

A model for the RNA-catalyzed replication of RNA[†]

(origin of life/ribozyme/RNA polymerase/intron/RNA splicing)

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ABSTRACT A shortened form of the self-splicing ribosomal RNA intervening sequence of *Tetrahymena thermophila* has enzymatic activity as a poly(cytidylic acid) polymerase [Zaug, A. J. & Cech, T. R. (1986) *Science* 231, 470–475]. Based on the known properties of this enzyme, a detailed model is developed for the template-dependent synthesis of RNA by an RNA polymerase itself made of RNA. The monomer units for RNA synthesis are tetra- and pentanucleotides of random base sequence. Polymerization occurs in a 5'-to-3' direction, and elongation rates are expected to approach two residues per minute. If the RNA enzyme could use another copy of itself as a template, RNA self-replication could be achieved. Thus, it seems possible that RNA catalysts might have played a part in prebiotic nucleic acid replication, prior to the availability of useful proteins.

The question about the origin of life often appears as [the] question . . . Which came first, the protein or the nucleic acid?—a modern variant of the old chicken-and-the-egg problem. The term “first” is usually meant to define a causal rather than a temporal relationship, and the words “protein” and “nucleic acid” may be substituted by “function” and “information.” The question in this form, when applied to the interplay of nucleic acids and proteins as presently encountered in the living cell, leads *ad absurdum*, because “function” cannot occur in an organized manner unless “information” is present and this “information” only acquires its meaning *via* the “function” for which it is coding. [M. Eigen (1)]

The finding of self-splicing RNA (2–8) and RNA with ribonuclease activity (9–11) has been widely interpreted to provide a possible resolution of the “chicken-and-the-egg problem” in favor of RNA (9, 12–17). That is, the presence of both “information” and “function” in the same RNA molecule might, in principle, allow it to catalyze its own replication. Yet neither self-splicing RNA nor RNase P has any obvious relationship to the process of nucleic acid replication as it occurs in contemporary cells. Thus, while it has been a logical extrapolation of known facts to envision RNA catalysis of prebiotic RNA recombination (2, 18, 19) or RNA processing (9, 13), it has been more difficult to envision RNA catalysis of prebiotic nucleic acid synthesis.

Arthur Zaug and I (20) recently described a system in which a 395-nucleotide form of the *Tetrahymena* ribosomal RNA intervening sequence (IVS) acts as an RNA cleavage–ligation enzyme. Using the same activity that it employs in the self-splicing and autocyclization reactions, the RNA enzyme converts pentacytidylic acid (pC₅) to poly(C) with multiple turnover. Thus, it acts as a poly(C) polymerase, synthesizing RNA in a 5'-to-3' direction. The enzyme could also be designated terminal cytidyltransferase or oligo(C) dismutase. The term “polymerase” is chosen to emphasize

the template dependence of the enzyme. The RNA enzyme differs from protein RNA polymerases in that it uses an internal rather than an external nucleic acid template.

I now extrapolate these findings to develop an entirely RNA-based model for prebiotic RNA replication. In so doing, I am in no way trying to present an historical account of the events that occurred early in evolution. Instead, I simply wish to establish the principle that an RNA polymerase, itself made of ribonucleic acid, might have played a key role in prebiotic nucleic acid replication independent of the availability of proteins. The model is intended to complement rather than compete with other models for prebiotic nucleic acid replication, such as those of Orgel, Usher, and colleagues (21–25).

The L – 19 IVS RNA Is a Poly(C) Polymerase. The L – 19 IVS RNA converts pC₅ (or any pC_n with $n \geq 4$) to both larger and smaller oligomers of C, with $K_m = 40 \mu\text{M}$ and a turnover number of 2 per min (20). Chain lengths up to 30 are produced after a 1-hr reaction, at which time the substrate is depleted. The reaction is specific for oligo(C) substrates, there being very little reaction with oligo(U) and none with oligo(A) or oligo(dC). The proposed mechanism is illustrated in Fig. 1. The individual steps in the mechanism are intermolecular versions of RNA self-splicing and IVS RNA cyclization.

At first glance, the activity of the L – 19 IVS RNA seems very different from that of RNA polymerase; the former catalyzes RNA recombination, giving no net change in the number of phosphodiester bonds, whereas the latter clearly gives net synthesis of nucleic acid. In fact, however, both reactions are strictly conservative with respect to the number of O–P bonds in the system. The L – 19 IVS RNA uses C₅ (or C₄) instead of CTP as a substrate. It incorporates pC units at the 3' end of the growing chain and releases C₄ (or C₃), which is analogous to the pyrophosphate released by RNA polymerase (Fig. 2).

The reaction shown in Fig. 1 has further similarities to contemporary RNA polymerase reactions. Chain elongation occurs in a 5'→3' polarity. All products have 3'-hydroxyl termini. The covalent linkages are exclusively 3',5'-phosphodiester bonds (20).

The L – 19 IVS RNA is thought to recognize oligo(C) substrates by Watson–Crick base-pairing to an oligonucleotide binding site (internal template) with the sequence GGAGGGA (26–29). This binding site was originally predicted from RNA structure models to be the part of the “internal guide sequence” that pairs with the 5' exon (ref. 30; see also

Abbreviations: IVS, intervening sequence (intron); L – 19 IVS RNA (read “L minus 19”), a 395-nucleotide RNA missing the first 19 nucleotides of the linear IVS RNA that is the direct product of *Tetrahymena* pre-ribosomal RNA splicing; ribozyme, an RNA molecule that shows intramolecular catalysis or acts as an enzyme; ribozyme*, a hypothetical ribozyme that has the known enzymatic activity of the L – 19 IVS RNA but is dependent on an external template and able to incorporate all four nucleotides.

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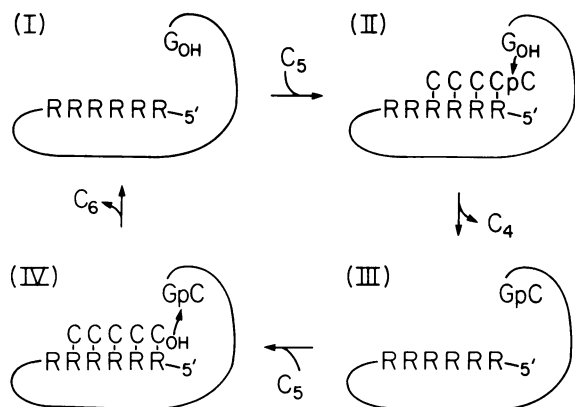


FIG. 1. Proposed mechanism of polymerization of cytidylic acid by a real RNA enzyme, the L - 19 IVS RNA (figure adapted from ref. 20, with permission of the copyright holder). The L - 19 IVS RNA enzyme (I) is shown with the pyrimidine oligonucleotide ("oligopyrimidine") binding site (RRRRRR, 6 purines) near its 5' end and guanosine-414 (G⁴¹⁴) 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 its substrate (C₅) by Watson-Crick base-pairing to form the noncovalent enzyme-substrate complex (II). Nucleophilic attack by G⁴¹⁴ leads to formation of the covalent intermediate (III), which contains a high-energy GpC bond (20, 26). If C₅ binds to the intermediate in the manner shown (IV), transesterification can occur to give the new product C₆ and regenerate the enzyme (I). As the concentration of the product C₆ increases, it can be used as a substrate to give C₇, and so on. With longer oligonucleotides as substrates, the enzyme is not restricted to attacking at the 3' end of the substrate; thus, the covalent intermediate can have more than one C residue esterified to G⁴¹⁴ (20).

ref. 31). The IVS can be altered to recognize a different 5' exon sequence by changing its internal guide (M. Been and T.R.C., unpublished data). Other group-I introns recognize quite different 5' exon sequences, presumably because they have different internal guide sequences (32, 33). Therefore, as RNA polymerases they are predicted to have different substrate specificity.

Although the 5' exon sequences of different group-I introns vary widely, there is one conserved position. The 5' splice is preceded by a conserved U residue that is thought to pair with a conserved G residue at the 5' end of the exon binding site within the intron (31-33). This interaction may be obligatory for the first step of RNA self-splicing (ref. 29; L. Barford and T.R.C., unpublished data), but recent results indicate that it may not be important for L - 19 IVS RNA catalysis (A. Zaug, R. Kierzek, M. Caruthers, and T.R.C., unpublished data). In any case, the ability of the L - 19 IVS RNA to polymerize RNA in a template-dependent manner with no restriction on the base sequence of its internal template is, at present, conjecture rather than established fact.

An RNA Enzyme as an RNA Polymerase. An effective RNA polymerase must not only be able to incorporate all four nucleotides into a growing chain, but it must also utilize an external rather than an internal template so that it can copy

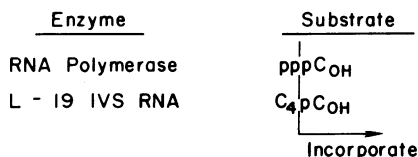


FIG. 2. Substrate requirements for contemporary (protein) RNA polymerases [using a poly(dG) template] and the L - 19 IVS RNA. Both polymerization reactions are conservative with respect to O-P bonds.

chains of any length and sequence. It seems possible that the L - 19 IVS RNA might retain activity if its template (internal guide sequence) were dissociated from its catalytic portion. The internal guide sequence is presumably oriented very precisely with respect to the critical conserved sequence elements (34-36) and the 3'-terminal G residue to allow self-splicing and L - 19 IVS RNA activity. It seems likely that this orientation is provided by multiple sequence-independent interactions, perhaps interactions with the phosphates or the 2'-hydroxyl groups of the ribose moieties. If its internal template were deleted, the molecule might assemble with an external template to give an active complex.

For the purpose of the RNA polymerase model, I define ribozyme* as an RNA enzyme with the known catalytic activity of the L - 19 IVS RNA but dependent on an external template and able to incorporate all four nucleotides. The proposed mechanism of RNA-catalyzed RNA polymerization is shown in Fig. 3A. The ribozyme* already has a nucleotide N loaded on its 3'-terminal guanosine (I). The ribozyme* is noncovalently bound to the template RNA, the template assuming the position occupied by the internal guide sequence in Fig. 1. The ribozyme* either slides along the template or transiently associates and dissociates. The solution also contains a collection of tetra- and pentanucleotides of random base sequence to provide monomer units. If one of these oligonucleotides binds to a complementary sequence on the template adjacent to the activated nucleotide N at the 3' end of ribozyme*, transesterification can occur to transfer N from the enzyme to the oligonucleotide (II). This reaction, which is thermodynamically favorable (26), is equivalent to reaction IV → I of L - 19 IVS RNA catalysis (Fig. 1). The

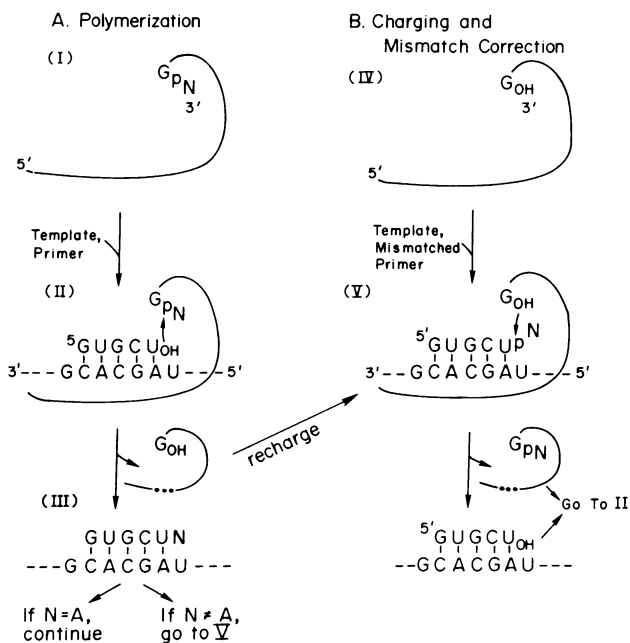


FIG. 3. Mechanism of template-dependent RNA polymerization by ribozyme*, a hypothetical RNA enzyme with enzymatic activity similar to that of the L - 19 IVS RNA. Ribozyme* is missing the oligopyrimidine binding site of the L - 19 IVS RNA (Fig. 1). (A) Charged ribozyme* (I) with a nucleotide N esterified to its 3'-terminal G residue, interacts with a template and primer to form complex II. Ternary complex II is envisioned to have the same structure as binary complex IV of Fig. 1. The enzyme facilitates the attack of the 3'-terminal hydroxyl group of the primer on the phosphate preceding N, transferring N to the growing chain (III) and releasing uncharged ribozyme*. (B) Uncharged ribozyme* (IV) interacts with a template-primer system containing a terminal mismatched nucleotide (V). The enzyme facilitates the attack of the 3'-terminal hydroxyl group of the ribozyme* on the phosphate preceding N, thereby recharging the enzyme.

transfer reaction is proposed to occur with highest efficiency if N can also base-pair with the template strand; i.e., if N = A in Fig. 3 (II). [The equivalent "rule" for RNA self-splicing would be for the first nucleotide of the IVS to be complementary to the nucleotide preceding the conserved G in the internal guide sequence. This rule is followed by most group-I introns (33), but the requirement for pairing of these bases has not yet been critically tested.]

If the newly added base N is properly paired with the template, another charged ribozyme* can bind and chain elongation can continue. On the other hand, if N is mispaired, the primer will be a very poor attacking group for further polymerization but a very good reactant for a charging reaction (Fig. 3B). Thus, the reversibility of the reaction assures attainment of equilibrium and gives an opportunity for mismatches to be corrected. Such a mechanism can give reasonable fidelity in copying ($<10^{-2}$ errors per step), provided that the rate of adding a matched nucleotide is at least 20-fold greater than the rate of adding a mismatched nucleotide, and that the rate of removing a mismatched nucleotide is at least 20-fold greater than the rate of removing a matched nucleotide. This is an energy-efficient correction process, because instead of removing the mispaired nucleotide by hydrolysis, it uses it to recharge a ribozyme*. However, it does not provide the high fidelity that can be obtained by proofreading mechanisms in which there is excess consumption of high-energy bonds.

The charging reaction (Fig. 3B) need not take place on the same template but could take place on a primer-template pair anywhere in the system. An uncharged ribozyme* with a free 3' hydroxyl (IV) associates with a template containing a primer that is not base-paired at its 3' end (V). The 3'-terminal G attacks the phosphate preceding nucleotide N, forming the covalent GpN bond and recharging the ribozyme*. The reaction is equivalent to reaction II \rightarrow III of L-19 IVS RNA catalysis (Fig. 1). It has an unfavorable equilibrium constant (26) but can be driven by an excess concentration of oligonucleotide.

Depending on the size distribution of oligonucleotide substrates, ribozyme* might be charged by more than a single nucleotide. This can be accommodated in the model. For example, if ribozyme* were charged with a dinucleotide, it would transfer two nucleotides to the primer in a single step. If both nucleotides could pair with the template, they would be retained. If neither paired, they would be removed by the correction mechanism. If the first nucleotide were matched but the 3'-terminal nucleotide were mismatched, the latter could be removed, giving a net extension of one nucleotide.

Early in the reaction, when the oligonucleotide primers are short, they would be expected to pair with the template only transiently, as in the reactions catalyzed by the L-19 IVS RNA. However, as the primer is lengthened it would become stably paired with the template. Because each step in the polymerization reaction is reversible, pairing would be important to help drive polymerization to completion. It would also be important for the accuracy of the replication process. As the primer becomes longer it will be able to pair stably even if there is an internal mismatch. If it were continually dissociating and reassociating, it could pair to sequences other than the one that served as its template. If the match were imperfect, errors would accumulate. On the other hand, if the primer remained bound, the only opportunity for introducing new errors would be at the 3' end of the growing chain, where the mismatch correction mechanism is operative. At all stages of the reaction, the ribozyme* would operate in a distributive rather than a processive manner, since it must dissociate from the template-primer to be recharged. In this manner the reaction is dissimilar to contemporary transcription and replication, which are processive.

RNA Self-Replication. The model for RNA-catalyzed, template-dependent RNA polymerization provides the key element for a complete model for RNA self-replication (Fig. 4). The general scheme is similar to one published recently by Sharp (15). The replication cycle begins with a double-stranded RNA (I). (This double-stranded form is a useful mental construct but, as described below, need not exist as an intermediate.) The double-stranded RNA undergoes strand-separation—e.g., thermal denaturation under the influence of the heat of the sun (21). One of the single strands (the plus strand) folds to form the ribozyme* (II). The complementary (minus) strand serves as the template. The oligonucleotides that serve as the primers and as the source of monomer units have been previously synthesized—e.g., by the template-directed nonenzymatic polymerization processes described by Orgel and colleagues (22–24).

Polymerization and mismatch correction proceed according to the mechanism described in Fig. 3 (III). The end result can be re-formation of a double-stranded RNA (I). Alternatively, it is attractive to envision strand-displacement taking place during replication (17), so that the intermediate (III) is directly converted to the single-stranded product (II) without going through a form that is double-stranded throughout its length (I). This might be accomplished if local regions of the RNA were able to form transient intramolecular base-pairing that competed with the intermolecular base-pairing, as occurs during the replication of single-stranded bacteriophage RNA (37, 38).

In a subsequent round of replication, one of the catalyst strands can serve as the template for the production of another minus-strand RNA. Thus, the same ribozyme* molecule can serve both as a functional catalyst and as an informational entity.

In considering the origin-of-life implications of RNA-catalyzed RNA polymerization, it is important to realize that a ribozyme* would not be restricted to using itself and its complement as templates. Other RNA molecules in its environment would also be replicated. Some of these might have useful enzymatic activities, for example as specific RNA-processing enzymes like RNase P (9–11). Other RNA molecules might be able to bind an amino acid as well as a portion of an RNA template, thereby serving as primitive transfer RNAs (39, 40). Still other RNA molecules might facilitate the binding of two transfer RNAs at adjacent sites

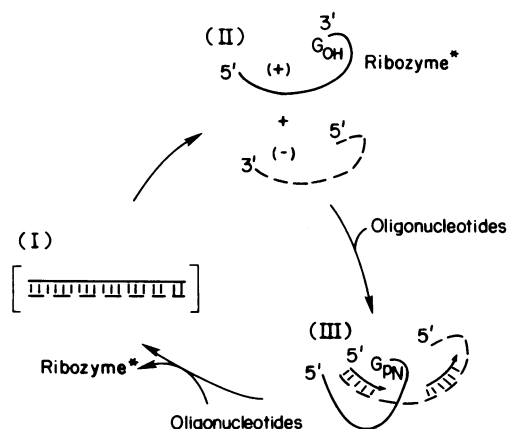


FIG. 4. Self-replication scheme. Double-stranded RNA (I) undergoes strand separation to give ribozyme* [(+)-strand] and the complementary (-)-strand (II). The ribozyme* catalyzes synthesis of a new (+)-strand, using the (-)-strand as a template (III). The detailed mechanism of replication is shown in Fig. 3. If more than one primer is elongated on the same template, the resulting fragments can be spliced together (15). Completion of replication gives a double-stranded RNA (I). Square brackets indicate that I is not an obligatory intermediate in the replication cycle (see text).

on an RNA template and catalyze peptide-bond formation, thereby serving as primitive ribosomal RNAs (41, 42). As peptides and proteins became available, some of them would interact with the RNA catalysts and enhance their activity or modulate it in useful ways. The ribozymes would begin to work as ribonucleoprotein particles. The major point of this paper, however, is that it is now reasonable to envision prebiotic nucleic acid replication in an entirely RNA-based system, prior to the advent of any translational machinery or other source of proteins.

Further Evaluation of the Model. The scheme for RNA-catalyzed RNA replication shown in Figs. 3 and 4 does not address some important details. To what extent would replication be blocked by structured regions in the RNA template? If replication proceeded simultaneously from two or more primers on the same template, could the fragments be ligated? [Such splicing could presumably be RNA-catalyzed; Sharp (15) has proposed a replication scheme based entirely on such RNA splicing.] How could the mismatch correction mechanism be restricted to working on the growing chain and prevented from catalyzing breakdown of the template? Although it is possible to invent solutions, there is not yet sufficient experimental basis for evaluating the gravity of these problems.

Further, it is not clear how small an RNA molecule could function as ribozyme*. Based on nucleotide-deletion studies with the *Tetrahymena* IVS RNA, it seems possible that efficient cleavage-ligation activity might require an RNA enzyme as large as 300 nucleotides (ref. 43; G. Dinter-Gottlieb, L. Dokken, and T.R.C., unpublished data). At the other extreme, the core structure of group-I introns (31–33) contains only about 100 nucleotides, so there is hope that smaller molecules might have substantial catalytic activity.

The scheme for RNA-catalyzed RNA replication appears to have several advantages over schemes for nonenzymatic polymerization of activated mononucleotides, in which the only catalysis involves the alignment of the monomer units by their interaction with the template (23). The most obvious is the rate acceleration. If ribozyme* could work at the rate of the L – 19 IVS RNA, it could achieve rates of chain elongation of two residues per minute (20), 1000 times the rate of polymerization of activated mononucleotides or oligonucleotides (23, 25). The higher rate might be necessary for the establishment and maintenance of a prebiotic replication system, because RNA is not infinitely stable and polymerization of reasonably long RNA molecules must occur fast enough to compete with random hydrolysis. Second, the L – 19 IVS RNA works efficiently in dilute solutions. It requires only micromolar concentrations of oligonucleotides, presumably because binding is facilitated by stacking and other interactions in the enzyme active site. The efficient nonenzymatic polymerization of activated mononucleotides or oligonucleotides, on the other hand, requires concentrations of 25–100 mM (23–25). Third, the enzymatic synthesis utilizes probable monomer units—oligonucleotides. It seems reasonable that oligonucleotide synthesis preceded polynucleotide synthesis, so that oligonucleotides were already present in the prebiotic environment where the first polynucleotides were synthesized (21). Finally, the enzymatic synthesis does not totally consume its monomer units but only reduces their length. Residual oligonucleotides that are too short to serve as monomers should be ideal reactants for extension by the nonenzymatic reactions, which in some cases have been shown to become more efficient once a dinucleotide or oligonucleotide has been formed (44). Thus, the enzymatic and nonenzymatic processes could be synergistic.

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