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THEORETICAL PAPER

Possible Ancestral Functions of the Genetic and RNA Operational Precodes and the Origin of the Genetic System

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Abstract

The origin of genetic systems is the central problem in the study of the origin of life for which various explanatory hypotheses have been presented. One model suggests that both ancestral transfer ribonucleic acid (tRNA) molecules and primitive ribosomes were originally involved in RNA replication (Campbell 1991). According to this model the early tRNA molecules catalyzed their own self-loading with a trinucleotide complementary to their anticodon triplet, while the primordial ribosome (protoribosome) catalyzed the transfer of these terminal trinucleotides from one tRNA to another tRNA harboring the growing RNA polymer at the 3´-end.

Here we present the notion that the anticodon-codon-like pairs presumably located in the acceptor stem of primordial tRNAs (Rodin et al. 1996) (thus being and remaining, after the code and translation origins, the major contributor to the RNA operational code (Schimmel et al. 1993)) might have originally been used for RNA replication rather than translation; these anticodon and acceptor stem triplets would have been involved in accurately loading the 3'-end of tRNAs with a trinucleotide complementary to their anticodon triplet, thus allowing the accurate repair of tRNAs for their use by the protoribosome during RNA replication.

We propose that tRNAs could have catalyzed their own trinucleotide self-loading by forming catalytic tRNA dimers which would have had polymerase activity. Therefore, the loading mechanism and its evolution may have been a basic step in the emergence of new genetic mechanisms such as genetic translation. The evolutionary implications of this proposed loading mechanism are also discussed.

Keywords RNA replication · Codon-anticodon interaction · RNA world · Origin of genetic translation · Genetic code · RNA operational code · Ribozyme

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Introduction

The notion that there was an early RNA-based stage in the evolution of life—the RNA world (Gilbert 1986)—is now widely accepted after the discovery of catalytic RNA (Cech et al. 1981; Guerrier-Takada et al. 1983) and the finding that ribosomes are macromolecular machines with a RNA-based catalytic center (Ban et al. 2000). Moreover, some recent findings have suggested efficient solutions to the problem of ribonucleoside formation through various pathways involving the synthesis of natural ribonucleosides under prebiotic conditions (Powner et al. 2009; Szostack 2009; Becker et al. 2016, 2019). These prebiotic routes of generation, in the absence of enzymes, reduce the metabolic complexity that would have been required and means that this synthesis –a prerequisite to create RNA- in the early RNA world would have been more feasible. Thus, at its start, the genetic system was likely based on RNA replication.

Moreover, in the molecular context of this RNA world, our understanding of the origin of the genetic code has changed. It is accepted that the genetic code arose during this step of life, but the question of how the relationship between RNA and amino acids came about in the absence of proteins remains. There are various examples of RNA structural motifs selected in vitro that interact specifically with amino acids. Some studies indicate that RNA sites that bind amino acids are composed predominantly of certain triplets that correspond to current codon assignments (Yarus 2005). However, there are reasons for caution in this interpretation (Ellintong 2000).

The establishment of reproducible RNA sequences through the emergence of an accurate RNA replication mechanism is a basic step to the generation of a primitive genetic system. That is, there can-not be a code until reproducible RNA sequences are available.

A basic element in the "RNA world hypothesis" (Gilbert 1986) is one ribozyme that catalyze its own replication. Thus, the absence to date of a demonstrable RNA replicase is one important problem. Even though, the generalized RNA-catalyzed RNA replication has not yet been achieved, there is one example of a pair ribozyme ligases that catalyze (each catalyze the formation of other) its own amplification with limited Darwinian evolution, and using four oligonucleotide substrates (Lincoln and Joyce 2009). Various models on the nature of the polymerase ribozyme have been proposed (Sharp 1985; Campbell 1991; Gordon 1995; Poole et al. 1998; Noller 2011).

A model by Noller proposes that two duplicator RNAs (dRNAs) —precursors of current tRNAs— with identical anticodon triplets formed a dRNA homodimer by base pairing of their self-complementary tails. It posits that these dRNA homodimers would have mediated RNA replication through indirect templating with dRNA anticodon triplets in primitive ribosomes with polymerase activity (Noller 2011). In this model, the RNA template would be duplicated without the formation of a complementary strand, and there would be an additional discriminatory mechanism -a precursor to the current-day decoding center (Noller 2011).

It is generally accepted that current tRNAs arose during the RNA stage by ancestral duplication of an RNA hairpin structure (Bloch et al. 1985; Moller and Jamssen 1990; Di Giulio 1992). Direct duplication of the precursor hairpin RNA molecule would have generated two regions (with two identical trinucleotide sequences); one could have evolved into the anticodon loop region and the other into the acceptor stem region, together forming the area that houses the main determinants of tRNA identity (Moller and Jamssen 1990; Di Giulio 2004). The first substantial, if indirect, evidence supporting this notion was presented as possible presence of the codon-anticodon pairs in the acceptor stem of primitive

tRNAs (Rodin et al. 1996). More recent and compelling evidence in support of a common ancestor for both codes (i.e., the RNA operational code embodied mostly in the acceptor stem and the genetic code per se embodied in the anticodon) was also reported (Rodin et al. 2009). This work suggests that the anticodon triplet and 3'-terminal triplet might have initially been identical or complementary, perhaps with the same origin. It has also been suggested that tRNA-like structures were first used for RNA replication by ribozymes in the RNA world before the advent of translation (Weiner and Maizels 1987; Maizels and Weiner 2000).

On the Nature of the Polymerase Ribozyme in the RNA World

A Model for the Genetic Loading Mechanism of tRNA with Trinucleotides

The model on the primitive RNA replicase proposed by Campbell suggests that ancestral transfer RNA (tRNA) molecules were originally loaded onto the 3'-end along with trinucleotide sequences complementary to their anticodon triplet. In this model the protoribosome, which would have initially functioned as a RNA replicase, would have then catalyzed the transfer of these terminal trinucleotides from the one tRNA to another tRNA harboring the growing RNA polymer at the 3´-end (Campbell 1991). According to this model, the addition of the CCA trinucleotide to present day tRNAs is a remnant of the original function of RNAs in replication, and also parallels the loading of tRNAs with amino acids. However, the author did not propose a detailed mechanism for tRNA loading with trinucleotides complementary to the anticodon. A model is proposed here for the possible charging of the primitive tRNA with trinucleotides, which is based in two central proposals;

- one proposal is that the anticodon-codon-like pairs presumably located in the acceptor stem of primordial tRNAs (Rodin et al. 1996) (thus being and remaining, after the code and translation origins, the major contributor to the RNA operational code (Schimmel et al. 1993)) might have originally been used for RNA replication, not translation, in accordance with Campbell´s hypothesis,
- the other proposal is that primitive tRNAs could have catalyzed their own trinucleotide self-loading by forming catalytic tRNA dimers (between tRNAs with identical anticodons and codon-anticodon pairs) which would have had polymerase activity (facilitated by possible presence of modified nucleosides in the anticodon loop).

Moreover, we proposed that the trinucleotide sequence loaded to the 3´-end strand and the anticodon triplet of the primitive tRNA would have been ancestors of two current codes; the operational code (acceptor stem) and the genetic classic (anticodon) code.

Since RNA replication in the RNA replisome, according to Campbell's model (1991), would have incorporated only three nucleotides per tRNA into the RNA product, it could only have evolved in an environment rich in tRNA substrates. The regeneration of substrates (tRNA) by repair seems to be a more efficient biological strategy compared with the de novo replication. In a primitive environment the high demand of substrates to be used in the RNA replication would have allowed the selection of tRNA substrates with capacity for self-repair. We propose that the emergence of tRNA-like molecules which harbored codon–anticodon pairs in the acceptor stem allowed the formation of catalytic dimers between two tRNAs with identical anticodon and codon–anticodon pairs in the

acceptor stem (with codon-like triplets located in the 3'-terminal strand). In accordance with our model we propose that the catalytic tRNA dimers could have possessed polymerase (limited) activity with single nucleotides which may have been used to repair the truncated tRNAs generated in the process of RNA replication in the protoribosome. Thus, a truncated tRNA produced in replication would have been able to form a dimer with an intact tRNA, both with identical anticodons. The catalytic tRNA dimer would have comprised two tRNA molecules (one truncated tRNA molecule and one intact tRNA molecule), which would have interacted through complementary base pairing of the anticodon loop on one molecule with the complementary sequence at the 3'-acceptor end of another identical molecule. There would have been two intermolecular interaction zones: one between the anticodon triplet of one molecule and the intact 3'-acceptor end of another molecule (which would have functioned to stabilize the dimer), and other would have been a zone of interaction between the anticodon triplet and the truncated 3'-acceptor end of another molecule (see Fig. 1b). Hence, in this model, the complementarity of sequences between the anticodon loop and the 3'-terminal acceptor strand in the tRNA molecule would have promoted dimerization. As in other RNA structures, divalent metals and 2'-hydroxyl groups (Strobel and Doudna 1997) might have played

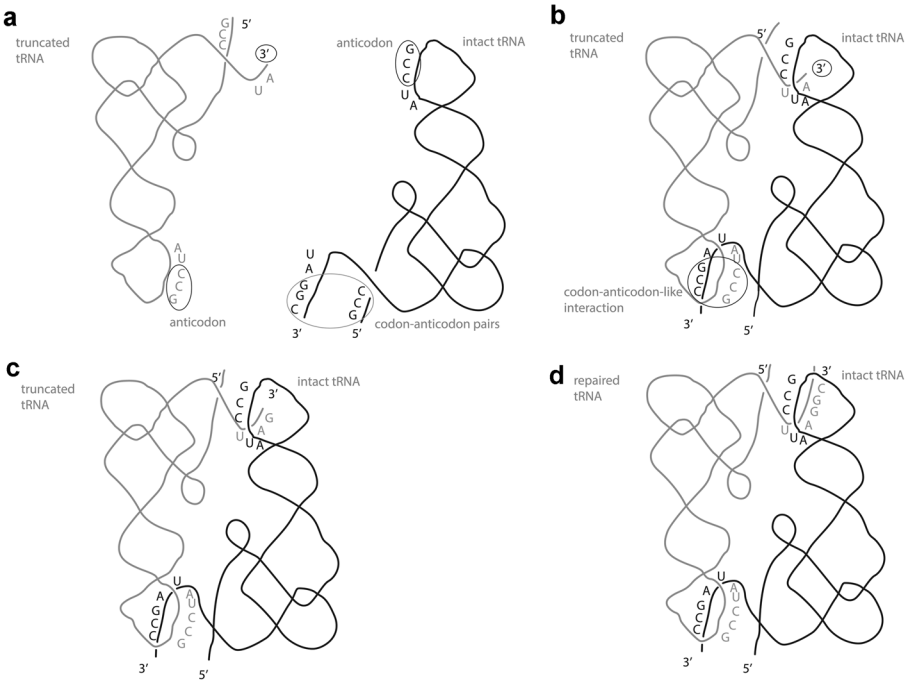

Fig. 1 The structure of primitive tRNAs is proposed to have been similar to current tRNAs. **b** , **c** and **d** Various steps in the proposed model for primitive genetic tRNA loading mechanism with trinucleotides based in a catalytic $tRNA_{intact}$–$tRNA_{truncated}$ dimer. **a** Two primitive tRNA molecules with identical anticodons; one truncated tRNA and one intact tRNA. **b** The structural model proposed for the $tRNA_{intact}$–$tRNA_{truncated}$ dimer with polymerase activity. **c** The addition of one nucleotide to the 3'-endcatalyzed by dimer. **d** The tRNA repaired by dimer

an important role in noncovalent interactions between the phosphate–ribose backbones of both tRNAs, thereby stabilizing the dimer.

In this model and with relation to the molecular mechanism of tRNA loading with trinucleotides complementary to their anticodon triplet we propose that these $tRNA_{intact}$–$tRNA_{truncated}$ dimers would have had one catalytic center located in intermolecular interaction zone formed between the anticodon loop and the truncated-acceptor strand. In this interaction zone, the anticodon of the intact tRNA molecule would have functioned as a template for activated nucleotides, with the 3'-end of the truncated tRNA molecule functioning as a primer. The ancestral duplication of tRNA precursors would have provided complementarity between some of the nucleotides next to the anticodon triplet and others close to the truncated 3'-end. This means that when the dimers formed, two terminal nucleotides at the truncated 3'-end would have interacted by base pairing with nucleotides close to anticodon triplet of intact tRNA. In the sequential catalytic addition of three nucleotides (one by one) to the truncated 3'-acceptor end, the ribose terminal 3'-OH group of the acceptor strand would have reacted with an incoming nucleotide guided by a template—the anticodon loop—to help it properly orient both substrates. Divalent metal ions have long been thought to have a direct catalytic role in ribozymes by activating the 3'-OH nucleophile (ribose terminal), and perhaps also through interactions with the monomer phosphate. Indeed, the intermolecular interaction between the anticodon loop and the truncated 3'-strand could produce a metal-binding site close to the 3'-terminal ribose. In favor of this idea, various metal ions have also been found in the anticodon loop structure of current tRNAs (Pan et al. 1993). In our proposed model, the anticodon loop could have acted as a template catalyst (Orgel 1986) by orienting complementary nucleotides (the 3'-truncated acceptor strand and incoming nucleotide) and as a metal ion-binding pocket (with a metal ion located between nucleotides 35 and 36 of the anticodon –bound to the phosphate group of nucleotide 36-). This would have facilitated binding of the metal ion close to the 3'-OH nucleophile group of the terminal ribose in the 3'-truncated acceptor strand in order to activate said nucleophile group. Thus, this 3'-hydroxyl would have been able to attack the 5'-triphosphate of the incoming nucleotide, thereby allowing a polymerization reaction to take place (formation of the phosphodiester bond) (see Fig. 1c).

In support of this model, an in vitro selection study produced a small, dual-catalytic RNA that promoted cleavage at one site and ligation of two RNA substrates at another site, thus suggesting that two conformations surrounded at least one unique divalent metal ion-binding site (Landweber and Pokrovskaya 1999). The same study showed that a change in the catalytic activity of this small ribozyme could be produced by a single metal switch. Moreover, the fact that such simple RNA structures may possess two types of catalytic activity (one –ligation- related with RNA replication) suggests that similar small metalloribozymes they arise easily during the prebiotic evolution (Landweber and Pokrovskaya 1999). Other reports consider the metal ion-catalyzed cleavage of tRNA-Phe to be an intramolecular version of a metallo-enzyme-catalyzed reaction (Brown et al. 1983) in which the D loop acts as the substrate and the remainder of the tRNA acts as the enzyme. Moreover, an Mg^{2+} ion near to anticodon (bound to the phosphate group of nucleotide 36) is involved in the tRNA self-cleavage reaction between nucleotides 35 and 36 (Jovine et al. 2000).

We suggest that catalytic tRNA dimers lacked full processivity (the ability to keep moving along the RNA being replicated) thus, the addition of successive nucleotides would have been progressively less efficient. Thus, the addition of the last (three) mononucleotide could be more error-prone (possibly due to its distance of catalytic ion). This could allow changes through point mutations and selection during RNA replication, which would facilitated the molecular Darwinian evolution, a key requirement for a genetic system.

Moreover, we posit that the proposed dimers we mentioned above would have been capable of a polymerase limited activity with dimer dissociation occurring after addition of the trinucleotide at the truncated tRNA, thereby producing two $tRNA_{intact}$ (see Fig. 1d).

The phylogenetic studies of modified nucleosides point out that some modifications (located in the tRNA and rRNA) are very ancient (Cermakian and Cedergren 1998). The synthesis of 5-substituted uracils, under potentially prebiotic conditions has been shown (Robertson and Miller 1995). The first example of a self-alkylating ribozyme has long been shown (Wilson and Szostack 1995). It is possible that analogous self-modification reactions were common in the RNA world (by generating more modified nucleosides). It have been suggested that some current modified nucleosides in the tRNA anticodon (including those that have amino acids in their structure) might be relics of an ancient code in the RNA world (Di Giulio 1998; Grosjean et al. 2004). These early modifications could have provided an expanded catalytic functionality to RNA enzymes (Cermakian and Cedergren 1998). It is possible that these modifications in the anticodon loop could have increased the catalytic capacity of the tRNA dimer (as long as the modifications did not interfere with the complementary base pairings -between anticodon and acceptor stem-). One possibility is that the chemical groups of modified nucleosides in the anticodon loop together with the polyelectrolyte character of RNA should make the coordination of metal ion at specific (catalytic) site quite feasible; other, by increasing the functional-group diversity of catalytic RNA.

Even assuming that other intermolecular forces would have been involved in dimer stabilization, we suggest that it might have been impossible for two tRNA molecules to form a stable dimer in the absence of three complementary base pairs in the stabilizing anticodon-3'-stem intermolecular interaction region. In other words, two truncated tRNAs would not have been able to form a stable dimer with catalytic activity. Thus, incorrect addition through interactions between two truncated tRNAs would have been unlikely, which would have led to a scenario with stable 'operational' and 'genetic' pre-codes.

In Campbell's original model (1991), triplet transfer by the RNA replisome was central to the origin of genetic replication; in our model this would require the addition of single nucleotides as a means to regenerate the 3'-end, thereby loading tRNAs with the substrate required for replication. In other words, triplet-based RNA polymerization first required the invention of effective polymerization with single nucleotides. The immediate question that arises is, if the latter works effectively, why would triplets be required at all? But this apparent paradox has a possible explanation. Ribozymes with ligase activity (a single condensation reaction with two oligonucleotide substrates) isolated in vitro can also catalyze the template-directed addition of only three successive nucleotides (Ekland and Bartel 1996; Joyce et al. 2004). That is, one ribozyme can possess the ability for both triplet- and mononucleotide-based polymerization. Thus, given that only four rounds in vitro were required for this class I ligase to 'evolve', it is possible that similar ribozymes easily arose in the RNA stage of life.

This idea could explain the functional significance and selection pressure for these codon-like triplets in the 70–71-72 (3'-end) positions of the tRNA acceptor stem. With the emergence of these tRNA molecules with codon-anticodon pairs in their acceptor stems, RNA replication would have produced RNA molecules identical to the RNA template. Therefore, this process would have provided a mechanism to store and maintain genetic information. Importantly, this RNA replication mechanism could only have evolved with the emergence of the tRNA repair process.

Experimental data that supports our structural model for tRNA dimers included the fact that a crystal structure comprising two bound RNA molecules, one tRNA and one T-box

riboswitch which is located upstream of the mRNA of the corresponding aminoacyl-tRNA synthetase (Zang and Ferré-D'Amaré 2013). Specific tRNA recognition by stem I of the T-box riboswitch in this complex seems to involve two main tRNA regions: the anticodon loop and the T- and D-loops. Moreover, the interactions between the three-nucleotide anticodon sequence (tRNA) and stem I in this structure are almost identical to the interaction between tRNA anticodons and mRNA on the ribosome. Thus, it appears that a few, small, structural motifs permit the recognition and promote the stability of this binary RNA complex. Another study showed that two different tRNAs can efficiently bind the specifier loop of a T-box riboswitch with two overlapping codons, both in vitro and in vivo (Saad et al. 2015). In addition, other models have been proposed to explain the complexes between two tRNAs (Yang et al. 1972; Eisinger and Cross 1974; Rodin and Ohno 1997; Grosjean et al. 1998).

RNA Replication by the RNA Replisome

According to Campbell's model (1991), reading in the 5' to 3'direction of a S1 sequence, (3') 1–2-3–4-5–6 (5'), translates to a new S1'sequence (3') 3–2-1–6-5–4 (5'). In other words, the S1 sequence is not complementary to the S1' sequence, although copying the latter would return the original S1 sequence. Because the RNA replisome must have read RNA in the same direction as the current ribosome (5'to 3'), it presumably copied RNA via an S1' intermediate. A problem with current polymerase is that the new complementary strand sequesters the template into a stable helix. The ancestral replisome with their copy mechanism avoids this difficulty and might have established the chemical basis for the difference between the phenotype (S1' sequence) and genotype (S1 sequence; Campbell 1991).

We propose that in the initial stage of the RNA world, the genotype comprised RNAs with simple strands. With the emergence of proteins during the transition to the RNA–protein world, the primitive genotype would have been copied by replicase proteins, producing RNAs with a double complementary strand. A large part of the genetic information stored in this primitive genotype would have been lost in the transition to the new double-stranded genotype, although it is possible that some elements of the phenotype were copied by replicases. We propose that only the more abundant functional RNAs or ribozymes (the phenotypes involved in RNA replication, such as tRNAs, RNase P and the RNA replisome) were copied, leading to conservation of their genetic information. This would explain the reduced presence of ribozymes in the current DNA–protein world. However, the genomic dark matter offers immense scope for discovery of novel RNA products, riboswitches, non-coding RNAs, with multiple functions, all of which has been repeatedly demonstrated. Therefore, a reduced presence of ribozymes may not entirely reflect the importance of RNA in extant biology, given the growing body of evidence suggesting an increasing role of RNAs in regulating biology.

Additionally, the genes (S1 sequence) would have lost their capacity for encoding ribozymes (with the S1' sequence) during the transition to the RNA–protein world. In the transition, the protein replicase would have synthesized complementary copies of the S1 sequence. However, the new protein-based genetic system could not have synthesized the S1' sequence (encoding a ribozyme) of the primitive phenotype. The S1' sequence would not have been complementary to the S1 sequence, and the S1' sequence would have only been synthesized by the primitive RNA replisome (Campbell 1991).

According to this model on the RNA replication in replisome, the trinucleotide added to the growing chain is the codon-like triplet located at the 3'-end of the tRNA substrate, and the addition only requires one excision. The main contribution of the replisome to triplet transfer catalysis might be that it positions within the active center the two substrates in the optimal orientation for the attack of the 3'-OH terminal ribose group of the incoming tRNA substrate on the phosphate group (70 position) of another tRNA substrate, rather than being a chemical catalyst.

In our model, the accuracy of RNA replication depends on the precision of two successive processes: first, the loading of truncated tRNAs with trinucleotides complementary to the anticodon (tRNA dimer), and second, the interaction between the anticodon of the loaded tRNAs and the codon of the RNA template during RNA replication (replisome). The latter would have required direct interaction between two short nucleotide sequences, the anticodon and codon, by complementary base pairing (codon-anticodon interaction). The former would have been based on the interaction between the anticodon loop and the 3'-truncated acceptor stem, with truncated tRNAs being repaired through the formation of catalytic tRNA dimers. Both processes are based on a simple mechanism which uses the language of nucleic acids, the base pairing complementarity. We propose that the initial function of the anticodon and acceptor triplets was not related to codon-aminoacid assignment (coding) or to genetic translation. According to our model, the functions of these triplets would have been related to the accuracy of the truncated tRNA loading with trinucleotides complementary to their anticodon triplet by a catalytic tRNA dimer. Thus, correctly repairing the tRNAs with the codon–anticodon pairs in the acceptor stem allowed the synthesis of an RNA product that was an accurate copy of the template during RNA replication in the replisome. The anticodon sequence would have established the identity of the tRNA molecule by specifying the triplet sequence that was loaded onto the tRNA, and the complementary relationship between the two triplets would have allowed accurate replication of RNA molecules. This complementary relationship might have been essential for the establishment of an RNA-based genetic system.

Moreover, our model also means that addition of every trinucleotide to the growing RNA by the replisome would have required a previous process through which the specific trinucleotide complementary to the anticodon would have been added to the acceptor 3'-end of a truncated tRNA using the anticodon of an intact tRNA as a template. This first process would have been catalyzed by a tRNA dimer and would have involved two regions of the tRNA molecule: the anticodon and the acceptor stem. We suggest that the genetic system might have been based on the function and complementary properties of these two small tRNA molecule sequences.

Of note, current genetic translation also requires the pre-loading of specific amino acids onto the corresponding tRNA by a specific protein aminoacyl-tRNAsynthetase (aaRS). In this process the identity determinants (signals) in the tRNA that trigger specific aminoacylation are mainly located in the anticodon loop and acceptor stem, even though tRNA recognition by aaRSs does not necessarily depend on anticodon and acceptor stem recognition; in most cases it depends only on one, and sometimes it does not require either.

Darwinian evolution and the genetic code were not possible until an accurate replication mechanism had been established. Thus, the establishment of reproducible RNA sequences through the emergence of an accurate RNA replication mechanism would have been basic in the generation of a primitive genetic system.

Origin of the Genetic Translation and the Genetic Code in the RNA World

The question is how, in an RNA world with reproducible sequences, the genetic code might have first appeared? Various models have been proposed to explain the origin of genetic translation and the genetic code (Woese 1965; Crick 1968; Orgel 1968, 1989; Weiner and Maizels 1987; Gibson and Lamond 1990; Campbell 1991; Szathmary 1993, 1999; Schimmel et al. 1993; Schimmel and Henderson 1994; Di Giulio 1994; Gordon, 1995; Poole et al. 1998; Yarus 1998; Knight and Landweber 2000; Brosius 2001; Wolf and Koonin 2007; Rodin et al. 2009; Chatterjee and Yadav 2019). The complex translation machinery must have evolved in a stepwise process (Wolf and Koonin, 2007) or perhaps the translation apparatus appropriated some RNA replication elements such as tRNAs (Weiner and Maizels 1987) and the RNA replisome—the ancestor of current ribosomes (Campbell 1991; Gordon, 1995; Poole et al. 1998). In the evolutionary process the hypothesis that the code emerged before genetic translation was presented long ago (Szathmary 1993). Moreover, the finding that self-aminoacylating ribozymes have been easily selected in vitro makes this evolutionary scenario towards translation more plausible (Illangasekare and Yarus 1999; Chumachenko et al. 2009).

We suggest that the primitive $tRNA_{intact}$–$tRNA_{truncated}$ dimer (with limited polymerase activity, related with tRNA loading with trinucleotides) involved in the primitive RNA replication could have evolved stepwise into the modern aaRS-based tRNA-protein complex, being the catalytic $tRNA_{intact}$–$tRNA_{intact}$ dimer (with self-aminoacylation activity) an intermediate stage in this molecular evolution.

We propose that the passing (change) from the polymerase function (based in the formation of phosphodiester bonds, and related with tRNA loading with trinucleotides) of the catalytic $tRNA_{intact}$– $tRNA_{truncated}$ dimer to the self-aminoacylation function (based in the formation of an aminoacyl ester bond, and related with the tRNA loading with amino acids) of the catalytic $tRNA_{intact}$– $tRNA_{intact}$ dimer could have been facilitated by the functional-group diversity available in the anticodon loop for having modified bases. The notion that reactions required for aminoacylation of tRNA chemically resembles RNA polymerization (that is, the first aminoacyl-tRNA synthetase was a variant activity of the early –ancestral- RNA replicase) has been suggested long ago (Weiner and Maizels 1987).

Indeed, a model has been proposed for a primitive, aminoacyl-tRNA synthetase comprises tRNA-like dimers ($tRNA_{intact}$–$tRNA_{intact}$ dimer which functions as a self-aminoacylating ribozyme) between two intact tRNAs with identical anticodons (and one codon-anticodon pair at the acceptor stem) which interact through base pairing of the anticodon loop of one molecule with the complementary sequence at the 3'-acceptor stem of another (Martínez Giménez and Tabares Seisdedos 2002). The resulting 'groove' formed by this interface would have allowed recognition (through H-bonds) of specific amino acids—according to the anticodon sequence (Martínez Giménez and Tabares Seisdedos 2002)—, by a structure similar to a complex of four nucleotides (termed the C4N model) consisting of a discriminator base and an anticodon triplet (Shimizu 1982). The Corey–Pauling–Koltun molecular model was used to show that noncovalent four-nucleotide C4N complex had a lock-and-key relationship with the cognate amino acid (Shimizu 1982). We propose that a selective complex of C4N-like type, with even a weak but sufficient stereochemical affinity between amino acids and anticodon-like triplets, would have occurred at the zone of intermolecular interaction formed by complementary pairing between the anticodon loop and the acceptor stem of $tRNA_{intact}$–$tRNA_{intact}$ dimer; thus, all the specificity for amino acid binding was provided by the charging RNA code –acceptor- and the genetic

code –anticodon-. Moreover, proposed that the type of 3´-acceptor:anticodon-amino acid stereochemical interaction could have been a progenitor of the genetic code (Martínez Giménez and Tabares Seisdedos 2002). Moreover, have been proposed that the tRNA dimer could form a structure capable of binding an amino acid close to the 3´-end of one tRNA, positioning the activated amino acid (aminoacyl-thioesters) for nucleophilic attack by the 3´-OH of terminal ribose to yield an aminoacyl-tRNA, with one catalytic metal ion located in the anticodon (by forming the ribose nucleophile); that is, the tRNA dimer could catalyze their own specific aminoacylation (Martínez Giménez and Tabares Seisdedos 2002).

Some studies with RNA aptamers indicate that amino acid RNA binding sites predominantly comprise a certain triplet that corresponds to the current cognate codon assignment (Yarus 2005). The aptamers for 8 amino acids, with one exception (glutamine), were enriched for codon and/or anticodon triplets at a statistically highly significant level (Ellintong et al. 2000; Yarus 2005). Moreover, tRNA ribose 2'-OH groups still significantly contribute to tRNA-assisted present-day aminoacylation process in some tRNAs (Manijigi and Francklyn 2008). A study showed that one RNA molecule with a similar structure to C4N complex can be weakly charged with the cognate amino acid in presence of a dipeptide catalyst (Shimizu 1995).

Dissociation of the dimer would have occurred after aminoacylation of both tRNAs, producing two aminoacyl-tRNAs. Hence, we posit that the emergence of a catalytic repair process may have increased the quantities of intact tRNAs available, and the genetic translation (facilitated by the increased formation of $tRNA_{intact}$–$tRNA_{intact}$ dimers) would have been favored over the RNA replication. Additionally, the polymerization of free amino acids into proteins would have decreased the osmotic potential of the protocell, which, perhaps, exerted a selective advantage in the form of increased nutrient transport and cellular stabilization.

We propose that the primitive tRNA molecules could have possessed both the RNA pairing function (that is, one tRNA molecule with coding functions in the primitive ribosome) and the ability to catalyze its own aminoacylation (aaRS functions, as catalytic tRNA dimer). Some data experimentally support the notion that the modern functions of aaRS (aminoacyl-tRNA formation) and tRNA (anticodon-codon pairing) were initially combined in the same RNA molecule (Illangasekare and Yarus, 1999).

In eubacteria, a paralog of glutamyl-tRNA synthetase (YadB) that can aminoacylate the $tRNA^{Asp}$ on a modified nucleoside at the wobble position of anticodon loop (not on the 3´-OH group of the acceptor stem as in the canonical aminoacylation) have been reported (Salazar et al. 2004). This intriguing discovery lends support to the theoretical models that point out a key role of the anticodon loop in the origin of the genetic code. According to our model on the origin of the genetic code the stereochemical relationships between codons or anticodons and amino acids was achieved through the formation of a C4N-like (anticodon loop-3´acceptor intermolecular interaction) complex on the tRNA dimer with self-aminoacylation activity. Since the elements that make up the anticodon loop-3´acceptor interaction were involved in the formation of the dimer, then we propose that tRNA dimers could have played a crucial role in defining the organization of the genetic code. In an extended view of our model, we propose that another type of dimer could have formed between two tRNAs with the similar, but 1^a-base different, anticodons (and a codon-anticodon pair at the acceptor stem)(see Fig. 2). We posit that one $tRNA^{Asp}$ molecule and one $tRNA^{Glu}$ molecule could form a $tRNA^{Asp}$–$tRNA^{Glu}$ dimer, which interact through the base-pairing of the anticodon loop of one molecule with the acceptor stem of another molecule (see Fig. 2.b). Assuming that other intermolecular forces are involved in the dimer stabilization, a single base mismatch in the anticodon loop-3´acceptor (interaction) region may not be sufficient to change the dimer stability. Given that this dimer would have

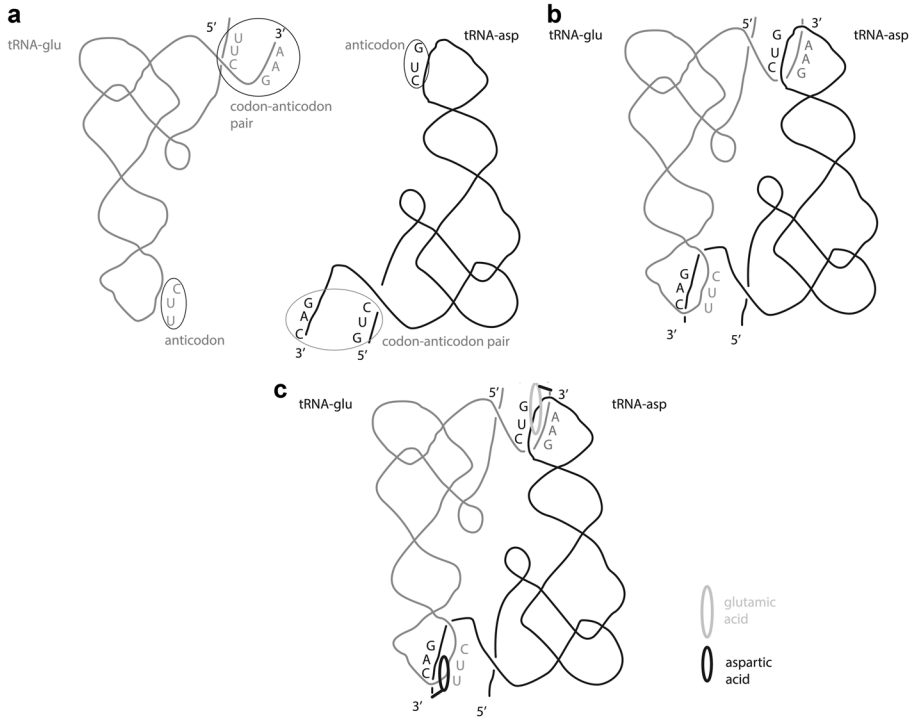

Fig. 2 The structure of primitive tRNAs is proposed to have been similar to current tRNAs. A structural model for a tRNA dimer between two tRNAs with similar, but 1[a]-base different, anticodons. **a** one $tRNA^{Asp}$ molecule and one $tRNA^{Glu}$ molecule, **b** the structural model proposed for the primitive catalytic $tRNA^{Asp}$–$tRNA^{Glu}$ dimer with self-aminoacylating function, **c** the primitive $tRNA^{Asp}$–$tRNA^{Glu}$ dimer charged with two related, "early" amino acids

possessed two similar, not identical, anticodon loop-3´acceptor regions then it is possible that it could have been able to bind two similar amino acids. We propose that the $tRNA^{Asp}$–$tRNA^{Glu}$ dimer could function as a self-aminoacylating ribozyme producing one glutamyl-$tRNA^{Glu}$ and one aspartyl-$tRNA^{Asp}$ (see Fig. 2b-c). The interactions between the anticodon loop-3´acceptor region and the glutamic acid could position said amino acid in the proximity of the terminal ribose at the ($tRNA^{Glu}$) 3'-acceptor stem. Glutamic acid in this position could react with the last ribose (in the 3' acceptor strand of $tRNA^{Glu}$), and also with the (wobble position) base of the ($tRNA^{Asp}$) anticodon; if well, it is more likely that it did so on ribose (in the 3´ acceptor end of $tRNA^{Glu}$) (see Fig. 2c). It is possible that this type of dimers would play a role in the addition of similar (related) amino acids during the code expansion. It has long been conjectured that the universal genetic code evolved from a simpler primitive code with fewer amino acids (Crick 1968). We posit that the $tRNA^{Asp}$–$tRNA^{Glu}$ dimer played a role the assignments of amino acids (Glu and Asp) to the codons in the 3[rd] column of the canonical table of code. Moreover, we suggest that this $tRNA^{Asp}$–$tRNA^{Glu}$ dimer could be the precursor of the current GluRS and AspRS enzymes and its paralogs. The discovery of new aaRS paralogs (similar to the YadB enzyme) could indicate the initial stages in the early evolution of the genetic code and the mechanisms involved in this process. Moreover, we suggest that a primitive $tRNA^{Gly}$–$tRNA^{Ser}$ dimer (which

could function as a self-aminoacylating ribozyme) could have played a role in the assignments of amino acids (Ser and Gly) to the codons in the 4th column of the canonical table of code.

The convergence in the results of various studies on the emergence and formation of amino acids suggests the 10 amino acids following (in order of abundance) can be confidently considered early; Gly, Ala, Asp, Glu, Val, Ser, Ile, Leu, Pro and Thr (Trifonov 2000; Zaia et al. 2008; Higgs and Pudritz 2009; Burton et al 2012; Koonin and Novozhilov 2017). These primordial amino acids could be incorporated in the code at an early stage. Various models suggest that genetic code started with the correspondence between the GNN codons and the five "early" amino acids then most abundant (Dillon 1973; Eigen et al. 1981; Hartman 1995; Brooks and Fresco 2003; Di Giulio 2008); that is, the primordial code used only these GNN codons. It has been suggested a rapid development of the code into a four-column code where all codons in the same column codes for the same amino acid; NUN = Val, NCN = Ala, NAN = Asp and/or Glu, and NGN = Gly (Higgs 2009). Other early structures of genetic code have been proposed (Di Giulio and Medugno 1999).

A few hypotheses have been presented to explain the selective advantages of the innovation of protein synthesis, but none are completely satisfactory. On the one part, this development would have bestowed ribozymes with simple structural functions (Orgel 1989), on the other, aminoacylation may have helped facilitate replication by fortifying the binding of the tRNA portion of the molecule to replicases (Poole et al. 1998). The identification of a 5-nt long RNA enzyme with a small active center that trans-aminoacylates a complementary 4-nt RNA supports the hypothesis that ribozymes with simple active structures participated in early forms of translation (Turk et al. 2010). Other studies have shown the generation of some in vitro selected ribozymes with activity related to genetic translation (Bessho et al. 2002; Saito et al. 2001).

Both trinucleotide and amino acid transfers involve ester transferase reactions (Campbell 1991). Moreover, the peptidyl transferase center of ribosomes can catalyze the formation of ester and polyester bonds (Fahnestock and Rich, 1971). Thus, this transition from replication to translation may have required only a minor structural alteration in the protoribosome. Moreover, tRNA ribose 2'-OH groups still significantly contribute to tRNA-assisted peptidyl transferase catalysis and the present-day aminoacylation process in some tRNAs (Weinger and Strobel 2006; Manijigi and Francklyn 2008). That is, the substrate (tRNA) still currently plays an important role in the catalysis of some genetic processes. This suggests that tRNA might have initially been the main player in translation, as anticipated by Woese (1980) and by Crick (1968) with the notion that primitive tRNA functioned as its own activating enzyme.

It is possible that the protoribosome could have initially catalyzed both replication and translation and we suggest the addition of a CCA trinucleotide to the 3'-terminal strand of intact tRNAs would have avoided the possibility of unwanted trinucleotide transfer reactions during the translation process.

Discussion

According to our model, a codon–anticodon-like interaction may have also been directly involved in other functions such as replication and genetic translation (coding). We postulate that codon–anticodon pairs were present on the acceptor stem (Rodin et al. 1996) and the complementary relationship between the anticodon and acceptor stem regions of the primitive tRNA molecule would have allowed the establishment of a primitive genetic tRNA loading mechanism: a basic requirement for the evolutionary development

of an RNA-based genetic system. In our view, this ancestral genetic tRNA loading mechanism might have been based on codon–anticodon-like interactions; these would have allowed the formation of catalytic tRNA dimers between two primitive tRNA molecules with the same anticodon and codon-anticodon pairs in their acceptor stems. In these proposed dimers, the anticodon loop of one molecule would have interacted by complementary base pairing with the 3'-acceptor stem of another identical molecule; that is, the codon-anticodon-like interaction.

We suggest that different genetic processes, such as replication or genetic translation, could have arisen from this initial tRNA loading mechanism which would only have required some minor structural variations using pre-existing structures. Thus, we suggest that the formation of tRNA dimers first catalyzed the restoration of triplets (which allowed RNA replication), which was later followed by the aminoacylation of tRNA molecules (which facilitated genetic translation). Thus, an evolutionary transition between two loading mechanisms (trinucleotides and amino acids) could have been possible (facilitated by possible presence of modified bases in the anticodon loop).

In our view on the RNA-based genetic system, the anticodon loop was able to read triplet codons and would have been also involved in the formation of the codon by successive addition of three nucleotides (guided by anticodon itself) at the acceptor 3´-end of one truncated tRNA molecule in a repair process catalyzed by $tRNA_{intact}$–$tRNA_{trucated}$ dimer. This codon (triplet) unit of genetic information would be deposited (added) in truncated tRNA molecules to be used later in RNA replication by RNA replisome.

Thus, it should be note the finding that the tRNAs marked with CCACCA by CCA-adding enzymes are targeted for degradation (Wilusz et al. 2011). It also seems that this addition is conserved across all three kingdoms of life. The authors suggest that enzyme adds CCACCA to tRNAs with unstable structures as universal mechanism for ensuring accurate protein synthesis (Wilusz et al. 2011).The addition of other triplet (-CCA) to CCA-end of current tRNAs could be a remnant of the original function of RNAs in replication.

We suggest that the quaternary assembly of smaller RNAs (as tRNAs) allowed the emergence of larger, more complex RNAs (as dimers) that could perform new functions by increasing the functional complexity of the system and maintaining the functions of the simpler RNAs. The complex RNAs would have formed more clefts and pockets than the smaller RNAs, some of which would have been binding pockets or catalytic centers. Importantly, the formation of these elaborate structures with specific cavities would have required only a few rudimentary RNA interactions. It is worth noting, however, that other possible mechanisms involved in the functional and structural evolution of tRNA have been proposed (Hayden and Lehman 2006; Manrrubia and Briones 2007; Schmidt, 1999). In our model, the complementarity between pre-codes would have increased the functional and structural complexity of RNA, allowing the formation of dimers.

Other works are interesting in support of the model. The basic chemistry needed for template-directed polymerization—in which the 3'-OH in a nucleotide or oligonucleotide5-triphosphate group phosphoanhydride bond is attacked—occurs within the group I intron of a natural ribozyme (Vincens and Cech 2009). The chemistry of polymerization has been discussed in a lot of detail in the academic literature, but one the most notable points is that dual metal-ion chemistry is leveraged for this reaction in both extant protein-based RNA and DNA polymerases and in vitro selected ribozyme RNA polymerases and ligases (Joyce 2004; Stahley and Strobel 2005; Strobel and Cochrane 2007).

Another study showed that UUU trinucleotide ribozymes are sufficient to promote ion-dependent cleavage of phosphodiester bond in trans between G and A bases of the GAAA

tetramer (Kazakov and Altman 1992). Moreover, the authors suggested that the main function of the UUU triplet is to act as a template catalyst (Orgel 1986) in complementary adenine base positioning to facilitate ion binding to these bases (Kazakov and Altman 1992), thus making RNA-based polymerase much more likely.

tRNAs and tRNA-like molecules play roles as templates or primers in a variety of replicative processes, including in RNA replication in bacteria and plantviruses, mitochondrial DNA, DNA plasmids, retroviruses, and modern chromosomal telomeres (Maizels and Weiner 2000; Negroni and Buc 2001). One feature common to most tRNAs (in all three Domains) is that the three 3'-end nucleotides are not encoded in the genome but rather, are added as part of the ongoing tRNA processing by nucleotidyltransferases without using a nucleic acid template.

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