

tRNA-like structures tag the 3' ends of genomic RNA molecules for replication: Implications for the origin of protein synthesis

(RNase P/CCA-adding enzyme/telomeres/tRNA synthase/*Tetrahymena* self-splicing intron)

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ABSTRACT Single-stranded RNA viruses often have 3'-terminal tRNA-like structures that serve as substrates for the enzymes of tRNA metabolism, including the tRNA synthases and the CCA-adding enzyme. We propose that such 3'-terminal tRNA-like structures are in fact molecular fossils of the original RNA world, where they tagged genomic RNA molecules for replication and also functioned as primitive telomeres to ensure that 3'-terminal nucleotides were not lost during replication. This picture suggests that the CCA-adding activity was originally an RNA enzyme, that modern DNA telomeres with the repetitive structure C_mA_n are the direct descendants of the CCA terminus of tRNA, and that the precursor of the modern enzyme RNase P evolved to convert genomic into functional RNA molecules by removing this 3'-terminal tRNA-like tag. Because early RNA replicases would have been catalytic RNA molecules that used the 3'-terminal tRNA-like tag as a template for the initiation of RNA synthesis, these tRNA-like structures could have been specifically aminoacylated with an amino acid by an aberrant activity of the replicase. We show that it is mechanistically reasonable to suppose that this aminoacylation occurred by the same sequence of reactions found in protein synthesis today. The advent of such tRNA synthases would thus have provided a pathway for the evolution of modern protein synthesis.

The discovery of the self-splicing rRNA intron of the ciliate *Tetrahymena*, and the subsequent discovery of many other catalytic RNA molecules, has made it seem likely that life began with RNA serving both as genome and as catalyst for its own replication (1). The chemical properties of RNA particularly suit it for such a dual role. Nucleic acids naturally form complementary base pairs, so RNA can serve as the template for its own replication using a minus-strand intermediate. RNA can also assume elaborate secondary and tertiary structures, as demonstrated by the crystal structure of modern tRNAs (2), and this enables RNA to function as an enzyme by accurately positioning chemically reactive groups. The 2'- and 3'-hydroxyl groups of RNA can play a direct role in catalysis, as is known to be the case for contemporary RNA enzymes (1, 3, 4), and the nucleic acid bases themselves could provide additional catalytic groups (1, 5). Finally, RNA molecules as small as 52 nucleotides have been shown to exhibit accurate enzymatic activities (6–9), and RNAs of this size could well have been synthesized under prebiotic conditions.

The realization that RNA can serve both as genome and as catalyst confirmed previous speculations that RNA preceded DNA in early evolution. These speculations were originally based on the essential role played by tRNA and rRNA in translation (refs. 11–13, reviewed in ref. 14). Later, two additional arguments were advanced in favor of RNA as the

original genetic material: All organisms synthesize the deoxyribonucleotide precursors of DNA by reducing the ribonucleotide precursors of RNA, and DNA synthesis is invariably primed by RNA (27, 42, 43). In this "RNA world" (15), the first living molecule would have been an RNA replicase that copied other RNA molecules, including other replicases, by way of a minus-strand intermediate. As the RNA world evolved, each primitive living system would have consisted of a population of RNA molecules that played different enzymatic or structural roles but that were all dependent on a common RNA replicase for their replication. This replicase performed a function analogous to that of the enzymes that replicate the RNA genomes of contemporary viruses as diverse as coliphage Q β and turnip yellow mosaic virus (16, 17).

tRNA-Like Structures at the 3' End of Modern RNA Genomes. Evidence has accumulated that the single-stranded RNA viruses of bacteria and plants contain a tRNA-like structure at their 3' ends (reviewed in refs. 16 and 17). The evidence that these structures are functionally similar to tRNA is compelling. The viral RNAs generally serve as substrates for enzymes of tRNA metabolism such as the CCA-adding enzyme (tRNA nucleotidyltransferase), the modifying enzymes (tRNA methyltransferase), and the peptidyl-tRNA hydrolase. The aminoacyl-tRNA synthases can specifically aminoacylate many of these viral RNAs, and elongation factor 1 (the eukaryotic analogue of prokaryotic elongation factor Tu) will in turn recognize these aminoacylated RNAs. Despite the many functional similarities between tRNA and the viral 3'-terminal tRNA-like structures, the predicted secondary structure of the 3' end of only some of these RNA viruses resembles the tRNA cloverleaf structure. However, the structural resemblance of the viral 3' ends to tRNA can be improved when the ability of RNA "pseudo-knotting" to generate tertiary structure is taken into account (18, 44).

Q β Replicase Uses Translation Factors for Initiation of Replication. A possible explanation for tRNA-like structures at the 3' end of viral RNAs was suggested by studies of the replicase of the single-stranded RNA coliphage Q β (reviewed in ref. 19). Q β replicase was found to be a tetramer consisting of a phage-encoded RNA polymerase (subunit II) complexed with three host proteins that were known to be required for protein synthesis—the elongation factors Tu and Ts and ribosomal protein S1. Although the secondary structure of the 3'-terminal sequence of the Q β genome does not resemble a typical tRNA cloverleaf, both the plus and minus strands have a 3'-terminal CCA, and this suggested a model for how the replicase might be able to initiate replication. During protein synthesis, Tu binds aminoacylated tRNAs, and Ts binds Tu, with GTP functioning as the cofactor. By analogy, the Tu and Ts subunits of Q β replicase might enable the enzyme to bind to 3'-terminal tRNA-like structures on the viral RNAs. Accurate initiation probably depends critically upon immobilization of the extreme 3' end of the RNA template, and the Tu and Ts subunits could facilitate initia-

tion by holding the 3'-terminal CCA in a fixed position relative to subunit II. Because synthesis of both the plus and minus strands initiates with GTP on the penultimate cytidine of the CCA 3' end, it was also plausible that a GTP bound to Tu (and/or Ts) might donate the initiating nucleotide triphosphate. Finally, because the 5' end of both the Q β plus and minus strands is 5'-triphosphoguananylyl(3'-5')guanosine (pppGG), the CCA-adding enzyme (or the replicase itself) would have to be able to add an untemplated 3'-terminal adenosine.

Functions of the 3'-Terminal Structure of Q β Replicase. Subsequent studies have shown that Q β replicase is far more complicated than this simple model would suggest (reviewed in ref. 19). Still, the experimental evidence is consistent with the notion that the 3'-terminal structure of Q β RNA functions both as an origin of replication (recognized by the host subunits of Q β replicase) and as a simple telomere (perhaps recognized by the CCA-adding enzyme) that prevents progressive loss of 3'-terminal RNA sequences during multiple rounds of replication.

From the start, it was difficult to imagine how an RNA phage had managed to misappropriate translation factors for use in RNA replication. One possibility was that Q β —and by implication all the other single-stranded RNA viruses of eukaryotes—had simply adopted the 3'-terminal tRNA-like structure as a way of using preexisting host proteins to stabilize the interaction of the phage replicase with the phage genome. However, so many different RNA viruses of bacterial and plant origin have 3'-terminal tRNA-like structures that these structures are most unlikely to have evolved independently. The more plausible interpretation is that these structures all derive from a common viral ancestor predating the divergence of prokaryotes and eukaryotes.

We suggest here that Q β did not borrow translation factors for RNA replication; rather, the translation apparatus appropriated RNA replication factors. Specifically, we will consider several disparate lines of evidence that support the hypothesis that the 3'-terminal tRNA-like structure of Q β RNA is a molecular fossil of an ancient RNA world, where such structures tagged the 3' end of genomic RNA molecules for replication by the RNA replicase. Such genomic organization suggests, furthermore, that the first step in the evolution of protein synthesis was the emergence of a variant RNA replicase that was able to aminoacylate the 3' end of another RNA molecule with a specific amino acid, using the same series of reactions that are found in protein synthesis today.

THE GENOMIC TAG MODEL

The model we propose here is based on the premise that just as the evolution of cellular life is written in the physical fossil record, so contemporary molecules and reactions provide a molecular fossil record of precellular life.

RNA Replication in the Early RNA World. The self-splicing intron of *Tetrahymena* rRNA provides a compelling contemporary example of a molecular fossil. This RNA molecule can be experimentally transformed with remarkable ease into a poly(C) polymerase, suggesting that the intron might itself be a direct descendant of an early RNA replicase (1). This fundamental insight inspired Cech (20) to propose that an early RNA replicase copied RNA by rearranging prebiotically synthesized oligonucleotides in a template-dependent fashion. Cech's elegant model has two great strengths. First, it is founded on experimentally verified properties of the intron. Second, it uses as the source of activated mononucleotides the same pool of relatively stable, prebiotically synthesized random oligonucleotides that must have given rise to the replicase itself (reviewed in ref. 21). Related models for the primitive replicase have been proposed by Sharp (3) and by Doudna *et al.* (45).

Any scheme for primitive replication immediately confronts two problems: identifying a specific site for initiation and distinguishing the template from other molecules. This is true whether random oligonucleotides or activated mononucleotides provided monomer units for polymerization. Without a specific initiation site, replication would be equally likely to initiate anywhere on a plus- or minus-strand template, and large tracts of template might be unreplicated. Without a mechanism for assuring specificity in the choice of substrate, the replicase would be as likely to squander its efforts on replication of random oligonucleotides as to copy other functional molecules—in particular, other replicases. Thus mechanisms for preferential and complete replication of useful RNA molecules were essential for the evolution of more sophisticated living systems.

3'-Terminal tRNA-Like Structures as the First Genomic Tags. The simplest way for an RNA molecule to be replicated efficiently and completely in the early RNA world would be for the active site of the replicase to have an affinity for the 3' end of the molecule. This would position the replicase to copy in the 5' to 3' direction, as in all modern replication reactions. We therefore suggest that genomic RNA molecules in the RNA world had a special 3'-terminal tag that served both to identify the molecule as a substrate for the replicase and to specify the initiation site for replication. RNA molecules lacking this tag would not be efficiently replicated and would ultimately be lost. We further suggest that this 3'-terminal tag was the first tRNA-like structure, and that both the sequence and the function of this structure have been preserved through more than 3 billion years of evolution in the form of the 3'-terminal tRNA-like structures found in modern RNA viruses of bacterial and plant origin (16, 17).

RNase P May Have Evolved To Distinguish Genomic and Functional RNA Molecules. Initially, there was probably no distinction between genomic and functional RNA molecules; the same RNA replicase molecule would have served both as genome and as enzyme. Later, as RNA enzymes with different activities evolved, each would have the same 3'-terminal tRNA-like structure to ensure that it was replicated by the common RNA replicase. But the 3' tag would obviously limit the variety of enzymatic reactions in which the 3' end of the RNA could participate. The 3' guanosine of the *Tetrahymena* rRNA intron, for example, serves as the enzymatically active residue for sequential transesterifications. Removal of the 3' tag from a fraction of the identical copies of each RNA species would have increased the potential variety of enzymatic and structural activities, and this would have provided the driving force for evolution of an RNA endonuclease that removed the 3'-terminal tRNA-like structure. For convenience, we shall designate those RNA molecules from which the 3'-genomic tag has been removed as "functional RNAs," with the understanding that some genomic RNA molecules may have been enzymatically functional despite—or even because of—the 3'-terminal tag.

We suggest that the RNA component of RNase P (5, 22) is a molecular fossil of this ancient untagging activity. Originally, RNase P would have functioned to liberate functional RNA enzymes from their genomic precursors by removing the 3'-terminal tRNA-like tags; the tags themselves would then have been degraded or recycled. Thus RNase P may have incidentally provided a population of essentially homogeneous tRNA-like molecules that could later be recruited to function as tRNAs in protein synthesis (see below). Apparently, then, the role of RNase P has suffered an historic reversal: an enzyme that evolved to liberate a functional RNA from its 3'-terminal tRNA-like tag now liberates the functional "tag" from a nonfunctional precursor RNA!

Was CCA the First Telomere? As Watson first pointed out for duplex DNA genomes (23), replication of the 3' end of a nucleic acid always requires a special device to prevent loss

of genetic information. This is as true for single-stranded RNA genomes as for double-stranded DNA. If an enzyme analogous to the contemporary CCA-adding enzyme evolved early in the RNA world, then the tRNA terminus would also function as a telomere to ensure that genomic RNA molecules would be faithfully replicated without progressive loss of 3'-terminal sequences.

The first eukaryotic telomere sequence to be identified was $(C_4A_2)_n$ in the ciliate *Tetrahymena*, and similar telomeric C_nA_m motifs have subsequently been found in a wide variety of organisms (24). We speculate that these telomeric sequences are evolutionarily related to the 3'-terminal sequence of tRNA and that conversion from linear RNA genomes to linear DNA genomes preserved the 3'-terminal CCA motif. But if telomeres descended from CCA termini, why does the *Tetrahymena* telomere terminal transferase add T_2G_4 rather than C_4A_2 ? We suggest that the CCA motif originally found at the 3' end of single-stranded genomic RNA molecules was transferred, later in evolution, to the 5' end of double-stranded genomic molecules by formation of the hairpin turnaround characteristic of modern telomeres. In fact, analogous primer-dependent but template-independent enzyme activities regenerate both the 3'-terminal CCA of tRNA and the *Tetrahymena* telomeres (25). The CCA-adding enzyme requires a tRNA-like structure as substrate, while the *Tetrahymena* T_2G_4 -adding enzyme requires a single-

stranded primer sequence $(T_2G_4)_n$, where $n \geq 1$. Interestingly, the *Tetrahymena* T_2G_4 -adding enzyme has an essential RNA component (C. Greider and E. H. Blackburn, personal communication), further implying that this activity is a molecular fossil of the RNA or ribonucleoprotein world.

A Variant RNA Replicase May Have Functioned as the First tRNA Synthase. If our speculations are correct, the active form of the RNA replicase possessed a binding site for a tRNA-like structure that positioned the 3'-terminal CCA group at the active site of the replicase. The presence of the 3'-terminal CCA of a tRNA-like structure at the active site of the replicase suggests to us that the first tRNA synthase was in fact a variant activity of the RNA replicase. We propose that tRNA aminoacylation by this early synthase would have followed the same series of reactions as in modern protein synthesis, as illustrated in Fig. 1.

The first step is the covalent addition of a mononucleotide to the variant RNA replicase (charging) exactly as documented for the poly(C) polymerase activity of the *Tetrahymena* intron (26) and as proposed by Cech (20) in his model for the original RNA replicase. However, the labile bond between the RNA replicase and the NMP (where N is any base) would be discharged by attack of an amino acid carboxyl group, rather than the 3'-hydroxyl group of an RNA, thus generating an enzyme-bound aminoacyl-NMP intermediate. Discharge would only occur at the active site,

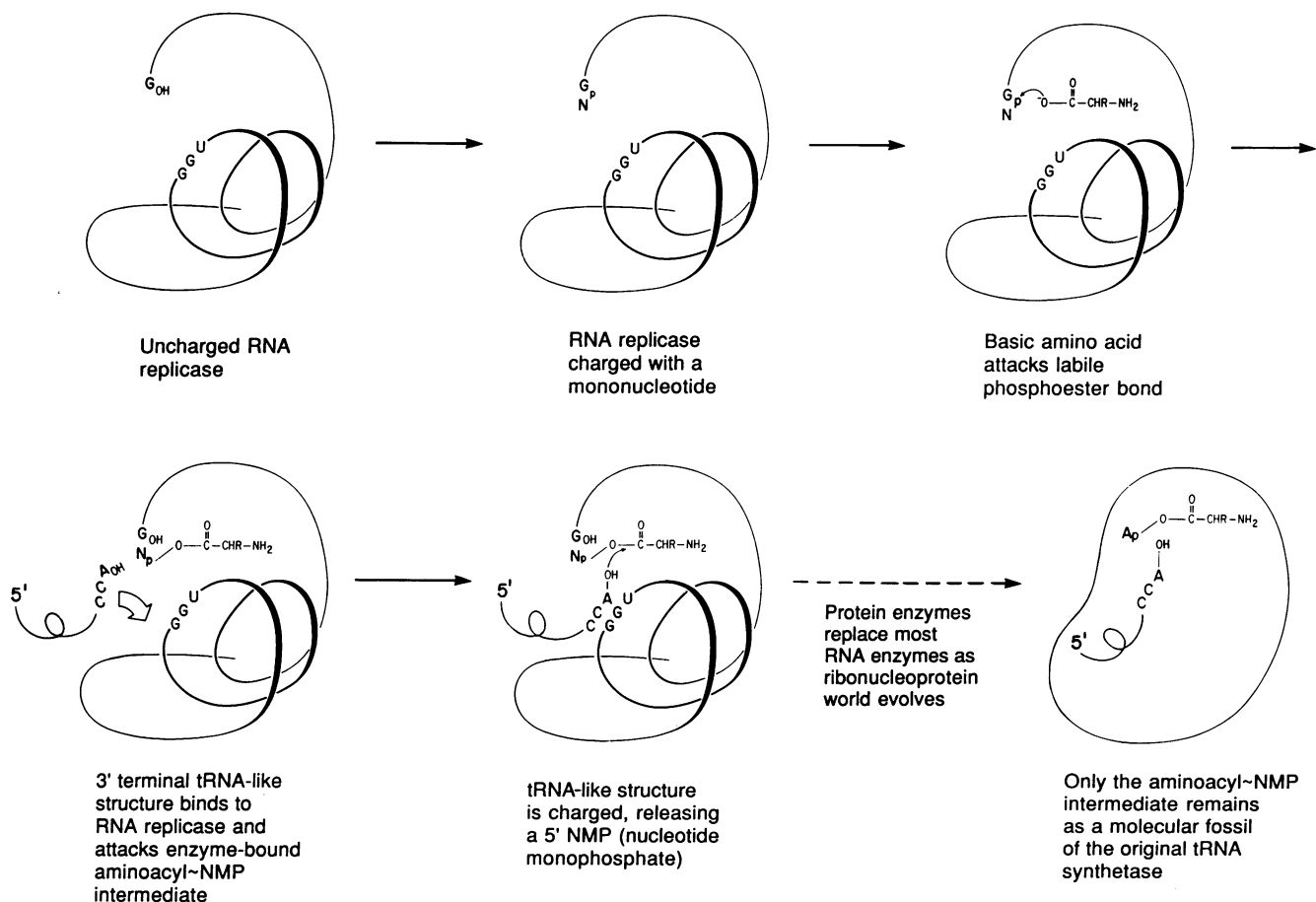


FIG. 1. An early RNA replicase could have functioned as the first tRNA synthase, using the same sequence of reactions found in modern protein synthesis. The functional form of the RNA replicase, lacking its 3'-terminal tRNA-like genomic tag, is shown aminoacylating a tRNA-like genomic tag. In principle, this tag could be free or attached to a genomic RNA molecule. For clarity, we show an RNA replicase in which addition of each new mononucleotide to the growing RNA chain proceeds through a covalent NMP-replicase intermediate (charged replicase) as originally proposed by Cech (20). Another possibility is that the primitive RNA replicase—like modern RNA polymerases—might have catalyzed direct addition of activated mononucleotides to the 3' end of the growing RNA chain. In this case, the enzyme-bound aminoacyl-NMP intermediate would have been generated—as it is today—by reaction of the amino acid with an activated mononucleotide that was noncovalently bound to the replicase. (Lower center, charged means aminoacylated.)

so the replicase would activate only those amino acids with a specific affinity for the active site. Transfer of the activated amino acid to the 3'-terminal CCA of a tRNA-like structure would complete the aminoacylation reaction. The CCA acceptor could be the terminus of a genomic RNA molecule or of a free genomic tag liberated by RNase P digestion. The sequence of reactions shown in Fig. 1 is essentially identical to the reactions of tRNA aminoacylation today.

We wish to emphasize that the aminoacylation reaction shown in Fig. 1 is not spontaneous but catalyzed by an enzyme, and thus the reaction can overcome the unfavorable equilibrium constant for formation in free solution of the mixed anhydride intermediate. This is true regardless of whether the source of energy for aminoacylation is a phosphodiester bond or a higher-energy mononucleotide oligophosphate. In protein synthesis today, formation of the aminoacyl-AMP intermediate is driven by hydrolysis of the α - β phosphoanhydride bond of ATP (27). Without a catalyst, however, the reaction of an amino acid with ATP to form an aminoacyl adenylate is extremely unfavorable ($K \approx 10^{-7}$ M); the reaction occurs readily only because aminoacyl-tRNA synthases use substrate binding energy to enhance catalysis. In particular, stabilization of the enzyme-bound tyrosyl-AMP intermediate on the *Bacillus stearothermophilus* tyrosyl-tRNA synthase has been shown to displace the unfavorable equilibrium constant for formation of tyrosyl-AMP by a remarkable factor of 10^7 (28). The use of substrate binding energy to enhance catalysis is unlikely to be restricted to protein enzymes. In fact, the ability of the *Tetrahymena* rRNA intron to bind guanosine nucleotides (reviewed in ref. 1) demonstrates that RNA enzymes can bind small substrate molecules with high specificity.

DISCUSSION

Previous models for the origin of protein synthesis have made the simplifying assumption that a homogeneous population of specifically aminoacylated tRNAs already existed (29–32). The genomic tag model naturally explains how such a population of aminoacylated tRNAs could have arisen. The tRNA-like structures would have been present already as tags on all genomic RNA molecules; they may even have existed as a stable population of free tRNA-like molecules cleaved from genomic RNAs by RNase P.

The genomic tag model also accounts for the specificity of tRNA aminoacylation. Previous efforts to explain the specificity of aminoacylation postulated a chemical "affinity" between the anticodon and amino acid that would somehow lead to uncatalyzed or prebiotic aminoacylation; however, experimental attempts to demonstrate such affinity have met with little success (33, 34). According to our model, the original association of a particular amino acid with a particular codon would simply reflect the fortuitous affinity of the amino acid for the active site of a particular tRNA synthase. Since positively charged amino acids might preferentially bind to the negatively charged sugar-phosphate backbone of the tRNA synthase, the first tRNA synthases might have been specific for basic amino acids (but see ref. 35). Later, the initially homogeneous population of tRNAs would begin to diverge and additional tRNA synthases would arise, each having a slightly different tRNA and amino acid binding site. Eventually, as proteins gradually replaced most RNA enzymes in the ribonucleoprotein world, the stepwise conversion of each tRNA synthase made of RNA into a tRNA synthase made of protein would have taken place independently. Assuming that there are many different ways of transforming an RNA enzyme into a protein enzyme (36, 37), this would account for the puzzling diversity of modern tRNA synthases (38, 39).

We suggest that specific activation of amino acids as the 3' esters of ribonucleotides allowed spontaneous polymerization of short peptides and that these particular short peptides were significantly more useful to the first living systems than were random prebiotically synthesized peptides. For example, if the original tRNA synthases were specific for basic amino acids, this might have resulted in synthesis of short basic peptides. Such peptides might have enhanced the activity of existing catalytic RNA molecules or have stabilized the lipid vesicles that may have served as cell membranes (29, 40).

The first primitive ribosome probably arose to accelerate spontaneous peptide bond formation. In fact, the absence of a demonstrable peptidyltransferase activity in the modern ribosome is often interpreted to mean that peptide bond formation occurs spontaneously after correct alignment of two charged tRNAs (reviewed in ref. 41). This first ribosome (protoribosome) might have been an entirely new enzyme, or it may have been derived from the replicase or the tRNA synthase by a gene duplication (resulting from a replication error or RNA recombination) that created two tRNA binding sites on a single molecule. While a ribosome with two identical tRNA binding sites would not have any mechanism for translocation of the growing peptide chain, its very symmetry may have allowed either (or both) sites to be occupied by a di- or oligopeptidyl-tRNA. In this way, repeated cycles of dissociation and reassociation could have provided a mechanism for the synthesis of peptides sufficiently long to play a useful biological role.

As we discuss in greater detail elsewhere (46), the synthesis of random peptides is unlikely to have been useful, and this suggests that early protoribosomes were peptide specific. At first the protoribosomes synthesized only one kind of peptide, because the tRNA synthase was unable to use more than one kind of amino acid. Later, as new tRNA synthases emerged that could use additional amino acids, new species of protoribosomes would evolve that could distinguish between the various species of aminoacylated tRNA. We suggest that one of the sequences within the tRNA that enabled it to bind to the protoribosome was the precursor of the contemporary anticodon and that the anticodon binding site on the protoribosome functioned as a built-in mRNA. Driven by selective pressure to synthesize peptides containing a greater variety of amino acids, the three components of the original translation apparatus—the tRNA, the tRNA synthase, and the protoribosome—would further diversify and coevolve. The resulting ribosomes would also be peptide-specific, and each ribosome would have its own built-in mRNA and its own corresponding tRNAs and tRNA synthases. This would define the rudiments of the genetic code. Ultimately, the advantage of making larger peptides would lead to replacement of the built-in mRNA on one particular peptide-specific ribosome with a loosely associated mRNA template, thereby creating the progenitor of the modern template-dependent ribosome.

In addition to providing a plausible pathway for the evolution of protein synthesis, the presence of 3'-terminal tRNA-like structures on ancient RNA genomes may also help to explain why tRNAs serve as primers for retroviral reverse transcription (47, 48); why tRNAs punctuate the primary transcripts of bacterial and chloroplast rRNA operons (49, 50); and why tRNA coding regions serve in DNA as promoters for transcription by eucaryotic RNA polymerase III (for review see ref. 51). These ideas will be discussed in more detail elsewhere.

Supremely sophisticated molecular machinery performs modern protein synthesis (reviewed in ref. 41). A working ribosome consists of two large RNA molecules, one or two small RNAs, at least 50 polypeptides, the mRNA template, numerous initiation, elongation, and termination factors, and

several dozen specifically aminoacylated tRNAs. Offstage, 20 different synthases must specifically aminoacylate the cognate tRNAs, while cellular metabolism provides GTP and ATP to drive peptide bond formation. The central problem in envisioning the evolution of this complex machinery has been that none of these components appears to be useful individually, and yet the apparatus must have evolved stepwise. If, as the contemporary molecular fossil record leads us to believe, tRNA-like structures predate protein synthesis in their role as genomic tags, then the emergence of an RNA enzyme with the function of a modern tRNA synthase could have served as the key step in the evolution of protein synthesis. The subsequent evolution of template-directed protein synthesis signaled the end of the RNA world.

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