Evolution of Protein Synthesis from an RNA World

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SUMMARY

Because of the molecular complexity of the ribosome and protein synthesis, it is a challenge to imagine how translation could have evolved from a primitive RNA World. Two specific suggestions are made here to help to address this, involving separate evolution of the peptidyl transferase and decoding functions. First, it is proposed that translation originally arose not to synthesize functional proteins, but to provide simple (perhaps random) peptides that bound to RNA, increasing its available structure space, and therefore its functional capabilities. Second, it is proposed that the decoding site of the ribosome evolved from a mechanism for duplication of RNA. This process involved homodimeric “duplicator RNAs,” resembling the anticodon arms of tRNAs, which directed ligation of trinucleotides in response to an RNA template.

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1 INTRODUCTION

Translation links the nucleotide sequences of genes to the amino acid sequences of proteins, establishing at the molecular level the correspondence between genotype and phenotype. The basic underlying mechanisms of translation must have arisen early in the history of molecular evolution, in some primitive form, before the existence of any genetically encoded protein. To understand how the ribosome, one of the most complex molecular structures in all of biology, and its associated translational ligands, could have emerged from an RNA world presents one of the most challenging problems in molecular evolution. Thanks to numerous fresh insights into the structure and function of ribosomes (and RNA in general), many of which are described in this collection, this once impenetrable problem can now be viewed as merely extraordinarily difficult. Among the central problems in reconstructing the molecular evolution of translation are: (1) The chicken-or-the-egg problem: If the ribosome requires proteins to function, where did the proteins come from to make the first ribosome and its translation factors? (2) What was the driving force for evolution of the ribosome? and (3) How did coding arise? Thanks to numerous advances in this field, we now have a likely answer to the first question, and a plausible answer to the second question (Noller 2004). Although the origins of coding remain a puzzle in spite of many decades of thought and speculation, a possible RNA World origin for the codon recognition function of the modern ribosome is suggested here. Another question, implicit in the RNA World hypothesis, is: (4) Can we account for all of the basic functions of translation in terms of RNA? The answer to this last question seems to be mainly “yes,” although some proteins, such as the type I release factors, may have taken over functional roles that were once played by RNA.

2 TRANSLATION OUT OF AN RNA WORLD

We begin with the question of how the first translational system could have arisen without proteins, a question that was raised in the years following the elucidation of the genetic code and the discovery of the general properties of the translational apparatus (Woese 1967; Crick 1968; Orgel 1968). The simplest ribosomes (those from bacteria and archaea) contain about 50 different proteins and three rRNAs (16S, 23S, and 5S rRNAs) comprising about 4500 nucleotides and two-thirds of the mass of the ribosome. In addition to the ribosomal proteins, many nonribosomal protein factors are required for the steps of initiation, elongation, termination, and ribosome recycling. But how could the first ribosome have depended on proteins for its function? The overall process of translation was from

the outset recognized to be centered around RNA—mRNA, tRNA, and the ribosome. In view of the fact that ribosomes contain large amounts of ribosomal RNA (rRNA), Crick asked whether the first ribosomes might have been made exclusively of RNA. Crick’s conjecture notwithstanding, the overwhelming preponderance of opinion in the translation field was that the functions of the ribosome were determined by its proteins, and by the translation factors.

The first proteins shown to be dispensable were the translation factors. Polypeptide synthesis could be initiated in the absence of initiation factors, by manipulating the ionic conditions (Nirenberg and Leder 1964). Aminoacyl-tRNA could be bound to the ribosome in the absence of elongation factor EF-Tu, albeit at greatly reduced rates (Lill et al. 1986). Peptide bond formation was shown to be catalyzed by the large ribosomal subunit itself (Monro 1967). And translocation of tRNA could occur without EF-G (Pestka 1968; Gavrilova et al. 1976). The isolation of deletion mutants showed that at least 17 ribosomal proteins were individually dispensable (Dabbs 1986). Moreover, early in vitro reconstitution studies showed that many small-subunit ribosomal proteins could be singly omitted without abolishing function (Nomura et al. 1969). Conversely, although mutations in certain proteins were known to confer antibiotic resistance or affect translational accuracy (Davies and Nomura 1972), no examples were found in which mutation or chemical modification of a ribosomal protein caused loss of ribosome function.

Around the same time, findings from several laboratories began to point to the possibility of a functional role for rRNA. Inactivation of ribosomes on cleavage of a single phosphodiester bond of 16S rRNA by colicin E3 (Bowman et al. 1971; Senior and Holland 1971), resistance to the antibiotic kasugamycin conferred by the absence of methylation of two bases in 16S rRNA (Helser et al. 1972), inactivation of ribosomes by kethoxal modification of a few bases in 16S rRNA (Noller and Chaires 1972), and the unusually high conservation of sequences within the rRNAs (Woese et al. 1975) were early warning signs. Crosslinking of the anticodon and acceptor ends of tRNA with surprisingly high efficiency to 16S and 23S rRNA, respectively, placed the two most important functional features of tRNA in close proximity to universally conserved features of the two large rRNAs (Prince et al. 1982; Barta et al. 1984). Inactivation of ribosomes by cleavage of a single phosphodiester bond in the large subunit rRNA by α -sarcin (Endo and Wool 1982) and the dominant lethal phenotype of point mutations of G530 of 16S rRNA (Powers and Noller 1990) were more in keeping with the notion of a functional rRNA than of a mere structural scaffold. The technique of chemical footprinting of RNA

quickly showed that tRNA, elongation factors, initiation factors and all major classes of ribosome-directed antibiotics interacted with 16S and/or 23S rRNA, often at universally conserved nucleotides [summarized in (Noller et al. 1990)].

In spite of the nearly overwhelming body of evidence, the idea that rRNA was a functional molecule, let alone *the* functional molecule of the ribosome, was met with widespread skepticism. The sole functional role for rRNA that was generally accepted was the Shine-Dalgarno mechanism for mRNA start-site selection (Shine and Dalgarno 1974), because of convincing supporting evidence (Steitz and Jakes 1975), but perhaps also because its straightforward base-pairing interactions put the mechanism in a comfortable context, in keeping with well-known properties of nucleic acids.

3 PEPTIDYL TRANSFERASE: THE RIBOSOME IS A RIBOZYME

To many outside observers of the field, the main function of the ribosome was considered to be the peptidyl transferase reaction, the sole chemical reaction known to be catalyzed by the ribosome itself. Although other ribosomal functions, including the crucial processes of aminoacyl-tRNA selection and translocation would seem to merit at least as much mechanistic interest, peptide bond formation is also a symbolic event—the point of entry of an amino acid into the protein world. Footprinting and crosslinking of tRNA and its CCA end (Barta et al. 1984; Moazed and Noller 1989; Moazed and Noller 1991), and localization of the sites of interaction of several peptidyl transferase inhibitors (Moazed and Noller 1987), had unambiguously placed 23S rRNA at the “scene of the crime,” although crosslinking studies had also shown that proteins L2 and L16 were nearby (reviewed in Wower et al. 1993). In vitro reconstitution experiments had eliminated all but a handful of large-subunit ribosomal proteins (Moore et al. 1975; Schulze and Nierhaus 1982). In one attempt to show the role of rRNA in catalysis of peptide bond formation, ribosomes were subjected to stringent protein-extraction procedures. *Thermus thermophilus* 50S subunits treated with 0.5% SDS and extensive digestion with protease K, followed by continuous vortexing for an hour or more with phenol, retained their full peptidyl transferase activity (Noller et al. 1992). Most, but not all, of the protein was removed by this procedure, leaving open the main question, but forcefully calling attention to the probable catalytic functionality of rRNA.

When the crystal structures of the ribosome and its subunits were solved, any remaining doubts about the functional role of rRNA were dispelled. Structures of the

30S and 50S subunits at 3.0 Å and 2.4 Å resolution, respectively, provided detailed descriptions of the folding of the RNA and protein components of the ribosome for the first time (Ban et al. 2000; Schlueder et al. 2000; Wimberly et al. 2000). The 5.5 Å resolution structure of the complete 70S ribosome, with mRNA and tRNAs bound, showed how the subunits fit together, and revealed the interactions between the ribosome and the P- and E-site tRNAs (Yusupov et al. 2001) (Fig. 1). A complex containing the complete A-site tRNA bound to the 70S ribosome at 7.0 Å, and another complex of a tRNA anticodon stem-loop bound to the 30S subunit at 3.1–3.3 Å provided the details of the interactions between the ribosome and the A-site tRNA (Ogle et al. 2001). Several different complexes containing the 50S subunit bound with a variety of tRNA acceptor-end mimics, including a transition-state analogue, provided insight into how the aminoacyl and peptidyl ends of the tRNAs interact with the peptidyl transferase catalytic site (Nissen et al. 2000).

The distribution of the rRNA and protein moieties on the surface of the ribosomal subunits (Fig. 2) makes a clear case for the functional importance of rRNA. Proteins are distributed more or less evenly over the external surface of the ribosome, filling nooks and crannies in the rRNA (Fig. 2B,D), but the subunit interface surface, which contains the tRNA binding sites as well as other functional features, is made up mainly of rRNA (Fig. 2A,C). The overall impression is that of an RNA structure that has gradually incorporated a number of proteins over evolutionary time, but has not allowed them to impinge on its crucial functional centers.

The high-resolution structure of the 50S subunit (Ban et al. 2000), together with the knowledge of the positions of the acceptor ends of the tRNAs (Nissen et al. 2000; Yusupov et al. 2001), provided the first look at the structure of the peptidyl transferase center. No protein moieties were found within 17 Å of the catalytic site, definitively demonstrating that peptide bond formation is indeed catalyzed by RNA. Although a more recent high-resolution structure of the *T. thermophilus* 70S ribosome bound with tRNAs shows interactions between the amino-terminal tail of protein L27 and the backbone of the 3' CCA end of the P-site tRNA (Selmer et al. 2006), no part of the protein is close enough to the catalytic site to play a direct (chemical) role in the reaction. Furthermore, early studies showed that *Escherichia coli* 50S subunits reconstituted in vitro without L27 were active in catalyzing peptide bond formation (Moore et al. 1975). Also, no counterpart to L27 is found in archaeal 50S subunits (Nissen et al. 2000). Comparison of structures of 50S complexes containing various substrate and transition-state analogs support an induced-fit model for catalysis of peptide bond formation based

Figure 1. Cross-section of the crystal structure of the *T. thermophilus* 70S ribosome (Yusupov et al. 2001), with the 30S subunit on the *left* and the 50S subunit on the *right*. The locations of the peptidyl-tRNA (orange) at the subunit interface, the mRNA (green-yellow-red) wrapping around the neck of the 30S subunit, and a modeled α -helical nascent polypeptide chain (green) in the polypeptide exit tunnel are indicated. The 16S rRNA is shown in cyan, 23S rRNA in grey, 5S rRNA in grey-blue, 30S subunit proteins in dark blue, and 50S subunit proteins in magenta.

solely on RNA (Schmeing et al. 2005). The present state of our understanding of this fundamental biological function is reviewed in detail by Moore and Steitz (Moore and Steitz 2010).

4 AMINOACYL-tRNA SELECTION: THE 30S SUBUNIT A SITE

Among the other basic functions of the ribosome are the binding of tRNA to its A, P, and E sites. tRNA binding carries functional implications well beyond the simple positioning of substrates for the catalytic step. Binding to the A site (aminoacyl-tRNA selection) is an important determinant of translational accuracy (Kurland et al. 1990). The crystal structure of the 30S subunit in complex with a U₆ mRNA and a tRNA anticodon stem-loop analog of tRNA^{Phe} by Ramakrishnan and coworkers provided profound insight into the mechanism by which the ribosome mediates tRNA selection (Ogle et al. 2001). Binding of tRNA to the 30S subunit A site results in movement of three bases of 16S rRNA - G530, A1492, and A1493 into contact with the codon-anticodon duplex. These same

three universally conserved bases had previously been identified in chemical footprinting experiments as the three main bases to interact with tRNA in the 30S A site (Moazed and Noller 1986; Moazed and Noller 1990); moreover, mutation of any one of them had been found to confer a dominant lethal phenotype (Powers and Noller 1990; Yoshizawa et al. 1999). The result of the tRNA-induced conformational rearrangement brings these bases into a remarkably close steric fit with the minor groove surfaces of the codon-anticodon base pairs, involving van der Waals contacts and hydrogen bonds to both the bases and backbone riboses (Fig. 3A). It is apparent that the close fit between 16S rRNA and the first two base pairs can be made only when perfect Watson-Crick pairing occurs. In contrast, only limited interactions are made with the third base pair, providing a structural explanation for the tolerance of noncanonical, or wobble pairing in the third position.

The full mechanistic significance of these interactions is not yet clear. Does the induced-fit interaction provide selectively enhanced thermodynamic stability to the cognate tRNA, thereby enhancing translational accuracy? Does the structural change in 16S rRNA initiate a signal that is

Figure 2. Interface and solvent views of the 30S and 50S subunits, as observed in the 70S ribosome crystal structure (Yusupov et al. 2001), showing the positions of the A-, P- and E-site tRNAs (yellow, orange, and red, respectively). *Top*, interface views of the 50S (*left*) and 30S (*right*) subunits, showing the relative absence of proteins surrounding the functional sites. *Bottom*, corresponding solvent surfaces of the subunits.

transmitted to the catalytic site of EF-Tu, accelerating hydrolysis of GTP? Or are both kinds of mechanisms involved? An additional observation explains the miscoding activity of aminoglycoside antibiotics. The crystal structure of the 30S subunit bound with paromomycin shows that this aminoglycoside causes A1492 and A1493 to rearrange from their normal locations stacked on the end of helix 44 to flip into almost exactly the positions induced by binding of the anticodon stem-loop (Carter et al. 2000; Ogle et al. 2001). Presumably, binding of noncognate tRNAs is stabilized by this largely prearranged conformational shift. Because the bases cannot form optimal minor groove interactions with the noncognate anticodon-codon complex, this result also suggests that there is more to this 16S rRNA interaction than simple thermodynamic stabilization of tRNA binding.

Most intriguing is that the ribosomal structure responsible for sensing whether true Watson-Crick pairs are made is formed from only three nucleotides of 16S rRNA. It is not difficult to imagine assembling such a mechanism from

small, rudimentary RNAs of the kind that have been suggested to have populated the RNA World. Moreover, this simple steric minor-groove calibration could provide a general mechanism to monitor the accuracy of base pairing in a variety of functional contexts, such as RNA recombination (splicing) and RNA replication, as discussed later. In fact, the plausibility of such scenarios is made clear by the common occurrence of these kinds of interactions in the structures of the ribosomal and other RNAs. The Yale group has termed them “A-minor” interactions (Nissen et al. 2001), and has assigned them to three different structural classes, called types I, II, and III (Fig. 3B). More than 130 examples of type I and type II A-minor interactions are found in the *Haloarcula marismortui* 23S rRNA alone (Nissen et al. 2001). In the 30S A site, A1493 of 16S rRNA makes a Type I A-minor interaction with the first codon-anticodon base pair (Fig. 3A,B; *top*). A1492 makes a type II interaction with the mRNA nucleotide of the middle base pair, and pairs with G530, which itself interacts with the tRNA nucleotide of the middle base pair in a

Figure 3. Steric minor-groove calibration of Watson-Crick codon-anticodon pairing by three conserved bases of 16S rRNA. (*left*) Contacts between G530, A1492, and A1493 of 16S rRNA and the codon-anticodon base pairs in the 30S A site (Ogle et al. 2001). (*right*) Type I and type II A-minor interactions (Nissen et al. 2001).

type II-like interaction (Fig. 3A,B; *middle*). The 30S subunit A site presents compelling evidence for the likelihood that the ribosome evolved from a purely RNA structure, and helps to explain why its function continues to be based on RNA.

5 THE 30S SUBUNIT P SITE: ANOTHER FUNCTION OF rRNA

One of the earliest indications of the functional role of rRNA was the implication of 16S rRNA in binding tRNA to the 30S P site. Inactivation of P-site binding by kethoxal modification of 16S rRNA (Noller and Chaires 1972), direct crosslinking of the wobble base of tRNA to C1400

of 16S rRNA (Prince et al. 1982), chemical footprinting of 16S rRNA by P-site tRNA (Moazed and Noller 1986; Moazed and Noller 1990) and modification-interference experiments (von Ahsen and Noller 1995) all pointed to the involvement of a constellation of 16S rRNA nucleotides in this function. The crystal structures directly showed the participation of 16S rRNA in tRNA binding to the 30S P site, providing further evidence for the RNA character of the ribosome (Wimberly et al. 2000; Yusupov et al. 2001). However, any hopes for a simple RNA-World picture were clouded by the intrusion of proteins S9 and S13 into the tRNA binding site (Wimberly et al. 2000). Both proteins contain extended carboxy-terminal tails, which contact phosphate 35 of the anticodon loop and the

backbone of the anticodon stem, respectively. Both tails contain basic amino-acid side-chains that appear to make electrostatic interactions with the tRNA backbone.

An RNA-World impression of S9 and S13 is one of two proteins that landed on and took root in 16S rRNA as a later evolutionary refinement of the basic RNA structure of the subunit. The functional requirement for their C-terminal tails was tested directly by replacing the genomic copies of the *E. coli* genes encoding S9 and S13 with versions in which their carboxy-terminal tails were deleted (Hoang et al. 2004). The result was that mutant strains bearing the deleted versions of the two proteins were viable, including strains in which the tails of both proteins were deleted. The phenotypes were relatively mild, amounting to a 40% reduction in growth rate for the double deletion. In this strain, all of the cellular proteins are synthesized by ribosomes whose 30S P sites are composed purely of RNA. Thus, 16S rRNA is able to support all of the essential functions of the 30S P site, including translational initiation, P-site tRNA binding, and maintenance of the translational reading frame.

6 RNA MOLECULAR MECHANICS AND TRANSLOCATION

Perhaps the most demanding step of translation is the coupled movement of mRNA and tRNA, called translocation, which follows formation of each new peptide bond. This step depends on elongation factor EF-G and is coupled to hydrolysis of GTP. It is coupled to large-scale molecular movements, including relative rotation of the two ribosomal subunits, emphasizing the structural dynamics of the ribosome. Because the pioneer ribosomes must have been capable of translocation, we can ask: (a) How could such a fundamental process have operated in the absence of EF-G, which is commonly referred to as the “translocase” of protein synthesis? And, what was the source of energy to drive movement of mRNA and tRNA, and intersubunit rotation? Studies by Pestka (Pestka 1968) and Spirin (Gavrilova et al. 1976) showed many years ago that poly(U)-dependent synthesis of polyphenylalanine could proceed in the absence of EF-G, under certain *in vitro* conditions, or by modification of ribosomes with thiol-directed reagents. Criticisms that the observed synthesis might have involved some sort of “slippage” of the poly(U) mRNA were addressed by Green and coworkers using a defined mRNA (Southworth et al. 2002). The requirement for GTP was shown not to be absolute by the demonstration that the peptidyl transferase inhibitor sparsomycin can trigger a single round of translocation *in vitro*, with high efficiency and accuracy, in the complete absence of EF-G or GTP (Fredrick and

Noller 2003). These studies suggest that translocation could have originated as a purely ribosomal, factor-independent process.

But in the absence of GTP hydrolysis, what is the source of free energy to drive the translocation reaction, and what keeps it from going backwards? The most obvious source of energy comes from peptide bond formation; peptidyl transfer results in formation of a peptide amide bond from an activated ribose ester linkage, accompanied by a large change in free energy. How can this free energy change be coupled to translocation? The answer most likely lies in the changing chemical nature of the acceptor end of the tRNA as it moves through the ribosome (Spirin 1985). It enters as an aminoacyl-tRNA, is transformed into a peptidyl-tRNA and then becomes completely deacylated. The 50S subunit contains three tRNA binding sites, the A, P, and E sites, which have specific affinities for the three forms of tRNA, providing at the same time a downhill energetic pathway and a unidirectional movement for the tRNA during translocation.

Translocation also appears to depend on rotation of the ~850 kDa 30S subunit relative to the 50S subunit for each step (Frank and Agrawal 2000; Frank et al. 2007; Horan and Noller 2007). In the absence of GTP hydrolysis, what is the source of energy to drive this massive intermolecular movement? Recent single-molecule FRET studies show that spontaneous intersubunit rotation can occur in a wide variety of mRNA-tRNA-ribosome complexes in the absence of EF-G or GTP, or even peptide bond formation (Cornish et al. 2008). This finding shows that thermal energy alone is sufficient to drive the intersubunit rotation underlying translocation. Translocation is also coupled to movement of a feature of the 50S subunit called the L1 stalk, which maintains contact with the elbow of the deacylated tRNA as it moves from the P/E to the E/E state. Single-molecule FRET experiments show that the L1 stalk can traverse through three different positions during translocation, corresponding to the P/E, E/E and vacant states of the E site (Cornish et al. 2009). Again, the L1 stalk was found to be able to move spontaneously in the absence of EF-G or GTP. These findings show that even the complex, large-scale molecular movements associated with translocation can be driven by thermal energy, obviating the need for special energy-generating steps in protein synthesis by the first ribosomes.

7 WHAT ARE RIBOSOMAL PROTEINS FOR?

If, as we suspect, the fundamental functions of the ribosome are based on its rRNA, why are there so many ribosomal proteins, some of which are highly conserved? So far, no one has reported observation of a ribosomal function

being carried out by a rigorously protein-free preparation of rRNA. One explanation for this is that rRNA does not fold into its functional state in the absence of r-proteins (Nomura et al. 1969; Stern et al. 1989). Another reason for the presence of proteins in ribosomes is that they improve the efficiency and accuracy of translation. For example, mutations in proteins S4 and S5 have long been known to cause increased translational error frequencies, implying that they help to improve the accuracy of translation (Davies and Nomura 1972; Kurland et al. 1990). The presence of the carboxy-terminal tails of S9 and S13 is not essential for 30S P-site function, as discussed above, but improves tRNA binding and increases the growth rate of *E. coli* (Hoang et al. 2004). A further point is that even small improvements in the speed and accuracy of translation bring strong selective advantages.

In contrast to histones, for example, the structures of the ribosomal proteins are extremely heterogeneous, representing a large number of different domain types, including helical bundles, α/β RRM folds, all- β OB folds, and so on. Many contain long unstructured tails that penetrate the structure of the rRNA (Ban et al. 2000; Wimberly et al. 2000). Some are essential for correct overall folding and assembly, whereas others are not (Nomura et al. 1969). A few are positioned at or near the subunit interface, where they can influence ribosomal function, whereas the majority are located on the solvent surface, remote from any functional site. Thus, the ribosomal proteins clearly did not arise by duplication of one another, to play a single role, or related roles, but give the impression that they were added one at a time over the course of evolution, as incremental refinements of an essentially RNA-based ribosome.

8 “STOP tRNAs” AND THE EVOLUTION OF TYPE I RELEASE FACTORS

All but three of the 64 possible triplet codons are recognized by tRNAs. The remaining three, the stop codons UAG, UAA and UGA are recognized by the type I release factors (RF1 and RF2 in bacteria). Recent crystal structures of 70S ribosome termination complexes show that the stop codons are directly recognized by the release factors and suggest that catalysis of peptidyl-tRNA hydrolysis is catalyzed by the $-NH$ group of the polypeptide backbone of a conserved Gln in the conserved GGQ motif (Korostelev et al. 2008; Laurberg et al. 2008; Weixlbaumer et al. 2008). The position of this $-NH$ group superimposes with the position of the 3'-OH group of a deacylated tRNA bound to the A site. This raises the possibility that stop codons were originally recognized by deacylated tRNAs, which were replaced during evolution by the type I release factors (Laurberg et al., 2008). In fact, it has been shown that

binding of deacylated tRNA to the A site catalyzes polypeptide release (Caskey et al. 1971). A potential shortcoming of a “stop tRNA” is that deacylated tRNAs are unable to bind elongation factor EF-Tu, which is critical for the accuracy of tRNA binding; the resulting high error frequency of translation termination may thus have driven the evolution of type I release factors.

9 dRNA AND THE ORIGINS OF THE RIBOSOMAL DECODING SITE

The molecular interactions involved in codon recognition shown in Figure 3 could, in principle, serve to monitor the accuracy of Watson-Crick base pairing in other RNA contexts. An obvious possible application of this type of quality control in the RNA World is the critical process of RNA replication. A-minor interactions are so simple and so widespread that it would be surprising if they were not put to use for this purpose. Is it possible that the 30S decoding site is a relic of an RNA replication mechanism from the RNA World? The following is a suggestion for how such a replication system may have worked, extrapolating from what we have learned from the ribosome.

Our starting assumption is that codon-anticodon interaction occurs at the site of the template-product interaction in an RNA World replicase, in which similar A-minor interactions were used to ensure the accuracy of RNA replication. The fact that the decoding site mediates triplet-triplet base pairing suggests that its RNA World role would have been to stabilize ligation of oligonucleotide triplets base-paired to a template; i.e., the replicase would have taken the form of an RNA ligase. The first critical question is whether the mRNA codon corresponds to the template (as it does in protein synthesis) or the product (in which case, the anticodon would be the template). Examination of the structure of the decoding site provides an unambiguous answer. The distance between phosphate and ribose groups of adjacent anticodons bound to the A and P sites is more than 30 Å, ruling out models in which the mRNA serves as template. Accordingly, we conclude that anticodons serve as short templates for positioning RNA triplets in the replicase active site.

This raises a second critical question: How can RNA be replicated using such short templates? Here, we introduce the idea of “duplicator RNA” (dRNA). dRNAs are small, tRNA-like structures that mediate *duplication* (as opposed to replication) of an RNA template (Fig. 4). dRNAs have a loop resembling an anticodon loop (here, it is shown as a seven-nucleotide loop, but loops of other sizes would be possible), and an unpaired self-complementary four-nucleotide tail, that allows dRNAs to form homodimers. One “anticodon” end of the dimer base pairs with a triplet

Figure 4. Duplicator RNA. (*left*) A schematic cartoon representing the structure of a duplicator RNA (dRNA) monomer, showing its two identity elements: A self-complementary tetramer tail and a degenerate anticodon triplet. There are 16 possible dRNAs. (*right*) A dRNA homodimer, formed by base pairing of its self-complementary tail. The wavy line indicates that other details of the structure between the anticodon and tail are not intended to be explicit.

sequence in the template RNA, and the other anticodon end pairs with either the nascent product RNA or the incoming triplet substrate (Fig. 5). The product interaction resembles the strong binding of the ribosomal P site and

the substrate interaction corresponds to that of the decoding site. Ligation of the substrate oligomer to the growing product chain occurs at the junction between these two binding sites.

Because there are 16 possible self-complementary tetramers, there are 16 possible different dRNAs. Interestingly, the same number of dRNAs is predicted from a completely independent argument. The geometry of the A-minor interactions used in the decoding site creates the basis for the degeneracy of the genetic code (Fig. 3); adjacent base pairs can interact with adjacent adenosines to form type I and type II interactions, but this does not extend to a third base pair (which, in the decoding site is partially taken over by a separate guanine, G530). Degeneracy in the third position of the replicase triplet-triplet interaction would thus limit the number of dRNA anticodons (which could themselves be degenerate) to 16. Thus, each dRNA has two distinct identity elements—a self-complementary tetramer tail, and a (degenerate) triplet anticodon—which establish the link between the template and product RNAs. (Implicit in this discussion is the idea that dRNAs are precursors to tRNAs. Besides their anticodon-like features, their self-complementary tails are reminiscent of tRNA identity elements that are often present in the acceptor stems of tRNAs.)

There are several properties of this form of indirect templating that distinguish it from normal RNA replication, some of which seem especially well suited to the

Figure 5. Indirect templating of RNA duplication mediated by dRNA homodimers. One dRNA dimer (*left*) is bound to the last triplet (GAN) in the product RNA, stabilized by a structure resembling the 30S ribosomal P site (blue box). A second dRNA dimer (*right*), bound to the next (CCN) triplet in the template by pairing with one of its GGN anticodons, binds the incoming substrate CCN trimer via base pairing with its other GGN anticodon. Discrimination of correct pairing with the incoming substrate trimer is promoted by A-minor interactions by a structure resembling the 30S ribosomal A site (red box). Binding of the upper anticodon triplets to the template RNA also uses structures resembling the 30S A and P sites (not shown).

challenges of the RNA World. First, the template is duplicated in a parallel fashion, eliminating the formation of an intermediate RNA duplex and the associated problem of unwinding a long duplex to allow release and folding of the product RNA. Second, the substrates are triplet oligonucleotides, which bind more stably to their templates than single nucleotides, yet are readily disrupted at ambient temperatures. These can be sourced from random-sequence triplet pools; because of third-position degeneracy, there are effectively only 16 different substrate oligomers.

The general scheme described here raises many detailed mechanistic questions. How are the substrate oligomers synthesized and activated? What is the mechanism of catalysis? How is the nascent chain translocated following addition of each oligomer? How is the secondary structure of the RNA template disrupted to allow dRNA binding? Would accidental binding of longer oligomers disrupt RNA duplication? Finally, the details of the structure of dRNA in the region linking the anticodon to the self-complementary tail (unspecified here) will be critical, so that the resulting geometry will allow binding of adjacent dRNAs to adjacent triplets and their parallel translocation with respect to the template and product RNAs at opposite ends of the dRNA dimers. These and other aspects of dRNA-mediated duplication are discussed elsewhere (Noller 2010 in preparation).

10 THE DRIVING FORCE FOR EVOLUTION OF TRANSLATION FROM AN RNA WORLD

It is challenging to ask how the structurally and functionally complex process of translation could have evolved from an RNA World. Most encouraging is the demonstration that small RNAs evolved *in vitro* are able to catalyze all of the principal chemical reactions of protein synthesis, including amino acid activation, aminoacylation of RNA, and peptidyl transfer (Zhang and Cech 1997; Illangasekare and Yarus 1999; Lee et al. 2000; Kumar and Yarus 2001). But quite apart from the mind-boggling prospect of evolving a structure as complex as the ribosome (even without its proteins), the probability of an early translational system producing a functionally capable protein, such as an enzyme, is vanishingly small (Woese 1967). Given these prospects, what was the driving force that led to the evolutionary selection of protein synthesis in the context of an RNA World? If we assume that some sort of Darwinian selection was in place, it must have provided a selective advantage to an RNA world system.

The diversity and efficiency of RNA function depends on the possible types of structure into which RNA can fold. Structural studies on naturally occurring and *in vitro*-selected RNAs have revealed a rich diversity

of RNA folds. Nevertheless, it can already be seen that the range of RNA structures is limited, probably because of the relatively modest chemical differences between the four nucleotide monomers, compounded by the strong inherent tendency for ribonucleotides to adopt conformations resembling those that are found in A-type double helices (Saenger 1984). We would therefore expect an expansion of the range of possible RNA structures to confer a strong selective advantage.

Studies on the structures of RNA-ligand complexes show that the binding of even quite small molecules to RNA can cause large-scale structural changes. RNAs that appear to be unstructured adopt well-defined three-dimensional folds, and structured RNAs undergo conformational rearrangements in the presence of bound ligands. An example is the AMP-dependent structuring of an *in vitro*-selected aptamer, in which the 11-nucleotide RNA wraps around the nucleotide into an intricate, well-defined fold, such that the AMP plays the role of the conserved A in a GNRA tetraloop (Dieckmann et al. 1996). Another example is the discovery of “riboswitches,” ligand-induced RNA structures that are found in naturally occurring mRNAs, influencing the expression of the mRNAs in which they are embedded (Mandal and Breaker 2004; Serganov et al. 2004; Breaker 2010). Thus, binding of small-molecule ligands is likely to have played an important part in expanding the structural repertoire of RNA.

Binding of peptides has also been shown to cause rearrangement of RNA structure. A vivid example is based on the HIV Tat-TAR complex, a protein-RNA interaction that is essential for viral function. Binding of the Tat protein to the TAR RNA could be mimicked by a nine-amino acid arginine-rich peptide derived from the binding site of the protein (Puglisi et al. 1992). Binding of this peptide causes a dramatic rearrangement of the structure of the TAR RNA, from a conventional hairpin stem-loop structure into a structure containing a bulge loop, resulting in formation of an A-U-U triple base pair tertiary interaction. At the core of the structure is an arginine side-chain that appears to play a crucial role in stabilizing the new fold. Remarkably, it was found that this same structural rearrangement occurred in the presence of a single arginine-amide (Puglisi et al. 1992) (Fig. 6), although the monomer bound with an affinity that was lower by five or six orders of magnitude. These findings show two points: (1) Simple peptides or even amino acid monomers can dramatically influence the structure of RNA, and (2) The extended structures provided by incorporating amino acids into peptides confer higher binding affinity (and concomitantly, increased specificity). Further examples of peptide-induced structural rearrangements of RNA have been observed for the HIV Rev-RRE, BIV Tat-TAR, and

Figure 6. Influence of the HIV Tat peptide on the folding of TAR RNA (Puglisi et al. 1992). (A) Secondary structure of TAR RNA; (B) NMR structure of the free TAR RNA; (C) NMR structure of the TAR RNA bound to a nine-amino acid peptide from Tat protein or bound to a single argininamide (shown). The argininamide is shown in orange and the three-nucleotide bulge loop in dark blue.

HTLV-1 Rex-aptamer interactions (Puglisi et al. 1995; Battiste et al. 1996; Jiang et al. 1999). Accordingly, the ability to synthesize small peptides could have provided a strong selective advantage to an RNAWorld system possessing such a capability. The kernel of the ribosome may therefore have arisen as a relatively simple RNA that was able to catalyze formation of simple peptides, to help expand the structure space of RNA. Poole et al. (Poole et al. 1998) have proposed a related idea, that primitive peptides could have acted as chaperones, to assist the folding of RNAs.

The general idea that polypeptides promote formation of the active conformations of functional RNAs is supported by the fact that most, if not all, present-day functional RNAs are found associated with proteins in vivo. Ribonuclease P, spliceosomes and ribosomes, whose functions have been ascribed to their respective RNA moieties (Guerrier-Takada et al. 1983; Sharp 1991), nevertheless require proteins to function in their physiological states. In the case of 16S rRNA, assembly of ribosomal proteins has been shown not only to be important for formation of local RNA tertiary structure (Stern et al. 1989), but has also been found to influence the relative orientation of adjacent RNA helical elements, thereby helping to establish even the large-scale geometry of the RNA (Orr et al. 1998). RNase P is thought to use its protein component to help overcome electrostatic repulsion between its catalytic RNA subunit and its RNA substrate, another potential selective advantage for synthesis of (cationic) peptides (Reich et al. 1988).

11 CONCLUSIONS

Evolution of coding remains the most difficult step to explain. It is easiest to think of the evolution of translation as

having begun with the synthesis of small peptides, possibly of random sequence. With short peptides containing a limited number of types of amino acids, useful amounts of peptides of defined sequence could be formed. In the absence of a coding mechanism, the substrates for the primitive peptidyl transferase would have been smaller proto-tRNAs, containing acceptor ends, but lacking anticodons (Maizels and Weiner 1993; Noller 1993; Schimmel et al. 1993). Coding would have evolved from a separate RNA-World mechanism, in which the Ramakrishnan A-minor calibration mechanism (Ogle et al. 2001) was used to check the accuracy of Watson-Crick base pairing in a completely different functional context, between short RNA sequences that were the counterparts of the codon and anticodon. At a later stage, the anticodon and proto-tRNA moieties would then be joined to form something resembling present-day tRNAs. It is interesting that the idea that the two halves of tRNA evolved separately has emerged independently, from three different laboratories, from three quite different lines of reasoning (Maizels and Weiner 1993; Noller 1993; Schimmel et al. 1993). How the coding of amino acids by specific nucleotide sequences emerged is harder to imagine. An interesting proposal has been put forth by Schimmel and coworkers (Schimmel and Henderson 1994), involving an intermediate stage of side-by-side interactions between adjacent amino acid-specific proto-tRNAs, whose identity elements were contained exclusively in their acceptor stems. This system of noncoded peptide synthesis would then be converted to a system for template-directed synthesis.

How did the present-day ribosome evolve? The early existence of an all-RNA ribosome of such a level of structural complexity is difficult to imagine. More likely, smaller functional units capable of carrying out the different

translational steps such as peptidyl transferase, decoding and so on evolved. These small functional RNA units then merged to form larger structures, which were incrementally refined by incorporation of additional RNA structural elements. Two features of RNA make such a process plausible. First is the fact that small RNAs, unlike most peptides, tend to retain their local structures when excised from larger RNA structures. For example, the anticodon stem-loop of tRNA interacts efficiently and accurately with the ribosome in the absence of the rest of the tRNA structure, and is even capable of accurate codon recognition and translocation (Rose et al. 1983; Joseph and Noller 1998; Ogle et al. 2001). This may be because RNAs do not have hydrophobic cores that are essential for their three-dimensional folding. Thus, building a large functional RNA out of pre-existing small ones can be accomplished while preserving structural and functional properties of the latter. Second, RNAs can readily interact with each other to form complexes, not only by base pairing, but by robust tertiary interactions such as the A-minor interactions described previously. RNAs that have associated noncovalently in this way can then become ligated together to form covalently stable RNAs of increasing size, by well-known ribozyme-catalyzed mechanisms. An explicit proposal for the hierarchical evolution of 23S rRNA was recently described by (Bokov and Steinberg 2009), who analyzed the distribution of donor adenosines and acceptor helices for the A-minor interactions of the 50S ribosomal subunit. They observed a striking asymmetry in domain V of 23S rRNA, which contains the elements of the peptidyl transferase center: in all but one case, domain V contributed the acceptor helices for adenosines from other domains. This suggests that 23S rRNA evolved by gradual addition of ancillary RNA domains to the catalytic core in domain V, exploiting A-minor interactions in positioning the added elements. Importantly, in an RNA World, a newly constructed RNA becomes its own gene, whose replication directly provides multiple copies of new functional RNAs. This cycle of noncovalent complex formation, followed by ligation, can then be repeated, while selecting for improvement of ribosome-like function. Finally, the influence of the peptides produced by a primitive ribosome on its own structure and assembly would also begin to impact its own evolution. Ribosome-binding peptides may thus have played an early role in shaping the ultimate form (and function) of the ribosome.

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