The ribosome as a missing link in prebiotic evolution II: Ribosomes encode ribosomal proteins that bind to common regions of their own mRNAs and rRNAs

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HIGHLIGHTS

- We propose that rRNA is likely the origin of the mRNAs that encode ribosome-binding proteins.
- We demonstrate that rb protein sequences are encoded in rRNA.
- We review studies demonstrating ubiquity of prokaryotic rb-proteins binding to their own mRNA.
- Ribosome-binding proteins have higher-than-expected incidence of arginine-rich modules.
- rRNA-binding regions of rb proteins are homologous to their mRNA binding regions.

GRAPHICAL ABSTRACT
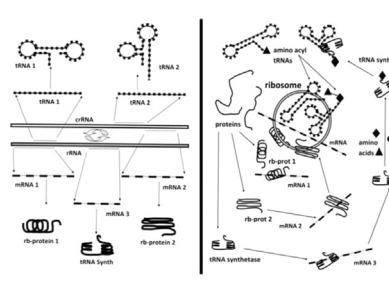
ABSTRACT

We have proposed that the ribosome may represent a missing link between prebiotic chemistries and the first cells. One of the predictions that follows from this hypothesis, which we test here, is that ribosomal RNA (rRNA) must have encoded the proteins necessary for ribosomal function. In other words, the rRNA also functioned pre-biotically as mRNA. Since these ribosome-binding proteins (rb-proteins) must bind to the rRNA, but the rRNA also functioned as mRNA, it follows that rb-proteins should bind to their own mRNA as well. This hypothesis can be contrasted to a "null" hypothesis in which rb-proteins evolved independently of the rRNA sequences and therefore there should be no necessary similarity between the rRNA to which rb-proteins bind and the mRNA that encodes the rb-protein. Five types of evidence reported here support the plausibility of the hypothesis that the mRNA encoding rb-proteins evolved from rRNA: (1) the ubiquity of rb-protein binding to their own mRNAs and autogenous control of their own translation; (2) the higher-than-expected incidence of Arginine-rich modules associated with RNA binding that occurs in rRNA-encoded proteins; (3) the fact that rRNA-binding regions of rb-proteins are homologous to their mRNA binding regions; (4) the higher than expected incidence of rb-protein sequences encoded in rRNA that are of a high degree of homology to their mRNA as compared with a random selection of other proteins; and (5) rRNA in modern prokaryotes and eukaryotes encodes functional proteins. None of these results can be explained by the null hypothesis that assumes independent evolution of rRNA and the mRNAs encoding ribosomal proteins. Also noteworthy is that very few proteins bind their own mRNAs that are not associated with ribosome function. Further tests of the hypothesis are suggested: (1) experimental testing of whether rRNA-encoded proteins bind to rRNA at their coding sites; (2) whether tRNA synthetases, which are also known to bind to their own mRNAs, are

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encoded by the tRNA sequences themselves; (3) and the prediction that archaeal and prokaryotic (DNA-based) genomes were built around rRNA "genes" so that rRNA-related sequences will be found to make up an unexpectedly high proportion of these genomes.

1. Introduction: Did rRNA-binding proteins evolve separately from rRNA or were they encoded in it?

We recently hypothesized that ribosomes were the first self-replicating entities, pre-dating the evolution of modern cellular life and providing the basis for both cellular metabolism and genetics (Root-Bernstein and Root-Bernstein, 2015). Our theory therefore integrates RNA-world and protein-world approaches to the origins of life question by proposing that the two evolved concurrently and interactively (Norris et al., 2012; Caetano-Anollés and Seufferheld, 2013). The formation of RNA-peptide and RNA-protein complexes would have stabilized them against degradative processes thereby increasing the probability that such complementary components would survive and that any functional entities encoded in the RNA be replicated. We further propose that as a result of this process, RNAs would be selected that encoded proteins that could bind to the encoding RNA or to the complementary sequence to that RNA. We have not yet addressed the origins of such self-replicating ribosomal entities in detail, but suggest that, in keeping with our previous work, such entities originated as a result of selection by molecular complementarity within a complex chemical ecology of peptide- or protein-RNA interactions (Root-Bernstein and Dillon, 1997; Hunding et al., 2006; Norris et al., 2009; Root-Bernstein, 2012). We propose that DNA evolved secondarily as a more stable way to store genetic information when not in use (Root-Bernstein and Root-Bernstein, 2015).

Our theory leads to a number of antidogmatic predictions such as the possibility that ribosomal RNA (rRNA) could act as its own self-replicating chromosome; that the positive and negative strands of such a chromosome would encode (in all possible reading frames) functionally active peptides and proteins; that these active peptides and proteins would more specifically have been selected for their ribosome-related functions (e.g., RNA and peptide polymerase activity; phosphotases to mediate energy utilization; ribosomal structural and functional proteins; tRNA synthetases; etc.); and that the rRNA would contain within it the transfer RNA (tRNA) sequences necessary to mediate between the genetic information and the protein sequences to be translated. In our previous paper, we presented evidence that ribosomal RNA encodes all twenty transfer RNAs as well as key functional fragments of ribosomal proteins (Root-Bernstein and Root-Bernstein, 2015).

The possibility that the ribosome is the descendent of a self-replicating entity leads to a host of new testable predictions, one of which we explore here: a self-replicating ribosome would have had to encode its own ribosome-binding proteins since these proteins are essential to ribosome function. A necessary corollary of this prediction is that rRNA would have had to function also as mRNA in order to encode the ribosome-binding proteins, in which case an evolutionary artifact would be that ribosome-binding proteins should be found to bind to, and regulate, their own mRNAs. Such an autogenous regulatory mechanism might then apply to all proteins essential to ribosomal function such as translation regulatory proteins and tRNA synthetases, which charge tRNAs with the amino acid appropriate to their anticodon. While modern organisms have generally shifted the genetic encoding of ribosomal proteins to genomic DNA sequences, several

testable consequences follow from our hypothesis. One is that the mRNA sequences encoding ribosomal proteins should exhibit homology to the rRNA itself. Another is that ribosome-binding proteins (rb-proteins) should bind to both the rRNA and their own mRNA, since these will be homologous. Third, in some cases, rRNA, or the corresponding rDNA that encodes it in modern organisms, should encode proteins that have ribosome-related functions (this, in spite of the fact that current dogma asserts that ribosomal genes encode no proteins). We provide five types of evidence below to support all three predictions.

Some basic background information provides the context in which we have devised the tests of our predictions. The prokaryotic ribosome is composed of several rRNA sequences (in prokaryotes, the 5S, 16S and 23S rRNAs) organized into two subunits. The smaller subunit is built around the 16S rRNA, which forms a scaffold for 21 ribosomal proteins that are identified by the prefix "S". Most, but not all of these proteins bind directly to the rRNA; however, a few of the proteins bind only to other ribosomal proteins. The 5S and 23S rRNAs interact to form a larger platform for an additional 31 ribosomal proteins that are identified by the prefix "L". Of these ribosomal proteins, fifteen of the small (S) and nineteen of the large (L) are universally found in all organisms and may thus be considered essential to ribosome function (Wilson and Nierhaus, 2006; Korobeinikova et al., 2012). The small and large subunits aggregate to form a highly ordered structure capable of carrying out the translation of mRNA-encoded information into protein sequences.

From the usual evolutionary perspective of either a cell giving rise to the ribosome or of a master molecule such as a self-replicating RNA or DNA giving rise to the ribosome, the problem of ribosomal self-organization is one of co-evolving separate genes for rRNAs and for the proteins that bind to them. The rRNA sequences would have evolved separately from the ribosomal protein sequences and been encoded in the cellular genome independently of each other, as is the case in all cellular organisms today. Evolution would then have selected for proteins that recognized specific regions of the rRNAs to produce stable, functional aggregates. The genes encoding these rRNA-binding proteins, and therefore their mRNAs, would not have any necessary sequence relationship with the genes encoding the rRNA sequences. It follows that rRNA-binding proteins (rb-proteins) would not bind to their own mRNA.

If the ribosome evolved before the cellular genome and gave rise to it, then the problem of ribosomal self-organization becomes quite different. The rRNAs in a self-replicating ribosomal entity would have had to function simultaneously as mRNAs that encoded the rb-proteins required for ribosomal self-assembly. Thus, the rb-proteins would have been encoded in the rRNAs that produced the mRNAs that produced the rb-proteins. Since these rb-proteins bind to rRNA and the rRNA also functioned as mRNA, it follows logically that ribosomal proteins would also have bound to their own mRNAs. In fact, at some point in evolution, rRNA and mRNA may have been a single entity. In short, a self-replicating ribosomal entity would have required the evolution of proteins that bound to both rRNA and their own mRNAs, and the sequences of the protein binding sites on these rRNAs and mRNAs would have had to have been shared. Remnants of such a system should still be apparent today in rb-protein binding to their own mRNAs.

The two scenarios for ribosome evolution just outlined above lead to very different, testable predictions. The possibility that the ribosome was not self-replicating leads to a "null hypothesis" in which the genes encoding its RNA structure and its protein functions evolved independently. In this case, there should be no binding of the rb-proteins to their own messenger RNAs and no relationship between the mRNA sequences that generate the ribosomal proteins and any ribosomal RNA sequence to which they bind. If, however, ribosomes evolved prior to cellular genomes and as self-replicating entities, then two mechanisms can be imagined for the evolution of self-organization. One mechanism would have permitted each rRNA sequence to produce a protein that bound to the RNA sequence that produced it. Such *an autogenous mechanism* assumes that the RNA encoding the protein is molecularly complementary to the protein itself. It also follows from this autogenous mechanism that the ability of a rb-protein to bind to its own rRNA would confer upon it the ability to bind to its own mRNA, since the mRNA and rRNA would have evolved from identical precursors. According to a second mechanism, the ribosome may have evolved as a closed system or network of rRNA "genes" such that each gene encoded a protein that could bind to a *different* rRNA sequence within the system. Since the rRNA of a self-replicating ribosome would produce its own mRNAs, in such a *complementary system*, ribosomal proteins would not bind to their own mRNAs but to mRNAs of other ribosomal proteins.

Assuming that rRNAs do encode proteins that bind rRNA and to some set of mRNAs, then two means of implementing such binding can be imagined. One means would involve the evolution of short, modular binding motifs that could be used as components of further protein evolution. The other means would involve the evolution of entire proteins specific for each rRNA sequence and entirely encoded in it.

Testing these alternative mechanisms is complicated by billions of years of evolution. Even if ribosomes were once self-replicating entities, modern cellular organisms have almost ubiquitously transferred ribosomal genes to the larger chromosomal structures of the cell, and to chloroplasts and mitochondria as well, so that today rRNA rarely functions simultaneously as mRNA to encode functional proteins. The exceptions are, however, noteworthy and instructive and will be reviewed at the end of this paper. More comprehensive tests of the possible mechanisms proposed, and the means to implement them, must look more broadly at whether ribosomal proteins bind to their own or other ribosome-related mRNAs and, if such rb-protein-mRNA binding does occur, whether the binding sites of the protein on the mRNA mimic the binding sites of the rb-protein on rRNA. If mRNA and rRNA share similar binding sites, the next test of the possible mechanisms is whether the rRNA encodes its own self-binding protein or a binding module. Demonstrating all of these phenomena provides a strong basis for arguing that rRNA once acted as its own genome to encode its own functional proteins, and may, in some cases, still does so today.

2. Most rRNA-binding proteins bind similar regions on their own mRNAs

Surprisingly, many studies already exist that are directly relevant to testing the predictions made by the self-replicating ribosome hypothesis, but these studies have never been interpreted in light of the possible evolutionary scenarios just elaborated nor, surprisingly, does a review of this quite extensive literature exist. We therefore begin by reviewing here what is already known about whether ribosomal proteins bind to their own messenger RNAs and whether these mRNA binding sites mimic the rRNA binding sites of these or other proteins. We add several novel tests

involving the possibility that rRNA encodes self-binding proteins or their modules.

Tables 1 and 2 summarize the many studies that have been performed showing that the vast majority of ribosomal proteins bind not only to rRNA but to their own mRNA as well. It should be noted immediately that while other examples exist of proteins that bind to their own mRNAs, these examples are extremely rare and almost always involve translation processes, as will be discussed below. Thus, the near ubiquity with which ribosomal proteins bind to their own mRNAs is exceptional.

The canonical examples of rRNA-binding proteins binding to similar structures on their own mRNAs are the S4 and S7 proteins first characterized by Nomura and his colleagues in the 1980s (Nomura et al., 1980). These canonical structures are often characterized by having large open rings of very similar sizes defined by complementary regions at both ends that are highly conserved sequentially. Subsequent research has verified Nomura's conjecture that proteins that bind to both rRNAs and mRNAs utilize shared binding determinants (e.g., Nomura et al., 1980; Gimautdinova et al., 1981; Changchien et al., 1988; Tang and Draper, 1990; Baker and Draper, 1995; Saito et al., 1994; Wimberly et al., 1997; Robert and Brakier-Gingras, 2001). This principle has been extended by experimental verification to almost all prokaryotic ribosomal proteins.

For example, S3 also binds to its own mRNA at a site similar to its rRNA binding site (Yusupova et al., 2001; Kim et al., 2010). S8 binds to a site on its own mRNA that contains "all of the characteristic features of the 16S rRNA binding site" (Olins and Nomura, 1981; see also Gregory et al. (1988), Wu et al. (1994) and Merianos et al. (2004)). S15 has also been demonstrated to bind to 16S rRNA and its own mRNA at sites that are molecular mimics of each other (Gimautdinova et al., 1981; Serganov et al., 2003; Ehresmann et al., 2004; Mathy et al., 2004; Ying et al., 2003; Philippe et al., 1990; Bénard et al., 1998; Scott and Williamson, 2001). S18 binds to sites on rRNA and its own mRNA that also exhibit molecular mimicry (Gimautdinova et al., 1981; Matelska et al., 2013; Fu et al., 2014). These and other ribosomal proteins including S1, S2, S7, S10, S15, L1, and L4, are known to act as autogenous regulators of their own mRNAs and are known to recognize similar targets on free rRNA and on mRNA (reviewed in Zengel and Lindahl (1994) and Stelzl et al. (2003); and references in Tables 1 and 2). L4 and L24 mRNAs mimic the rRNA binding sites of their proteins (Stelzl et al., 2003; Allen et al., 1999, 2004; Williams, 2008; Zengel et al., 1980; Mattheakis et al., 1989). The L20 mRNA has two sites that mimic the protein's rRNA binding site (Guillier et al., 2002, 2005; Raibaud et al., 2003; Allemand et al., 2007; Macias et al., 2008).

In addition, some rRNA-binding proteins are promiscuous for mRNAs. For example, S1 acts as a chaperone for many mRNA species and also helps to melt double-stranded and helical portions of such mRNAs (Skouv et al., 1990; Boni et al., 2000, 2001; Delvillani et al., 2011; Duval et al., 2013). S3 is part of the mRNA binding site on both prokaryote and eukaryote ribosomes and binds most mRNAs, including its own (Yusupova et al., 2001; Kim et al., 2010; Graifer et al., 2014).

3. Some rRNA-binding proteins regulate expression of other rRNA-binding proteins

In light of the fact that some rb-proteins bind to multiple mRNAs, it is not surprising to find examples of one rb-protein regulating the expression of another. In some cases, such regulation occurs even though the other protein already autogenously regulates itself. For example, S18 also regulates S6, even though each protein can regulate itself (Matelska et al., 2013; Fu et al.,

Table 1
Articles demonstrating ribosomal proteins making up the 30S (S1–S21) subunits of the prokaryote ribosome bind not only to rRNA but also to their own mRNA. If no references are listed, there is no evidence at present of binding of that particular ribosomal protein to its mRNA.

Prokaryote ribosomal S protein	References to mRNA binding
S1	Skouv et al. (1990), Boni et al. (2000, 2001), Delvillani et al. (2011) and Duval et al. (2013)
S2 (S0, SA)	Aseev et al. (2008, 2009)
S3	Yusupova et al. (2001) and Kim et al. (2010)
S4	Nomura et al. (1980), Gimautdinova et al. (1981), Changchien et al. (1988), Tang and Draper (1990) and Baker and Draper (1995)
S5	Gimautdinova et al. (1981)
S6	Matelska et al. (2013) and Fu et al. (2014)
S7	Gimautdinova et al. (1981), Nomura et al. (1980), Saito et al (1994), Wimberly et al. (1997) and Robert and Brakier-Gingras (2001)
S8	Olins and Nomura (1981), Gregory et al. (1988), Wu et al. (1994) and Merianos et al. (2004)
S9	Gimautdinova et al. (1981)
S10	Stelzl et al. (2003) and Allen et al. (2004)
S11	Gimautdinova et al. (1981)
S12	Cukras et al. (2003) and Holberger and Hayes (2009)
S13	Gimautdinova et al. (1981) and Zanzoni et al. (2013)
S14	
S15	Gimautdinova et al. (1981), Philippe et al. (1990), Portier et al. (1990), Philippe et al. (1995), Bénard et al. (1998), Scott and Williamson (2001), Serganov et al. (2003), Ying et al. (2003), Ehresmann et al. (2004) and Mathy et al. (2004)
S16	
S17	
S18	Gimautdinova et al. (1981), Matelska et al. (2013) and Fu et al. (2014)
S19	Schuster et al. (2010)
S20	Wirth et al. (1982); but: Donly and Mackie (1988)
S21	Gimautdinova et al. (1981)
S26	Ivanov et al. (2014)
S28	Badis et al. (2004) and Garneau et al. (2007)

Table 2
Articles demonstrating ribosomal proteins making up the 50S (L1–L33) subunits of the prokaryote ribosome bind not only to rRNA but also to their own mRNA. If no references are listed, there is no evidence at present of binding of that particular ribosomal protein to its mRNA.

Prokaryote ribosomal L protein	References to mRNA binding
L1	Gimautdinova et al. (1981), Köhrer et al. (1998), Kraft et al. (1999), Tishchenko et al. (2006, 2007, 2008) and Nevskaya et al. (2006)
L2	Gimautdinova et al. (1981)
L4	Stelzl et al. (2000), Stelzl et al. (2003), Allen et al. (2004), Williams (2008) and Stelzl et al. (2003)
L6	Gimautdinova et al. (1981) and Stelzl et al. (2000)
L7/L12	Fukuda (1980), Gimautdinova et al. (1981) and Brot and Weissbach (1981)
L10	Fukuda (1980), Yates et al. (1981), Johnsen et al. (1982) and Iben and Draper (2008)
L11	Hanner et al. (1994) and Mayer et al. (1998)
L15	
L18	
L19	Gimautdinova et al. (1981)
L20	Guillier et al. (2002), Raibaud et al. (2003), Guillier et al. (2005), Allemand et al. (2007) and Mangeol et al. (2011)
L22	
L24	Williams (2008)
L25	Aseev et al. (2015)
L29	
L30	Macias et al. (2008) and Vilardell et al., (2000)
L31	Gimautdinova et al. (1981)
L32	Gimautdinova et al. (1981) and Dabeva and Warner (1993)
L35	Guillier et al. (2002)

2014). Similarly, the L4 rRNA-binding protein also regulates the S10 operon (Stelzl et al., 2003; Zengel et al., 1980). These results suggest that that S4 and L10 may have had a common ancestor molecule, as may S6 and S18. Alternatively, the two proteins may have undergone convergent evolution due to similar selection pressures.

Another twist on autogenous regulation involves one ribosomal protein controlling the transcription or translation of several other ribosomal proteins, one or more of which lack their own autogenous regulation. For example, the L1 rRNA-binding protein regulates the L1 operon, which also controls the transcription and translation of L10 and L12 in some bacteria (Kraft et al., 1999) and L11 in others (Barreiro et al., 2001). Similarly, S8 regulates L5, L14 and L24 (Olins and Nomura, 1981; Mattheakis et al., 1989); L10 autoregulation also controls L12 activity since they are on the same operon (Johnsen et al., 1982; Iben and Draper, 2008); and L20 regulates itself and L35 (Guillier et al., 2002, 2005). Whether the control of several ribosomal proteins by one is a result of gene duplication followed by sequence divergence due to mutation, or due to a process of eliminating competing or redundant regulatory mechanisms will require further investigation.

The upshot of these observations is that although most ribosomal proteins bind to their own mRNA as well as to rRNA, some ribosomal proteins also regulate other ribosomal proteins by binding to their mRNAs, but the occurrence of such regulation is much less common than is autogenous regulation. Thus, it is most likely that rb-proteins evolved to bind to the RNAs that encoded them rather than to RNAs of other proteins, but in some cases, either as a result of divergent evolution from common sequences, or through convergent evolution toward common regulatory sequences, some rb-proteins evolved to bind to other rRNAs and to regulate the expression of other mRNAs as well.

4. Autogenous control of ribosomal proteins by binding to their own mRNA in eukaryotes

The kinds of autogenous control mechanisms reviewed above are very highly conserved among prokaryotes (Allen et al., 1999; Köhrer et al., 1998; Gourse et al., 1981; Cerretti et al, 1988), which raises the question of whether they are similarly conserved in the evolution of eukaryotes. In some cases, prokaryotic rb-proteins can perform their activities on eukaryotic ones, arguing for at least some evolutionary conservation (Gourse et al., 1981). More generally, autogenous control of ribosomal protein expression by binding of the protein to its own mRNA certainly occurs to some extent in lower eukaryotes such as yeast (Saccharomyces cerevisiae) (Antúnez de Mayolo and Woolford, 2003; Dabeva and Warner, 1993; Li et al., 1995, Presutti et al., 1995a, 1995b; Vilardell and Warner, 1997), but may not be nearly as universal as observed in prokaryotic ribosomal proteins (Tsay et al., 1988). Claims have been made that such autogenous control does not occur at all in higher eukaryotes such as Trypanosoma brucei (Wilson et al., 2000) or Xenopus laevis (Pierandrei-Amaldi et al., 1985a, 1985b), but evidence exists that the L1 ribosomal protein does bind to similar sites on rRNA and its own mRNA in various Xenopus species in order to auto-regulate its own production (Prislei et al., 1992; Fragapane et al., 1990; Gultyaev and Shestopalov, 1988). Moreover, there are reports that various human ribosomal S proteins are autogenously regulated by binding to their own mRNAs (Macias et al., 2008; Tasheva and Roufa, 1995; Ivanov et al., 2005; Malygin et al., 2007; Hemmerich et al., 1997; Neumann et al., 1995; Russo

et al., 2005; Witte et al., 1996). It seems likely, therefore, that such autogenous control, which is essentially ubiquitous among pro-karyotes, has been superseded in most eukaryotes by other forms of regulation, but survives in some instances.

These observations strongly suggest that during the origins of life, autogenous control of translation was a primitive and possibly foundational mode of metabolic regulation. Over the course of evolution, this control increasingly has been superseded by non-autogenous mechanisms. These observations are again consistent with the hypothesis that at some point in evolution, ribosomes regulated their own production through direct feedback mechan-isms involving protein-RNA binding.

5. Tests of the evolution of autogenous rRNA-mRNA protein binding 1: arginine-rich modules

One of the implications of the self-replicating ribosome theory is that the rRNA should encode its own rb-proteins. We have provided some evidence for such encodings in a previous paper, demonstrating that more than half of all of the protein sequences encoded in the 5S, 16S and 23S rRNAs map onto proteins with known ribosome-associated functions, and that these mappings correspond more often than would be expected by chance to the active sites of the modern protein equivalents. We have also demonstrated that these mappings occur statistically significantly more often for *E. coli* rRNA than for random mRNA sequences drawn from the *E. coli* genome (Nomura et al., 1980). The obser-vations made here regarding binding of rb-proteins to both rRNA and their own mRNAs suggests two additional tests of whether rRNA may once have functioned as a ribosomal genome. These two tests concern the nature of RNA-binding protein motifs.

Many laboratories have characterized RNA-binding proteins, concluding that the majority of naturally occurring RNA-binding proteins are arginine-rich (reviewed in Änkö (2014), Risso et al. (2012), Reddy and Shad Ali (2011) and Godin and Varani (2007)). These include most rb-proteins, which are known to contain such arginine-rich modules (ARMs) (Swiercz et al., 2005; Furumoto et al., 2000; Pelletier et al., 2000; Gustafson et al., 1998). ARMs have, in fact, been produced synthetically as a means for creating protein-RNA complexes (Kim et al., 2013; Crowet et al., 2013; Crombez et al., 2009; Ryu et al., 2012; Peng et al., 2015; Weiss and Naryana, 1998).

The existence of ARMs in most ribosomal proteins provides a means for further testing the likelihood that rRNA may once have encoded its own ribosomal proteins. On the one hand, if ribosomal proteins were encoded in genes existing outside rRNA, then rRNA would be no more likely than any other sequences in the pro-karyote genome to be rich in arginines. Stated another way, if rRNA did not function as a genome for its own self-replication, then the incidence of arginine codons within rRNA should be no different from that found anywhere else in the genome. If, how-ever, the rRNA once represented an autogenously controlled gen-ome encoding its own proteins, then since these proteins needed to bind to the rRNA to form functional ribosome complexes, rRNA would have encoded an unusually high proportion of ARM.

To test whether there are an unexpectedly high proportion of ARM within rRNA or not, the incidence of ARM was determined for the 5S, 16S and 23S rRNAs of *E. coli* K12 and compared with the incidence of ARM in the average of several *E. coli* genomes as a whole. The Arg codon usage in *E. coli* was determined using the Codon Usage Database (http://www.kazusa.or.jp/codon/) which employs the NCBI-GenBank Flat File Release 160.0 [June 15 2007]. The codon usage of the complete genomes of four strains of *Escherichia coli* were determined: CFT073 [gbbct]: 5379 CDS's (1581056 codons); O157:H7 str. Sakai [gbbct]: 5442 CDS's

(1639585 codons); O157:H7 EDL933 [gbbct]: 5347 CDS's (1611503 codons); APEC O1 [gbbct]: 4890 CDS's (1598551 codons). Arg codon (CGU, CGG, CGA, CGC, AGA, AGG) abundance ranged from 5.57 to 5.69 percent of the total codon usage, so that on average about 1 in 18 amino acids appearing in *E. coli* proteins should, by chance, be an Arg (see also Blake and Hinds (1984)). For compar-ison, the same calculation was made for *E. coli* lysine (Lys or K) usage, since Lys is also a positively charged amino acid like Arg, but is not associated with binding of proteins to RNAs. Lys usage ranged from 4.40 to 4.50 percent, or about 1 in 22 amino acids.

The results for *E. coli* K12 rRNA were significantly different from the global Arg and Lys usages. Since we do not know what reading frame, or reading frames, may have been employed by a prebiotic ribosome, we calculated the average number of ARM for all six possible reading frames of each rRNA. On average, Arg accounts for 9.8 percent of the amino acids (about 1 in 10) rather than the 5.6 percent (1 in 18) found in the overall *E. coli* genome (see Table 3). More specifically, the 5 S rRNA encodes 11.4 percent Arg – twice as many Arg as the rest of the genome – (ranging from 7.7 to 15.4 percent, depending on the reading frame); the 16S rRNA encodes 9.6 percent Arg (ranging from 7.0 to 12.2 percent depending on reading frame); and the 23S rRNA encodes 9.6 per-cent Arg (ranging from 8.6 to 11.1 percent). (See Supplemental Data for additional information.) Notably, every reading frame for every rRNA exhibits a larger number of Arg than would be expected by chance.

Additionally, the probability that these Arg would occur in doublets across the combined 5S, 16S and 23S is significantly greater than expected by chance, suggesting that these pairings have been selected by evolution for some function (Table 3 and Supplemental data). There is also an increase in the number of Arg triplets, although this result did not reach statistical significance due to the small number of triplets found. For comparison, there was a significant decrease in Lys usage compared with the rest of the *E. coli* genome (2.9% versus 4.45%). These results suggest that there has been selection specifically for Arg-rich codings in the rRNA (see Table 3), a result that is difficult to explain if rRNA did not, at one time, encode self-binding proteins.

6. Tests of the evolution of autogenous rRNA-mRNA protein binding 2: Do autogenous RB-protein binding sites on rRNA encode their own proteins?

A second test of the plausibility that rRNA functioned simul-taneously as mRNA at some time in the past is to compare the protein sequences of modern r-b proteins with those that might have been encoded in rRNAs. If the hypothesis of a common self-replicating ribosome is correct, then the sequence to which an mRNA-binding protein binds must also encode that binding region. The same would go for the rRNA sequence. Thus, if ribo-somes were once self-replicating entities in which the rRNA encoded its own ribosomal proteins, the shared binding regions of the rRNA and mRNA should encode homologous protein sequen-ces. Such homology would not be expected according to the null hypothesis since the ability of a protein to bind to any RNA sequence would have evolved independently of the RNA sequence itself. Such homology would also not be predicted based on the complementary binding version of the self-replication hypothesis in which the protein should bind to its complementary RNA, not to its coding sequence.

The rRNA and mRNA binding site of the S7 rb-protein for 30S rRNA have been sufficiently characterized to attempt the type of analysis just suggested. Nomura et al. (1980) and Robert and Brakier-Gingras (2001) each independently identified the putative binding sites of ribosomal protein S7 on its own mRNA and on the

30S subunit of rRNA (see also: Gimautdinova et al. (1981), Saito et al. (1994) and Wimberly et al. (1997)). The S7 binding region on the 30S rRNA identified by Nomura et al. (1980) is:

GUGGAGCAUGUACGUGCUACAAUGGCGCAUACAAAGAGAAGCG ACCUCGCGAGAGCAAGCGGACCUCAUAAAGUGCGUCGUAGUCCGGA UUGGAUCUGCAACUCGACUCCAUGAAGUCGGAAU

The S7 binding region on its own mRNA is: GUGAAGCGUC-CUAAGGCUUAAUGGUUCUCCGUUAAGUAAGGCCAAACGUUUUAAC UUAAAUGUCAAACUAAACUCGUAGAGUUUUGGACAAUCCUGAAUU AACAACGGAGUAUUUCCAUGCCACGUCGUCGC.

These sequences encode 41 and 44 amino acids respectively. As expected for an RNA-binding sequence, the incidence of Arg in the possible translations (in all six reading frames) of these sequences is unusually high (11.3%, or 1 in 9, for the rRNA sequence; and 8.3% or 1 in 11 for the mRNA sequence), and both sequences may contain ARM, though statistics are inappropriate for testing such short sequences so the significance is open to question. In addition, using a LALIGN search (BLOSSUM 80, 20 pairings, other settings on default) the sequences share several homologies (Fig. 1).

Similar results are obtained by comparing the ribosomal protein S4 binding sites on 16S rRNA: AGAAGAAGCACCGGCU AAUCCGUGCCAGCAGCCGCGGGUAAUACGGAGGGUGAAAGCGUUA AUCGG

and its own mRNA as elucidated by Nomura et al. (1980) (see also Gimautdinova et al. (1981), Changchien et al. (1988), Tang and Draper (1990) and Baker and Draper (1985)): GUAUCCU-GAAAACGGGUUUUUCAGCAUGGAACGUACAUAUCAAAUAGUAGGA-GUGCGGCCCGUAUAGCAGCG

Once again, results are shown in Fig. 1.

In other words, it is possible that at some time in the distant past, the rRNA region to which S7 binds was also the same sequence from which the S7 mRNA was derived; and the rRNA region to which S4 binds was also the same sequence from which the S4 mRNA was derived. We predict that comparison of other rb-proteins mRNA with their rRNA binding regions will reveal that most of these rRNA regions also encode proteins homologous to the rb-protein.

7. Tests of the evolution of autogenous rRNA-mRNA protein binding 3: does rRNA encode ribosome-binding proteins at a rate significantly higher than chance?

Unfortunately, the short nature of the sequences just compared in the previous section, and the fact that they have been preselected for binding the same protein rather than randomly selected, mediates against using statistics to validate the observed homologies. We have therefore employed a different strategy to establish the plausibility of these homologies. Instead, we investigated whether ribosomal proteins in general are more likely than other proteins of equivalent lengths to yield homologies to rRNA-encoded protein sequences. If our hypothesis that ribosomal protein mRNAs evolved from rRNAs is correct, we would expect that ribosomal proteins would be significantly more likely than non-ribosomal proteins of equivalent lengths to contain homologies with the protein sequences encoded by rRNAs. We therefore used the mRNA sequences of the eighteen small subunit ribosomal proteins for which there is evidence of binding to their own mRNAs (see Table 1) and used LALIGN (BLOSSUM 80; 20 pairings; and the other settings on default) to search for possible homologies with the 16S rRNA to which they bind. Three of S proteins were not utilized in the search (S14, S16 and S17) because there is no evidence that they bind to rRNA (they are known to bind, instead, to other ribosomal proteins) and are not, therefore relevant to testing the hypothesis that rRNA-encoded proteins bind to RNA. The S1 protein was also omitted, although it is known to bind to rRNA, because it is almost three times the length of any other S protein and would have dramatically skewed the resulting homology results. The resulting alignments were then categorized by the Waterman-Eggert score, a measure of the probability that an alignment of any given quality will appear randomly, high scores being rarer and of higher quality than lower scores (Table 4).

Waterman and Eggert, however, caution that in order to derive proper statistics from their scores, an appropriate control group must be used (Waterman and Eggert, 1987). Therefore, we chose two sets of 18 proteins, one set from the first 300 sequences listed in the SwissProt protein catalog when the search term "E. coli K12" was entered via the expasy.org website, and the other set from the second 300 sequences that were listed. In order to match the ribosomal proteins, the thirty-six mRNAs chosen as controls were limited to the range of proteins lengths found among ribosomal proteins (71–241 amino acids) The control sequences were also culled to eliminate any ribosomal protein sequences, DNA and RNA-related enzymes; tRNA-synthetases and ligases; phosphotases; replicases, etc., that may have evolved from, or could be expected to bind to RNA. The first eighteen proteins that fell within the combined length and non-RNA-binding parameters in the first and second 300 sequences were utilized as the controls. The total number of amino acids in the sets of sequences that were

Table 3

Statistical study (chi-squared analysis) of the frequency of arginine and arginine-rich modules (ARM) in the three rRNA subunits of the prokaryote ribosome (5S, 16S, 23S) and considered in sum) as compared with the frequency of arginine (R) use in the *E. coli* genome as a whole (see text for calculation). Calculations are provided for R, pairs of R (RR), occurrence of three R in a string of four amino acids (3R/4), and (for comparison) the frequency of lysine (K). Modules incorporating R are more frequent than would be expected by chance; K is significantly depleted in the ribosomal RNA as compared with the rest of the *E. coli* genome. The statistics are provided in raw form. Since the same dataset was analyzed in multiple ways, the results need to be interpreted accordingly: Sidak's adjustment, for each test: lower the acceptable p value to 0.0127; Bonferroni's adjustment: lower the acceptable p value to 0.0125. In light of these corrections, significant values are bolded. Arginines by themselves and in groups (ARM) occur significantly more frequently in rRNA-encoded protein sequences that would be expected by chance.

rRNA	Seq Length	R Exp	R Find	R Stats	RR Exp	RR Find	RR Stats
5S	234 aa	13	27	$\chi^2 = 4.6199$, $p = 0.0316$	0.7	4	$\chi^2 = 1.137$, $p = 0.2863$
16S	3084 aa	167	295	$\chi^2 = 37.7379$, $p = 8.092e - 10$	9	21	$\chi^2 = 4.053$, $p = 0.0441$
23S	5708 aa	319	546	$\chi^2 = 34.5588$, $p = 4.136e - 09$	18	40	$\chi^2 = 5.4457$, $p = 0.0209$
SUM	9026 aa	556	868	$\chi^2 = 73.1143$, $p < 2.e - 16$	28	65	$\chi^2 = 11.2861$, $p = .00078$

rRNA	Seq length	3R/4 Exp	3R/4 Find	3R/4 Stats	K Exp	K Find	K Stats
5S	234 aa	0.2	1	$\chi^2 = 0$, $p = 1$	10	2	$\chi^2 = 4.1908$, $p = 0.0406$
16S	3084 aa	2	7	$\chi^2 = 1.7804$, $p = 0.1821$	140	94	$\chi^2 = 8.9951$, $p = 0.0027$
23S	5708 aa	4	13	$\chi^2 = 3.7695$, $p = 0.0522$	264	191	$\chi^2 = 26.3122$, $p = 2.904e - 07$
SUM	9026 aa	6	21	$\chi^2 = 7.269$, $p = 0.0071$	409	287	$\chi^2 = 38.6145$, $p = 5.163e - 10$

```
16S S4 bs rRNA 5'3' Frame 1 (17 aa)
                          10
rp S4             LKLSRREGTD
                  |::| |X |:
16S S4 bs         LRASSRGNTE
                          10
16S S4 bs rRNA 5'3' Frame 2  (17 aa)
                           150
rp S4             EKAKKQSRVKAAL
                  |:|  :|/  ||:
16S S4 bs         EEAPANSVPAAAV
                          10
16S S4 bs rRNA 3'5' Frame 1 (18 aa)
                  10
rp S4             LKLSRREGTDL
                  |:::   ||:|
16S S4 bs         LRITAAAGTEL
                          10
16S S4 bs rRNA 3'5' Frame 1 (18 aa)
                           160
rp S4             RVKAAL--ELA
                  |: ||:  |||
16S S4 bs         RITAAAGTELA
                          10


30S S7 bs rRNA Frame 1 (41 aa) vs. S7 mRNA (P02359)
                      10        20
S7 rRNA           AHTKRSDLARASGPHK
                  |  |||:|: :  :|
S7 mRNA           AVKKREDVHRMAEANK
                        140
30S S7 bs rRNA Frame 3 (41 aa) vs. S7 mRNA (P02359)
                    20          30
S7 rRNA           ESKRTSSAS---SGLD-LQLDSMKSE
                  : |: X /|   |:|: |\  | |||
S7 mRNA           DGKKSTAESIVYSALETLAQRSGKSE
                              40        50
30S S7 bs rRNA Frame 6 (41 aa) vs. S7 mRNA (P02359)
                      20
S7 rRNA           ALYEVRLLSRGRFS
                  |: : | ||\/ ||
S7 mRNA           AFAHYRWLSLRSFS
```

Fig. 1. Homologies between the protein region encoded by the binding site (bs) sequences of the S4 and S7 ribosomal proteins on their mRNAs as compared with the protein region encoded by the corresponding rRNA binding site of the same protein. These homologies were obtained by translating the mRNA sequence and the six possible reading frames of the corresponding rRNA sequence as provided in (see text for sequences and Nomura et al. (1980) and Robert and Brakier-Gingras (2001) for original data). The resulting protein sequences were then compared with each other using LALIGN (www.expasy.org; BLOSSUM 80; 20 sequences; other factors on default). See Table 3 for an indirect statistical validation of these sequences.

compared turned out to be statistically comparable to the total number of amino acids found in the ribosomal proteins by a Kolmogorov–Smirnov test (Table 4).

Our hypothesis that rRNA may be the origins of ribosomal protein mRNAs appears to be supported by the results: 1.33% of the 16S ribosomal proteins had homologies with 16S rRNA-encoded protein sequences having Waterman–Eggert scores of 50 and above while only 0.44% of random proteins did. Similarly, 10.1% of the 16S ribosomal proteins had homologies with the 16S rRNA-encoded protein sequences having Waterman–Eggert scores of 40 and above, while only 5.9% of the random proteins did. So the higher the degree of homology, the greater the difference between the rb-proteins and the random proteins. These results are statistically significant (see Table 4).

Because the method employed in deriving these data was extremely labor- and time-intensive, we did not attempt to verify the results by examining the 30S rRNA.

As a possibly important aside, we want to draw attention to the uniformly short lengths of ribosome-binding proteins. Our sense

from the SwissProt database is that the average protein must be in the range of 400–500 amino acids in length. The average for the S proteins is 134. We speculate that the extremely short length of these ribosomal proteins might itself be an argument for their primitive origin within a highly constrained genome such as rRNA. The S1 protein is an exception.

8. Test 4: Autogenous regulation of protein synthesis by protein binding to mRNA is rare among proteins without ribosome-related functions

One final and very important point needs to be made concerning the results reported here, and that is autogenous control of protein synthesis by the protein binding to its own mRNA may be limited almost completely to proteins associated with ribosome function (e.g., rb-proteins, RNA synthetases, ligases, and polymerases; tRNA synthetases; elongation factors; protein initiation factors; etc.), or somewhat more generally, translation and transcription. In other words, proteins binding to their own mRNA is not a general phenomenon but rather a distinct exception. It is not, of course, possible to prove a negative, so the question is whether there exist proteins that exert autogenous control that are not related to ribosome function. Thus far, an intensive search for such proteins has not yielded any exceptions. Even if a small number of exceptions are found, the fact that such instances must be rare is critical for validating our tests of our null hypothesis. Our null hypothesis, recall, was that ribosome-associated proteins evolved independently of rRNA and therefore that their mRNAs should share no more than probabilistic similarities with rRNAs; binding to rRNA should not, therefore, predict binding to self-mRNA. The null hypothesis actually does appear to hold for most, if not all, proteins that do not have either translation or transcription-related functions, thus suggesting that ribosome-associated proteins evolved by means of a process significantly different than did most cellular proteins.

9. Test 5: Modern examples of functionally active rRNA-encoded peptides and proteins

One final test of our hypothesis that ribosomes once encoded genetic information related to their own functions is that some traces of such encodings should still exist in some modern organisms, as suggested previously by Seligmann (2013a). This assertion runs counter to current dogma. Coelho et al. (2002) are one of many investigators to state the generally accepted wisdom that, "In eukaryotes, it is widely assumed that genes coding for proteins and structural RNAs do not overlap." This belief has persisted, as Tenson and Mankin (1995) note, although short open reading frame (ORF) segments have been known for some time to exist in rRNA and the rDNA that encodes it: "The possibility that some smaller open reading frames in rRNA may have functional significance has been essentially ignored." In recent years, exploration of open reading frames in both prokaryotes and eukaryotes has, however, yielded the surprising observation that some of their rRNA and rDNA sequences can be translated into peptides and proteins that have functional activity. For example, Tenson and Mankin (1995), Tenson et al. (1996) and Dam et al. (1996) have demonstrated in various bacteria that there is a, "short open reading frame in the 23S rRNA that encodes a pentapeptide (E-peptide) whose expression *in vivo* renders cells resistant to erythromycin." Tenson and Mankin (1995) and Tenson et al. (1996) have shown that this peptide binds directly to the 23S rRNA, exerting its function through this interaction.

Table 4

Statistical study of the distributions of homology or similarity scores (Waterman–Eggert) resulting from LALIGN alignments of eighteen of the S ribosomal proteins with 16S rRNA-encoded protein sequences, compared with two control groups of 18 randomly chosen protein sequences with equivalent length distributions (one from the first 300 proteins listed in the SwissProt protein database and one from the second 300 proteins listed there). The 16S rRNA was translated into each of its possible six reading frames. All six of the resulting proteins sequences were then compared with each S protein and each control protein (www.expasy.org; BLOSSUM 80; 20 sequences; all of other factors on default). All three sets of proteins varied in length from between 71 and 241 amino acids and the total number of amino acids in each group was also comparable as verified by a two-sample Kolmogorov–Smirnov test. A Wilcoxon signed rank test was used to test whether the differences in incidence of protein homology scores were significantly different between the S protein-derived scores and the control groups. The S proteins are significantly more likely to yield more higher-scoring homologies than the controls. See Fig. 1 for some examples.

W–E Score	> 60	55–59	50–54	45–49	40–44	35–39	Total	TOT > 34	# SEQ	TOT AA	KS Test	Wilcoxian
Controls 1	0	2	9	27	86	261	385	770	18	2624		
											$D=0.2222, p=0.7658$	$V=21, p=0.0312$
S proteins	2	6	16	42	116	358	540	1080	18	2433		
											$D=0.2222, p=0.7658$	$V=21, p=0.0312$
Controls 2	1	0	4	21	65	193	284	568	18	2219		

A second, very well-characterized example of an rRNA-encoded protein involves homing endonucleases. "Homing" refers to the ability of the enzyme to induce lateral transfer of a polynucleotide or polypeptide sequence encoded as an intron (gene spacer) or intein (protein spacer) to a homologous sequence (gene or protein) that lacks that sequence. Homing endonucleases are encoded in open reading frames (ORF) embedded within the intron or intein (Chevalier and Stoddard, 2001). Homing endonucleases, in other words, are encoded within regions generally considered to be "non-coding" and, like the many rb-proteins already described here, bind back on the sequences that encode them. Their function is to facilitate recombination of genetic segments and to act as retrotransposable elements (Jakubczak et al., 1991; Brett et al., 2002). The first rRNA-encoded homing endonucleases were described in the 1970s in the yeast *Saccharomyces cerevisiae* in the rRNA gene of the mitochondrial genome (Bos et al., 1978; Dujon, 1980; Colleaux et al., 1986; Dujon et al., 1986). This intron is now called I-SceI and, according to Chevalier and Stoddard (2001) was the first of over 250 homing endonucleases having transposase activity that have since been identified. DNA sequences encoding homing endonucleases have been found in a very wide range of bacteria including *E. coli, Salmonella sp., Haemophilus, Neisseria, Proteus, Pasteurella* (Liu et al., 1993), *Chlamydomonas* (Dürrenberger and Rochaix 1991; Heath et al., 1997), and many fungal species (Hafez et al., 2013). Many of these homing endonucleases are located within introns of mitochondrial rRNA (e.g., Bos et al. 1978; Dujon, 1980; Michel and Cummings, 1985; Colleaux et al., 1986; Hafez et al., 2013) or chloroplast rRNA (Rochaix et al., 1985; Dürrenberger and Rochaix, 1991; Dürrenberger and Rochaix, 1993; Thompson et al., 1992; Heath et al., 1997), and they are also found in archaea (Lykke-Andersen et al., 1997; Kjems and Garrett, 1988; Barzel et al, 2011). Such evidence suggests that homing endonucleases have very ancient origins (as old as ribosomes themselves, and therefore pre-dating cellular life) and may have played key roles in the generation of rRNA-encoded genetic diversity during the origins of life.

Two additional proteins are known to be encoded by the rDNA sequence encoding rRNA. One is ribin, a protein that modulates ribosomal transcription in a wide range of eukaryotes (Kermekchiev and Ivanova, 2001; Barthélémy et al., 2010). The second, Tar1p, discovered by Coelho et al. (2002), is a mitochondrial protein found in many eukaryotes, that is encoded in an open reading frame (ORF) named TAR1, which stands for Transcript Antisense to Ribosomal RNA. Tar1p appears to regulate mitochondrial gene expression through an RNAase-mediated mechanism (Coelho et al., 2002; Bonawitz et al., 2008; Galopier and Hermann-Le Denmat, 2011).

Notably, all known rRNA- and rDNA-encoded peptides and proteins exercise their activity by binding to RNA, which is in keeping with our hypothesis that at one time the ribosome encoded its own structural and regulatory molecules (Figs. 2 and 3). In addition, as predicted in our first paper (Root-Bernstein and Root-Bernstein, 2015), such proteins are encoded not only in the native rRNA but also in the complementary strand (in this case the rDNA) as well.

Many other open reading frames possibly encoding functional peptides and proteins within rRNA and rDNA sequences are known to exist in both prokaryotes and eukaryotes, but are currently written-off as being meaningless anomalies that need to be excised from genome sequencing studies (Tripp et al., 2011) or have simply not been investigated for possible functionality. As Johansen et al. (2007) note: "Although rDNA-embedded protein genes are widespread in nuclei, organelles and bacteria, there is surprisingly little information available on how these genes are expressed. Exceptions include [as described above] a handful of HEGs [homing endonuclease genes] from group I introns." Investigating these other rRNA- or rDNA-encoded genes might be warranted by the evidence presented here. Additionally, in bacteria at least, rRNA is known to exist in translation-accessible fragments or short rRNA (reviewed in Evguenieva-Hackenberg (2005)), and rDNA sequences are known to form myriad small plasmid-like circles (Einvik et al., 1998) that may function as translation regulators through transcription and/or translation (reviewed in Poole et al. (2012)). In short, the dogma that rRNA do not encode proteins may be incorrect and due mainly to the failure to search for such encodings rather than to their absence.

10. Summary

In sum, five types of evidence reported here support the plausibility of the hypothesis that the mRNA encoding ribosomal proteins evolved from rRNA: (1) the ubiquity of rb-protein binding to their own mRNAs and controlling their own translation autogenously; (2) the higher-than-expected incidence of Arginine-rich modules (ARM) in rb-proteins; (3) the fact that rRNA-binding regions of rb-proteins are homologous to their mRNA binding regions; (4) the higher than expected incidence of rb-protein sequences encoded in rRNA that of a high degree of homology to their mRNA as compared with the lower incidence of equally good homologies between a random selection of other proteins; and (5) evidence that the rRNA (or rDNA) of modern prokaryotes and eukaryotes contain genes encoding at least six, functional ribosome-related peptides or proteins. None of these results can be explained by the null hypothesis that assumes independent evolution of rRNA and the mRNAs encoding ribosomal proteins. It is also evident from the data summarized above that rRNA does not encode entire ribosomal proteins, but the modules specifically associated with the binding of the protein to RNAs. The rarity of proteins binding to their own mRNAs that are not associated with
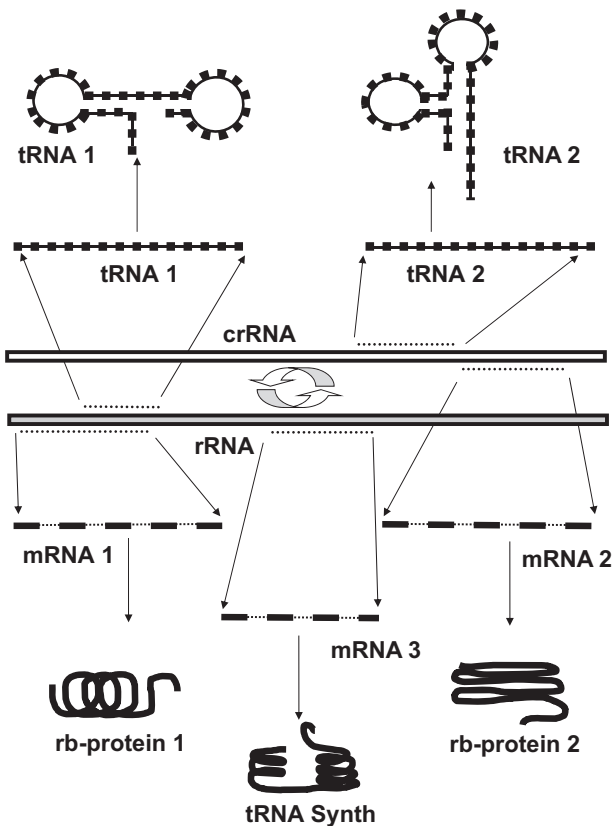
Fig. 2. Cartoon providing an overview of the major information-encoding features of the self-replicating ribosome theory. The rRNA (center, thick gray line) encodes both tRNA and mRNA and can also be transcribed into a complementary rRNA sequence (crRNA, white line) that also encodes tRNA and mRNA (see Root-Bernstein and Root-Bernstein (2015)). The mRNA (and thus the original rRNA) encode various protein modules that include various ribosomal proteins, tRNA synthetases, and enzyme active sites. The rRNA and the tRNAs, mRNAs and proteins that it encodes provide the key elements necessary for the ribosome to replicate itself.

Fig. 3. Cartoon providing an overview of how the components described in Fig. 2 interact to create a functioning ribosome capable of carrying out translation and simple metabolic control. The rRNA (thick gray circle, center) folds into a conformation appropriate for binding ribosome-binding proteins (rb-proteins). These rb-proteins not only provide ribosomal functions such as aiding in the binding of tRNAs, but also autogenously regulate their own production by binding to mRNA sequences homologous to their rRNA binding sites. Some of these proteins also carry out additional metabolic functions such as attaching amino acids to their appropriate tRNAs to create amino acyl-tRNAs that are used by the ribosome to synthesize peptides or proteins. The result of this is an auto-regulated system of ribosome-directed replication, transcription and translation that encodes all of the components necessary for the ribosome to replicate itself.

ribosome function may also be interpreted as being consistent with our hypothesis and the unusually short length of ribosome-binding proteins may be a clue worth further investigation as well.

11. Further testable predictions of the self-replicating ribosome theory

A large number of further testable predictions can be made from the self-replicating ribosome theory. Some follow directly from the results reported here. For example, there is evidence that RNA polymerases (which logically would have had to co-evolve with a self-replicating ribosome) also exhibit autogenous control, binding to their own mRNAs or gene promoters (Fukuda et al., 1978; Dykxhoorn et al., 1996; Passador and Linn, 1992; Steward and Linn, 1992; van Gemen et al., 1989; Steinmetz et al., 2001; Roth et al., 2005). We make all the same predictions regarding RNA polymerases that we have demonstrated here for ribosomal proteins: (1) that RNA polymerases will ubiquitously be under autogenous control; (2) that they will display RNA binding motifs such as ARM at unusually high rates; (3) that the binding sites on growing RNA chains and on their own mRNAs will be similar; and (4) that RNA polymerases will be found encoded in rRNA at much higher rates and with much better homologies than any set of randomly chosen proteins of equivalent length.

A similar set of predictions can be made for other proteins with ribosome-related functions such as LIN28; thymidylate synthase (TS) and dihydrofolate reductase (DHFR); IF3; and EF-Tu (tRNA-
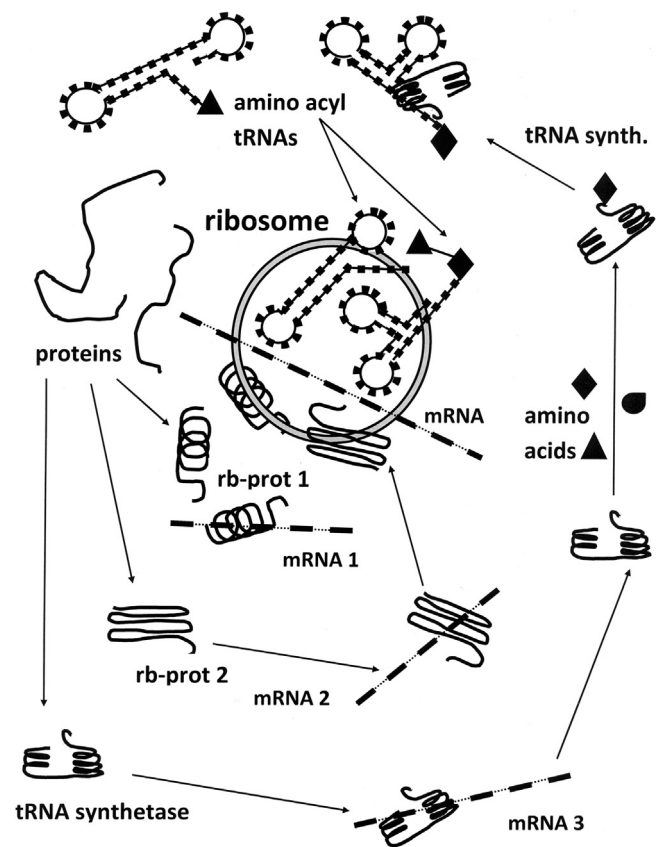
tufB). "LIN28A Acts as a 'translational enhancer', driving specific mRNAs to polysomes and thus increasing the efficiency of protein synthesis. Its association with the translational machinery and target mRNAs results in an increased number of initiation events per molecule of mRNA and, indirectly, in stabilizing the mRNAs. Binds IGF2 mRNA, MYOD1 mRNA, ARBP/36B4 ribosomal protein mRNA and its own mRNA" [http://www.phosphosite.org/proteinAction.do?id=22107&showAllSites=true]. TS and DHFR are folate-dependent enzymes critical for providing the requisite nucleotide precursors for maintaining synthesis and repair of polynucleotides. They, too, regulate their production by binding to their own mRNA (Tai et al., 2004). IF3 is a protein synthesis initiation factor in *E. coli* that autogenously regulates its own production (Butler et al., 1986) and EF-Tu binds to ribosomes mediating tRNA binding and acting as another regulator of protein translation (van der Meide et al., 1983; Van Delft et al., 1988). Both autoregulate their own production by binding to their mRNA.

Another testable implication follows from our previous paper (Root-Bernstein and Root-Bernstein, 2015), in which we demonstrated that all twenty tRNAs are encoded in both the 16S and 23S RNAs. One implication is that rRNAs may have originated from tRNA-like modules so that protein translation evolved in tandem with the ribosome itself. This hypothesis has been suggested

Table 5
Articles demonstrating that some tRNA synthetases bind to their own mRNAs.

Prokaryote tRNA Synthetases	References to mRNA binding
Asp-tRNA synthetase	Frugier and Giegé (2003) and Ryckelynck et al. (2005)
Glu-tRNA synthetase	Schray and Knippers (1991)
Met-tRNA synthetase	Romby et al. (1992)
Thr-tRNA synthetase	Springer et al. (1989), Moine et al. (1990), Brunel et al., (1992), Romby et al. (1992, 1996), Nogueira et al. (2001), Torres-Larios et al. (2002) and Romby and Springer (2003)

previously by several other investigators as well (e.g., Waterman and Eggert, 1987; Bloch et al., 1989; DiGiulio, 2004; Seligmann, 2013a, 2013b, 2014; de Farias et al., 2014; Nasir and Caetano-Anolles, 2015). What has not previously been suggested by us or anyone else is that tRNA might represent primitive genes that produce gene products under autogenous control. It is known, in fact, that many tRNA synthetases (or ligases), which function to bind to tRNA and add the amino acid that matches its anticodon, also autogenously regulate their own mRNAs (reviewed in Romby and Springer (2003) and Ryckelynck et al. (2005)). Space does not permit a full discussion here (which we will leave for a future publication), but autogenous regulation of tRNA synthetases has been demonstrated for threoninyl-, aspartic acid-, histidine-, methionine-, and phenylalanine-tRNA synthetases (Table 5). What has not yet been tested is the further prediction that these tRNA should also encode the key protein binding region of the synthetase so that, like the rb-proteins characterized above, the tRNA should encode "genes" for the functional modules necessary to their own function. If this is true, then it follows that tRNAs may, at one time, have also functioned as mRNAs and as such, tRNA sequences may have encoded other key ribosomal protein modules as well. The tRNA-synthetase story may turn out to be more complicated than the rb-protein story, however, because many tRNA-synthetases bind not to their own mRNAs, but to other regulatory elements (e.g., Ryckelynck et al., 2005). A further testable implication is that these protein modules should form stable complexes with their tRNA and that these complexes should protect their components against degradation, thereby making them more likely to survive prebiotic conditions.

Additional predictions include the possibility that archael and prokaryotic (DNA-based) genomes were built around ribosomal RNA "genes"; that the ribosome should therefore have metabolic roles that extend far beyond mere protein translation, such as energy production and regulation; and that viruses co-evolved as degenerate or symbiotic proto-ribosomes, perhaps forming a primitive disseminated ecology of such "organisms" long before the emergence of cells, an idea being independently explored by Nasir and Caetano-Anolles (2015). The overall implication of this view of the origins of ribosome function is that protein-RNA interactions long preceded the evolution of the completely-integrated translation ribosome, and may have resided in a distributed set of tRNA-like modules that incorporated RNA-binding as well as encoding enzymatic functions associated with translation and its associated metabolic prerequisites (see also Seligmann and Raoult (in press)).

There are so many possibilities to explore here that we strongly urge any investigator in possession of the relevant skills and tools to address these predictions and we will, of course, do so ourselves.

Disclosures

The authors declare that they have no conflicts of interest to report.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtbi.2016.02.030.

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