Minireview

Translocation of tRNA during protein synthesis

Harry F. Noller[a,*], Marat M. Yusupov[a,1], Gulnara Z. Yusupova[a,1], Albion Baucom[a], J.H.D. Cate[a,b]

[a] *Center for Molecular Biology of RNA and Department of Molecular, Cellular and Developmental Biology, Sinsheimer Laboratories, UCSC, Santa Cruz, CA 95064, USA*

[b] *Berkeley Center for Structural Biology, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*

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Abstract Coupled translocation of tRNA and mRNA in the ribosome during protein synthesis is one of the most challenging and intriguing problems in the field of translation. We highlight several key questions regarding the mechanism of translocation, and discuss possible mechanistic models in light of the recent crystal structures of the ribosome and its subunits. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ribosome; Hybrid state; tRNA; mRNA; Translocation; Spirin

1. Introduction

During protein synthesis, messenger RNA and transfer RNA move in a coordinated way through the ribosome. The large-scale and precise macromolecular rearrangements that accompany the movements of tRNA are surely one of the most impressive examples of molecular movement in living cells. They are all the more interesting because they are universally conserved and, unlike most other cellular machine-like movements, most likely involve movement of RNA (ribosomal RNA). More than three decades have elapsed since Spirin originally proposed the first explicit mechanical model for tRNA translocation [1]. During that time, a tremendous number of papers have been published on this problem, based on genetic, biochemical and physical approaches. Suffice it to say that our understanding of the molecular mechanism of translocation nevertheless remains incomplete, and controversy is rampant, in spite of a fairly complete understanding of ribosome structure.

Here, our intent is to try to summarize what is known about some aspects of translocation that have been studied in our laboratory and elsewhere, attempting to view them in the context of recent X-ray structures of the ribosome, and to pose some questions that are raised in the process of bringing the translocation problem into three dimensions. The contributions of Alexander Spirin and his co-workers to this field are well known. Besides his fundamental discoveries and insights concerning the mechanism of translocation, pioneering studies on crystallization of *Thermus thermophilus* 70S ribosomes in his laboratory [2] paved the way for solution of the crystal structures of functional complexes of the complete ribosome [3,4].

2. tRNA binding sites on the ribosome

Originally, ribosomes were envisaged as having two binding sites for tRNA – the A (aminoacyl) site and the P (peptidyl) site [5]. This was, first of all, the minimum number of binding sites that could account for the known mechanism of protein synthesis (attack of the amino group of aminoacyl-tRNA at the carbonyl group of peptidyl-tRNA), and secondly, it was consistent with virtually all of the available data at that time. The classical two-site model began to give way to the modern three-site model with the discovery of the E (exit) site by Nierhaus and co-workers [6]. At least some of the early resistance to the E site was the lack of any theoretical reason to invoke a third site, and the tradition of favoring the simplest possible model (Why bother with an E site? Why not just discard the deacylated tRNA?). Eventually, the original findings were confirmed by several groups, and the paradigm expanded to include the E site.

Meanwhile, the molecular nature of the A, P and E sites began to emerge. Since the mRNA bound to the 30S subunit, it was assumed that the anticodon ends of the tRNAs also bound there; since peptidyl transferase was located on the 50S subunit, the acceptor ends of the tRNAs must bind there. This was confirmed by numerous biochemical crosslinking and footprinting experiments (reviewed in [7]). These studies also provided evidence that the tRNAs were surrounded mainly by rRNA, although some proteins were also implicated. Chemical modification and modification-interference experiments provided evidence that the surrounding rRNA was part of the tRNA binding sites, and identified specific elements of rRNA that could be assigned to each tRNA binding site [8–11]. From the base-specific footprinting experiments in our own laboratory, we concluded that there were at least five distinct binding sites for tRNA on the ribosome – A and P sites on the 30S subunit, and A, P and E sites on the 50S subunit. The recent co-crystal structure of the 70S ribosome bound with tRNAs shows that there is indeed a 30S E site

*Corresponding author. Fax: (1)-831-459 3737.
E-mail address: harry@nuvolari.ucsc.edu (H.F. Noller).

[1] UPR 9004 de Biologie et de Genomiques Structurales du CNRS, IGBMC B.P. 163, 1 rue L. Fries, 67404 Illkirch Cedex – CU de Strasbourg, France.

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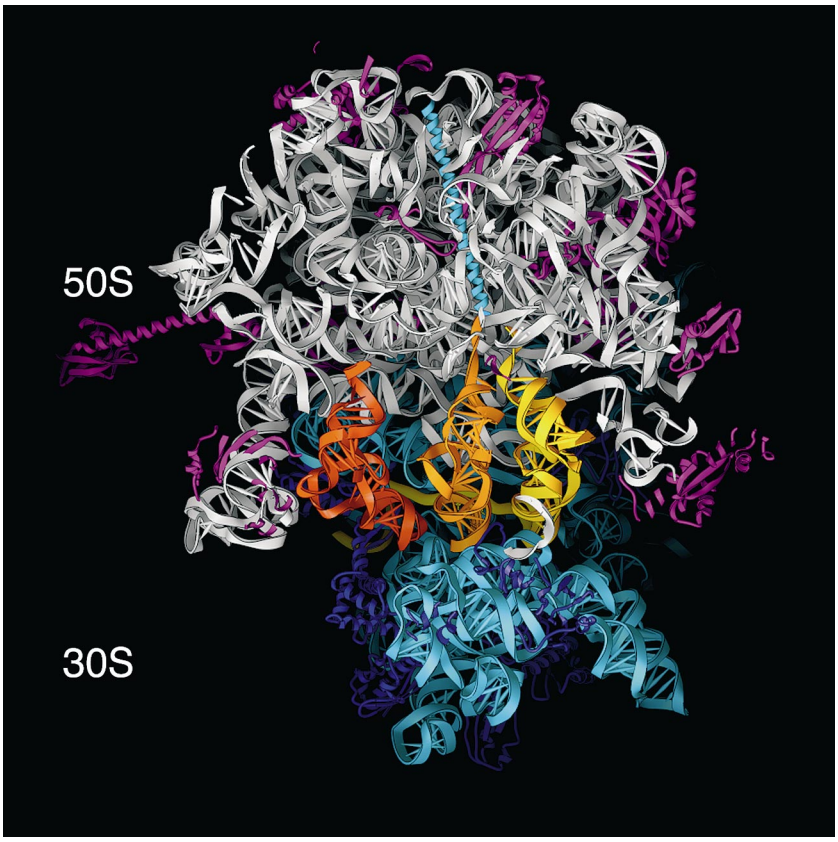

Fig. 1. Cutaway view of the *T. thermophilus* 70S ribosome [4] containing tRNAs bound in the A/A (yellow), P/P (light orange) and E/E (dark orange) states, corresponding to the classical A, P and E sites. The 16S and 23S rRNAs are shown in cyan and gray, and the 30S and 50S subunit proteins in blue and magenta, respectively. The nascent polypeptide chain (cyan) is modeled as an α-helix occupying the polypeptide exit channel in the 50S subunit. Movement of tRNA through the ribosome is from right to left.

[3,4], as had been proposed by others [12,13], so we now know that there are binding sites for A, P and E tRNAs on both subunits (Fig. 1). The absence of footprinting evidence for the 30S E site could be explained by the fact that the E-tRNA interacts with the RNA backbone and r-proteins in the 30S subunit, which were not detectable by the base-specific probing methods used.

3. Hybrid states

Chemical footprinting was next used to monitor the progress of tRNA through the ribosome, relative to tRNA-protected bases on the 16S and 23S rRNAs (on the 30S and 50S subunits, respectively). The first experiment was simple and gave an unambiguous result [14]. *N*-acetyl-Phe-tRNA was bound to the ribosomal P site, as confirmed by its full reactivity with puromycin. The bases in 16S and 23S rRNA that were protected by the *N*-acetyl-Phe-tRNA could therefore be assigned to the 30S and 50S P sites, respectively. After reacting the complex with puromycin, the complex was again footprinted. The 16S rRNA footprint was virtually unchanged, but surprisingly, the 23S rRNA footprint was completely different. The 23S rRNA P-site bases were no longer protected, but a new set of bases, previously identified as E-site bases, became protected. Since only a single equivalent of tRNA was bound, it meant that the acceptor end of the tRNA had moved spontaneously from the 50S P site to the E site, while the anticodon end of the tRNA remained bound to the 30S P site. Accordingly, this was called the P/E hybrid state. Movement of the tRNA from the P/P state (the classical 'P site') to the P/E hybrid state occurred, at least under our in vitro conditions, in the absence of endocrine factor (EF)-G or GTP.

Complexes in which *N*-acetyl-Phe-tRNA was bound in the P site and aminoacyl-tRNA introduced to the A site showed a similar behavior. Following peptidyl transfer, the 50S A-site footprint disappeared, and an E-site footprint appeared, indicating that the two tRNAs had rearranged from their A/A and P/P states to the A/P and P/E hybrid states. Again, the rearrangement was independent of EF-G or GTP. A third complex, previously believed to result in binding of *N*-acetyl-Phe-tRNA to the A site, was also probed. Deacylated tRNA was first bound to ribosomes, followed by binding of *N*-acetyl-Phe-tRNA. The footprinting results showed A and P footprints on 16S rRNA and P and E footprints on 23S rRNA, again indicating binding in the A/P and P/E hybrid states [14].

It was then found that incubation of these hybrid-state complexes with EF-G and GTP resulted in footprints that indicated P/P and E binding. In other words, movement of the tRNAs with respect to the 30S subunit required EF-G and GTP, whereas movement with respect to the 50S subunit was spontaneous (Fig. 2). Movement of tRNA in two steps was first suggested by Bretscher [15], and one of the two models he proposed bears close resemblance to the one shown in Fig. 2.

The discovery of the P/E state offered a possible explanation for the existence of the E site. The affinity of the E site

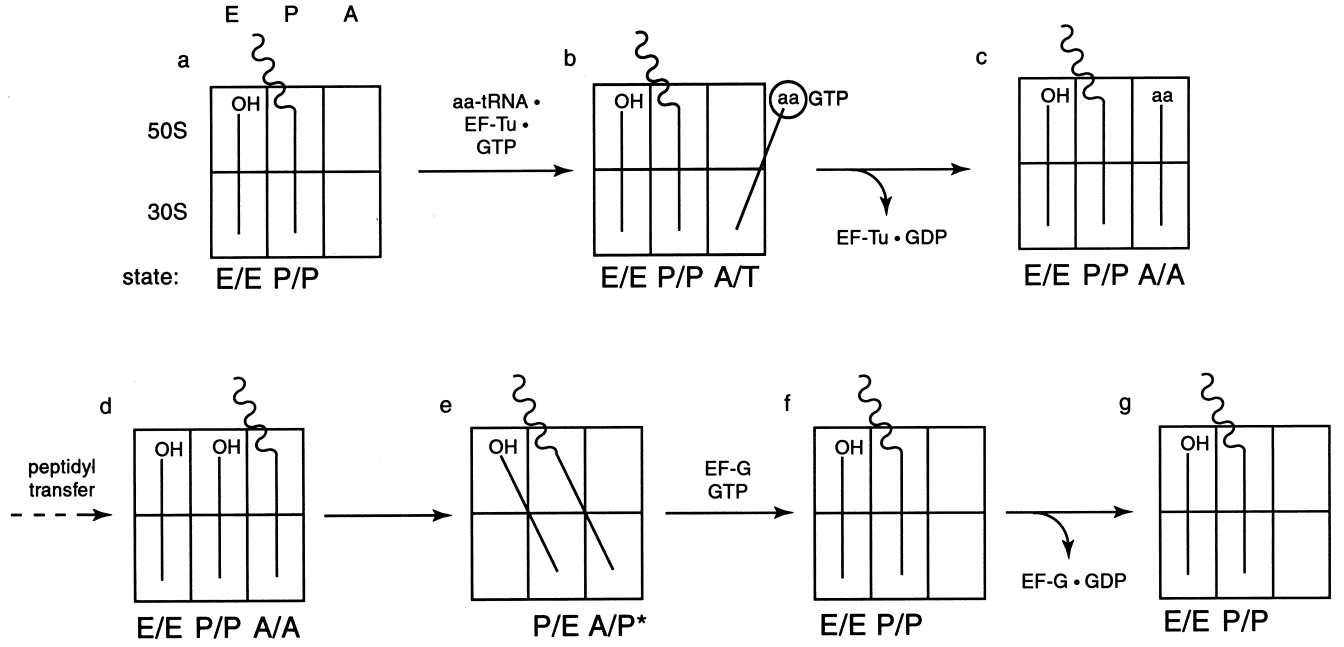

Fig. 2. Schematic representation of our current understanding of the main steps of the hybrid-states translocation cycle. The 70S ribosome is schematized as a rectangle, divided into 30S and 50S subunits, each of which has an A, P and E site. The tRNAs are shown as vertical lines, and the mRNA is not shown.

for the 3′-end of the deacylated tRNA [16] could provide the driving force for the spontaneous step of translocation from the P/P to the P/E state. Similarly, transfer of the peptidyl moiety to the aminoacyl-tRNA could provide the driving force for spontaneous translocation from the A/A to the A/P state, since the 50S P site has a specific affinity for the peptidyl moiety. Although it was not clear at the time whether the spontaneous movement occurred in a concerted fashion, simultaneously with peptide bond formation, or followed sequentially, there is now evidence that these two steps are sequential, as shown schematically in Fig. 2. First, an A-site tRNA analog covalently crosslinked to nucleotide G2553 in the 50S A site is fully active in the peptidyl transferase reaction, although the crosslink suggests that it would be unable

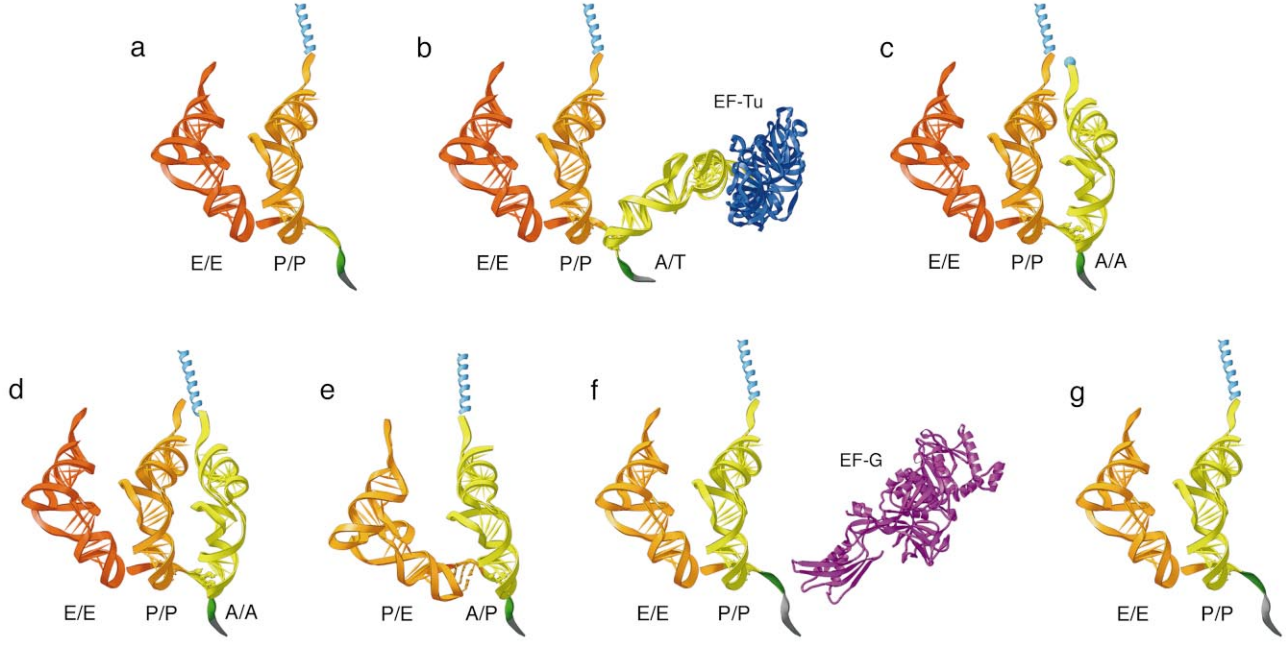

Fig. 3. Three-dimensional representation of the hybrid-states translocation cycle shown in Fig. 2. The positions of the A/A, P/P and E/E tRNAs and mRNA codons are as determined crystallographically [3,4], and the A/T, A/P and P/E states were modeled as described. The binding states of the tRNAs are indicated. The nascent polypeptide chain is shown as an α-helix (cyan) and the incoming aminoacyl group as a sphere (cyan).

to move into the P site [17]. In addition, reaction of f-Met-tRNA with puromycin, under certain in vitro conditions, has been observed to result in binding of the deacylated initiator tRNA in the P/P, rather than the P/E state [18].

A third type of hybrid state was observed when the EF-Tu–tRNA–GTP ternary complex was bound to ribosomes, stabilized either by using a non-hydrolyzable GTP analog, or in the presence of kirromycin [14,19]. In this state, termed the A/T state (T for EF-Tu), the tRNA footprint on 16S rRNA was identical to that observed for the A/A and A/P states, but no tRNA footprint was observed on 23S rRNA. Instead, protection of bases in the sarcin/ricin loop of 23S rRNA was found that was similar, although not identical, to that found for binding of EF-G [19]. This was interpreted to mean that EF-Tu prevents entry of the aminoacyl end of tRNA into the peptidyl transferase site during the tRNA initial selection process, while its anticodon is free to interact with the mRNA codon in the 30S subunit A site.

Independent evidence for the spontaneous rearrangement of tRNA from P/P to P/E states was obtained by Hardesty and co-workers [20]. Using fluorescence resonance energy transfer, they showed that the 5′-terminus of *N*-acetyl-Phe-tRNA moves by more than 20 Å toward protein L1, following reaction with puromycin, consistent with the crystallographically observed positions of tRNA in the 50S P and E sites [3,4].

4. Hybrid states in three dimensions

Three of the six binding states of tRNA shown in Fig. 2 are seen in the X-ray structures of the 70S ribosomal complexes (Fig. 1) [3,4]. They are the classical A, P and E states (called A/A, P/P and E/E in Fig. 2). Although ribosomal complexes containing tRNAs bound in hybrid states have not been solved so far by X-ray crystallography, structures containing tRNAs bound in states resembling the predicted properties of the A/P and P/E hybrid states have been observed directly in cryo-EM reconstructions [21–23].

Fig. 3 shows a representation of the hybrid-states translocational cycle of Fig. 2 in three dimensions. The positions of tRNA in the classical A/A, P/P and E/E states are as observed crystallographically [3,4]. The predicted positions of tRNAs in the A/T, A/P and P/E hybrid states were modeled in the 70S ribosome using a combination of crystallographic and biochemical constraints, maintaining the tRNAs as rigid bodies. The crystallographically observed positions of the tRNAs in their classical A/A, P/P and E/E states provide the magnitudes of the overall distances moved by a tRNA as it traverses the ribosomal interface. Some estimate of the distances moved by tRNA in each step of the translocation cycle can be predicted from the modeled hybrid-states positions (Table 1) (given that the hybrid-states positions are modeled, rather than experimentally observed, the values given should be taken as approximate). The largest movements are clearly that of the acceptor arm in the A/T to A/A transition, and the elbow in the P/P to P/E transition, both of which amount to more than 50 Å.

The A/T state was modeled by superimposing common features of the respective G domains of EF-Tu and EF-G, which was docked in the 70S structure using constraints obtained by chemical footprinting and directed hydroxyl radical probing [24]. Orientation of the EF-Tu–GTP ternary complex was then adjusted to juxtapose the anticodon of A/T tRNA with its A-site codon. The A/P state was modeled by pivoting the A/A tRNA slightly around the middle of its anticodon, to orient its CCA end toward the 50S P site. The P/E state was modeled in a similar way, pivoting around the middle of the P/P tRNA anticodon, to bring its CCA end into contact with the 50S E-site pocket. All three of the hybrid-states conformations resemble those observed by cryo-EM reconstructions [21–23]. However, cryo-EM studies have not so far shown tRNA bound in the crystallographically observed E/E state; instead, an additional state, in which the tRNA appears to be bound to the 50S, but not the 30S, E site is found.

The modeling exercise raised a number of interesting questions. Although it was straightforward to place the two ends of the A/T tRNA in the 30S A site and in complex with EF-Tu, respectively, the large angle of rotation between the A/T and A/A states made it necessary to re-orient the A codon by rotation around the phosphodiester linkage between the A and P codons, relative to that of the classical-state codons [25,26]. The resulting orientation of the A/T codon is in a position that is mid-way between that of a continuation of the A-form path of the P codon and that of the A codon. This predicts that the deviation of the geometry of the junction between the A and P codons from that of A-form RNA increases progressively as the tRNA moves from the A/T to the A/A state. In contrast, modeling the hybrid-state A/P and P/E codons required only modest movements of the mRNA backbone from the observed A and P codon positions.

Surprisingly, it was not possible to simultaneously satisfy the A/P state constraints by rigid-body modeling of the tRNA in a rigid ribosome, even though the distance between the 50S A and P sites is relatively small. If the tRNA is pivoted around its anticodon in the 30S P site, its CCA end falls about 7 Å short of reaching its 50S P-site contacts. Thus, the conformation of either the tRNA or the ribosome (or both) is likely to change in undergoing the A/A to A/P transition.

In contrast, the extremities of the P/E tRNA can be placed simultaneously in the 30S P and 50S E sites, even though the P/P to P/E movement is much greater than the A/A to A/P movement (Table 1). However, the resulting position of the elbow of P/E tRNA is about 20 Å from the part of the L1 region of the 50S subunit that it must contact to satisfy the biochemically observed P/E interactions with 23S rRNA. A likely explanation is that the L1 region itself is re-positioned in the hybrid state, consistent with its disorder in the *Haloar-*

Table 1
Predicted distances between tRNA hybrid-states positions (Å)

tRNA position (nucleotide number)	A/T to A/A	A/A to A/P	A/P to P/P	P/P to P/E	P/E to E/E
Anticodon (34)	9	1	21	1	19
Elbow (56)	31	26	21	58	22
Acceptor arm (72)	56	14	8	34	15

cula marismortui 50S subunit structure [27], and with its dramatically shifted location in the *Deinococcus radiodurans* 50S crystal structure [28]. Movement of this region of the ribosome would explain the observed enhanced reactivity toward dimethyl sulfate of nucleotide A702 in hybrid-states complexes. In the crystallographically observed classical state, A702 contacts helix 68 of 23S rRNA, which contacts the L1-region RNA; if coupled movement of the L1 RNA and helix 68 accompany hybrid-states formation, contact with A702 in the interface bridge B7a would be disrupted. Previously, we noted that the deacylated tRNA, bound in the E/E state, is trapped inside the ribosome by the surrounding structures of the 30S platform and head, and the L1 region of the 50S subunit [4]. Movement of the L1 region could thus explain how the deacylated tRNA escapes from the ribosome.

5. The molecular basis of translocation

What is the source of the molecular movement inherent in the translocation process? This question can be broken into two parts: What provides the energy for the movement, and what are the moving parts of the machine? A further question is, how is translocation coordinated and regulated – i.e. how is movement of tRNA coupled to mRNA movement, and how is the translational reading frame accurately maintained while they move? And, how does the ribosome (and EF-G) know when to translocate? Finally, what constrains the directionality of tRNA movement?

Since it has been shown that translocation can occur spontaneously [29], the energy for movement need not come from GTP. This implies that each sequential binding state must have a progressively lower free energy. Movement of tRNA through the 50S subunit can be explained by the different substrate specificities of the 50S A, P and E sites for the different chemical states of the acceptor end of tRNA. The 50S A site is specific for the aminoacyl group, the P site for the peptidyl group and the E site for a free, deacylated 3′ end. Thus, when the aminoacyl-tRNA becomes a peptidyl-tRNA, its movement into the 50S P site is energetically favored. And when peptidyl-tRNA becomes deacylated following peptide bond formation, its affinity for the E site provides the energy for movement. In both cases, the energy comes ultimately from the chemical step of peptide bond formation – in other words, the difference in free energy between the peptidyl-tRNA ester bond and the resulting peptide bond, on the one hand, and that of the deacylated tRNA on the other. At the same time, the substrate specificities of the three 50S binding sites constrain the directionality of the movement, from A to P to E.

Movement in the small subunit is more complicated. First, it must ensure coupling of mRNA movement to tRNA movement, and second, except in special circumstances [29], it requires participation of EF-G. The fact that it *can* proceed spontaneously, suggested to Spirin [1,30] that the requirement for EF-G and GTP is to overcome a kinetic barrier, which he described as a 'locking and unlocking' of the ribosome that occurs cyclically during elongation. This poses a question, so far unanswered: what is the molecular basis of locking and unlocking? And, what does EF-G actually do when it breaches the kinetic barrier? There is accumulating evidence that the true substrate for EF-G is the hybrid-state ribosome. The original hybrid-states experiments showed that, although movement with respect to the 50S subunit could proceed spontaneously, movement on the 30S subunit required EF-G and GTP [14]. There is increasing evidence that an important role of the E site is to promote translocation, first by allowing movement on the 50S subunit into the A/P and P/E states, and second by promoting the second, EF-G-dependent step.

Feinberg and Joseph [31] have shown that the presence of a single methyl group on the 2′-hydroxyl of nucleotide 71 of peptidyl-tRNA prevents EF-G-catalyzed translocation. The only contact observed between ribose 71 and the ribosome is with the minor groove of helix 68 in the 50S E site [4]; therefore, the observed inhibition of translocation must be caused by defective interaction of the hybrid-state P/E tRNA with the 50S E site. Interestingly, the ribose 71 contact with helix 68 is a short distance away from the interaction made by A702 of 16S rRNA that becomes disrupted in the hybrid state, as discussed above. It is difficult to explain the effect of methylation of ribose 71 on the action of EF-G in a direct way, since its interaction with the ribosome is about 70 Å away from the closest approach of EF-G, and about 100 Å from the GTPase center of EF-G. Methylation of a single ribose either prevents movement of the tRNA into the P/E state, or the presence of a free 2′-hydroxyl group at position 71 of P/E tRNA can be detected over a very large distance through the ribosome structure.

Earlier studies by Wintermeyer and co-workers [16] showed that modifications of the 3′ adenosine that decrease the affinity of deacylated tRNA for the E site interfere with EF-G-dependent translocation. These results can be interpreted as interfering with movement of tRNA into the P/E state (and, therefore, into the A/P state). More recently, studies from the Wintermeyer laboratory have shown that movement of tRNA from the A/A to the A/P state dramatically decreases both the affinity of the tRNA for the ribosome and the activation energy for EF-G-catalyzed translocation [32]. These findings suggest that the affinity of tRNA and its associated mRNA codons for the 30S subunit presents an energy barrier to movement between the 30S A and P sites, and that the lowered affinity resulting from movement into hybrid states reduces this barrier, promoting translocation. Dependence on hybrid-states formation would also ensure that EF-G-catalyzed translocation is coordinated with peptide bond formation.

Coupling of tRNA movement with mRNA movement is one of the most important aspects of the translocation mechanism, and perhaps the least well understood. Failure of this process will result in a shift in the translational reading frame, almost always causing premature termination at an out-of-frame stop codon. An important question is whether the 30S translocation mechanism acts on the mRNA, the tRNA or both. Spirin and co-workers showed that EF-G-dependent translocation can occur in the absence of mRNA, demonstrating that the mechanism must act directly on the tRNA, rather than indirectly through the mRNA [33]. The remaining issue, then, is whether it also acts directly on the mRNA, or whether the mRNA is dragged through the translocational cycle by virtue of its base pairing with tRNA. Since codon–anticon pairing is stabilized by interactions with the ribosome, the latter option would introduce the danger of frame-shifting during translocation, if contacts with the codon and anticodon are not simultaneously maintained.

It therefore seems most unlikely that movement of parts of

the ribosome would not accompany movement of tRNA and mRNA. But, movement of structural elements of the ribosome would require some sort of ratcheting event, in order to begin the next round of translocation; contacts between the ribosome and tRNA/mRNA would need to be disrupted at the end of each round of translocation. To avoid disruption of interaction with the ribosome, there would therefore need to be some sort of redundancy in ribosomal contacts with mRNA and tRNA.

What are the moving parts of the machine? Some likely candidates have already been identified. The head, platform and penultimate stem of the 30S subunit, and the bridges B1a and B1b, the L1 ridge, the L11 region, and helices 68 and 69 of 23S rRNA in the 50S subunit have all been implicated repeatedly as dynamic elements of the ribosome. Earlier work implicated protein L7/L12 in tRNA movement associated with EF-Tu and EF-G [34]. Moreover, the subunits themselves appear to move with respect to one another during translocation [35]. A further, even more daunting problem is how movement of all these different molecular components is coordinated.

Ultimately, the answers to these questions will appear in the form of a 'movie' at atomic resolution. Although we are still far from being able to view the high-resolution movie, cryo-EM reconstruction experiments have already been carried out on ribosomal complexes trapped in many different functional states [21–23,35–42]. The low-resolution movies that are emerging from these studies (see, for example, [35]) will provide a preview to the ultimate description of this ancient molecular machine.

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