Identification of the prebiotic translation apparatus within the contemporary ribosome

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Authors contributions
CD performed all stages of the biochemical experiments
IA developed the hypothesis and wrote the manuscript
AB analyzed the structures, designed and prepared the figures
AY designed the study, analyzed the results and co-wrote the manuscript
All authors discussed the results and commented on the manuscript

Summary

A structural element that could have existed independently in the prebiotic era was identified at the active site of the contemporary ribosome. It is suggested to have functioned as a proto-ribosome catalyzing peptide bond formation and non-coded elongation in the same manner that contemporary ribosomes exert positional catalysis, namely by accommodating the reactants in stereochemistry favourable for inline nucleophilic attack. This simple apparatus is a dimer of self-folding RNA units that could have assembled spontaneously into a symmetrical pocket-like structure, sufficiently efficient to be preserved throughout evolution as the active site of modern ribosomes, thus presenting a conceivable starting point for translation.

Here we discuss the proto-ribosome emergence hypothesis and show that the tendency for dimerization, a prerequisite for obtaining the catalytic centre, is linked to the fold of its

two components, indicating functional selection at the molecular level in the prebiotic era and supporting the existence of dimeric proto-ribosome.

Evolution of translation is a key issue in understanding the emergence of life. Contemporary translation is a complex process by which the genetic information is converted into proteins. This process takes place at the ribosome, a universal ribonucleoprotein catalytic machine, composed of two subunits. The ribosome's central task, peptide bond formation, occurs in the peptidyl transferase centre (PTC), the active site of the large subunit, located in the large subunit and composed of RNA. In the contemporary ribosome the PTC is located in the midst of a symmetrical region[1-3] (Fig.1a) that contains the A- (amino acylated) and P (peptidyl) loops that accommodate the 3'ends of the two A- and P-site tRNA molecules. This internal symmetry provides the framework for the primary catalysis that the ribosome exerts on its substrates, namely their positioning in a stereochemistry favourable for peptide bond formation[1-8]. The symmetry relates two RNA structural elements, called A and P sub-regions[1,3], having matching three dimensional RNA backbone folds and nucleotide conformations but unrelated sequences. This symmetrical region was identified in all known ribosomal structures[9-13] and its nucleotide sequences the secondary structures are highly conserved throughout kingdoms and organelles, indicating its vital function.

The assumption underlying this study is that the current ribosome evolved gradually from a far simpler primordial proto-ribosome, which could catalyze non-coded peptide bond formation and simple elongation. This hypothesis is based on the identification of a region within the contemporary ribosome, which is suggested here to be the remnant of a proto-ribosome. This hypothesis presents an advancement on our previous proposition that the ancient ribosome was built as an RNA dimer resembling the modern ribosome symmetrical region, which evolved by gene duplication or gene fusion[3,14], which was recently extended by comprehensive structural analysis[15].

Here we identify the proto-ribosome as an internal segment of the contemporary ribosome and discuss the functional, structural and thermodynamic aspects of its emergence as a self dimerized RNA unit. In support of our dimeric proto-ribosome hypothesis we present

chemical results correlating specific sequences and sizes with the tendency of RNA oligomers to form structures resembling the ribosomal active site.

Extraction of the suggested proto-ribosome

The proto-ribosome was primarily an apparatus catalyzing peptide bond formation. In the contemporary ribosome this activity takes place in the RNA domain V (Fig 1a) by the PTC, located within the symmetrical region encircling it from the non-symmetrical extensions, radiating from it. These extensions interact with peripheral ribosomal components, elongation factors and the modern large tRNA substrates, but not directly with the peptide bond formation site[3], therefore are functional only in the context of the later evolved versions of the modern ribosome. The redundancy of the non-symmetrical extensions in the context of the proto-ribosome is further strengthened by the absence of the majority of these extensions in mitochondrial ribosomes[17] or by their replacement by r-proteins[18], supporting the assumption of ribosomal rudimentary function without them.

It is conceivable that in the initial stage in the evolution of the proto-ribosome, it accommodated minimal substrates, such as single amino acids, amino acid-nucleotide or amino acid-oligonucleotide conjugates. In the modern ribosome, the amino acid carriers, i.e. the 3´ends of A, P-tRNAs, are accommodated in a fashion allowing for efficient peptide bond formation, dictated mainly by the tRNA remote interactions that allow for base pairing with the universally conserved nucleotides G2553 and G2251 (*E.coli* numbering throughout) in the A- and P-loops[2], respectively. These symmetrically positioned nucleotides are located in the PTC about 22Å apart, therefore are useful only for accommodating only larger substrates, namely amino acid-oligonucleotide conjugates and tRNAs. In general, peptidyl transferase activity does not involve structural elements concerned solely with modern ribosomal functions. Consequently, the A, P-sites and the non-symmetrical extensions can be eliminated from the primal apparatus (Fig. 1b). This extraction yields a ‘pocket-like’ entity (Fig. 2a,b) possessing an approximate 2-fold symmetry and containing the ribosomal nucleotides adjacent to the reacting amino acids in the modern ribosome.

This ‘pocket-like’ structure is suggested here to be the remnant of the proto-ribosome. It is composed of two highly conserved (Fig. 1a) L-shaped RNA elements, similar to each other

in fold but differing in sequence, named hereafter the A- and P- ribosomal core units (Fig. 1b,2c), and containing ~60 nucleotides, folded into a stem-elbow-stem (SES) form. The A-core unit is composed of helices H90 and H93 whereas the P-core unit- is composed of H74 and most of H89, with elbow regions created *via* a network of hydrogen bonds involving nucleotides from the Central loop of domain V.

Based on the structure of the symmetric pocket in the contemporary ribosome, we hypothesize that the proto-ribosome was self-assembled dimer, obtained from two core RNA units of similar size, each having the SES fold. In the prebiotic world SES folds could have been obtained by spontaneous folding of randomly sequenced RNA chains of about 60-70 nucleotides. The dimers that established the proto-ribosome pockets could accommodate single amino acids, and/or other small substrates suitable to form peptide bonds, like amino acid nucleotide conjugates or short peptidyl chains obtained during previous cycles of the apparatus function. Peptide bonds could be formed when the two reactants were accommodated, each attached to one core unit within the dimeric pocket, in a fashion resembling their positioning on the modern ribosome, namely at a favourable distance and mutual orientation.

Following the formation of a peptide bond, the newly obtained dipeptide could have either remained attached to one of the proto-ribosomal sites or could have dissociated and later re-accommodated on the apparatus. This enabled the positioning of a new reactant in the vacant site, followed by the formation of a subsequent peptide bond, thus adding another amino acid to the peptidyl chain. Reiterations of this process would have resulted in a simple mechanism for non-coded elongation, equivalent to the elongation mechanism proposed elsewhere[19].

Substantiation of the hypothesis

The hypothesis that the proto-ribosome, which is still embedded in the core of the modern ribosome large subunit, was a self-assembled, dimeric RNA enzyme[3,14] was recently further investigated by addressing key issues concerning spontaneous folding and dimerization prospect, as well as its ability to accommodate substrates. Sequence dependent predictions of the secondary structures of the A- and P-core units, as found in all known structures, based on free energy minimization[20] consistently generated SES secondary structure with free

energy in the range of ΔG=-26 $\pm$ 3 kcal/mol. In all but one case unique solutions were found, indicating low likelihood of obtaining alternative secondary structures. Particularly, in all cases the sequence of helix H93 was predicted to fold into a secondary structure identical to that found within the contemporary ribosome. Helix H74 and the single strand of the Central loop of domain V could have been made to fold correctly by constraining few nucleotides into specific interactions. In contrast, helices H89 and H90, which contain a complex network of uncoupled bulged nucleotides (Fig. 1b), did fold into a form of a helix, but the predicted scheme for the uncoupled nucleotides differed from the ribosomal secondary structure even when constraints were applied.

Overall, the predictions of the constrained folds, aimed at reproducing secondary structures closer to those found within the ribosome, were less stable by 3.1 kcal/mol on average compared to the non-constrained folds. This may suggest that the current active site structure, which contains a large number of nucleotides involved in the modern peptidyl transferase activity, adapted to its advanced functions through mutating the original proto-ribosome, at the expense of increasing the free energy. Secondary structure predictions indicate that folding of such a 60-70 oligomers into SES fold is likely to be a thermodynamically 'downhill' process, suggesting that folding of an oligonucleotide into the molecules comprising the ribosome core could have occurred spontaneously. Furthermore, as self-folded RNAs form stable molecules *in vitro* across a large range of conditions[21], the ancestor of the proto-ribosome could have been a stable construct at the prebiotic era.

Further support for prebiotic existence of core units can be obtained by relating it to another known SES molecule, the tRNA, which is widely believed to be a relic from the prebiotic world[22,23]. In particular, if one tRNA helix is overlapped with one of the helices of the core unit, their remaining helices have similar arrangement in space, although they point to opposite directions (Fig. 2c). The two forms of SES molecules could have existed, side by side, in the prebiotic environment and were then recruited for their separate roles in translation. The later recruitment of existing prebiotic molecules towards evolving tasks is supported by the variety of roles tRNA-like molecules perform in replication[23]. Within the ribosome, the association of the two core units is partially stabilized by a highly conserved GNRA interaction motif[24] between the stem loop of helix 93 and a receptor area on H74, including an A-minor tertiary interactions between A2598 of the H93 stem loop and G2436:C2073 of H74. The association of the two core units to form the proto-ribosome,

accords with the suggestion[15] that A-minor interactions were used by evolution to add new RNA elements to the proto-ribosome.

Dimerization tendency of similar size RNA molecules having similar structural motifs has been observed, some of which were shown to form stable dimers spontaneously[25-28]. In several cases symmetrical self-dimerization of two RNA molecules, obtained via GNRA tertiary interactions, including an A-minor motif, was found to contribute significantly to the stability of these molecules[26,29]. This suggests that self-assembly of the proto-ribosome core units into dimers is a favourable process that could have occurred spontaneously in a symmetrical manner in a primordial world. This spontaneous dimerization that formed a 'pocket-like' structure could have occurred either between identical duplicates or between two SES units, similar in fold but having a different sequence, leading later on to RNA gene duplication or gene fusion, respectively. Divalent metal ions could have been essential for maintaining the folds of the SES units as well as their dimerization product, as observed for other RNA molecules[29-33].

Experimental results indicate that some, albeit not all, short RNA units with sequences resembling those observed in current ribosome, are capable of forming dimers that may have a 'pocket-like' structure under mild conditions. Gel electrophoresis, performed on various constructs, such as P1 and A1 (Figures 3 and S1), under non-denaturing conditions (Figure S2) showed a correlation between the tendency to dimerize and the sequence of the RNA unit as well as the presence of Mg^{++} ions. Among the pair A1 and P1, slow migrating band was detected only for P1 (Figure S2), indicating its dimerization, consistent with results of Electrophoresis Mobility Shift Assay (EMSA), which showed dependency of the intensity of the slow migrating band and the RNA concentration (Figure S3).

The involvement of A-minor interactions in the stabilization of the proto-ribosome dimer was demonstrated by obtaining a significantly higher dimer concentration (Fifure S2) when using a modified P1 construct (called P1c), obtained by the incorporation of a GNRA tetra loop instead of the CTTCGG loop in H89 at P1 (Figure S1). Further modification of P1c, namely the mutation G53U in which the third nucleotide of the GNRA motif is replaced, did not yield dimers (Figure S4). This result emphasizes the crucial contribution of the GNRA tetraloop to dimer formation, implies the existence of structural selection and is consistent with functional selection.

Indications for a compact 'pocket-like' structure formation *via* loop-receptor interactions rather than *via* loop-loop interactions, were obtained by a series of deletion/insertion of a base-pair (Figures 3 and S1), designed to slightly shorten or extend helix H89 in P1. The findings that the longer and the shorter constructs did not dimerize (Figure S4) imply that the P1 and P1c dimers are not made of randomly interacting RNA units, but have specific structures that can have the 'pocket-like' motif. Further functional experiments, exploring the peptidyl transferase (PT) activity of a large variety of the RNA dimers are in accord with the structural analysis (Davidovich et al., to be published).

Independently of its exact nature, the proto-ribosome should have positioned the amino acids in a spatial arrangement similar to the modern one, allowing the atoms participating in the nucleophilic attack to interact closely and effectively for inline attack. The proto-ribosome should have also exhibited non-specific affinity towards amino acids, regardless of the type of amino acid carrier, allowing for the largest variety of amino acids to utilize equally the ancient translation apparatus. Both requirements are fulfilled if one assumes that the ancient substrates were accommodated on the proto-ribosome analogously to their positioning in modern ribosomes. Moreover, such positioning facilitates envision a continuous evolutionary path from the proto-ribosome towards the modern machine. The contemporary substrates are primarily positioned in the PTC *via* the interactions of the modern tRNA acceptor stem with the components of the cavity leading to the PTC that facilitate base pairing between C74, C75 of the tRNA 3´ends, and PTC nucleotides. Such accommodation mode could not have been used by the smaller reagents, namely single amino acids or amino-nucleotide conjugates. Nevertheless, these substrates suit the characteristics of amino acid-RNA non-covalent bonding[34] occurring mostly at the interface between single stranded RNA segments and amino acids, and are sufficiently weak to be readily reversible. This basic property would have facilitated the release of the di- or oligo-peptides from the active site while concurrently allowing the accommodation of new reactants, thus enabling a dynamic process, which is a basic requirement for elongation.

In short, careful investigations of the basic assumption of the proto-ribosome hypothesis presented here confirmed the possible existence of an ancient dimer of a 'pocket-like' structure, similar to the modern ribosomal active site. We suggest that such 'pocket-like' templates were involved in providing positional catalysis for peptide bond formation and for

non-coded elongation. Programmed translation evolved later, in correlation with larger ribosomes, more complex substrates and the involvement of non-ribosomal factors.

Discussion

A crucial step in the emergence of life was the appearance of the proto-ribosome, an event that marks the transition into the present era in which proteins and nucleic acids together form life. A main drawback in the usual way these questions are tackled originates from the current lack of knowledge concerning the conditions during the prebiotic period. This leads to multiple hypotheses, each based on different axioms, mostly not experimentally refutable. The novelty of the present research stems from its factual starting point, *i.e.* from the known structure of the contemporary ribosomal rRNA. As all living cells have a common mechanism for protein synthesis, it is conceivable that the emergence of this simple translation system preceded life as we know it. By analyzing the currently available structures and focusing on their conserved regions, we are attempting to look into the origin of peptide bond formation. This study is based on our assumption that the proto-ribosome was a simple apparatus with the ability to catalyze peptide bond formation and to allow simple elongation, and that this apparatus could have materialized spontaneously under prebiotic conditions. The system presented herein, a self-assembled dimer of SES RNA units that is embedded within the ribosome active site, is in remarkable correlation with a plausible assumptions expressed previously, namely: "the essence of the primitive apparatus remains at the heart of modern translation" and that "there is little doubt that translation began with simple tRNA-like entities..."[35].

The evolution of the suggested proto-ribosome could have advanced by the reproduction of the most efficient constructs, which were likely to be found among the dimeric organizations that were formed initially and could function as proto-ribosomes[3,14]. Efficient reproduction could have been performed by the RNA itself since RNA can act as an enzyme as well as its own template for replication[36], either by gene duplication, if the sequence of two RNA units forming the dimer were the same, or by gene fusion, in case RNA units with different sequences formed the initial dimers. Although the proto-ribosome can act as a ribozyme, on its own it provides only a modest level of activity, mainly owing to its seemingly limited

structural variability and relatively low stability. Additionally, because of their size the initial dimers could accommodate only small reactants, therefore offered rather loose substrate accommodation.

A substantial increase in the catalytic rate could have been generated by peripheral RNA elements, as observed for other ribozymes[37]. The following stage in the ribosome evolution could involve the addition of the A-site and P-site, which could have extended from the far ends of helices H74 and H90 for P-, A-core units, respectively (Fig. 1a,b). Such larger proto-ribosome could have accommodated larger substrates through base pairing, achieving enhanced stability of the reactants positioning, thus allowing a better functioning non-coding translation apparatus. Further addition of RNA structural elements and mutational variations in the proto-ribosome, adapting it to the advanced functions, could have ultimately given rise to the formation of structural pockets (e.g. A-minor) suitable for the incorporation of additional RNA elements as well as of polypeptides or small proteins, which by then could be made by the advanced proto-ribosome. The addition of the new structural elements contributed to the stability and functional efficiency of the growing proto-ribosome eventually leading to the current translation mechanism. The emergence of the genetic code followed once proteins started to fulfil functional tasks that could not be achieved by RNA machines. However, the transition from non-coded into coded translation is still incomprehensible.

Efforts to identify a precursor of the contemporary ribosome have been endeavoured elsewhere by several approaches, such as comparative sequence analysis[38] and investigating the active site neighbourhood, which led to an attempt to synthesize a minimal rRNA, based on the domain V sequence, which can catalyze peptide bond formation. Additionally, RNA constructs exposed to *in vitro* selection, were tested for substrates accommodation and peptide bond formation but none was PT active[39]. A continuous patch of about 200 nucleotides, including most of the modern active site, was identified as a self-folding entity. The unit, however, contains only the A-site, while the P-site is absent, raising issues concerning the substrates that can be accommodated in it[40]. Recently, based on the role of A-minor interactions, a region containing the entire symmetrical region combined with the non-symmetrical part of H75 was implicated as a possible nucleolus for assembling the contemporary ribosome[15]. Apart from the non-symmetric part of H75, this system accords with the second stage in the evolution of the proto-ribosome presented here.

The current hypothesis offers a considerable explanatory power. It provides a simple and feasible origin for the complex and extremely important genetic code translation. The starting point for ribosome evolution described here, and the path it took from a small passive template to the current complex molecular machine assembled around it, enables to envision a continuous path from the primordial world to contemporary life, while respecting the basic survival and reproduction rules.

Conclusions

Only two prerequisites are sufficient for showing that prebiotic formation of a proto-ribosome could be conceived, namely the primordial existence of minimal substrates, and of oligoribonucleotides of moderate size (60-70 nucleotides), combined with the application of self-assembly of small RNA units. This dimeric primitive apparatus can be directly linked to the contemporary ribosome, as it was identified in its heart. Experimental results, obtained by using contemporary RNA sequences, show that such RNA units can form dimers under mild conditions and indicate correlation between the RNA structural properties and their tendency to dimerize into defined structures, suggesting the existence of selection rules at the molecular level in the prebiotic era. Overall, the proto-ribosome proposed here could offer a fitted template for favourable positioning of substrates and facilitate peptide bond formation and simple elongation, thus acting as an all-in-one apparatus for non-coded prebiotic protein synthesis.

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Figure legends

Figure 1:

a. Secondary structure of 23S 3´ half from *E. coli* showing phylogenetic conservation in three domains and two organelles[17].

Nucleotides marked by capital letters are more then 98% conserved. Domain V is shown on grey background. The A- and P- sub-regions of the symmetrical region (the symmetry is not portrayed by this 2D scheme drawing) are marked in blue and green, respectively (same colour scheme maintained in all figures). The non-symmetrical extensions are marked in yellow.

b. **2D scheme of the symmetrical region from the structure of *Deinococcus radiodurans*, D50S, drawn in a manner portraying the 3D symmetry**. The suggested proto-ribosome, constructed from the two symmetry related ribosomal core units, each composed of about 60-70 nucleotides forming two helices connected via a single stranded region, is shown on blue and green background.

Figure 2: The SES structure of the ribosome core and its similarity to the tRNA fold.

In all, the A- and P- components of the proto-ribosome as well as the sub-regions of the symmetrical region are shown in blue and green, respectively.

a. The suggested remnant of the proto-ribosome within the ribosomal large subunit from *D. radiodurans* shown as a grey body (PDB code 1NJP).

b. Superposition of the pocket suggested to represent the remnant of the proto-ribosome in *D. Radiodurans* and *Haloarcula marismortui*.. The eubacterial ribosome is shown in dark blue and green, using the structure of D50S (PDB code 1NJP) and the archaeal ribosome is of *H. marismortui* (PDB code 1VQN), shown in cyan and olive green. A model for an amino acid, obtained by cutting out (computational) from the crystal structure of complexes of the above ribosomal particles with substrates mimicking the tip of the tRNA 3'end, is positioned within the pocket. The P-site amino acid in the D50S structure was derived from the A-site amino acid by applying the rotatory motion [1,3].

c. Overlap of the A- and P- core units from the structure of D50S (PDB code 1NKW), obtained by a rotation of 178.6^0 around the symmetry axis, as in[3]. The projection direction is

perpendicular to that shown in Fig. 2b. The symmetry relating the A-, P- core units is reduced at the elbow regions[3], as these form the entrance to the tunnel, whereas the symmetry is needed for positioning the reactants. The anticodon helix of a tRNA molecule (in gold) is overlapped on helices H89, H93.

Figure 3: an example of sequences used for a minimal dimeric construct, and their 2D fold within the contemporary eubacterial ribosome.
RNA units A1 and P1, shown in panels a and b, respectively, are of the eubacteria *D. radiodurans* (and *Thermus thermophilus)*. Nucleotides that were added for pasting the truncated stems are shown in green. A->G "mutation" performed for facilitating transcription is shown in cyan. The region that was modified forming construct P1c (Figure S1) is circled in red.

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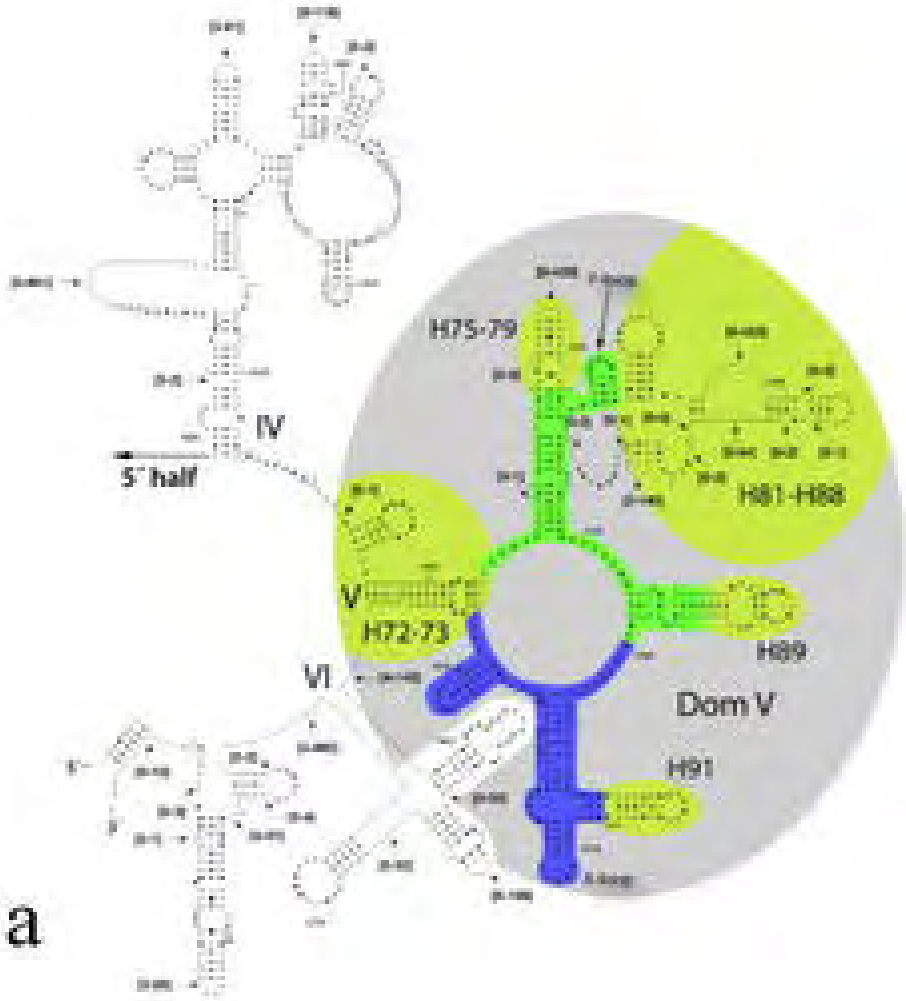
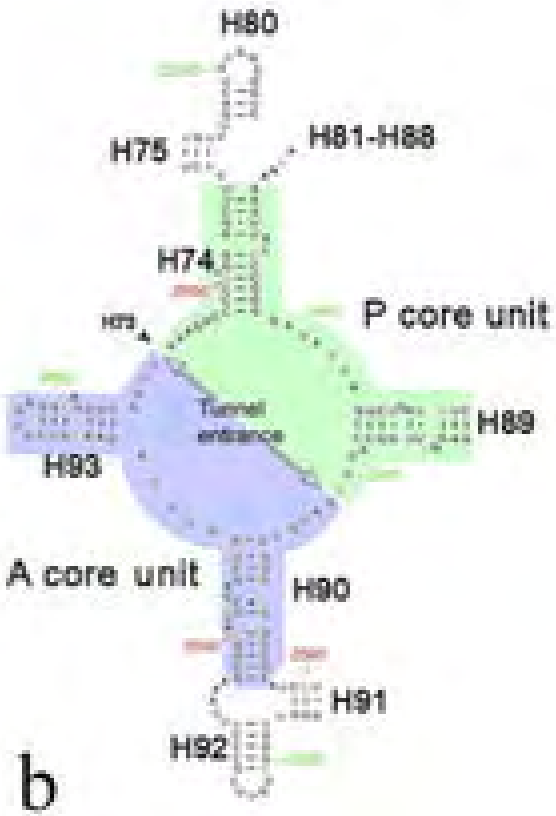
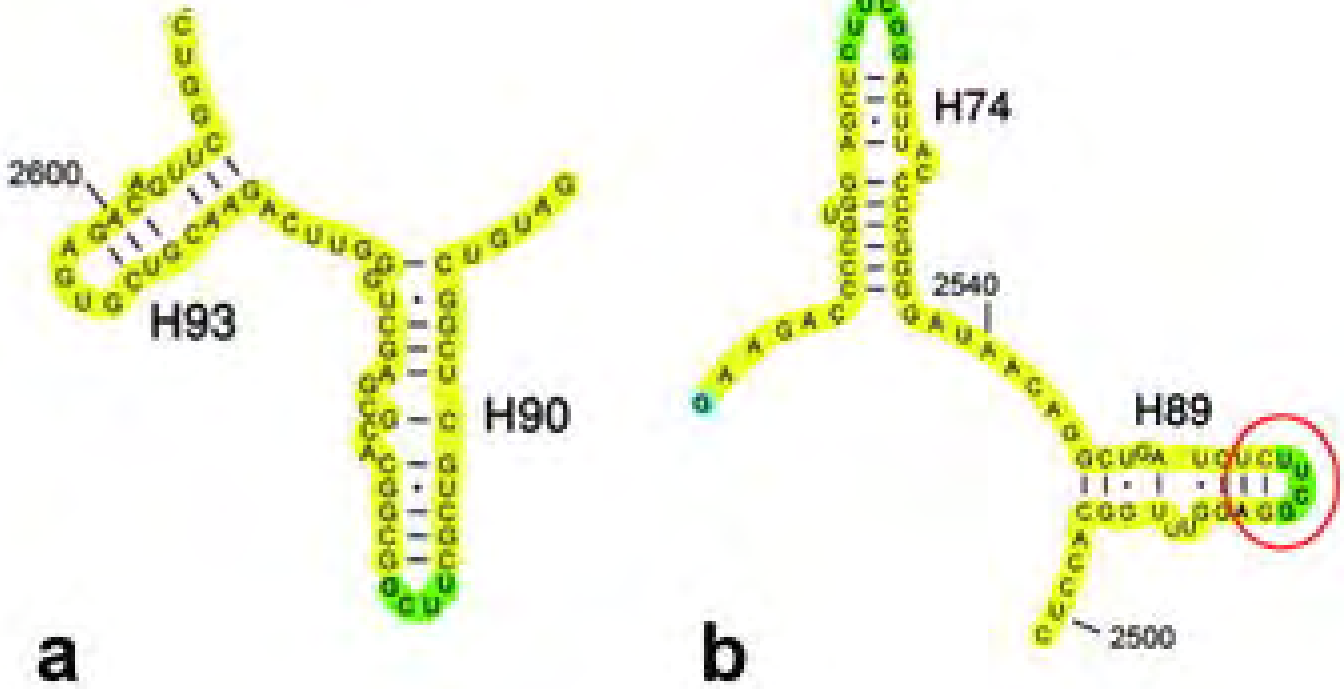

IV
5´ half
H75-79
H81-H88
V
H72-73
H89
VI
Dom V
H91
a


H80
H75
H81-H88
H74
P core unit
H89
Tunnel entrance
H93
A core unit
H90
H91
H92
b


a
b
tRNA 3'end
tRNA acceptor stem
tRNA
anticodon
H89, H93
H74, H90
c


2600
H93
H90
a
H74
2540
H89
2500
b