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REVIEW

The Peptidyl Transferase Center: a Window to the Past

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SUMMARY In his 2001 article, "Translation: in retrospect and prospect," the late Carl Woese made a prescient observation that there was a need for the then-current view of translation to be "reformulated to become an all-embracing perspective about which 21st century Biology can develop" (RNA 7:1055–1067, 2001, https://doi.org/10.1017/s1355838201010615). The quest to decipher the origins of life and the road to the genetic code are both inextricably linked with the history of the ribosome. After over 60 years of research, significant progress in our understanding of how ribosomes work has been made. Particularly attractive is a model in which the ribosome may facilitate an ~180° rotation of the CCA end of the tRNA from the A-site to the P-site while the acceptor stem of the tRNA would then undergo a translation from the A-site to the P-site. However, the central question of how the ribosome originated remains unresolved. Along the path from a primitive RNA world or an RNA-peptide world to a proto-ribosome world, the advent of the peptidyl transferase activity would have been a seminal event. This functionality is now housed within a local region of the large-subunit (LSU) rRNA, namely, the peptidyl transferase center (PTC). The PTC is responsible for peptide bond formation during protein synthesis and is usually considered to be the oldest part of the modern ribosome. What is frequently overlooked is that by examining the origins of the PTC itself, one is likely going back even further in time. In this regard, it has been proposed that the modern PTC originated from the association of two smaller RNAs that were once independent and now comprise a pseudosymmetric region in the modern PTC. Could such an association have survived? Recent studies have shown that the extant PTC is largely depleted of ribosomal protein interactions. It is other elements like metallic ion coordination and nonstandard base/base interactions that would have had to stabilize the association of RNAs. Here, we present a detailed review of the literature focused on the nature of the extant PTC and its proposed ancestor, the proto-ribosome.

KEYWORDS PTC, peptidyl transferase center, accretion model, evolutionary history, protein synthesis, ribosomes, translation

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INTRODUCTION

The ribosome has a unique place in efforts to understand the origin and early evolution of life (1). This reflects the fact that its history predates the last universal common ancestor (LUCA) and may "have evolved in an RNA world before the evolution of the genetic code and proteins" (2). The ribosome houses an activity center that catalyzes peptidyl transfer during protein synthesis. The general location where this activity is located is referred to as the peptidyl transferase center (PTC). The present review will focus on recent developments in our understanding of the evolution of the PTC, which is where peptide bonds are formed without the direct involvement of proteins.

The modern ribosome is a complex molecular machine that is responsible for coded protein synthesis. It is comprised of a small subunit (SSU), which contains the decoding center, and the large subunit (LSU) that contains the PTC (3), which in turn is responsible for peptide bond synthesis (4–8). The subunits are ribonucleoprotein complexes comprised of rRNA and ribosomal proteins (rProteins) (9). Ribosome function is directly facilitated by tRNAs and proteins such as elongation factors and initiation factors and indirectly facilitated by proteins such as the aminoacyl-tRNA synthetases that attach amino acids to the tRNAs. Since many of the core components are universally found, a functional ribosome is considered to be an important component of LUCA (10–16). Secondary structures deduced by comparative sequence analysis initially revealed that the LSU rRNA has six domains (17, 18). An additional domain referred to as domain 0 was recognized later (19). Atomic resolution studies show that domain 5 houses the PTC region (Fig. 1) (6, 20, 21), which includes an RNA pore (22, 23). The pore serves as the entrance to the exit tunnel through which a growing peptide leaves the ribosome.

THE PEPTIDYL TRANSFERASE CENTER: LOCATION AND COMPOSITION

PTC is a somewhat vague term that refers to the region of the ribosome formed by approximately 180 nucleotide residues of the LSU rRNA. Of these, at least 18 noncontiguous residues (24) facilitate the PTC activity. Collectively, these residues and their nearest neighbors are the most conserved and therefore likely the oldest sections of the LSU rRNA (25) (Fig. 1). Together they form a molecular platform for the ideal relative positioning of the reaction substrates (26), ensuring the efficient accommodation of the aminoacyl-tRNA 3′ end into the 50S A-site (27–29) and thereby enabling the transpeptidation activity (30–33). It is noteworthy that the structures of the PTC in the 50S subunit and in intact ribosomes are very similar (34, 35).

MUTATIONAL STUDIES AND RIBOSOMAL MUTATIONAL FLEXIBILITY

The PTC region is positioned to accommodate the acceptor ends of the tRNAs (36) (Fig. 2). This may in fact be far more vital than the nature of the conserved residues themselves, as demonstrated by mutation experiments. For example, individual mutations at positions C2063, G2447, A2450, A2451, A2453, and U2585 and the combination of A2451U and A2602G did not eliminate the transpeptidation activity (26, 37–45). Positions A2451, U2506, U2585, and A2602 were defined as forming part of an "inner shell" of the PTC. Mutations at these positions affected the peptide release significantly but did not equally affect the PTC activity (44). Three of these residues, namely, A2451, U2506, and U2585, along with G2252, are important for the binding of peptidyl-tRNA to the P-site of the large ribosomal subunit (38). Likewise, A2572 is in the immediate neighborhood of the aminoacyl-tRNA in the A-site (46) and is thought to be a sensor of conformational changes in the PTC (47, 48). It was shown to be important for PTC activity (49). The next adjacent base, C2573 (in collaboration with U2492 and C2556), facilitates the placement of the aminoacyl-tRNA at the A-site, tentatively labeled the "accommodation gate" (50–52). Mutations at A2572 and C2573 affect the peptide release but not the PTC activity (52). Several highly conserved residues are particularly important for their roles in positioning the tRNA at the PTC center. G2251 is essential for positioning the P-site tRNA during peptidyl transfer, and its mutation severely affects the PTC activity (53). Mutations of G2252, which base pairs with C74 of the P-site tRNA (54), can severely impact peptide release while not affecting the PTC activity itself (55). G2553

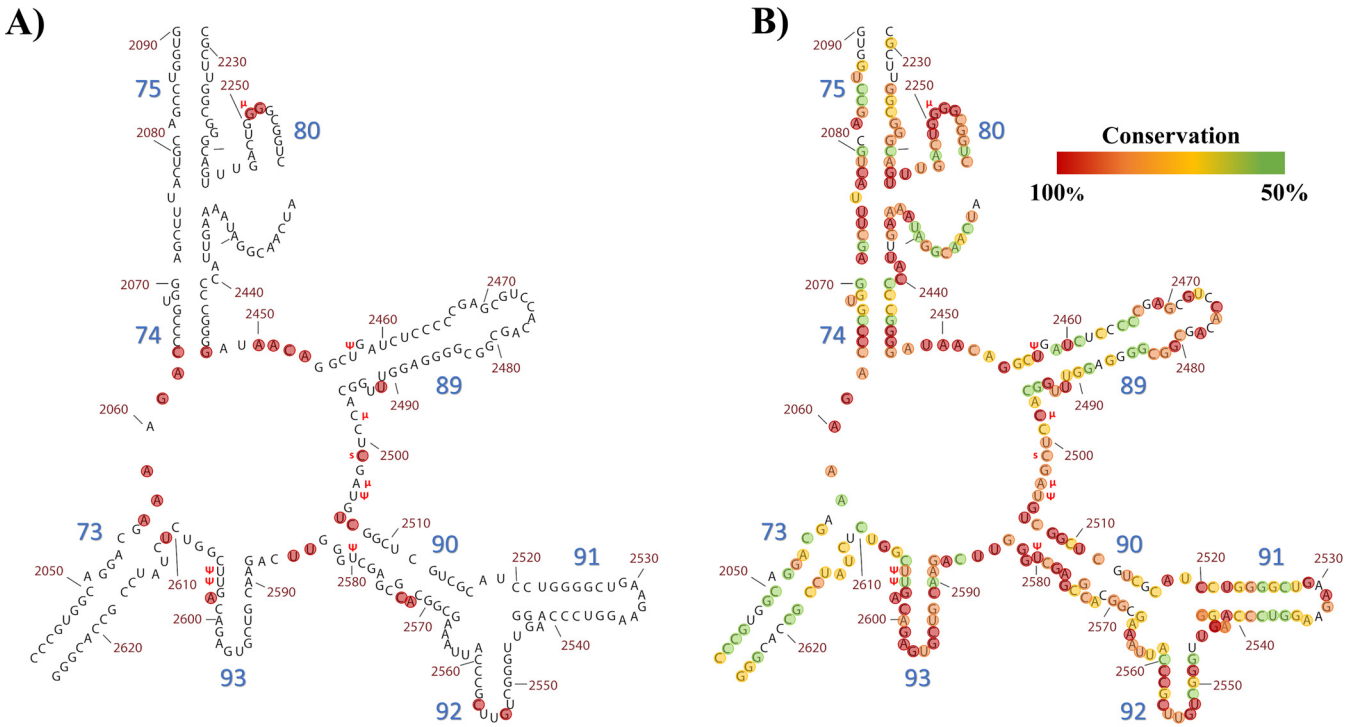

FIG 1 Fragment from the complete secondary structure of the LSU of *Thermus thermophilus* derived from crystallographic data (19), which contains the nucleotides within and around the PTC. Modified bases described in the text are marked with a red symbol in front of the corresponding base, "Ψ" for pseudouridine, "μ" for methylation, and "s" for 2-thiocytidine. Helix numbers are in large blue font, while position numbers are in smaller red font. (A) PTC residues mentioned throughout the text with a previously described functional role are highlighted within red circles. Details on functional role and/or implication for the ribosome activity are summarized in Table 1. As can be seen, PTC functional residues are not represented by a single contiguous chain of residues; instead, the PTC is formed from a diverse collection of distant residues that, when the RNA is folded into their tridimensional shape, act together as a catalytic unit, the PTC. (B) Conservation of the nucleobases within this fragment according to crystallographic data and corresponding alignments from Bernier et al. (275). This fragment comprises 282 nucleotides (nt). Red circles highlight those with 100% conservation (78 nt). Orange circles highlight those with 90 to 99.9% conservation (68 nt). Yellow and green circles highlight those with 70 to 89.9% and 50 to 69.9% conservation, respectively (52 and 49 nt). Thirty-five nucleotides from this fragment have shown less than 50% conservation.

makes base-pairing interactions with C75 of the A-site tRNA (56), and its mutation reduces the PTC activity (57).

In addition, there are nine modified residues of interest in the PTC. However, these residues are not universally conserved at their respective locations. Six pseudouridine (Ψ) residues are found around the PTC of bacterial and eukaryotic ribosomes, with roles in A-site tRNA positioning, PTC activity, translocation, initiation, termination, and tunnel composition (6, 58, 59) (Fig. 1). In this context, only 2 of these 9 modified residues are found among the 41 modified residues in the cytoplasmic LSU rRNA of *Euglena gracilis* (60). Furthermore, mutational studies of Ψ2504, Ψ2580, Ψ2604, and Ψ2605 (A-site) and Ψ2457 (P-site) suggest that they are not necessary for the overall activity (61). Other modified nucleotides in the PTC (Fig. 1) are reported as having some function. Methylated guanosine (G2251) is useful for positioning the P-site tRNA (53), while 2-methyladenosine (A2503) and Ψ2504 are integral parts of the tunnel entrance near the A-site (62). 2′-O-methylated C2498 is thought to help stabilize the PTC loop (62, 63). Finally, 2-thiocytidine (C2501) was found to be the only partially modified base in the PTC (64). Overall, of all the posttranscriptional modifications on RNAs, N^6-methyladenosine (m^6A) is shared by all the RNA types (namely, mRNA, rRNA, tRNA, and snRNA [small nucleolar RNA]) (65). The PTC is enriched in modified residues in most cases, and yet the variation in the type of modified residues is clearly evident. Although these modifications are useful in extant ribosomes, they are likely the outcome of evolutionary improvement. None of them seem to be essential for peptide bond formation.

In a more recent study (66), a detailed mutational analysis employing a single nucleotide mutation(s) within the PTC region was performed. More than 85% of the PTC nucleotides were found to be mutationally flexible. The residues classified as the least mutationally flexible,

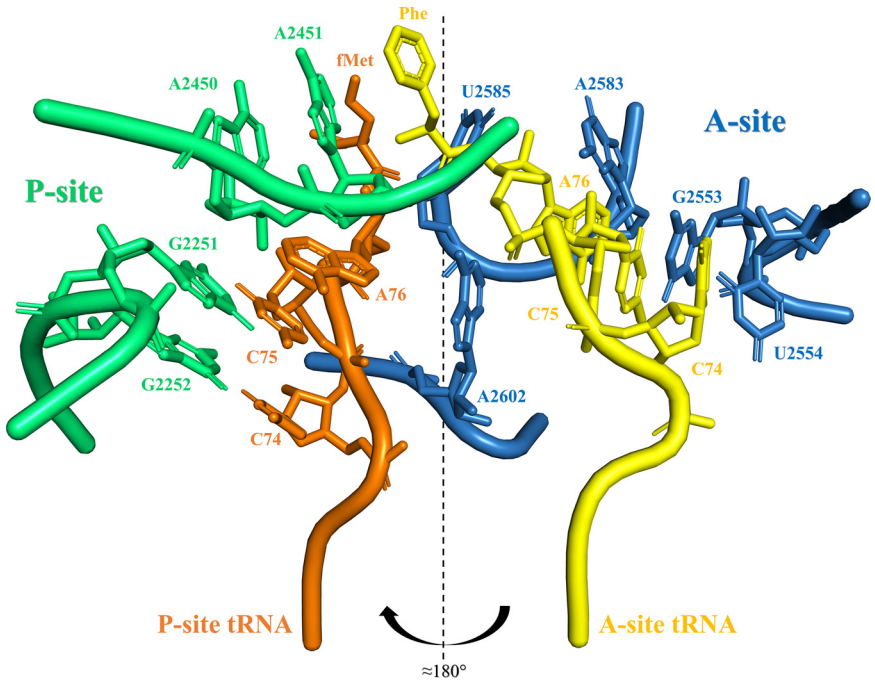

FIG 2 Closeup view of PTC with P-site and A-site tRNAs associated. View of some of the nucleotides that coordinate the CCA end and the amino acid residue that is carried by the P-site and A-site tRNAs. P-site rRNA chain and residues are colored green, and A-site rRNA chain and residues are colored blue. P-site tRNA is colored orange, while A-site tRNA is colored yellow. The CCA end of P-site tRNA is mainly coordinated by the Watson and Crick (WC) base pairs of G2252:C74 and G2251:C75, while an A-minor interaction of A76 with A2450 from the P-site tRNA is established. Both P-site residues A2450 and A2451 are considered important to the transpeptidation reaction. The CCA end of A-site tRNA is mainly stabilized by WC base pair G2553:C75, while U2554 establishes a hydrogen bond with C74. A2583 creates an A-minor interaction with A76 from the A-site tRNA. The "rotatory motion" mechanism implies that the 3′ end of the tRNA passage moves in a helical motion of almost 180° coupled with peptide bond formation. The tRNA transitions from the A- to the P-site along a rotation axis and around U2585 and A2602. This figure was created from the crystal structure NCBI ID 4WPO, using the PyMOL Molecular Graphics System, version 2.0 (Schrödinger, LLC).

namely, C2063, A2450, and C2501, are in very close proximity to the P-site tRNA. These residues form a spatial configuration with G2252 and G2251 of the P-loop of the 23S rRNA, both of which interact with the CCA end of the P-site tRNA (53, 54) (Fig. 2). A2450 and C2063 form a wobble pair and along with C2501 form a base triple setup, which forms stacking interactions with another base triple formed by G2447-A2451-G2061 (67). Furthermore, C2063 forms base stacking interactions with G2061, A2059, A2058, and A2503 (68). *In silico* simulation experiments have shown that C2063 forms an intricate network of H bonds with water molecules (69). C2063 (along with C2064 and A2435) is also part of helix 74. Helix 74 is an important contact region for the 3′-CCA end of the tRNA in the conformational rearrangements during translocation (70). Meanwhile, G2447 forms noncanonical interactions with U2504 and C2452 (68). Thus, the complex noncanonical interaction network involving C2063, A2450, and C2501 likely contributes to their very limited mutational flexibility. The importance of C2063 for the PTC is further demonstrated by the observation that its interaction with A76 of the P-site tRNA is disrupted by an antibiotic like bactobolin A. The antibiotic displaces the CCA backbone of the P-site (71). Likewise, C2507 in H90 interacts with multiple partners. It base pairs with G2582 (H90) and interacts with G2553. Most importantly, the 2′OH of C2507 forms hydrogen bonds with the 2′OH and O2 of C75 in the tRNA (72). This four-base interaction is thought to stabilize the 3′ end of the tRNA in the A-site. Its importance was first established when a mutation at C2507 was found to have an adverse effect on PTC activity (73). This was further corroborated by the findings of d'Aquino et al. in 2020 (66).

In 2012, 15 residues were reported to define the entrance to the exit pore (22). When placing these residues in the context of the mutational flexibility scale described by d'Aquino et al.

TABLE 1 Functional implications/roles of nucleobases that form the PTC (*E. coli* numbering system)[a]

Position[b]	Functional role/implication
A2057	Mutation confers macrolide resistance
A2058	Mutation confers macrolide resistance
A2059	Mutation confers macrolide resistance
G2061	Mutation reduces transpeptidation activity
A2062	Mutation confers macrolide resistance
C2063*	Mutation reduces transpeptidation activity; involved in peptide bond formation
G2251	Involved in accommodation of the P-site tRNA during peptidyl transfer; mutation reduces transpeptidation activity
G2252	Binding of P-site tRNA; mutation impacts peptide release
G2447	Mutation reduces transpeptidation activity
A2450	Mutation reduces transpeptidation activity
A2451*	Has role in peptide release; binding of P-site tRNA; mutation reduces transpeptidation activity; involved in peptide bond formation
C2452	Mutation confers macrolide resistance
A2453	Mutation reduces transpeptidation activity
U2492	Involved in accommodation of the A-site tRNA
C2501	Mutation reduces transpeptidation activity
U2506	Has role in peptide release; binding of P-site tRNA; mutation reduces transpeptidation activity
C2507	Mutation reduces transpeptidation activity
G2553	Interacts with A-site tRNA; mutation reduces transpeptidation activity
C2556	Involved in accommodation of the A-site tRNA
G2572	Sensor of conformational changes at the PTC; involved in transpeptidation activity; has role in peptide release
C2573	Involved in accommodation of the A-site tRNA; has role in peptide release
U2584*	Involved in peptide bond formation
U2585	Has role in peptide release; binding of P-site tRNA; mutation reduces transpeptidation activity
A2602*	Has role in peptide release; involved in transpeptidation activity; involved in peptide bond formation
C2611	Mutation confers macrolide resistance

[a]Updated from reference 24 with permission of Taylor & Francis Ltd.
[b]Asterisks indicate nucleobases involved in the proton wire reaction mechanism for peptide bond formation, as proposed by Polikanov et al. (206).

(66), the following was observed. P-site residues, namely, G2061, C2063, A2450, and A2451, and two A-site residues, namely, U2506 and U2585, would rank as the least flexible. U2506 interacts with residue A76 (74). The 2′OH and the O2 of U2506 hydrogen bond with the 2′OH of A76 in the A-site (72). Its mutation severely affects PTC activity (44), and its lack of mutational flexibility was further established by d'Aquino et al. (66).

On the other hand, three P-site residues, A2062, A2451, and C2452, and three A-site residues, G2505, U2586, and A2587, are the most flexible of the 15 residues lining the exit pore. Overall, the 23S rRNA residues that are the most mutationally flexible are found near the entrance to the exit tunnel (66, 75). The mutational flexibility seen was directly proportional to the distance from the PTC region that surrounds the aminoacyl end of the P-site tRNA (66). This work corroborated the findings from several earlier lines of inquiry, including a chemical footprinting assay mapping the interaction sites of macrolide antibiotics on the 23S rRNA (76). In that study by Vester and Douthwaite (76), residues A2062, A2057, A2058, A2059, C2611, and C2452 were found to confer macrolide resistance via mutations, thus establishing their mutational flexibility (Table 1).

Assuming that the mutational flexibility of these residues was similar in the proto-ribosome, the pore at the PTC that would eventually evolve into the exit tunnel was likely formed from residues of varying mutational flexibility. Thus, the "plasticity" observed in the PTC in extant ribosomes could well be the signature of a phenomenon with ancient origins.

In one study on yeast ribosomes, the rRNA mutations involving universally or highly conserved bases in the PTC region resulted in subtle structural rearrangements (51). However, these changes had a minimal effect on the overall translational accuracy, aminoacyl-tRNA binding, or PTC activity. Thus, the extant PTC rRNA could accommodate a certain level of plasticity without affecting the overall PTC function (51). Such plasticity could well be attributed to the functional groups and likely be a clue to its functionally generalized pasts. Several significant studies highlight this "plasticity." Two studies have shown that it was not base A2451 but rather the 2′-hydroxyl group that was important for the reaction to occur (45, 77). Likewise, in the case of A2602, the role of the ribose moiety at that location in aiding peptide release has been demonstrated (78). This "plasticity" in effect

confers functional "versatility" to the PTC. This can be seen in its demonstrated ability to support different substrates such as esters and thioesters (79, 80). It has been suggested that this capability may be reminiscent of the PTC's ancient abilities, when it may have had to accommodate multiple substrates (81). This could also be traced to the fact that the extant ribosomes can accommodate 20 different amino acids from aminoacyl-tRNA carriers. Overall, the structural organization of the extant PTC is characterized by a property defined as an "induced-fit mechanism" (conformational change) (82). This is an extension of the induced-fit mechanism originally proposed to explain general enzyme-substrate specificity (83). Such a mechanism, in turn, is thought to confer an extraordinary ability to the ribosome to handle substrates of various sizes (84).

RIBOSOMES ARE HETEROGENOUS WITH THE PTC CORE UNCHANGED OVER TIME

In parallel with cellular evolution, the proto-ribosome evolved to give rise to the present-day ribosome that is much larger in size and complexity than its ancestral state. During this process, it also became structurally and functionally heterogenous. In several small bacterial genomes, the loss of rRNA segments (thus leading to reduction in rRNA size and complexity) has been shown to be coupled with the loss of ribosomal proteins (85). The deletions in the 23S rRNAs in these genomes were commonly located in helices H10, H63, H79, and H98, thus preserving the PTC core. Diversity and specialization have both been observed at the interspecies and intraorganismal levels (86). Both eukaryotic cells and eukaryotic cellular organelles (mitochondria) show substantial heterogeneity in their overall ribosomal structure and composition (87–91). The evolutionary origins of the mitochondria are thought to be rooted in alphaproteobacteria through endosymbiosis (92–94). Seldom discussed is the fact that this would place mitochondrial origins well after LUCA. In fact, mitochondria possess ribosomes, which show substantial divergence from their roots (95). The differences in their structures, functions, and compositions have been described in detail. Mitoribosomes are diverse in size among different lineages (96–100). Substantial sections of the rRNA have been replaced by additional protein sections (101–105). In the (55S) mitoribosome (which is specific to animals), the rRNA has been shown to have been reduced to an inner core retaining the functionally important sites, namely, the PTC active site and the decoding site (106). A detailed structural study of the yeast mitoribosome suggests that a base pairing between U2877 (C2610 in *Escherichia coli*) and A1958 (A2058 in *E. coli*) causes the narrowing of the exit tunnel entrance (107). However, this narrowing is far removed from the actual PTC activity site. The key regions of interactions between the 3′ CCA ends of the tRNAs and the PTC in the mitoribosomes, which are vital for the peptide bond formation, are not different from what is observed in the corresponding bacterial and eukaryotic (cytosolic) ribosomes (102, 108–110). In one study on the human cytosolic ribosome, cross-linking methods were used to show the proximity of an rProtein to the P-site tRNA. The rProtein rpL36a-like (rpL36AL) was found to be directly contacting the CCA 3′-terminal adenosine of the P-site tRNA (111). Otherwise, the arrangements of the PTC regions found in the eukaryotic nuclear ribosomes are not much different than those found in the ribosomes of bacteria (112, 113).

THE PTC HAS BEEN "FOSSILIZED" IN THE RIBOSOMAL LARGE SUBUNIT SINCE PRE-LUCA TIMES

Early efforts to understand ribosome evolution were inspired by the observation that the rRNAs are far too large to have emerged in a single step. Thus, some portions of the rRNAs must precede others (114), but how can we tell which are the oldest? A proposed solution was to identify timing events that provide insight into the relative age of different regions (115). The first example that was utilized was connectivity. It was argued that highly connected regions of the rRNA are likely older than less-connected regions. With this in mind, the number of long-range interactions between domains was identified (115). This approach was extended to individual helical elements by looking at A-minor interactions where adenines interact with a necessarily preexisting helical element (116–118). In the onion model (119), the PTC was assumed to be the oldest region and then other regions were dated by their distance from the PTC.

A significant breakthrough came with the realization that there was evidence of past accretion events even in the conserved regions of the rRNA (120, 121). From structural comparisons, it was inferred that new RNA was added to "older" RNA (120). Using this accretion approach, the various regions of the rRNA have been used to create a chronological hierarchy (12, 15, 120–125). This accretion model provides a detailed helix-by-helix model of LSU rRNA evolution in which each addition is assigned to one of six phases. In this model, the PTC is thought to have been assembled in phases one and two. Thus, much of the evolution of the rRNAs likely occurred well before LUCA. This approach has been extended to include the SSU rRNA as well (126).

The accretion model can also be explained and related to the function of the extant ribosome. The ribosome has been defined as a ratcheting motor (127, 128) and a Brownian machine (129) that undergoes several intermediate stages of motion described as "hybrid" states (130–134). These states include the rotation of the 30S (SSU) with respect to the 50S (LSU) (127, 135–138), translocation of the transfer RNAs and mRNA during the reverse rotation of the 30S subunit by the elongation factor G (EF-G) (139–146). It is now fairly well established that just before peptide bond formation, the ribosome is "locked" in the nonrotated state. Thus, the entire ribosomal evolution and accretion around the PTC have occurred in such a manner to accommodate all these motions associated with the extant ribosome, without in any way compromising the oldest part of the ribosome, the PTC. Thus, these subtle motions are paused during that "locked" nonrotated state, ostensibly to promote catalysis at the PTC (147). In fact, it is peptidyl transfer itself which is thought to induce the rotated conformation (136, 138, 148). At the molecular level, these various motions are associated with weak sites such as nonstandard base-base interactions and three-way junctions in the rRNAs (51, 149, 150). A comprehensive search for pivot points associated with EF-G and EF-Tu binding to the ribosome has been reported (151, 152). The importance of weak sites was found to be a general rule.

COEVOLUTION OF THE PTC AND THE tRNA

The origins of the tRNA are also linked with the origins of the PTC. The choice of the amino acid that were likely involved in early PTC activity has implications for the origins of coding. The ancestral small RNAs of extant tRNAs were aminoacylated based on the amino acid affinity of the CCA stem. This was most likely facilitated by an ancestral operational genetic code (153–155). Evidence for the same can be found in the examples of minihelix RNA comprising the acceptor stem that could be charged (156, 157) by extant aminoacyl-tRNA synthetases without the need for the anticodon. The modern tRNA has the universal CCA sequence at its 3′ end, with the anticodon segment of the tRNA at a molecular distance of around 70 Å. Depending on which tRNA is associated with the PTC, the CCA end carries the incoming amino acid or the growing peptide chain. During the initial stages of evolution, the PTC and the CCA end of the tRNA likely coevolved together (155). Recently, an ancient insertion fingerprint in the tRNA was discovered that gives credence to the idea of the addition of the anticodon section (along with the D arm) to the older fragment comprising the CCA stem (and the T arm) (120).

PROTEINS STABILIZE THE RIBOSOMES BUT ARE NOT ESSENTIAL FOR PTC ACTIVITY

Ribosome stability is achieved through interactions between bases, metal ion coordination, and the interaction with rProteins. RNA-protein interactions are significant in the ribosome as a whole, as well as in the PTC. However, none of the rProteins alone seem to be indispensable for peptide bond formation (4). There are several rProteins, such as uL2, uL3, and uL4, that have unstructured segments which dive deep into the ribosome in areas near the PTC (6, 158–160). Other rProteins, namely, uL6, L10e, uL14, bL27, and L15e/L31, are also found in the general vicinity of the PTC (6, 21, 161). Structural studies on bacterial ribosomes suggest that P-site tRNA likely interacts with the tails of rProteins such as uL5, uL16, bL27, uS9, and uS13 (34, 161). In a photoaffinity labeling experiment, it was shown that while L1 and L33 are close to the 3′ end of the deacylated tRNA at the E-site, bL27 likely interacts with the 3′ end (A76) of the P-site tRNA (162). Deletion of only three residues of the

N-terminal tail of bL27 resulted in the reduction of the PTC activity (159), as does the deletion of uL16 (163, 164). However, in both cases, the PTC activity is not abolished entirely. It should also be noted that bL27 is a bacterium-specific protein with no homolog in Archaea or Eukaryota and hence not available to facilitate peptidyl transfer until after LUCA. It has been shown that uL16 is important for the proper placement of the tRNA in the A-site (34). Furthermore, in a more recent study, when 5S rRNA is depleted, uL16 is not incorporated into the LSU, which in turn disrupts the correct assembly of the PTC region in the modern ribosomes (165). However, this may not have been a necessity in the rudimentary stages of the PTC, when it was far from the excellent protein factory, which it later evolved to be. Thus, all of these rProteins are possibly the product of ribosome evolution in a protein-dominated world (158, 166, 167). Therefore, it is natural to consider other more elemental forces, like base-base interactions and metal ion coordination, that likely played supportive roles at the very early stages of PTC evolution.

METAL IONS AT THE PTC IN EXTANT RIBOSOMES

It is well established that metal ions such as magnesium, calcium, sodium, and potassium are biologically important in general and particularly for RNA (168). Among monovalent cations, potassium (K^+) seems to be the most common and the most effective in stabilizing RNA structures in early folding stages or in the absence of divalent cations (169). It has been suggested that K^+ ions play a major role in RNA condensation because they promote the formation of secondary structure (168). This most likely occurs because early neutralization of the intrinsic negative charge from the phosphate backbone allows RNA molecules to collapse into more compact structures. In the context of the ribosome, the importance of K^+ ions was established when mammalian ribosomes were observed to lose polymerizing activity in the absence of potassium (170, 171). The specific occurrence of K^+ ions in the PTC region was first reported in the 50S subunit of the archaeon *Haloarcula marismortui*. In this peculiar case, the K^+ ion at the PTC region showed planar coordination with ion base distances that are consistent with the presence of other K^+ ions (35). Recently, the relative abundance of K^+ ions in the *Thermus thermophilus* ribosome and within its PTC has been determined using long-wavelength X-ray crystallography (172). Two independent studies, one by Klein et al. (166) and another by Rozov et al. (172), arrived at similar inferences about K^+ ions. One K^+ ion was assigned to the PTC (coordinating to G2061, G2447, C2501, and U2503), and another was assigned to the H11 of the 23S rRNA (coordinating to C192, U193, and A202) (166, 172). Overall, K^+ ions interact with either the carbonyl groups on bases or the hydroxyl group of a ribose (173).

Among divalent cations, magnesium (Mg^{2+}) is thought to be the most suitable for neutralizing the intrinsic negative charge of the RNA phosphate backbone due to its abundant intracellular concentrations (174). In addition, Mg^{2+} ions possess the highest charge density amid biologically available ions, a feature that seems to enhance its bonding to RNA (175, 176). Two types of magnesium bonding to RNA have been described, and they represent the current basis for the understanding of the Mg^{2+} ion interaction with rRNA (166, 168). The first type involves "diffuse binding," which provides long-range charge screening that overcomes electrostatic repulsion between the RNA phosphate backbone and the hexahydrate Mg^{2+}, essentially following Coulomb's law. The second type is "site binding," which involves specific coordination of anionic ligands to a particular dehydrated state of Mg^{2+}. Site binding involves two subtypes. In the first, the "site-bound, outer sphere" subcategory, Mg^{2+} ions interact with the RNA but do not form direct contacts. Instead, the water ligands around Mg^{2+} ions interact with the bases and the phosphate backbone to stabilize specific RNA motifs. The second, the "site-bound, inner sphere" subcategory, is where Mg^{2+} ions directly interact with the bases, the sugar, and the backbone of RNA.

The role of magnesium in the structure and function of the ribosome has been well documented (177). As early as in the 1950s, it was reported that the association of a functional ribosome depended heavily on Mg^{2+} concentrations (178, 179). Magnesium was next established as indispensable for the cellular ribosomal maintenance (180), the structural integrity of the ribosome (181), and eventually for the PTC reaction (182).

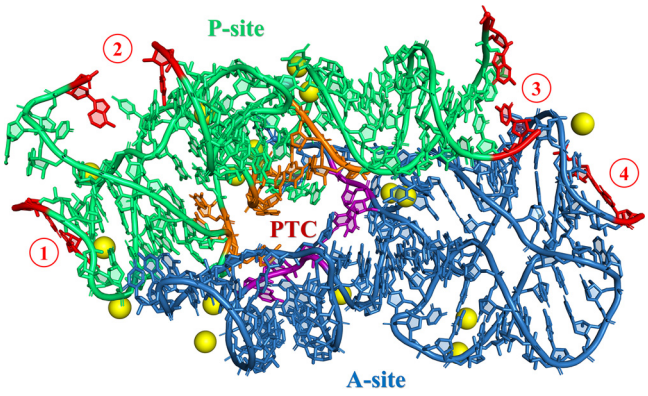

FIG 3 Three-dimensional representation of the pseudosymmetrical region (SymR; Agmon et al. [250]) extracted from the *Thermus thermophilus* crystallographic structure (NCBI ID 4WPO) using PyMOL (The PyMOL Molecular Graphics System, version 2.0; Schrödinger, LLC). The residues from the P-site and the A-site are colored green and blue, respectively. Residues delineating the entrance to the PTC pore are colored orange (P-site) and purple (A-site), as defined by Fox et al. (22). Sixteen conserved magnesium ions that are around the contact area of the SymR residues are shown as yellow spheres, as described by Rivas and Fox (68). Regions where the addition of fragments is required for the ligation of smaller fragments are highlighted by red numbers within red circles at the end of each helix, three at the P-site and one at the A-site. Also, the last residues of each ligation point are colored red. A secondary structure depiction of these elements is presented in Fig. 4.

The presence of hydrated Mg^{2+} ions was first reported in the *Deinococcus radiodurans* (bacterium) 50S subunit (74). In a further analysis of ribosome crystallographic structures, the role of Mg^{2+} ions in assisting the interaction of distal segments of rRNA by coordination of phosphate groups and its ability to mediate protein interaction with rRNA was recognized (183). Mg^{2+} ions are particularly abundant in the region surrounding the PTC, where they form magnesium microclusters (184). In one model featuring the structure of the *E. coli* (bacterial) ribosome at 3.5-Å resolution, 170 Mg^{2+} ions per ribosome were reported (185). Overall, metal ions like Mg^{2+} are concentrated in functional regions, such as the PTC, where they stabilize RNA chains at places that rProteins cannot access (166) (Fig. 3). Recently, the conservation of these Mg^{2+} interactions in and around the PTC across diverse phylogenetic groups has been confirmed, and a plausible role of these Mg^{2+} interactions in the early evolution of the PTC has been proposed (68). Going forward, a more judicious use of stereochemical principles applied to structures with higher resolution is expected to better resolve the nature and identity of the ions in ribosomes (186).

METALS AND THE PROTO-RIBOSOME ON THE EARLY EARTH

Although Mg^{2+} is recognized as an essential cofactor with diverse structural and functional roles within the modern ribosome, conditions on the early Earth may have favored other ions. In particular, the ancient Earth would have provided environments with low O_2 concentrations (187–189) and high ferrous iron (Fe^{2+}) and manganese (Mn^{2+}) availability (190, 191). By replacing Mg^{2+} with Fe^{2+}, the catalytic abilities of several RNA molecules are expanded, including a previously uncharacterized ability to catalyze single-electron transfer (192). Under anaerobic conditions, Mg^{2+} can degrade RNA (193). Evidence that the role in folding and catalysis of Mg^{2+} can be replaced by Fe^{2+} has been presented from both quantum mechanical calculations and experiments with several ribozymes (194). Fe^{2+} and Mn^{2+} not only could aid rRNA folding but also could replace Mg^{2+} as the cation during translation under experimental conditions (195). These results suggest that within the anoxic environment of the early Earth, other metallic ions like Fe^{2+} could have been interacting with earlier versions of the ribosome, including its catalytic core, the PTC. Irrespective of the metal involved, what matters is the positioning of the various players, including the CCA end of the tRNAs and the highly conserved residues of the PTC. If experimental evidence supports the notion that the PTC activity is flexible with its metal occupants, then it would provide further support for the idea that the PTC was present during the anoxic chemical era of early

Earth. It would also show that its metal-accommodating ability is a function rooted in its early origins.

THE PTC IS A DYNAMIC PEPTIDE SYNTHESIS MACHINE

Multiple lines of evidence have established that the extant PTC region is an RNA "engine" powering the peptide bond formation reaction (196–198). The PTC has not only virtually survived unchanged over 4 billion years of evolution but hasn also remained central to modern biology (15, 24, 119, 125, 199, 200). The modern PTC is postulated to act as an entropy trap platform, which ensures a certain three-dimensional placement/orientation of the substrate molecules (201, 202).

Molecular biology has been described as "a brilliantly staged molecular theater" (203). If so, the PTC sets the stage for a molecular "theater" of a kind, involving the following molecules, namely, the incoming tRNA charged with an amino acid and the nascent peptide linked to the 3′ hydroxyl end of the 3′ terminal ribose of the P-site by an ester bond (24, 204). The nucleophilic α-amino group of the A-site aminoacyl-tRNA breaks the electrophilic ester carbonyl carbon of the peptidyl-tRNA (aminolysis) (205). This results in the transfer of the peptidyl chain to the aminoacyl-tRNA at the A-site. This process is thought to involve the movement of three protons in the PTC along a proton wire (206). An initial deprotonation of the α-NH_3^+ of the A-site tRNA creates the α-NH_2. Then, a proton is further removed from this amine along the proton wire, and at the end of the reaction, another proton is made available to the 3′-oxygen of the A76 P-site tRNA ribose.

In a significant study using quantum *ab initio* calculations, supported by crystal structure data, conserved water molecules "trapped" in the PTC region have been shown to be important players as well. One water molecule plays a mediatory role in this proton shuttle, while another stabilizes the negative charge on the substrate oxyanion (207). Water molecules at the PTC are thus thought to be important for maintaining the thermodynamic parameters of the reaction. Another player is the 2′-OH of the peptidyl-tRNA substrate, which is thought to ensure an efficient proton movement via a network of hydrogen bonds (208, 209). The P-site A76 2′-OH is placed in close proximity to the attacking α-amine and the O3′ leaving group and is thus optimally positioned to enable efficient catalysis (69, 82, 210, 211) (Fig. 2). This 2′-OH is important, irrespective of whether the LSU is by itself or is part of the whole ribosome unit along with the SSU (209, 212, 213). While the 2′-OH of the A76 ribose is essential for maintaining the reaction rate, the catalysis itself is attributed to the reduction of the activation free energy, achieved via electrostatic effects (214). A76 is also essential for peptide release and EF-Tu binding (215, 216).

This entire process goes on cyclically (3), and the peptidyl transferase reaction is thus pushed forward, resulting in the fairly rapid synthesis of 15 to 50 peptide bonds/s in a modern organism (217). This continues until a stop codon is reached, at which point ester hydrolysis breaks the bond between the P-site tRNA and the peptide. At each step, the growing peptide is released into an RNA pore (22, 23), which forms the entrance of the exit tunnel (218) that ultimately allows the finished peptide to get out of the way and leave the ribosome. Thus, the nanopore at the PTC acts like a molecular womb, where not only is the peptide formed, but it is also given an exit. The uniqueness of this pore at the PTC was demonstrated in a study which examined multiple "pores" formed and found in rRNAs as well as other RNAs (23). The nanopore at the PTC region is formed by RNA residues in which the nitrogenous bases are exposed. The phosphate backbone is twisted away from the lumen of the pore. Significantly, in contrast with the PTC pore, other known pores feature the nitrogenous bases twisting away, leaving the phosphate backbone to line the lumen of the pore (23, 219). Although much progress has been made in understanding ribosome structure and function, the actual chemistry of the PTC continues to be deciphered (215, 220–224).

In the modern ribosome when there are obstacles in the tunnel, peptide growth is inhibited. The coordinated train of movement of the peptide then stalls and prevents the PTC from functioning. This has been amply demonstrated with antibiotics (225–232). Antibiotic-enabled disruption of any of the players in the ribosome PTC arena, including their respective three-dimensional positioning of acceptor or donor substrates, results in a stalled or dysfunctional ribosome

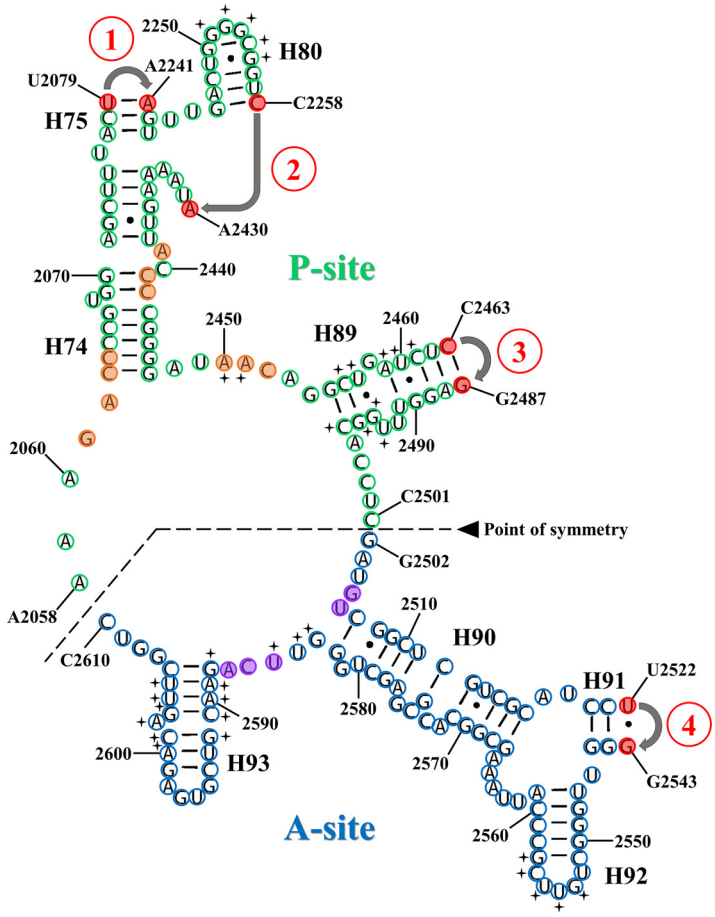

FIG 4 Secondary structure of the pseudosymmetrical region (SymR; Agmon et al. [250]), derived from the LSU secondary structure of *Thermus thermophilus* (Petrov et al. [19]). A black dotted line indicates the boundary between the P-site and the A-site. Bases from the P-site are highlighted by green circles, and bases from the A-site are highlighted by blue circles. The orange (P-site) and purple (A-site) colored circles highlight residues that delineate the entrance to the exit pore of the PTC, as defined by Fox et al. (22). In order to dissect SymR from the extant ribosomal structure, one must artificially cut and anneal the four sites highlighted by red numbers within red circles, three at the P-site (H75, H80, and H89) and one at the A-site (H91). Also, the last residues of each ligation point are highlighted by red circles. Bases involved in the "rotatory motion" mechanism from the front and rear walls and the P- and A-loops are marked by a black plus symbol in front of each base according to Agmon et al. (251) and Bashan et al. (74, 256).

(24, 233, 234). Growing nascent peptides can stall the PTC activity by interacting with the exit tunnel (234–240). In its early stages, an evolving PTC would not have the fully grown ribosome with an exit tunnel to contend with. Therefore, its efficiency in making peptides would have been centered on a relatively "simple" process of a "substrate in" and a "product out." However, when accretion events occurred, the exit tunnel might have been blocked, thereby preventing peptide synthesis. Thus, selection would favor mutations that would accommodate the peptide chain's exit, through any obstructive expansion segments. The exit tunnel's interactions with the growing peptide and the related stalling are thus likely consequences of ribosomal evolution (241). As the genetic code was established, the nature of the nascent peptide as well as the quality of the mRNA also played vital roles in stalling events (242–245). In order to solve the problems arising from such stalling, systems like ribosome rescue may have evolved (243, 246–249). The early PTC likely did not have to deal with such problems.

DISSECTING THE STRUCTURE OF THE PTC REVEALS ITS ORIGINS FROM SIMPLER FORMS

Close examination of the high-resolution structure of the entire domain 5 revealed the presence of a region of pseudosymmetry now known as SymR (250, 251) (Fig. 3 and 4). The

two LSU rRNA fragments that define this region correspond to the A-site and P-site segments of the extant PTC (250, 251). These segments are listed as occurring in phase 1 and phase 2 in the accretion model (120). In addition, within the ribosome, the complex forms an RNA pore (22, 23). Overall, the level of conservation observed among the residues of the extant PTC (112, 252, 253) is at many locations extremely high (Fig. 1).

This initial observation immediately led to the hypothesis that an ancient duplication and dimerization event had created a functional proto-ribosome (2, 250, 251, 254, 255) that was "frozen" and is now preserved in the modern PTC. In an elegant series of papers (250, 251, 254, 255), the Yonath group have proposed a detailed model of how the modern ribosome works. Grounded on the analysis of several ribosomal crystallographic structures, they proposed a "rotatory motion" mechanism that describes the tRNA passage from the A-site to the P-site. This requires two independent motions: (i) a helical rotation of ~180° of the A-site tRNA-3′ coupled with the peptide bond formation (Fig. 2) and (ii) an A-to-P-site translation of the tRNA acceptor steam (74, 256). As stated by Yonath in 2003 (257), the most significant biological implication of the rotatory motion seems to be the establishment of favorable stereochemistry for peptide bond formation without drastic conformational rearrangements at the PTC. Due to its helical nature, the entrance of the nascent peptide into the ribosomal exit tunnel is ensured. Peptide bond formation, translocation, and nascent protein progression seemed to be the result of this rotatory motion, although it is not clear if peptide bond formation is triggered by the motion or the motion is triggered by the reaction (257). Yonath and coworkers have recognized the participation of several nucleotides in the proposed rotatory motion, most of which are contained within SymR (74, 250, 251, 256, 257) (Fig. 4).

From an evolutionary perspective, the rotation model depends strongly on the ability of the two fragments to anneal to one another. Each fragment by itself is stabilized by several standard base pairs and Mg^{2+} ions (Fig. 3). However, a cursory observation fails to find standard base pairs that could stabilize the proposed association of the two pieces. Likely as a result of this and the difficulty until recently of synthesizing RNAs in the 80–100-nucleotide size range, the hypothesis has largely been ignored. However, in fact, six conserved regions of nonstandard interactions have been identified that could facilitate association of the two RNAs (68, 250, 254). Among these, long-range interactions seem to be a dominant theme. Most of the interactions are represented by splayed-apart bases that due to their peculiar nature are able to establish several hydrogen bond contacts between the A and P fragments. In addition, there are several highly conserved Mg^{2+} ions that interact with the two fragments (Fig. 3). This includes two conserved Mg^{2+} ions that connect with both regions and that might have played a role in the association and stabilization of the two segments. Even further, one of these Mg^{2+} ions is in a position where it not only fixed the end of the P-site chain but can also have played a role as a potential catalytic element that enhanced the ligation of the two aboriginal fragments (68). It has been proposed that together, these interactions can stabilize an association between the two fragments. To test this hypothesis, computational studies of such an RNA pair were conducted using quantum kernel energy parameters. It was found that the two fragments could indeed be expected to hybridize (2). Adding to the optimism is the fact that examples exist in extant biology, where the LSU rRNA is expressed as multiple fragments that come together to assemble the ribosome (258, 259). This includes the PTC being formed by the association of two independent RNA pieces in both the *Euglena gracilis* cytosolic ribosome (260) and the *Chlamydomonas reinhardtii* mitochondrial ribosome (261).

Like its modern version, the hypothetical proto-ribosome would actually perform two tasks, not just peptide bond formation. In the prebiotic world, after the initial task of formation of the first peptide dimer, the question that follows is whether it will be extended to a tripeptide or be released as a dipeptide. Extension seems favorable, as the rate of synthesis is higher than the rate of peptide release (218). However, the ancient machine would soon clog unless there was a mechanism to keep the growing peptide out of the way. Thus, after a peptide bond is formed between two starter amino acids, the newly made dipeptide might begin to enter the RNA pore that is formed by the association of the two segments of the pseudosymmetrical region and

then move along via the pore as synthesis continues. Thus, in the proto-ribosome and its modern descendants, the process would again not be just synthesis but polymerization as well. The primitive "entropy trap" would only need activated amino acids to make peptide bonds to extend the peptide.

However, as they exist in the modern ribosome, the two fragments are connected to the remainder of the LSU rRNA. To experimentally test the dimerization theory, it is necessary to synthesize RNAs that in effect remove the two hypothesized regions from the PTC rRNA as two independent RNAs. This requires the addition of three small RNA connectors to the P-site RNA in order to shorten helixes 75 and 89 and to connect H80 to H74 and one addition to the A-site RNA to shorten H91 (Fig. 3 and 4). By shortening H75, one removes the L1 stalk, and by dissecting and connecting H80 to H74, one removes the rRNA that corresponds to the central protuberance. Deletion of the whole helix 73 would be the final step in dissecting the SymR from the LSU rRNA. It is not clear how critical the specific choice of the additions and cuts would be.

CAN THE PTC BE BUILT FROM ITS SIMPLER COMPONENTS WHILE RETAINING ACTIVITY?

Recently, Xu and Wang have shown that a slightly larger version of two fragments encompassing the proto-ribosome can in fact form a stable complex (262). Subsequently, a seven-residue fragment ending with CCA and carrying lysine at its 3′ end was incubated with the two-piece RNA construct. A nine-residue polylysine was apparently formed (263). If this is correct, in essence, both peptide bond formation and polymerization had occurred as in the modern ribosome (263). If these findings survive scrutiny, our understanding of the prebiotic world will be enhanced. There is, however, concern that products of shorter or longer size were not observed.

Having previously shown that the large subunit alone could catalyze peptide bond formation (31), Noller pointedly observed in 1993 that the intricately intertwined RNA-protein "cage" in the large subunit would make further progress toward separating the RNA from the protein and still make the former retain its catalytic activity, a worthy challenge (264). This has proven to be true, as several groups have reportedly made multiple attempts with at best partial success. However, with the exception of the effort of Xu and Wang (262), they have not reached the mainstream literature. In terms of future studies, much depends on a convincing demonstration that the still hypothetical proto-ribosome would have been functional. This would, for example, strengthen arguments for the operational code (153, 154, 265). Also, the menu of likely follow-on studies will have to be directed toward understanding how two initially independent fragments came to be covalently linked and when, in the larger scheme of ribosomal evolution, this occurred. Such linkage would likely be an important step in stabilizing the evolving proto-ribosome. It has been suggested that translation evolved to extend the structural and functional capabilities of the hypothetical RNA world (167) and, during the course of its evolution, might have terminated the RNA world (219). How does the evolving system accrete what ultimately becomes the ribosome? And, of course, can one simplify the system further?

The existence of an active proto-ribosome would also set the stage to inquire about the potential role of the peptides produced. Nothing dramatic is thought to have been associated with early peptide functionality, and a compelling argument might be that the proto-ribosome would simply make peptides because that is what it did, in a random "nonutilitarian"/"nonspecific" fashion, and not because they were useful. But they might have been. Recent studies have shown that even extremely small peptides can perform a large variety of functions (266, 267). At the least, peptides made by the proto-ribosome might simply have augmented the ancestral PTC. For example, this could have been accomplished by building layers (7) and using the peptides to stabilize the emerging PTC. Thus, the activity would represent the possible beginnings of a transition from an "RNA world" to an "RNA/peptide world." Studies of the ribosomal protein structures support this, as they become increasingly more complex as one proceeds from the older regions of the ribosome to the newer (268–271).

In summary, all of this reminds us that there must have been a predecessor to the modern PTC. This premise is based on the nature of RNA structure itself, since small

RNAs derived from larger RNAs can still maintain their local structures (7). Thus, in the prebiotic context, simpler, smaller RNAs could have given rise to the PTC through association (272, 273). Sulima and Dinman in 2019 referred to the evolution of a proto-ribosome, forming the primordial ribosomal core, approximately 4 billion years ago as the "Big Bang" of the riboverse (86). Understanding the origin and properties of that ancestor will be a major advance and should be obtainable if the community continues to pursue it. Quoting Darwin ". . . from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved . . ." (274).

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George E. Fox earned his B.Sc. and Ph.D. in chemical engineering at Syracuse University. As a postdoctoral fellow with Carl R. Woese at the University of Illinois, he was the codiscoverer of the Archaea, the progenote concept, and the use of comparative sequence analysis to predict RNA secondary structure. In 1977, Fox joined the University of Houston, where he remains an Emeritus Moores Professor of Biology and Biochemistry. In 1980, Fox, Woese, and collaborators published the first comprehensive tree of bacterial relationships. In "How Close Is Close: 16S rRNA Sequence Identity May Not Be Sufficient To Guarantee Species Identity" (G. E. Fox, J. D. Wisotzkey, and P. Jurtshuk, Jr., Int J Syst Evol Microbiol 42:166–170, 1992, https://doi.org/10.1099/00207713-42-1-166), Fox recognized the limitations of 16S rRNA sequence comparisons. His current research is focused on ribosome function and evolution. He is an elected fellow of the American Academy of Microbiology, the American Association for the Advancement of Science, the American Institute for Medical and Biological Engineering, and the International Astrobiology Society. He chaired the 2010 Origin of Life Gordon Conference and has over 180 peer-reviewed publications.