleotides in self-splicing RNA? The answer, in part, is that the IVS RNA contains binding sites for both the guanosine nucleoside cofactor and the 5′ and 3′ splice sites (*16, 25, 26, 43, 44*). The binding sites explain the great specificity of the reaction. By aligning the reacting groups with a very special orientation and in close proximity, the binding sites can explain part of the rate acceleration as well. The loss of entropy and the orientation of reactive groups achieved by formation of an enzyme-substrate complex appear to be universally important for enzyme catalysis (*9*). The folded RNA structure has yet another catalytic strategy. Whether or not the relevant nucleophile (G-OH or YYU-OH) is present, the phosphates at the reaction sites are somehow designated as "special" and are particularly prone to nucleophilic attack. Thus, while all phosphodiester bonds are created equal by

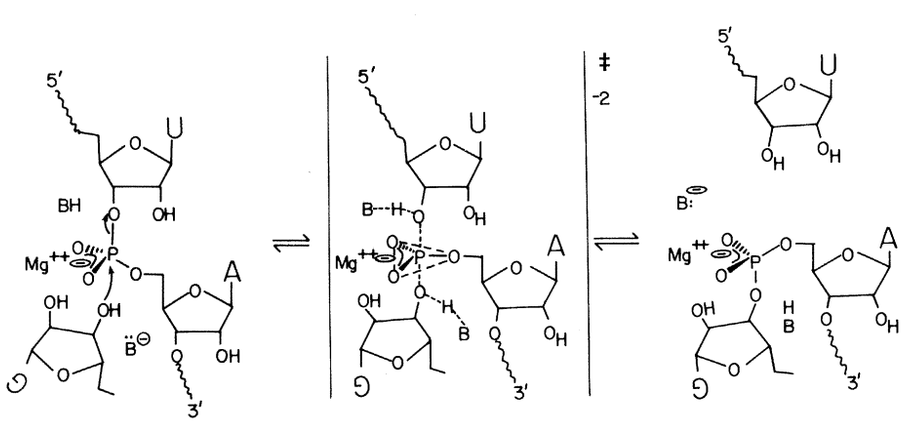

Fig. 3. Model for the initial step of pre-rRNA self-splicing involving nucleophilic attack by the 3′-hydroxyl group of guanosine on the phosphorus atom at the 5′ splice site. The hypothesis of an S_N2 (P) reaction with inversion of configuration around phosphorus has not been tested. The identities of the hypothetical acid (BH) and base (B^-) are unknown. It is possible that the 2′-oxygen of guanosine serves as the initial base. Coordination of Mg^{2+} to the phosphate would enhance the electrophilicity of the phosphorus atom and could stabilize the trigonal bipyramid transition state (*76*). In addition to its interaction with the phosphate, the metal ion could activate the 3′-hydroxyl as a nucleophile by enabling the oxyanion to exist at a *p*H far below *p*H 12.5, the normal pK_a of the hydroxyl function (*77*). ‡, Transition state, with a net charge of −2.

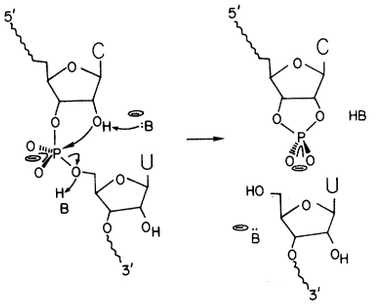

Fig. 4. Common mechanism of cleavage of an RNA chain by transphosphorylation, leaving 2′,3′-cyclic phosphate and 5′-hydroxyl termini. In many cases, the cyclic phosphate undergoes hydrolysis to give a 3′-phosphate monoester or a mixture of 2′- and 3′-phosphate monoesters (not shown). The mechanism shown here is applicable to alkaline cleavage, in which case the base B^- is OH^-; ribonuclease-catalyzed cleavage (*63*), in which the base and acid are enzyme substituents; and Pb(II)-catalyzed cleavage of tRNA (*64*), in which the base is a hydroxyl group coordinated to the metal ion. It is also applicable to self-cleavage of certain plant and newt RNAs, as described in the text.

RNA polymerase, they can have vastly unequal stability in the context of a particular folded RNA structure.

The lability of certain phosphodiester bonds was first noted in the circular form of the IVS RNA. The circle undergoes site-specific hydrolysis exactly at the bond formed during cyclization, producing a linear RNA with 5′-phosphate and 3′-hydroxyl termini (*5*). A 3′-hydroxyl is the same end group generated by the self-splicing reactions (Figs. 1 to 3), but differs from the 2′,3′-cyclic phosphates and ultimately 2′- and 3′-phosphate monoesters normally produced by alkaline hydrolysis of RNA (Fig. 4). Thus, the site-specific hydrolysis reaction appeared to be relevant to self-splicing. The relationship was clarified when it was found that the pre-rRNA had a similar labile phosphodiester bond at its 3′ splice site (*34*). Our interpretation is as follows: the phosphate at the 3′ splice site is activated for attack by the 5′ exon in the exon-ligation reaction (second step of Fig. 1) and, in the absence of the guanosine needed to initiate splicing, the phosphate is subject to attack by hydroxide ion from the aqueous solvent. The 3′ splice site of pre-rRNA and the cyclization junction of the circular IVS RNA both follow the 3′-terminal guanosine of the IVS (G^{414}) and are presumably structurally equivalent.

There is also some genetic evidence that the lability of the phosphodiester bond at the 3′ splice site is relevant to splicing. Mutations in sequence elements 9L and 2, elements which form a base-paired structure that is part of the catalytic core of the IVS, result in a greatly reduced rate of 3′ splice site hydrolysis and a similarly reduced rate of exon ligation (*45*).

The rate of hydrolysis of the bond following G^{414} increases linearly with the concentration of OH^- in the range *p*H 7.5 to 9.5, consistent with direct attack by OH^- (*46*). The second-order rate constant for hydrolysis is 12 orders of magnitude higher than that of a simple phosphate diester and about 10 orders of magnitude higher than that calculated for an average phosphodiester bond in RNA (Fig. 5).

The phosphate at the 5′ splice site is also prone to hydrolysis, although at a rate slower than that at the 3′ splice site (*34, 47*). Unlike hydrolysis at the 3′ splice site, which is nonproductive for splicing, hydrolysis at the 5′ splice site produces a 5′ exon ending in a 3′-hydroxyl and could therefore give exon ligation. In fact, a small amount of ligated exon RNA appears to be produced in a guanosine-independent reaction (*34*). Around neutral *p*H, however, hydrolysis is a slow reaction; at *p*H 7.5 and 42°C, 3′ splice site hydrolysis has a half-life ($t_{1/2}$) = 8 hours (*46*) while guanosine-dependent splicing has $t_{1/2} < 1$ minute at saturating guanosine concentration (*43, 48*). The phosphates at the major and minor cyclization sites of the linear IVS RNA also undergo site-specific hydrolysis (*44*), making it a general feature of all the biologically relevant reaction sites in the molecule.

Protein catalysts facilitate the formation of the transition state by providing active site groups that bind the transition state more tightly than the ground state of the substrate (*49*). The enhanced hydrolysis of certain phosphates in the self-splicing pre-rRNA may reflect the stabilization of their trigonal bipyramidal transition state (Fig. 3) by functional groups within the IVS RNA (*50*). Thus, RNA catalysts may be recapitulating this important catalytic strategy of proteins, or vice versa.

Multiple Turnover

After excising itself from the pre-rRNA, the IVS RNA undergoes a series of cyclization and site-specific hydrolysis reactions (*5*). The final product is the L − 19 IVS RNA, a linear RNA missing the first 19 nucleotides of the IVS. These 19 nucleotides contain the major and minor cyclization sites. In their absence, the L − 19 IVS RNA is left with no intramolecular substrate to attack, so it undergoes no further self-processing. Yet the RNA retains activity; when supplied with exogenous oligoribonucleotides as substrates, it catalyzes nucleotidyl transfer reactions (Fig. 6A). The L − 19 IVS RNA is regenerated in each reaction cycle, and is therefore acting as a true catalyst (*11*). Once again, the reactions have a divalent cation requirement.

The enzymatic nucleotidyltransferase mechanism (Fig. 6A) involves steps that are intermolecular versions of IVS RNA cyclization (Fig. 6B) or pre-rRNA self-splicing (Fig. 6C). Thus, from a mechanistic viewpoint there is little to distinguish intramolecular catalysis (self-splicing or cyclization) from catalysis on external substrates (the L − 19 IVS RNA reactions). Conversion of the former to the latter can be accomplished simply by cleaving the linear IVS RNA after nucleotide 19, thereby separating its substrate and enzyme portions (*51*). Even the reaction kinetics are similar. The first-order rate constant for IVS RNA cyclization is 0.8 min^{-1} at 42°C (*46*), and the catalytic rate constant for L − 19 IVS RNA-catalyzed nucleotidyl transfer is 1.7 min^{-1} at the same temperature (*11*). Thus, as long as there is a saturating concentration of substrate

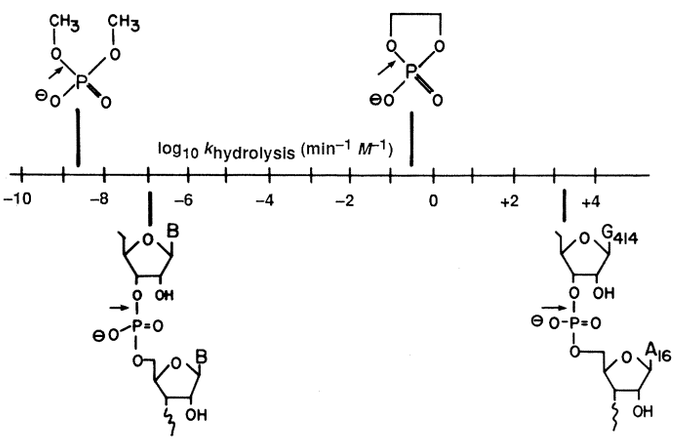

Fig. 5. Second-order rate constants for the alkaline hydrolysis of phosphate diesters are displayed on a logarithmic scale. Rate constants determined at 42°C or extrapolated to that temperature are given. Arrows designate the P-O band that undergoes cleavage. Data are given for dimethyl phosphate and ethylene phosphate (*78*), random hydrolysis of RNA leaving a 5′-phosphate terminus calculated as described (*11*), and hydrolysis of the labile phosphodiester bond of the circular IVS RNA (*46*).

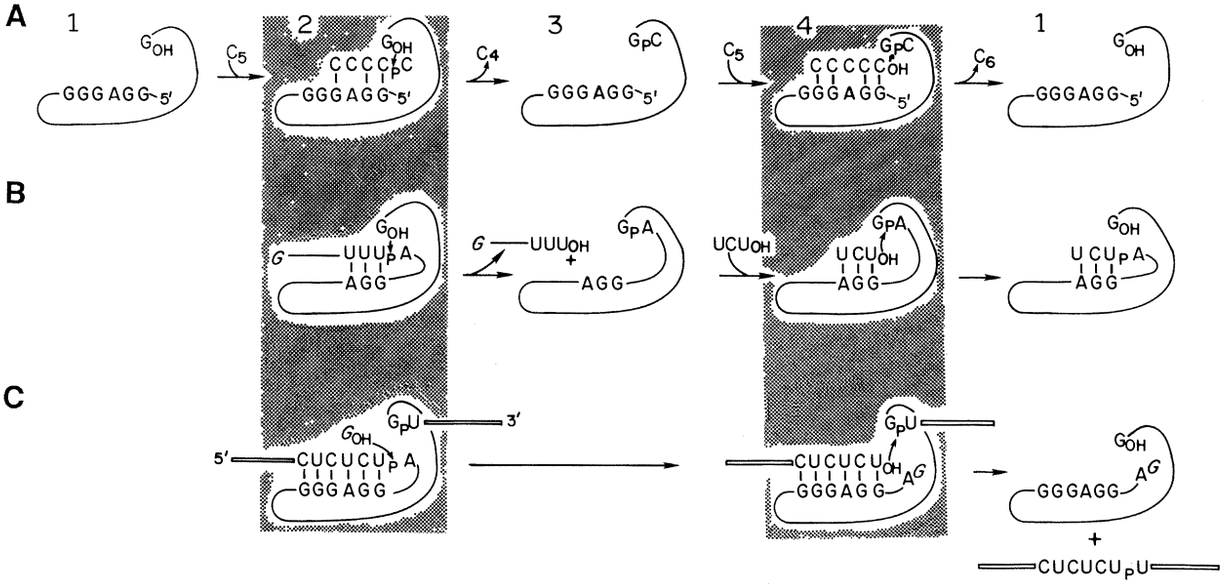

Fig. 6. Enzymatic mechanism of the L − 19 IVS RNA (A) is compared to IVS RNA cyclization (B) and self-splicing (C). Shaded regions emphasize analogous reactions. (**A**) The L − 19 IVS RNA enzyme (**1**) is shown with the substrate-binding site (GGAGGG) near its 5′ end and G^{414} with a free 3′-hydroxyl group at its 3′ end. The complex folded core structure of the molecule is simply represented by a curved line. The enzyme binds the oligoribonucleotide substrate (C_5) by Watson-Crick base-pairing to form the noncovalent enzyme-substrate complex (**2**). Nucleophilic attack by G^{414} leads to formation of the covalent intermediate (**3**). If C_5 binds to the intermediate in the manner shown in (**4**), transesterification can occur to give the product C_6 and regenerate the enzyme (**1**). All reactions are expected to be reversible. Taken from Zaug and Cech (*11*) and Been and Cech (*26*). (**B**) Cyclization of the excised IVS RNA (*16, 27, 34*) and reversal of cyclization by the oligopyrimidine UCU (*36*). The GGA that serves as the substrate-binding site for cyclization begins four nucleotides downstream from the GGA shown in (A); nearby tripurine sequences can also serve this function (*27*). (**C**) Self-splicing of the *Tetrahymena* pre-rRNA (*16*). Details of the base-pairing interaction between CUCUCU at the end of the 5′ exon and GGAGGG within the IVS have been described (*25, 26*). The guanosine nucleotide shown in italics is not encoded by the DNA, but added to the IVS during the splicing reaction.

(*52*), the enzymatic reaction is at least as fast as the intramolecular self-reaction.

If one ignores for a moment the polyribonucleotide composition of the catalyst, the reaction scheme of Fig. 6A does not appear unusual for an enzyme-catalyzed reaction. The RNA enzyme, like a protein enzyme, forms a specific noncovalent complex (**2**) with its oligonucleotide substrate. A covalent enzyme-substrate complex (**3**) is then formed via nucleophilic attack of the 3′-hydroxyl of G^{414} on a phosphorus atom of the substrate. Covalent intermediates are prevalent in enzyme-catalyzed group transfer reactions, including those involving phosphate esters. Thus G^{414} of the L − 19 IVS RNA is analogous to the active-site histidine of galactose-1-phosphate uridylyltransferase, another nucleotidyltransferase (*53*).

The phosphodiester bond following G^{414} in the covalent intermediate (**3**) is subject to the same site-specific hydrolysis reaction as the equivalent bond in the circular IVS RNA or pre-rRNA (*11*). The nucleotide following G^{414} is different in each case; cytidine in the covalent intermediate, adenosine in the circular IVS RNA, and uridine in pre-rRNA. Furthermore, the covalent intermediate has a labile phosphodiester bond even if there is only one nucleotide after G^{414}. It thus appears that a localized catalytic apparatus is responsible for enhancing the reaction at whatever phosphodiester bond follows G^{414} of the IVS RNA. The apparatus might provide functional groups that stabilize the trigonal bipyramidal transition state and might help coordinate a Mg^{2+} ion.

Phosphotransferase

In the reactions of the L − 19 IVS RNA discussed above, the substrates have all been phosphate diesters. The finding that the L − 19 IVS RNA also has activity toward phosphate monoesters was due to serendipity. We prepared oligo(cytidylic acid) 3′-phosphate to study the formation of the covalent L − 19 IVS RNA-substrate complex, reasoning that blockage of the 3′-hydroxyl of the substrate with a phosphate would not interfere with substrate cleavage (Fig. 6A, **2** → **3**) but would prevent the substrate attacking the covalent complex (**4** → **1**). When the oligo(cytidylic acid) 3′-phosphate was mixed with the L − 19 IVS RNA, however, the predominant reaction was no longer nucleotidyl transfer, but rather phospho transfer (*12*). The phospho group was transferred from the 3′ terminus of the substrate to the 3′-terminal guanosine of the L − 19 IVS RNA.

Phosphorylation of the L − 19 IVS RNA is reversible by C_5-OH and a variety of other pyrimidine oligonucleotides that terminate with a 3′-hydroxyl. Thus, the RNA enzyme can act as a phosphotransferase, transferring the 3′-terminal phospho group from one oligonucleotide to another with multiple turnover. Accomplishing transfer by way of a phosphoenzyme intermediate has ample precedent among protein phosphotransferases (*39, 54*). At *p*H 4 and 5, the phosphoenzyme undergoes slow hydrolysis to yield inorganic phosphate. Thus, the L − 19 IVS RNA also has acid phosphatase activity (*12*).

Dephosphorylation is specific for the 3′-phosphate of oligo(cytidylic acid). For example, the 5′-phosphate of pC_5 is not reactive, neither phosphate is removed from pCp, and A_6Cp is not a substrate. The substrate specificity and other features of the dephosphorylation reaction indicate that it takes place in the same active site as the nucleotidyl transfer reaction (*12*).

Nonenzymatic reactions of phosphate monoester monoanions in aqueous solution usually proceed by way of a dissociative, metaphosphate-like transition state, a pathway entirely different from that of phosphate diesters (*39, 55*). Thus, it was somewhat surprising to observe the L − 19 IVS RNA catalyzing reactions on both monoester and diester substrates. RNA-catalyzed phosphotransfer is optimal at *p*H 5, where the monoester ($pK_a \simeq 6$) is presumably

protonated (*12*). Protonation of a phosphate monoester makes it possible for it to react like a diester (*55*). A reasonable hypothesis, therefore, is that the RNA-catalyzed reaction of the monoester uses the path of the RNA-catalyzed diester reaction, rather than the dissociative pathway one would expect for the uncatalyzed reaction.

Finding an enzyme with both phosphodiesterase and phosphomonoesterase activity is unusual but not unprecedented. Exonuclease III, P1 nuclease and mung bean nuclease all have 3′-phosphatase activity (*56*). The L − 19 IVS RNA is unique among known enzymes in its ability to remove 3′-phosphates from RNA with high sequence specificity.

The Importance of Being Ribose

Could DNA, if freed from its rigid double-helical structure, have catalytic activity like its RNA cousin? No examples of DNA catalysts are known, and a single-stranded DNA copy of the *Tetrahymena* pre-rRNA does not undergo self-splicing in vitro. What, then, is the importance of the 2′-hydroxyl group when a nucleic acid acts as a catalyst? The answer is likely to be different for each reaction examined. Structural differences certainly exist between the two classes of nucleic acid, although it is not at all clear that A-form double-helical regions in RNA should be any better suited to catalysis than the B-form double helices of DNA. On the other hand, the 2′-hydroxyl group, specific to RNA, is a hydrogen bond donor and acceptor that gives RNA more versatility than DNA in formation of tertiary structure (*57*). RNA is also more chemically reactive than DNA. The 2′-hydroxyl participates directly in the common mechanism of RNA hydrolysis (Fig. 4) and, when it does not enter into covalent bond formation, it can serve to enhance the reactivity of the adjacent 3′-hydroxyl (*58*). Ribose has a $pK_a \simeq 12.5$ while deoxyribose, like other secondary alcohols, is a much weaker acid ($pK_a \simeq 16$).

The importance of being ribose has been examined in some detail for the guanosine nucleotide that initiates the pre-rRNA self-splicing reaction (*59*). Deoxyguanosine is a competitive inhibitor of the reaction of guanosine, but it binds only weakly (K_i = 1.1 m*M* for deoxyguanosine; K_m = 0.03 m*M* for guanosine). Thus, the 2′-hydroxyl of guanosine contributes binding free energy worth an estimated 2 kcal/mol. Deoxyguanosine itself is unreactive even at very high concentrations. Thus, in addition to its importance in binding, the 2′-hydroxyl of guanosine is required for reactivity. One reasonable explanation is that the 2′-oxygen serves to facilitate deprotonation of the 3′-hydroxyl, increasing its nucleophilic character. The inability of deoxyguanosine to substitute for guanosine has also been demonstrated for the guanosine that serves as the nucleophile in the IVS RNA cyclization reaction (Fig. 6B) (*44*).

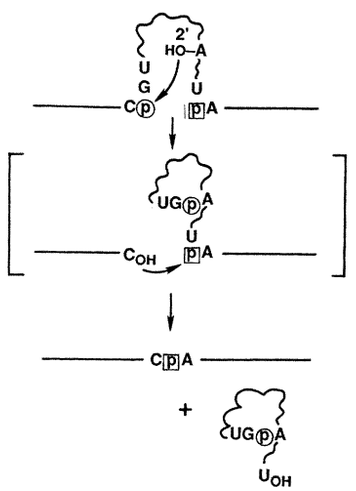

Fig. 7. Model for self-splicing of mitochondrial pre-mRNA containing a group II IVS (*3, 62*). ___, Exons. ~~~, IVS. Square brackets designate an intermediate which does not accumulate except in certain mutants (*79*).

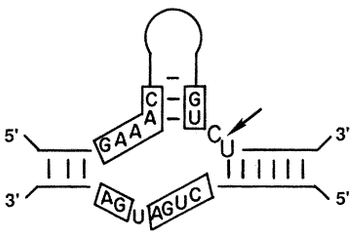

Fig. 8. Conserved nucleotide sequences and secondary structures in the vicinity of sites of self-cleavage of certain plant viroids, viral satellites, and virusoids (*7*). This is the smallest self-processing RNA structure known at this time. The arrow shows the autolytically cleaved phosphodiester bond.

In the L − 19 IVS RNA nucleotidyl transfer reactions, dC_5 cannot substitute for C_5 as a substrate. Once again, the DNA version of the substrate does bind to the active site of the RNA catalyst, as judged by competitive inhibition. In this case, the DNA version is a good competitive inhibitor [K_i = 260 μ*M*, compared to K_m = 42 μ*M* for C_5 (*11*)]. Surprisingly, a chimeric substrate molecule consisting of five deoxyribonucleotides followed by a single ribonucleotide (dC_5rC) is cleaved (*60*), even though the phosphate that must be attacked has a deoxyribose environment. Thus, in this case it appears that the inactivity of DNA as a substrate is not due to any chemical deficiency, but simply because the DNA binds to the active site in a nonproductive manner.

Although there are no established examples of DNA acting as a catalyst, it certainly seems possible that it could have such activity. However, both because of its more limited structural repertoire, and because of its intrinsically lower chemical reactivity, DNA might be a much less efficient catalyst than RNA.

Other Examples of RNA Catalysis

Self-splicing of the *Tetrahymena* pre-rRNA and other precursor RNAs containing group I intervening sequences comprise one category of intramolecular catalysis by RNA. As discussed above, the diagnostic features of this category are the guanosine nucleotide requirement, the presence of 3′-hydroxyl groups on the product RNAs, and the two-step transesterification mechanism.

A second category is self-splicing of mitochondrial mRNA precursors that contain group II intervening sequences. These intervening sequences were recognized as forming a structurally distinct class before anything was known about their splicing mechanism (*23*). Two of the group II intervening sequences have been reported to undergo self-splicing in vitro by a mechanism that involves formation of a branched "lariat" RNA and has no requirement for an external nucleotide substrate (*3, 61*). The proposed mechanism is shown in Fig. 7; precedent for this mechanism is provided by the larger class of nuclear pre-mRNA intervening sequences (*15*), which do not self-splice but require proteins and small nuclear ribonucleoproteins for splicing. In the model of Fig. 7, splicing is initiated by nucleophilic attack of the 2′-hydroxyl group of an internal adenosine on the phosphate at the 5′ splice site. Transesterification results in formation of the branched adenosine nucleotide and leaves the 5′ exon with a 3′-terminal hydroxyl group. This hydroxyl group then attacks the 3′ splice site, ligating the exons and releasing the intervening sequence as a lariat. Fundamental similarities to the mechanism of group I self-splicing include the two-step transesterification mechanism, with the first step occurring at the 5′ splice site and the second at the 3′ splice site, and the use of the 3′-hydroxyl of the 5′ exon as the nucleophile in the second step (Fig. 1) (*62*). The fundamental difference concerns the use of the 2′-hydroxyl as the nucleophile in the first step of group II RNA self-splicing; such a

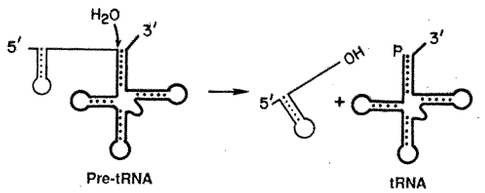

Fig. 9. Site-specific cleavage of tRNA precursors as catalyzed by the RNA enzyme, RNase P. Thin line, precursor RNA segment removed by the processing reaction. Thick line, mature tRNA molecule. Portions of the mature domain of the precursor are important for it to be recognized as a substrate.

mechanism precludes invoking the special reactivity of the *cis*-diol of ribose.

The third category of intramolecular RNA catalysis is site-specific self-cleavage of RNA with generation of a 2′,3′-cyclic phosphate. The likely mechanism for such cleavage is transesterification via attack by the adjacent 2′-hydroxyl group, as shown in Fig. 4. Precedents for such a mechanism are abundant. They include alkaline hydrolysis of RNA, pancreatic ribonuclease-catalyzed cleavage of RNA (*63*), and Pb(II)-catalyzed cleavage of RNA (*64*). Self-cleavage of RNA by such a mechanism appears to contribute to a natural RNA processing reaction in the case of a bacteriophage T4 RNA (*4*). Furthermore, a number of small RNAs associated with plant infections, including the satellite RNA of tobacco ringspot virus, the avocado sunblotch viroid, and the virusoid associated with lucerne transient streak virus, undergo site-specific self-cleavage leaving 2′,3′-cyclic phosphate termini (*6, 7*). The cleavage reaction and its reversal, in which the 3′-hydroxyl group attacks the cyclic phosphate to reform a 3′-5′-phosphodiester bond (*6*), may be important in the replicative cycle of these infectious agents (*6, 7, 65*). These plant RNAs share the structural motif shown in Fig. 8. This rather small structure has been shown to be necessary and sufficient to direct efficient site-specific cleavage (*66*). Recently an RNA of vertebrate origin, a dimeric satellite DNA transcript from the newt, has been found to undergo self-cleavage in vitro; the RNA sequence in the vicinity of the cleavage site can be folded into the structure shown in Fig. 8 (*8*). Thus, this particular self-cleaving cassette of RNA is widespread, occurring in plants and animals.

The first example of RNA acting as a true, recycling catalyst was the tRNA-processing enzyme ribonuclease P (RNase P). This enzyme is remarkable in that it precisely cleaves the 5′ leader sequences from the entire array of tRNA precursors, which have little sequence homology around the site of cleavage (Fig. 9). In addition, the composition of the enzyme is unusual. Since the late 1970s it has been known that ribonuclease P from *Escherichia coli* consists of both a 377-nucleotide RNA and a polypeptide; both subunits are required for enzyme activity in vivo (*67*). In 1983 Altman, Pace, and coworkers found that the RNA component by itself could catalyze the precise cleavage of pre-tRNA in vitro under certain conditions such as elevated Mg^{2+} concentration (*10*). The protein subunit had no nuclease activity under any condition tested. Thus, the RNA is the catalytic subunit of the enzyme, and the protein serves auxiliary functions such as stabilizing the active structure of the RNA under intracellular conditions and modifying the substrate specificity of the enzyme (*10, 68*). The RNA components of RNase P from *Bacillus subtilis* and *Salmonella typhimurium* also act as enzymes in vitro (*10, 68, 69*).

Cleavage of pre-tRNA by RNase P requires Mg^{2+} or Mn^{2+}, and leaves 5′-phosphate and 3′-hydroxyl termini on the cleaved substrate. These features are identical to those of group I RNA self-splicing, prompting the question of whether cleavage might occur by a mechanism analogous to that shown in Fig. 1. That is, RNase P could in principle make a nucleophilic attack on the substrate RNA, forming a covalent intermediate that could later undergo hydrolysis to restore the active enzyme. However, modification of the 3′-hydroxyl of either the *B. subtilis* or *Escherichia coli* RNA enzyme does not affect reactivity, eliminating the possibility that the 3′-hydroxyl acts as a nucleophile (*70, 71*). This of course does not rule out the participation of any of the numerous 2′-hydroxyl groups of the RNA, which would give a branched RNA intermediate analogous to that shown in Fig. 7. No such enzyme-substrate intermediate has been detected, however, suggesting that the nucleophile is H_2O or hydroxide ion rather than a substituent of the enzyme.

Thus, the proposed reaction mechanism for RNase P cleavage of pre-tRNA resembles that of Fig. 3, except that nucleophilic attack is by solvent instead of the ribose of a nucleotide. A specific proposal for the identity of the acid and base (BH and B in Fig. 3) has been made. Guerrier-Takada *et al.* (*71*) suggest that the reaction could be catalyzed by a solvated Mg(II) coordination complex. A hydroxyl ligand of the Mg(II) would act as the base B, facilitating deprotonation of a water molecule that acts as the nucleophile. A water ligand of the same Mg(II) would act as the acid BH, donating a proton to the 3′-oxygen of the leaving group.

One year ago, RNase P and the L − 19 IVS RNA were the only two RNA catalysts known to work with multiple turnover. Recently, however, it was announced that a polysaccharide branching enzyme, 1,4-α-D-glucan:1,4-α-D-glucan-6α-(1,4-α-glucano) transferase, isolated from rabbit skeletal muscle, has a catalytic subunit composed of RNA (*14*). The RNA is only 31 nucleotides in length and contains 10 modified bases of the sort found in tRNA (*72*). The level of activity of the RNA alone is actually higher than that of the holoenzyme (RNA + protein) when assayed at 37°C in a 2 m*M* sodium carbonate buffer, *p*H 7.2. It has yet to be demonstrated that the RNA catalyzes formation of the same α-1,6 linkage characteristic of glycogen synthesis in vivo. This is a particularly exciting system because, if confirmed by more detailed studies, it will mark the first example of an RNA enzyme working on a substrate other than RNA.

Additional examples may be close at hand. Two different enzymes involved in the process of mammalian mitochondrial DNA replication have been reported to have essential RNA components (*17, 18*). For one of these, an endonuclease that cleaves the RNA primer for heavy-strand DNA synthesis, the RNA component of the enzyme has been identified and sequenced (*73*). It is a 138-nucleotide RNA molecule that is encoded by the nuclear genome and imported into the mitochondrion. It has yet to be shown if this RNA has some auxiliary role in the reaction or, as in the case of RNase P, contains the active site.

Nuclear pre-mRNAs do not undergo self-splicing in vitro, but do splice in a reaction mixture containing proteins and small nuclear ribonucleoproteins (snRNPs). Each snRNP consists of a small RNA, usually in the range of 90 to 220 nucleotides, complexed with several polypeptides (*74*). As speculated by Kruger *et al.* (*1*) and others (*20, 62, 75*) it is possible that the snRNPs, when assembled with the pre-mRNA, form a catalytic RNA complex equivalent to the structure that self-splicing RNAs can form by themselves. Such a model could be disproven by the identification of an endonuclease or ligase involved in nuclear pre-mRNA splicing; however, no such enzyme has yet been found.

In all living cells, protein synthesis takes place on ribosomes, each of which contains three or four RNA molecules and more than 50 polypeptides. The most fundamental activity of protein synthesis, the peptidyltransferase reaction, has never been pinned down to a particular ribosomal protein or group of such proteins. Thus, the possibility of RNA catalysis must be considered (*16, 19*). One concern is that the peptidyltransferase center might be such an intimate congregation of protein and nucleic acid as to make it

impossible ever to assign catalytic function to either component. The same limitation could apply to the snRNPs and to other ribonucleoproteins that participate in biochemical reactions. With RNase P and many of the group I and II intervening sequences, however, RNA clearly works in concert with proteins in vivo; nonetheless, biochemical studies have made it possible to assign catalytic function to the RNA.

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