ARTICULO DE REVISION

Studies of accuracy during protein synthesis involving the E-tRNA and the Shine-Dalgarno interaction

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ABSTRACT

The ribosome contains three tRNA binding sites, the A, P and E sites. Although the E site is separated from the A *via* the intervening P site, there is a striking communication between these sites. This cross-talk plays an important role for the accuracy of the decoding process. Codon-anticodon interaction at the E site seems to be the signal to switch into the post-translocational (POST) state characterized by a low affinity of the A site. This low affinity state forces the ternary complexes aminoacyl-tRNA•EF-Tu•GTP to enter the A site *via* the decoding center preventing the selection of non-cognate aminoacyl-tRNAs and incorporation of the amino acid. This has the important consequence that only 1 in 400 mis-incorporations affects protein functions. Another aspect of the three tRNA binding sites is that at least two tRNAs are present on the ribosome. Since the tRNAs are firmly bound by the ribosome whereas the mRNA practically only *via* codon-anticodon interaction during the elongation phase, the movement of the tRNA during translocation pulls the mRNA through the ribosome. In fact, the six base pairs of two adjacent codon-anticodon interactions are instrumental for maintaining the reading frame. Without the codon-anticodon interaction of the E tRNA the reading frame would be lost at least after the incorporation of about 50 amino acids into the nascent chain.

Key words: Ribosome, tRNA, E-site, Shine-Dalgarno

RESUMEN

Estudios de fidelidad durante en la síntesis de proteínas que involucran al ARNt unido al sitio E y la interacción Shine-Dalgarno

El ribosoma contiene tres sitios de unión para ARNt, los sitios A, P y E. Aunque el sitio E está separado del sitio A por el sitio P, hay una importante comunicación entre ambos. Esta comunicación juega un papel importante en la exactitud de los procesos de decodificación. La interacción codón-anticodón en el sitio E parece ser la señal para cambiar al estado post-translocacional (POST) caracterizado por una baja afinidad del sitio A. Esta baja afinidad determina que los complejos ternarios aminoacil-ARNt•EF-Tu•GTP solo puedan entrar al sitio A por el centro de decodificación previniendo la selección de aminoacil-ARNts no-complementarios al codón del sitio A y la incorporación de un amino ácido incorrecto. Como consecuencia importante, solo una en 400 incorporaciones erróneas afecta la función de la proteína sintetizada. Otro aspecto de los tres sitios de unión para ARNt es que al menos dos ARNts

están presentes en un ribosoma activo. Puesto que durante la elongación los ARNts están fuertemente unidos al ribosoma mientras que el ARNm lo está solo por las interacciones codón-anticodón, el movimiento de los ARNts durante la translocación determina el correspondiente movimiento del ARNm a través del ribosoma. De hecho, los seis pares de bases de las interacciones codón-anticod-on adyascentes son esenciales para el mantenimiento del marco de lectura. Sin la interacción del ARNt-E el marco de lectura probablemente se perdería después de la incorporación de aproximadamente 50 aminoácidos en la cadena polipeptídica naciente.

Palabras clave: Ribosoma, ARNt, sitio E, Shine-Dalgarno

INTRODUCTION.

All ribosomes have three tRNA binding sites

During the elongation phase, the central functional phase of the ribosome, a tRNA moves through all three tRNA binding sites on the ribosome. Our current understanding of the elongation cycle is pictured in **Figure** 1 (for review see 1).

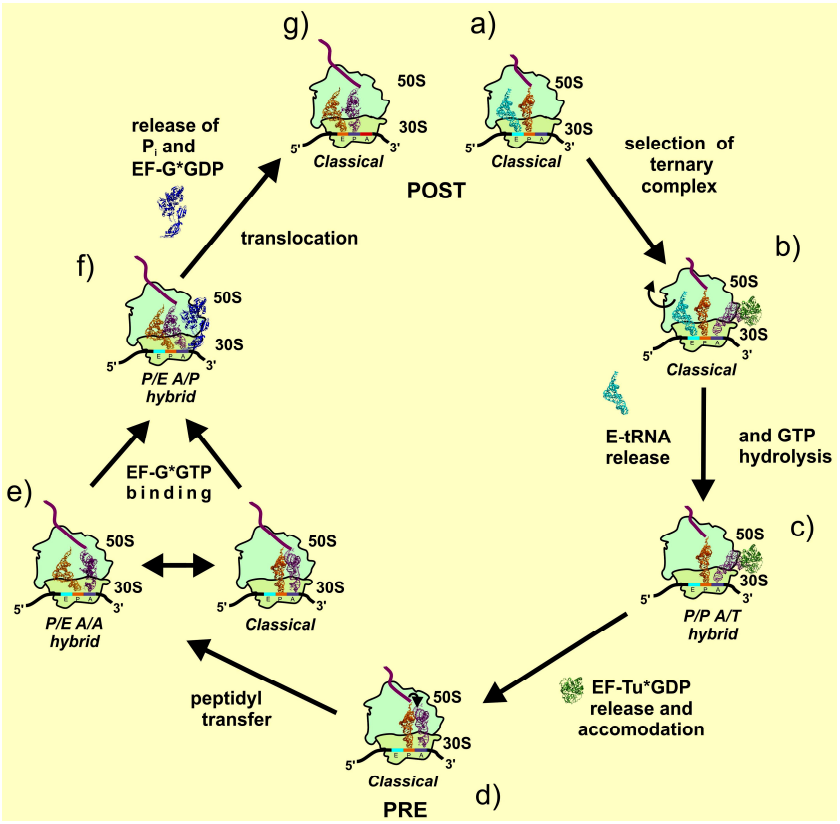

Figure 1: The elongation cycle of protein synthesis. For explanations see text.

We start with a ribosome carrying already two tRNAs, a peptidyl-tRNA at the P site (P for peptidyl-tRNA) with the already synthesized nascent peptide and a deacylated tRNA at the E site (E for exit). This ribosome state is called post-translocational state or POST state (Figure 1a). An aminoacyl-tRNA enters the ribosome at the A site, A for aminoacyl-tRNA (aa-tRNA). It binds to this site in the form of a ternary complex aa-tRNA•EF-Tu•GTP containing EF-Tu, one of

the two universal elongation factors, both being G-proteins, and is selected at the decoding center of the A site according to the codon of the mRNA exposed at this site (Figure 1b). Decoding occurs with the ternary complex allowing codon-anticodon interaction at the A site but preventing most of the contacts of the aa-tRNA with the A site outside the decoding centers. After this step three tightly coupled steps happen (Figure 1c and d): (i) The ribosome triggers the EF-Tu GTPase activity and EF-Tu•GDP having changed its conformation from the GTP to the GDP conformer falls off the ribosome. (ii) The E site releases the deacylated tRNA from the ribosome, and (iii) the aa-tRNA moves fully into the A site (accommodation). Now the ribosome is in the pre-translocational state (PRE-state) characterized by two tRNAs at the A and P sites.

The peptidyl-residue is transferred from the P-tRNA (the tRNA in the P site) to the adjacent aminoacyl-tRNA yielding the peptidyl-tRNA at the A site prolonged by one amino acid and leaving a deacylated tRNA at the P site (Figure 1e). This PRE state seems to be in equilibrium with a state, where the tRNA can move already to the E site on the 50S subunit but maintaining codon-anticodon interaction still on the 30S subunit in the P site (2), a tRNA position termed P/E hybrid site. Next the second elongation factor EF-G•GTP binds and brings the A-tRNA (tRNA at the A site) into a hybrid position A/P (3). The energy for this tRNA movement might be paid by the binding energy of this large factor (Figure 1f). The tRNA movement is accompanied or caused by a ratchet movement (forward ratcheting) of the 30S subunit by about 4° relative to the 50S subunit (4,5). After the ribosome has triggered the GTP hydrolysis, the P_i release triggers the release of EF•GDP from the ribosome and the full translocation of the two tRNAs into the classical P and E sites (back-ratcheting; POST state; Figure 1g). The ribosome is now ready to enter the next round of the elongation cycle

Features of the E site. As the description of the elongation cycle in the preceding section implies the three classical ribosomal tRNA binding sites A, P and E are characterized by a strikingly different capacity to bind different kinds of tRNAs: (i) The A site binds aminoacyl-tRNA and peptidyl-tRNA in each elongation cycle and deacylated tRNA only under defined stress conditions, the stringent response. (ii) The P site accepts deacylated tRNA and peptidyl-tRNA during an elongation cycle and aminoacyl-tRNA in the form of initiator Met-$tRNA_i^{Met}$ during eukaryotic and archaeal initiation. (iii) The E site is most restricted in this respect, it binds exclusively deacylated tRNA. The structural reason is a hydrogen-bond network around the ultimate A of an E-tRNA, where the residue C2394 plays a central role (Figure 2). This residue is located at the 3'-base of helix H88, and whenever this helix-landmark is present in the 23S-type rRNA the corresponding C residue is observed including mitochondrial ribosomes. At the other tip of the L-shaped tRNA, i.e. the anticodon site, the situation is clearer: tRNA in all three sites and their hybrid variations undergo codon-anticodon interactions.

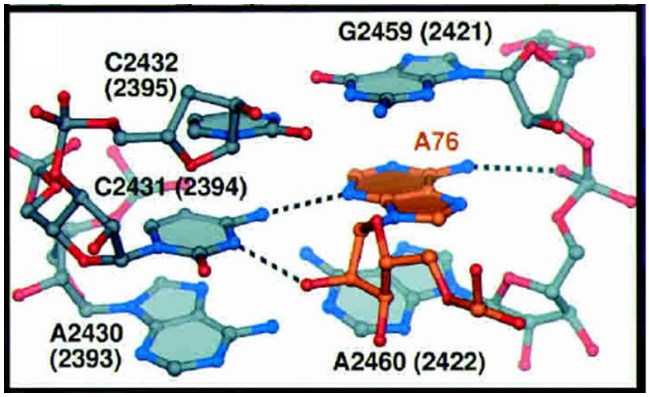

Figure 2: The ultimate residue A76 of the E-tRNA stacks in between 23S Rrna nucleotides G2421 and A2422 (*E. coli* nomenclature), and hydrogen bonds with universally conserved C2394. Taken from Schmeing et al. (66).

Codon-anticodon interaction at the A site has to occur, since at the decoding site – a part of the A site – codons of an mRNA are deciphered. In the seventies codon-anticodon interaction at the P site was still a controversial issue, and could be settled only in 1979 (6,7). Shortly after the E site was detected (8,9), but only after about ten years later it became slowly accepted by the scientific community, when its existence was demonstrated also in archaeal and eukaryotic ribosomes (10 and 11,12, respectively). However, the existence of codon-anticodon interaction continued to be controversially discussed, and the issue was only recently settled, when previous biochemical evidence (12,13) could be confirmed by genetic and structural data (14,15,16).

Although the E site is separated from the A site *via* the intervening P site, there is a striking communication between these sites. This cross-talk plays an important role for two accuracy aspects:

(i) Codon-anticodon interaction at the E site seems to be the signal to switch into the post-translocational (POST) state characterized by a low affinity of the A site (17). As we will point out later, the low affinity A site is instrumental for both an accurate aminoacyl-tRNA selection and prevention of incorporating an amino acid from a non-cognate aminoacyl-tRNA; the chemical nature of which is sharply different from the cognate one and thus would likely affect folding, stability and function of the mature protein. If the E site is so important for accuracy of decoding, there should be an accuracy problem at the codon just following the initiation codon AUG, where the initiator (f)Met-tRNA is present at the P site and the E site is free. How the bacterial ribosome solves this problem is discussed later.

(ii) A and E sites interact in a sense of negative cooperativity with each other (18,19,12). This means that in addition to the already low affinity state of the A site triggered by the E-tRNA also an occupation of the A site induces a low-affinity state of the E site leading to a loss of the E-tRNA from the ribosome (20). A consequence of this interaction is that on average two tRNAs are on the ribosome, either at A and P site in the pre-translocational (PRE) state or in at P and E sites in the POST state (21,22). Since the tRNAs are firmly bound by the ribosome, whereas the mRNA is fixed only *via* codon-anticodon interaction during the elongation phase (23), the movement of the tRNA during translocation pulls the mRNA through the ribosome. In fact, the six base pairs of two adjacent codon-anticodon interactions are instrumental for maintaining the reading frame, without the codon-anticodon interaction of the E tRNA the

reading frame would be lost at least after the incorporation of about 50 amino acids into the nascent chain (24).

A cognate E-tRNA prevents incorporation of non-cognate aminoacyl-tRNAs The binding of aa-tRNAs to the ribosome is dictated by the complementarity between the anticodon of the tRNA and the codon of the mRNA. To ensure the high fidelity of translation, the correct stereochemistry of the mRNA-tRNA codon-anticodon interaction is monitored by components of the small ribosomal subunit, in a process known as decoding (reviewed in 25).

During decoding, the first and second nucleotide positions (in terms of the codon) of the mRNA-tRNA duplex are closely monitored, whereas interaction at the third or wobble position is less strictly recognized. Consistently, the mis-incorporation of the wrong amino acids into polypeptide chains usually occurs through the binding of near-cognate aa-tRNAs, i.e. those tRNAs carrying an anticodon similar to that of the cognate aa-tRNA, rather than non-cognate aa-tRNAs, which carry dissimilar anticodons.

Nascent polypeptide chains are surprisingly tolerant to mis-incorporation, with only one in ~400 mis-incorporations being deleterious for the protein's activity (reviewed in 26). The reason for this is that usually near-cognate aa-tRNAs are selected instead of the cognate aa-tRNA, and the genetic code lexicon is organized in such a way that near-cognate tRNAs bear amino acids that are chemical similar to those carried by the cognate tRNA. For example, the mis-incorporation of an aspartate (codon: GAU/C) by near-cognate Asp-tRNA, instead of glutamate (GAA/G) by the cognate Glu-tRNA, both incorporate acidic amino acids. The middle and, also in most cases, the first position of a codon are almost never misread, even under error-inducing conditions such as high magnesium or the presence of aminoglycosides, and are thus considered as being non-cognate (for review and references see 27). The terms cognate, near-cognate and non-cognate are also defined functionally, viz. the mis-incorporation of near-cognate amino acids *in vivo* and *in vitro* require higher GTP consumption than for cognate, whereas non-cognate amino acids are never incorporated and no GTP is consumed (17,28), or if incorporation is observed, the rate is greatly reduced (29,30).

Aa-tRNAs can, however, occupy the A site without being subjected to the decoding process. For example, the tRNA moiety of Ala-tmRNA does not even have an anticodon, but still binds efficiently to the A site in complex with EF-Tu•GTP and the SmpB protein (31). Another example is when the ribosome has an empty E site, i.e. a peptidyl-tRNA occupies the P site while the A and E sites are free. In such a situation, even a non-cognate aa-tRNA can enter the A site leading to an incorporation of the non-cognate amino acid into the nascent peptide chain (32,17). After translocation a peptidyl-tRNA resides at the P site and the E site is tightly occupied by a deacylated tRNA (18,24). The E-tRNA is released through an active mechanism, whereby interaction of a ternary complex aminoacyl-tRNA•EF-Tu•GTP at the A site is coupled to the release of the E-tRNA (19,12). E-tRNA release follows the decoding step, but occurs before accommodation of the aa-tRNA into the A site (20).

The presence of an E-tRNA has been shown to be important for - as mentioned above - preventing the selection of non-cognate aa-tRNAs. In the latter experiment, Geigenmüller et al. (17) demonstrated that when the E site was unoccupied, the non-cognate acidic Asp (codon GAC/U) could be mis-

incorporated in place of the cognate aromatic hydrophobic Phe (codon UUU/C), however, no mis-incorporation of Asp was observed when the E site was occupied. Since tRNA near-cognate to the E site could not prevent the incorporation of a non-cognate amino acid, the conclusion was that codon-anticodon interaction at the E site is required to prevent mis-incorporation at the A site (17). This is consistent with the genetic (33,16), biochemical (34,35,12), and structural evidence (14) demonstrating the likelihood of codon-anticodon interaction at the E site.

We note that the recognition of the codon-anticodon duplex in the E site is very different from that at the decoding site in the A site. In the A site the correctness of the Watson-Crick base pairs is checked, i.e. whether or not the bases and sugars are aligned according to a correct base pair. Reference of this checking process are for example the 2'OH groups of the pairs in the first and second position of the codon (25); **Figure 3**.

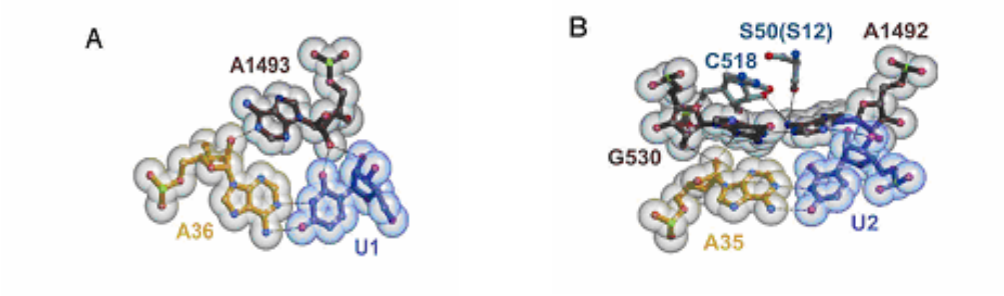

Figure 3: Codon-anticodon interaction at the decoding center of the A site in the first two positions of the codon. A, in the first position, A1493 binds in the minor groove of the A36-U1 base pair. B, in the second position, G530 and A1492 act in concert to monitor the A35-U2 base pair. According to Ogle et al. (67).

For example, the universally conserved residue A1493 flips into the shallow grove of the first base pair of codon-anticodon and forms H-bridges with the 2'OH groups of the participating nucleotides. Non-Watson-Crick pairs would form H-bridges of lower energy if at all thus defining the recognition mode. The middle base-pair is even checked with an higher effort involving A1492, G530 and the Ser50 residue of the ribosomal protein S12. Giving a structural basis for the known fact that the middle position is practically never misread, the first position very rarely if at all, and quite often the third position (wobble position), where more space provides more freedom to accommodate incorrect base pairings. It follows that the kind of base pair of the type A:U or G:C is of now importance, a surprising observation 25 years ago (36). However, this is precise the recognition mode of the E site, since it was demonstrated that the stability of the codon-anticodon interaction influencing the affinity of the E-tRNA is reversely proportional to the accuracy at the A site (16,15).

A more recent demonstration of preventing the incorporation of non-cognate amino acids by an occupied E site is shown in **Figure** 4 (32). *E. coli* 70S ribosomes carried an AcPhe-tRNA at the P site and displayed an AAA codon. A stoichiometric mixture of the cognate basic hydrophilic Lys-tRNA and the non-cognate hydrophobic Leu-tRNA (codon UUA/A) was added and the dipeptides formed identified *via* HPLC chromatography. In the absence of an E-tRNA a significant amount of the deleterious dipeptide AcPhe-Leu is observed, whereas in the presence of an E-tRNA only the cognate product AcPhe-Lys was found

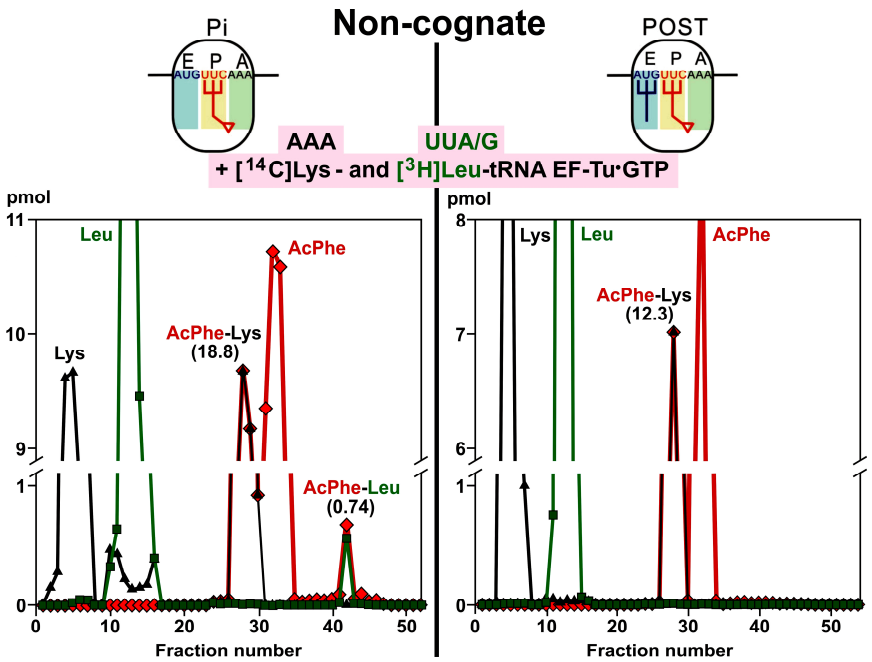

Figure 4: Noncognate misincorporation levels. The influence of the E-tRNA: HPLC analysis of dipeptides formed by the addition of a stoichiometric mixture of ternary complexes containing cognate [^{14}C]Lys-tRNA and noncognate [^{3}H]Leu-tRNA to either (i) Pi-state ribosomes (Left) containing AcPhe-tRNA at the P-site or (ii) POST-state ribosomes (Right) carrying AcPhe-tRNA at the P-site and deacylated [^{32}P]tRNAfMet at the E-site, generated via EF-G-dependent translocation. The codons are given above the amino acids. According to Di Giacco et al. (32).

So how does the presence of an E-tRNA influence decoding at the A site? An occupied E site dramatically increases (almost three-fold) the activation-energy barrier for A-site occupation, namely from ~40 kJ/mol to ~115 kJ/mol (in a physiological buffer with 3-6 mM Mg2+ and polyamines (37). These findings were incorporated into the allosteric three-site model (28,18,12) stating that the A and E sites are reciprocally linked, such that occupation of the E site induces a low-affinity A-site, and vice versa. This model explains, why in native polysomes from both eukaryotes and bacteria precisely two tRNAs per ribosome are observed (21,22).

The next question is how the low-affinity A-site excludes the selection of non-cognate aa-tRNAs? One possibility is that the low-affinity A-site restricts the binding of the ternary complex (aa-tRNA•EF-Tu•GTP) with the ribosome to only the interaction between the A-site codon and the anticodon of the tRNA, until successful decoding is completed. In this model, contacts outside of the codon-anticodon interaction would not contribute to the selection precision because they are common to all ternary complexes, regardless of cognate or non-cognate, and therefore would allow even non-cognate aa-tRNAs to interfere with the selection process, as well as leading to the occasional mis-incorporation before the decoding potential of the codon-anticodon interaction has been exploited (38). Indeed, cryo-electron microscopic (cryo-EM) studies reveal that during A-site decoding the incoming ternary complex aa-tRNA•EF-Tu•GTP binds in an initial A/T state, where codon-anticodon interaction is checked in the decoding centre of the A site before the aa-tRNA fully moves into the classic A site (39,40,3). Interestingly, the anticodon loop is kinked

relative to the anticodon stem by ~40° to allow decoding, while simultaneously preventing interaction of the tRNA outside the anticodon loop with the A site **(Figure 5, A-D).**

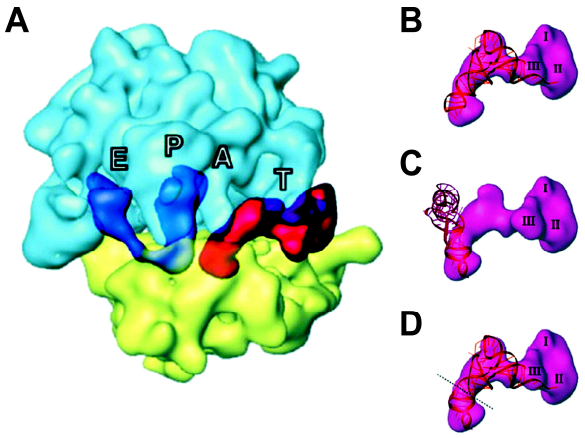

Figure 5: The ternary complex interacting with the decoding center of the A-site as seen by using cryo-EM. (A) The ribosome position of the ternary complex during the decoding process (A/T-site). (B–D) Fitting the aminoacyl- tRNA within the ribosomal-bound ternary complex. To satisfactorily fit the crystal structure of a tRNA into the corresponding cryo-EM density requires the introduction of a kink codon stem of the aminoacyl-tRNA. (According to Valle et al. (40), modified)

However, EF-Tu interaction with the ribosome visualized in these complexes probably reflects a state after the decoding process has been completed, and therefore it is unclear whether EF-Tu interacts with the ribosome prior to or during the selection process. We assume that EF-Tu contacts the ribosome only after the decoding process (38), but we note that this point remains controversial (29,30).

Since non-cognate aa-tRNAs in the cell are in five- to ten-fold excess over a cognate aa-tRNA, it is clear that the absence of the beneficial effects of cognate E-tRNA and in particular its codon-anticodon interactions would practically exclude the synthesis of a protein of a length of about 400 amino acids with an undisturbed structure and function.

Shine-Dalgarno sequence can take over the function of the E site. If an occupied E site is important for translational fidelity by reducing near-cognate or preventing non-cognate mis-incorporation at the A site, as explained by the allosteric three-site model, this raises the question as to how accuracy is maintained when the first aa-tRNA binds to the A site directly following the initiation phase. This is a unique situation, in which ribosomes contain only one tRNA, namely an initiator-tRNA bound at the P site, referred to as a Pi state. Therefore, directly following initiation the binding of ternary complex to the ribosome and decoding at the A site occurs with an empty E site and according to the allosteric three-site model, should be error-prone. This would be surprising, since there is a strong codon bias at the second position for GCN codons in highly expressed genes (41), i.e. the codon directly following the start codon, and this position has been shown to have a strong influence on the efficiency of translation initiation (42). Indeed, stable cognate codon-anticodon interaction at this position has been proposed to be important for preventing premature peptidyl-tRNA drop-off (41). Furthermore, the first few N-terminal

amino acids modulate the stability of proteins as well as providing determinants for the cleavage of the N-terminal formyl-methionine residue from nascent peptide (42,43). Collectively, this suggests that accurate decoding at the second position is important for gene expression and therefore bacteria must have developed a mechanism to ensure accurate decoding at the A site in the absence of an E-tRNA.

Recently, we have demonstrated that the presence of a Shine-Dalgarno (SD) sequence located in the 5' untranslated region of an mRNA can functionally compensate for the lack of a cognate tRNA at the E site, a situation that occurs directly following the initiation phase of translation. Ribosomes carried an fMet-tRNA and exposed a GUA codon at the A site cognate for Val-tRNA (**Figure** 6). Similar to the above described experiment in Figure 4 a stoichiometric mixture of cognate hydrophobic Val-tRNA and non-cognate acidic hydrophilic Asp-tRNA were added. The HPLC analysis revealed that the presence of the Shine-Dalgarno interaction does suppress the formation of non-cognate fMet-Asp in a similar way then the presence of an E-tRNA (32).

We thus demonstrated that the SD sequence confers similar beneficial effects as an E-tRNA, in terms of accuracy during the selection of ternary complexes aa-tRNA•EF-Tu•GTP at the decoding center: The selection of the near-cognate aa-tRNA is moderately improved by a factor of two (32), but - most significant – the mis-incorporation of detrimental non-cognate amino acids is abolished. [Figure 6]

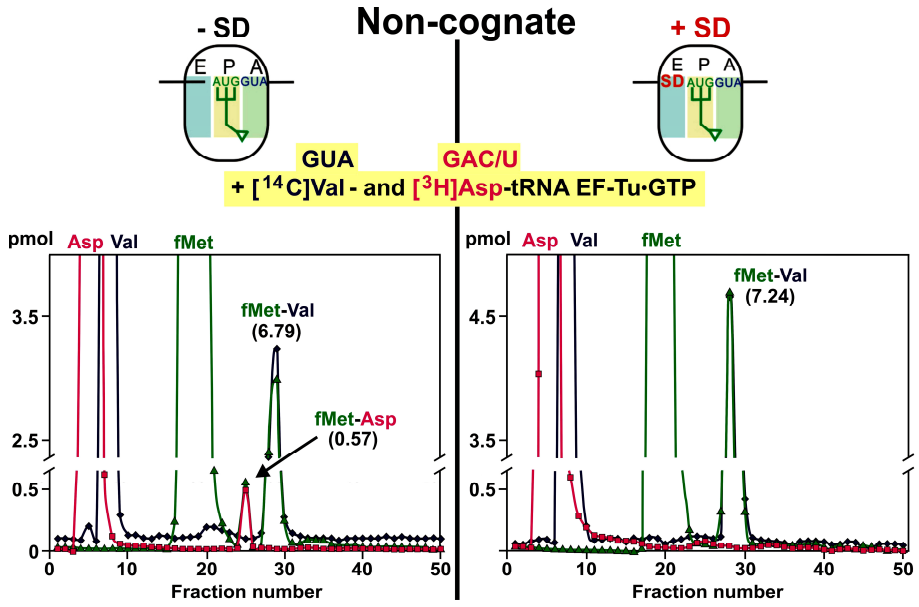

Figure 6: Noncognate misincorporation levels. The influence of SD on selection of noncognate aminoacyl-tRNA in the presence of MVF-mRNA: HPLC analysis of dipeptides. After filling the P-site with fMet-tRNA, a mixture of ternary complexes was added containing cognate [^{14}C]Val-tRNA (codon GUA) and noncognate [^{3}H]Asp-tRNA (GAC/U). In the absence of the SD sequence, an error of 7.7% was observed (Left), whereas in its presence, a significant amount of noncognate fMet-Asp is not observed (Right).

Recent X-ray crystallography studies have visualized the interaction of the SD sequence with the anti-SD sequence located in the 3´-end of the 16S rRNA on the ribosome (14,45-47). These studies reveal that the SD-helix sits in a pocket located between the head and platform of the 30S subunit, adjacent to but not directly in the E site. The SD-antiSD interaction probably reduces the time necessary for mRNA-ribosome programming, since it helps to guide the mRNA from an initial stand-by site into a position whereby the AUG start codon is correctly positioned in the presence of initiator tRNA (48,49,45). Interestingly, the conformation of the mRNA in the E site appears to be influenced by the state of the ribosome. In the initiation state, the mRNA is considered to be in a structurally constrained conformation, such that codon-anticodon interaction would not be possible in the E site (14,47).

However, following initiation the whole SD-helix rotates on the ribosome towards the E site, which leads to conformational relaxation in the mRNA, such that the A-form helix adopted by the E-codon of the mRNA that now allows codon-anticodon interaction at the E site (14). The recent observation that the SD helix appears to fix the orientation of head of the 30S subunit (46) might provide the first structural hint as to how the SD helix (or E-tRNA) influences A site accuracy, however further work utilizing both in vitro and in vivo experimental systems will be required to fully elucidate this mechanism.

We do not know if or how the eukaryotic 80S ribosomes overcome the accuracy problem during the first decoding step, since their mRNAs do not contain SD sequences. Eukaryotic translation systems require about 12 initiation factors, some of which are composed of several different subunits (for reviews see 50,51), whereas bacterial systems use only three monomeric initiation factors. Thus, we can only speculate that the more complicated system required for the formation of both the 40S and subsequent 80S initiation complexes solves the accuracy problem of the first aa-tRNA selection.

Finally, archaeal mRNAs often contain an identifiable SD, but their set of initiation factors is similar - although somewhat simpler – to that in eukaryotes (52). Curiously, archaea contain a number of leaderless mRNAs, i.e. mRNAs that have no 5' untranslated region (and therefore no SD sequence), and start directly with an AUG start codon. Based on the findings presented here, we would predict that leaderless mRNAs are error prone at the step of forming the initial dipeptide. Whether the corresponding proteins can tolerate an increased error at the N-terminus, or whether another mechanism operates, remains unknown. At least in bacteria, only a fraction (below 0.1%) of mRNAs are leaderless and do not comprise mRNAs of essential genes (53), therefore the accuracy problem might not pose a significant problem towards cell viability in these cases.

In summary, the SD sequence, in addition to its canonical function related to mRNA positioning, has a second important function. This is seen in the fact that the SD-antiSD interaction can functionally replace the E-tRNA to confer accurate decoding of the codon following the AUG. Specifically, the SD sequence reduces near-cognate mis-incorporation and precludes the selection of non-cognate aa-tRNAs, thereby protecting the cell from amino acids substitutions detrimental to protein folding, stability and function.

Maintaining the reading frame. The ribosome must ensure that the binding of the tRNAs remains faithful to the codon of the mRNA displayed at the A site and that the correct reading frame of the mRNA is maintained during translation

(reviewed in 54). During translation the error frequency associated with frameshift events is extremely low and has been estimated to be not higher than one event per 30,000 amino acids incorporated (55). However, in specific mRNAs there are loci designated recoding sites, where the efficiency of these frameshift events is significantly higher (reviewed in 56). A classic example of such a site is located within the mRNA of the E. coli prfB gene, which encodes the termination release factor 2 (RF2). Translation of the full-length and active RF2 protein requires a +1 frameshift at the 26th position of the mRNA, in order to bypass an in-frame UGA stop codon. In fact, this programmed frameshifting site acts as an auto-regulatory mechanism, since RF2 terminates translation at UGA stop codons. Therefore, when the intracellular levels of RF2 are high, termination at the 26th position in the prfB mRNA predominates producing an inactive truncated RF2 protein that is rapidly degraded. However, when RF2 levels are low, the stop codon is bypassed via the +1 frameshifting event, leading to the production of full-length protein. What is extraordinary is that the frameshifting rate was determined to be ~30% (57-59) and could be modulated to occur with up to 100 % efficiency (60), i.e. frameshifting on the prfB mRNA occurs with a frequency that is more than four orders of magnitude higher than normal.

Several features have been identified that contribute to this efficiency; Frameshifting is facilitated because (i) translation termination occurs slowly at a weak UGAC stop signal (61,62), particularly when the intracellular levels of RF2 are low, (ii) of the weak G:U wobble base pair of the oligopeptidyl-tRNALeu at the P site, which promotes slippage into the new +1 frame (63), (iii) a perfect realignment of the peptidyl-tRNA at the P site with the new aminoacyl-tRNA Asp-tRNA in the new frame is acquired after the frameshifting (63), and, (iv) a Shine-Dalgarno(SD)-like sequence precedes the UGA stop codon, which has complementary to the anti-SD sequence found at the 3' end of 16S rRNA (59).

We have noted that the complementarity between the SD-like sequence of the mRNA and the anti-SD sequence of the 16S rRNA extends into the ribosomal E site. This prompted us to establish an *in vitro* translation system that allows both the efficiency of frameshifting to be measured as well as the extent of deacylated-tRNA release from the ribosomal E site. It could be demonstrated that the SD-antiSD interaction enhances frameshifting by causing the release of the deacylated-tRNA from the ribosomal E site. Indeed, we could show by monitoring dipeptide-formation within a model of the prfB +1-frameshift window, that the presence of a tRNA at the E site, and probably codon-anticodon interaction at this site, prohibits slippage of the tRNAs in the +1 frame and also stable binding of the A-site tRNA out of frame (24). This suggests that the occupation of the E site by a tRNA is instrumental for maintaining the reading frame, and that modulation of this dependence is exploited for the highly efficient feedback regulation of the translation of the RF2 mRNA.

It is likely that codon-anticodon interaction at the E site plays a decisive role for the observed effects. There is a growing body of evidence for the presence of such interaction. (i) Chasing experiments of labelled E-tRNAs from the ribosome are only effective if the chase tRNA carries an anticodon complementary to the E site codon (reviewed in 64). (ii) The distances between anticodons of adjacent tRNAs on the ribosome are comparable of tRNAs at A and P sites and tRNAs at P and E sites (20±3 Å and 16±3 Å, cryo-electron-microscopic study, (65). Since simultaneous codon-anticodon interaction of

tRNAs at A and P sites is a generally accepted feature, the same feature should therefore hold for tRNAs at P and E sites. (iii) The X-ray structure of 70S ribosomes during the initiation and elongation phase demonstrated that after initiation the E-site codon adopted a classical A-helical conformations ready to form codon-anticodon interaction (14). (iv) Recently three groups demonstrated *in vivo* that the strength of codon-anticodon interaction at the E site is reversely proportional to the accuracy/frameshift efficiency in a system containing the RF2 frameshift window (15,16).

It follows that codon-anticodon interaction at the E site is a standard feature during protein synthesis, essential for maintaining the reading frame. One can estimate that without an E-tRNA translation would run into a frameshift after incorporation of 20 to 50 amino acids making it prohibitively difficult to synthesize proteins of a length of 300 to 500 amino acids, the average length of proteins.

New understanding of antibiotics action and perspectives on light of the E site The features of the E site and its involvement on aminoacyl-tRNA selection at the A site are also useful for studying the action of a number of important antibiotics. For example, the mechanism of action of aminoglycosides, thiostrepton, and viomycin could be fully understood when the features of the E site and its role in the elongation cycle are considered (27, 68). The activity of novel ribosomal inhibitors like oxazolidinones involve also binding to structural motifs of the E site, documented via footprinting techniques (69), and more recently via crystal structure studies together with other new antibiotics (70, 71). As one should expect from a key structure for ribosomal function, the E site is also a hot spot for attack with novel drugs specifically constructed to inhibit the ribosomes of an infectious agent. The recent tendencies for ribosome structure-based antibiotic design (72, 73) will make use of the available biochemical and molecular information about the structure and function of the E site.

BIBLIOGRAPHY

1. Wilson, D.N., and Nierhaus, K.H. (2006). The E-site story: the importance of maintaining two tRNAs on the ribosome during protein synthesis. Cell Mol Life Sci 63, 2725-2737.
2. Munro, J.B., Altman, R.B., O'Connor, N., and Blanchard, S.C. (2007). Identification of two distinct hybrid state intermediates on the ribosome. Mol Cell 25, 505-517.
3. Valle, M., Zavialov, A., Sengupta, J., Rawat, U., Ehrenberg, M., and Frank, J. (2003). Locking and unlocking of ribosomal motions. Cell 114, 123-134.
4. Frank, J., and Agrawal, R.K. (2000). A ratchet-like inter-subunit reorganization of the ribosome during translocation. Nature 406, 318-322.
5. Spahn, C.M., Blaha, G., Agrawal, R.K., Penczek, P., Grassucci, R.A., Trieber, C.A., Connell, S.R., Taylor, D.E., Nierhaus, K.H., and Frank, J. (2001). Localization of the ribosomal protection protein Tet(O) on the ribosome and the mechanism of tetracycline resistance. Mol Cell 7, 1037-1045.
6. Luhrmann, R., Eckhardt, H., and Stoffler, G. (1979). Codon-anticodon interaction at the ribosomal peptidyl-site. Nature 280, 423-425.
7. Wurmbach, P., and Nierhaus, K.H. (1979). Codon-anticodon interaction at the ribosomal P(peptidyl)-tRNA site. Proc Natl Acad Sci USA 76, 2143-2147.
8. Rheinberger, H.-J., and Nierhaus, K.H. (1980). Simultaneous binding of the 3 tRNA molecules by the ribosome of E coli. Biochem Internatl 1, 297-303.
9. Rheinberger, H.-J., Sternbach, H., and Nierhaus, K.H. (1981). Three tRNA binding sites on Escherichia coli ribosomes. Proc Natl Acad Sci USA 78, 5310-5314.

10. Saruyama, H., and Nierhaus, K.H. (1986). Evidence that the three-site model for ribosomal elongation cycle is also valid in the archaebacterium Halobacterium halobium. Mol Gen Genet 204, 221-228.

11. El'skaya, A.V., Ovcharenko, G.V., Palchevskii, S.S., Petrushenko, Z.M., Triana-Alonso, F.J., and Nierhaus, K.H. (1997). Three tRNA binding sites in rabbit liver ribosomes and role of the intrinsic ATPase in 80S ribosomes from higher eukaryotes. Biochemistry 36, 10492-10497.

12. Triana-Alonso, F.J., Chakraburtty, K., and Nierhaus, K.H. (1995). The elongation factor 3 unique in higher fungi and essential for protein biosynthesis is an E site factor. J Biol Chem 270, 20473-20478.

13. Rheinberger, H.-J., Sternbach, H., and Nierhaus, K.H. (1986). Codon-anticodon interaction at the ribosomal E site. J Biol Chem 261, 9140-9143.

14. Jenner, L., Rees, B., Yusupov, M., and Yusupova, G. (2007). Messenger RNA conformations in the ribosomal E site revealed by X-ray crystallography. EMBO Rep 8, 846-850.

15. Liao, P.Y., Gupta, P., Petrov, A.N., Dinman, J.D., and Lee, K.H. (2008). A new kinetic model reveals the synergistic effect of E-, P- and A-sites on +1 ribosomal frameshifting. Nucleic Acids Res 36, 2619-2629.

16. Sanders, C.L., and Curran, J.F. (2007). Genetic analysis of the E site during RF2 programmed frameshifting. RNA 13, 1483-1491.

17. Geigenmüller, U., and Nierhaus, K.H. (1990). Significance of the third tRNA binding site, the E site, on E. coli ribosomes for the accuracy of translation: an occupied E site prevents the binding of non-cognate aminoacyl-transfer RNA to the A site. EMBO J 9, 4527-4533.

18. Rheinberger, H.-J., and Nierhaus, K.H. (1983). Testing an alternative model for the ribosomal peptide elongation cycle. Proc Natl Acad Sci USA 80, 4213-4217.

19. Rheinberger, H.-J., and Nierhaus, K.H. (1986b). Allosteric interactions between the ribosomal transfer RNA-binding sites A and E. J Biol Chem 261, 9133-9139.

20. Dinos, G., Kalpaxis, D.L., Wilson, D.N., and Nierhaus, K.H. (2005). Deacylated tRNA is released from the E site upon A site occupation but before GTP is hydrolyzed by EF-Tu. Nucleic Acids Res 33, 5291-5296.

21. Remme, J., Margus, T., Villems, R., and Nierhaus, K.H. (1989). The third ribosomal tRNA-binding site, the E site, is occupied in native polysomes. European Journal of Biochemistry 183, 281-284.

22. Warner, J.R., and Rich, A. (1964). The number of soluble RNA molecules on reticulocyte polyribosomes. Proc Natl Acad Sci USA 51, 1134-1141.

23. Alexeeva, E.V., Shpanchenko, O.V., Dontsova, O.A., Bogdanov, A.A., and Nierhaus, K.H. (1996). Interaction of mRNA with the Escherichia coli ribosome: Accessibility of phosphorothioate-containing mRNA bound to ribosomes for iodine cleavage. Nucl Acids Res 24, 2228-2235.

24. Marquez, V., Wilson, D.N., Tate, W.P., Triana-Alonso, F., and Nierhaus, K.H. (2004). Maintaining the ribosomal reading frame: The influence of the E site during translational regulation of release factor 2. Cell 118, 45-55.

25. Ogle, J.M., and Ramakrishnan, V. (2005). Structural insights into translational fidelity. Annu Rev Biochem 74, 129-177.

26. Kurland, C.G., Jørgensen, F., Richter, A., Ehrenberg, M., Bilgin, N., and Rojas, A.-M. (1990). Through the accuracy window. In The Ribosome- Structure, Function, and Evolution, A. Dahlberg, W.E. Hill, R.A. Garrett, P.B. Moore, D. Schlessinger, and J.R. Warner, eds. (Washington, D. C., Amer. Soc. Microbiol.), pp. 513-526.

27. Szaflarski, W., Vesper, O., Teraoka, Y., Plitta, B., Wilson, D.N., and Nierhaus, K.H. (2008). New features of the ribosome and ribosomal inhibitors: Non-enzymatic recycling, misreading and back-translocation. J Mol Biol 380, 193-205.

28. Nierhaus, K.H. (1990). The allosteric three-site model for the ribosomal elongation cycle: features and future. Biochemistry 29, 4997-5008.

29. Cochella, L., and Green, R. (2005). Fidelity in protein synthesis. Curr Biol 15, R536-R540.

30. Daviter, T., Gromadski, K.B., and Rodnina, M.V. (2006). The ribosome's response to codon-anticodon mismatches. Biochimie 88, 1001-1011.

31. Moore, S.D., and Sauer, R.T. (2007). The tmRNA system for translational surveillance and ribosome rescue. Annu Rev Biochem 76, 101-124.

32. Di Giacco, V., Marquez, V., Qin, Y., Pech, M., Triana-Alonso, F.J., Wilson, D.N., and Nierhaus, K.H. (2008). Shine-Dalgarno interaction prevents incorporation of noncognate amino acids at the codon following the AUG. Proc Natl Acad Sci U S A 105, 10715-10720.

33. Leger, M., Dulude, D., Steinberg, S.V., and Brakier-Gingras, L. (2007). The three transfer RNAs occupying the A, P and E sites on the ribosome are involved in viral programmed -1 ribosomal frameshift. Nucleic Acids Res 35, 5581-5592.

34. Gnirke, A., Geigenmüller, U., Rheinberger, H.-J., and Nierhaus, K.H. (1989). The allosteric three-site model for the ribosomal elongation cycle. J Biol Chem 264, 7291-7301.

35. Rheinberger, H.-J., and Nierhaus, K.H. (1986a). Adjacent codon-anticodon interactions of both tRNAs present at the ribosomal A and P or P and E sites. FEBS Lett 204, 97-99.

36. Andersson, S.G.E., Buckingham, R.H., and Kurland, C.G. (1984). Does codon composition influence ribosome function? The EMBO J 3, 91-94.

37. Schilling-Bartetzko, S., Bartetzko, A., and Nierhaus, K.H. (1992). Kinetic and thermodynamic parameters for transfer RNA binding to the ribosome and for the translocation reaction. J Biol Chem 267, 4703-4712.

38. Nierhaus, K.H. (1993). Solution of the ribosomal riddle: How the ribosome selects the correct aminoacyl-tRNA out of 41 similar contestants. Mol Microbiol 9, 661-669.

39. Stark, H., Rodnina, M.V., Wieden, H.J., Zemlin, F., Wintermeyer, W., and Van Heel, M. (2002). Ribosome interactions of aminoacyl-tRNA and elongation factor Tu in the codon-recognition complex. Nat Struct Biol 15, 15-20.

40. Valle, M., Sengupta, J., Swami, N.K., Grassucci, R.A., Burkhardt, N., Nierhaus, K.H., Agrawal, R.K., and Frank, J. (2002). Cryo-EM reveals an active role for aminoacyl-tRNA in the accommodation process. EMBO J 21, 3557-3567.

41. Tats, A., Remm, M., and Tenson, T. (2006). Highly expressed proteins have an increased frequency of alanine in the second amino acid position. BMC Genomics 7, 28.

42. Stenstrom, C.M., Holmgren, E., and Isaksson, L.A. (2001). Cooperative effects by the initiation codon and its flanking regions on translation initiation. Gene 273, 259-265.

43. Solbiati, J., Chapman-Smith, A., Miller, J.L., Miller, C.G., and Cronan, J.E., Jr. (1999). Processing of the N termini of nascent polypeptide chains requires deformylation prior to methionine removal. J Mol Biol 290, 607-614.

44. Varshavsky, A. (1996). The N-end rule: Functions, mysteries, uses - Inaugural paper. Proc Natl Acad Sci USA 93, 12142-12149.

45. Kaminishi, T., Wilson, D.N., Takemoto, C., Harms, J.M., Kawazoe, M., Schluenzen, F., Hanawa-Suetsugu, K., Shirouzu, M., Fucini, P., and Yokoyama, S. (2007). A snapshot of the 30S ribosomal subunit capturing mRNA via the Shine-Dalgarno interaction. Structure 15, 289-297.

46. Korostelev, A., Trakhanov, S., Asahara, H., Laurberg, M., Lancaster, L., and Noller, H.F. (2007). Interactions and dynamics of the Shine Dalgarno helix in the 70S ribosome. Proc Natl Acad Sci USA 104, 16840-16843.

47. Yusupova, G., Jenner, L., Rees, B., Moras, D., and Yusupov, M. (2006). Structural basis for messenger RNA movement on the ribosome. Nature 444, 391-394.

48. Gualerzi, C.O., Brandi, L., Caserta, E., Garofalo, C., Lammi, M., La Teana, A., Petrelli, D., Spurio, R., Tomsic, J., and Pon, C.L. (2001). Initiation factors in the early events of mRNA translation in bacteria. Cold Spring Harb Symp Quant Biol 66, 363-376.

49. Smith, D.W.E. (1996). Problems of translating heterologous genes in expression systems: The role of tRNA. Biotechnol Progr 12, 417-422.

50. Gebauer, F., and Hentze, M.W. (2004). Molecular mechanisms of translational control. Nat Rev Mol Cell Biol 5, 827-835.

51. Pestova, T.V., Kolupaeva, V.G., Lomakin, I.B., Pilipenko, E.V., Shatsky, I.N., Agol, V.I., and Hellen, C.U.T. (2001). Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA 98, 7029-7036.

52. Londei, P. (2005). Evolution of translational initiation: new insights from the archaea. FEMS Microbiol Rev 29, 185-200.

53. Moll, I., Grill, S., Gualerzi, C.O., and Blasi, U. (2002). Leaderless mRNAs in bacteria: surprises in ribosomal recruitment and translational control. Mol Microbiol 43, 239-246.

54. Wilson, D.N., and Nierhaus, K.H. (2003). The ribosome through the looking glass. Angew Chem Int Ed Engl 42, 3464-3486.

55. Jorgensen, F., and Kurland, C.G. (1990). Processivity errors of gene expression in Escherichia coli. J Mol Biol 215, 511-521.

56. Atkins, J.A., Herr, A.J., Massire, C., O'Connor, M., Ivanov, I., and Gesteland, R.F. (2000). Poking a hole in the sancity of the triplet code: Inferences for framing. In The Ribosome: Structure, Function, Antibiotics, and Cellular Interactions, R.A. Garret, S.R. Douthwaite, A. Liljas, A.T. Matheson, P.B. Moore, and H.F. Noller, eds. (Washington, DC, ASM Press, American Society for Microbiology), pp. 369-384.

57. Curran, J.F., and Yarus, M. (1988). Use of tRNA Suppressors to Probe Regulation of Escherichia coli Release Factor 2. J Mol Biol 203, 75-83.

58. Weiss, R.B., Dunn, D.M., Atkins, J.F., and Gesteland, R.F. (1987). Slippery runs, shifty stops, backward steps, and forward hops: 2, 1, +1, +2, +5, and +6 ribosomal frameshifting. Cold Spring Harbor Symp Quant Biol 52, 687-693.

59. Weiss, R.B., Dunn, D.M., Dalhberg, A.E., Atkins, J.F., and Gesteland, R.F. (1988). Reading frame switch caused by base-pair formation between the 3' end of 16S rRNA and the mRNA during elongation of protein synthesis in Escherichia coli. EMBO J 7, 1503-1507.

60. Donly, B.C., Edgar, C.D., Adamski, F.M., and Tate, W.P. (1990). Frameshift autoregulation in the gene for Escherichia coli release factor-2 - Partly functional mutants result in frameshift enhancement. Nucleic Acids Res 18, 6517-6522.

61. Major, L.L., Poole, E.S., Dalphin, M.E., Mannering, S.A., and Tate, W.P. (1996). Is the in-frame termination signal of the Escherichia coli release factor-2 frameshift site weakened by a particularly poor context? Nucleic Acids Res 24, 2673-2678.

62. Poole, E.S., Brown, C.M., and Tate, W.P. (1995). The identity of the base following the stop codon determines the efficiency of in vivo translational termination in Escherichia coli. EMBO J 14, 151-158.

63. Curran, J.F. (1993). Analysis of effects of tRNA:message stability on frameshift frequency at the Escherichia coli RF2 programmed frameshift site. Nucleic Acids Res 21, 1837-1843.

64. Blaha, G., and Nierhaus, K.H. (2001). Features and functions of the ribosomal E site. Cold Spring Harbor Symposia on Quantitative Biology 65, 135-145.

65. Agrawal, R.K., Spahn, C.M.T., Penczek, P., Grassucci, R.A., Nierhaus, K.H., and Frank, J. (2000). Visualization of tRNA movements on the Escherichia coli 70S ribosome during the elongation cycle. J Cell Biol 150, 447-459.

66. Schmeing, T.M., Moore, P.B., and Steitz, T.A. (2003). Structures of deacylated tRNA mimics bound to the E site of the large ribosomal subunit. RNA 9, 1345-1352.

67. Ogle, J.M., Brodersen, D.E., Clemons Jr, W.M., Tarry, M.J., Carter, A.P., and Ramakrishnan, V. (2001). Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science 292, 897-902.

68. Hausner TP, Geigenmüller U, and Nierhaus KH. (1988). The allosteric three-site model for the ribosomal elongation cycle. New insights into the inhibition mechanisms of aminoglycosides, thiostrepton, and viomycin. J Biol Chem. 263, 13103-1311.

69. Matassova NB, Rodnina MV, Endermann R, Kroll HP, Pleiss U, Wild H, and Wintermeyer W. (1999). Ribosomal RNA is the target for oxazolidinones, a novel class of translational inhibitors. RNA 5, 939-946.

70. Ippolito JA, Kanyo ZF, Wang D, Franceschi FJ, Moore PB, Steitz TA, and Duffy EM. (2008). Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. J Med Chem. 51, 3353-3356.

71. Schroeder SJ, Blaha G, Tirado-Rives J, Steitz TA, and Moore PB. (2007). The structures of antibiotics bound to the E site region of the 50 S ribosomal subunit of Haloarcula marismortui: 13-deoxytedanolide and girodazole. J Mol Biol. 367, 1471-1479.

72. Franceschi F. (2007). Back to the future: the ribosome as an antibiotic target. Future Microbiol. 2, 571-574.

73. Franceschi F, and Duffy EM. (2006). Structure-based drug design meets the ribosome. Biochem Pharmacol. 71, 1016-1025.