Further in Vitro Exploration Fails to Support the Allosteric Three-site Model

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Background: E-site tRNA and Shine-Dalgarno interactions have been proposed to increase the fidelity of protein synthesis.

Results: Neither E-site tRNA nor the Shine-Dalgarno interactions impact the fidelity of tRNA selection during protein synthesis.

Conclusion: The allosteric three-site model for the ribosome cannot be confirmed.

Significance: This work will contribute to understanding the molecular mechanisms that dictate fidelity during protein synthesis.

Ongoing debate in the ribosome field has focused on the role of bound E-site tRNA and the Shine-Dalgarno-anti-Shine-Dalgarno (SD-aSD) interaction on A-site tRNA interactions and the fidelity of tRNA selection. Here we use an in vitro reconstituted Escherichia coli translation system to explore the reported effects of E-site-bound tRNA and SD-aSD interactions on tRNA selection events and find no evidence for allosteric coupling. A large set of experiments exploring the role of the E-site tRNA in miscoding failed to recapitulate the observations of earlier studies (Di Giacco, V., Márquez, V., Qin, Y., Pech, M., Triana-Alonso, F. J., Wilson, D. N., and Nierhaus, K. H. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 10715–10720 and Geigenmüller, U., and Nierhaus, K. H. (1990) EMBO J. 9, 4527–4533); the frequency of miscoding was unaffected by the presence of E-site-bound cognate tRNA. Moreover, our data provide clear evidence that the reported effects of the SD-aSD interaction on fidelity can be attributed to the binding of ribosomes to an unanticipated site on the mRNA (in the absence of the SD sequence) that provides a cognate pairing codon leading naturally to incorporation of the purported “noncognate” amino acid.

A series of in vitro studies over several decades has argued that occupation of the E-site by a deacylated cognate tRNA allosterically regulates affinity for A-site substrates, thus increasing the overall fidelity of tRNA selection (2–4). A recent study extended these ideas by arguing that just following initiation, when no E-site tRNA is available to occupy the E-site, the SD-aSD[^3] pairing interaction functionally replaces the E-site codon-anticodon interaction, similarly increasing the fidelity of tRNA selection (1). These studies were all biochemical in nature, using well defined reconstituted systems with either homopolymeric or defined mRNA sequences and with direct analysis of miscoding events by HPLC. The observed effects in these studies are generally striking, and it has been difficult to identify a conceptual error.

Despite the appeal of such allosteric models, other studies have argued strongly against such a role for the E-site tRNA (5). Most compellingly perhaps is a recent single-molecule study performed under relatively normal translation conditions (that include physiologically relevant concentrations of ternary complex and EFG) that failed to show any coupling between tRNA binding events in the E- and A-sites, arguing against models for allosteric coupling (6). Another more recent single-molecule study under less physiological conditions also argued for uncoupled activity between E- and A-sites during some stages of translation elongation (7). Also, although x-ray structures of the ribosome often contain E-site-bound tRNA, there is little, albeit some, evidence for the existence of defined codon-anticodon interactions in these structures (8–11), consistent with earlier biophysical studies (12, 13). It should be acknowledged, however, that the E-site tRNA in these structures has typically not been cognate, but instead a heterogeneous collection of tRNAs that happened to copurify with the ribosomes. We note that although the role for E-site tRNA during tRNA selection is highly contested, its role in frame maintenance is less controversial and has been documented by multiple groups both in vitro and in vivo (14–16). Moreover, our own studies of post-peptidyl quality control suggested that the quality of codon-anticodon interactions in the E-site might impact interactions with both aminoacyl-tRNA and release factor substrates in the A-site (17).

Here we examined the effects of the SD-aSD interaction on the fidelity of the decoding process and found no evidence supporting a role for this interaction in increasing the fidelity of protein synthesis. Instead, in the absence of the SD-aSD interaction, the ribosome fails to quantitatively initiate on the first AUG of the mRNA, hence leading to multiple initiation events, both of which lead to the incorporation of cognate amino acids.
corresponding to the codon poised in the A-site. We also examined the role of E-site-bound tRNA on the decoding process and failed to observe the dramatic effects previously reported by Nierhaus and colleagues (1–4). We further provide experimental evidence to suggest that direct competition in the A-site by excess decylated tRNA may account for some of the previously reported surprising effects on fidelity.

EXPERIMENTAL PROCEDURES

Buffers and Reagents—Buffers used were: (i) polymix buffer (95 mM KCl, 5 mM NH₄Cl, 5 mM magnesium acetate, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, pH 7.5, 1 mM DTT) (18); (ii) polyamine buffer (20 mM HEPES-potassium hydroxide, pH 7.6, 150 mM NH₄Cl, 4.5 mM MgCl₂, 2 mM spermidine, 0.05 mM spermine, 4 mM β-mercaptoethanol) (19); and (iii) buffer A (50 mM Tris-HCl, pH 7.5, 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM DTT).

Escherichia coli MRE600 (ATCC29417) tight couple ribosomes were prepared as described previously (20). For 30 S and 50 S subunits, crude 70 S ribosomes were first isolated by pelleting over sucrose cushions and dialyzed 2× in 1 M MgCl₂. The subunits were then separated using a 10–40% sucrose gradient on a Ti-15 rotor. Over-expressed native IF1, IF3 and His-tagged IF2, EFTu, and EFG were purified on a 5-mL His-Trap FF (GE Healthcare) as described previously (21).

tRNA<sub><sup>Met</sup></sub>, tRNA<sub><sup>Lys</sup></sub>, tRNA<sub><sup>Glu</sup></sub>, and tRNA<sub><sup>Phe</sup></sub> were purchased from Chemical Block Ltd. tRNA<sub><sup>Leu</sup></sub> was from Subriden RNA, and tRNA<sub><sup>bulk</sup></sub> was from Sigma (all from E. coli). mRNAs were transcribed by T7 RNA polymerase from double-stranded DNA templates. mRNA used for the SD experiments had the sequence at the 3′-end to allow the radiolabeled oligonucleotide primer to anneal and be extended by reverse transcriptase (23).

Identification of Peptides—Samples were treated with KOH (0.3 M) at 50 °C for 30 min to hydrolyze the peptidyl-tRNAs. Peptides were then resolved using electrophoretic cellulose TLC in pyridine acetate buffer, pH 2.7 (submerged in Stoddard solvent) and applying a voltage of 1200 V over a distance of 8 cm for ~30 min (22).

For the HPLC experiments, ICs were prepared by mixing 30 S and 50 S subunits (2 μM each) with N-Ac-[<sup>14</sup>C]Phe-tRNA<sub><sup>Phe</sup></sub> (4 μM) and mRNA (6 μM) with or without the deacylated E-site tRNA (tRNA<sub><sup>Met</sup></sub> or tRNA<sub><sup>Tyr</sup></sub>); 4 μM for 30 min at 37 °C in polyamine buffer. The ICs were then pelleted over sucrose cushion and resuspended in the appropriate concentration (2 μM). The ICs were then incubated with an equivalent volume of ternary complex containing 40 μM of each aa-tRNA.

RESULTS

The Shine-Dalgarno-Anti-Shine-Dalgarno Interaction Is Not Critical in Specifying Fidelity of First Round of Elongation—A previous study argued that the SD-aSD interaction between mRNA and the 16 S rRNA functionally compensates for the lack of codon-anticodon interaction at the E-site during the first round of elongation (1). Using a defined mRNA species, the authors argue...
that the incorporation of noncognate Asp-tRNA_{Asp} on a valine codon (GUA) in the A-site is reduced in the presence of a functional SD sequence in the mRNA.

We began to explore this phenomenon in our own E. coli in vitro reconstituted translation system, utilizing mRNAs and tRNA isoacceptors similar to those used in the earlier study. In these experiments, ribosomes were programmed with heteropolymeric mRNAs either with or without a Shine-Dalgarno sequence (GGAGGU) five nucleotides upstream of the coding sequence AUG-GUA-UUC (MVF) (Fig. 1A), and the P-site was loaded with f-Met-tRNA_{fMet} through a standard initiation process (24). These ICs were subsequently reacted with two different ternary complexes, EFTu-GTP-Val-tRNA_{Val} or EFTu-GTP-Asp-tRNA_{Asp}, or both together, and the products were resolved by electrophoretic TLC (22). As reported previously (1), when mRNAs encoding MVF, with and without a SD sequence, were compared, there was a greater extent of incorporation of Asp (into MD product), a noncognate species for the GUA codon, in the absence of the SD sequence (Fig. 1B, lanes 8 and 9). Indeed, when both Val-tRNA_{Val} and Asp-tRNA_{Asp} are provided, in the presence of the SD sequence, primarily MV product is generated, whereas in the absence of the SD sequence, a near equal mixture of MD and MV product is generated (Fig. 1B, lane 9). These data might suggest that there is considerable promiscuity of the ribosome in decoding the second amino acid of a protein sequence when there is no SD sequence found in the mRNA. However, we noted that in the absence of the SD sequence, the reactions with either Val-tRNA_{Val} or Asp-tRNA_{Asp} failed to go to completion, and there appears to be no competition between the two aa-tRNAs when both are added together in the same reaction. In contrast, in the presence of the SD sequence, the reaction with Val-tRNA_{Val} proceeded to near completion where most of the fMet-tRNA_{fMet} was consumed (Fig. 1B, lane 2). These observations suggest that in the absence of the SD sequence, there exists a population of ribosomes that preferentially react with a distinct aa-tRNA (Asp-tRNA_{Asp}), thus explaining the observed end-point defects.

**Toeprinting Analysis Identifies Two AUG Codons in the mRNA Sequence**—To interrogate ribosome positioning on the mRNA during this unusual decoding event (Asp-tRNA_{Asp} efficiently decoding a noncognate valine codon, GUA), a “toeprinting” assay was performed in which primer extension by reverse transcriptase from the extended mRNA 3'-tail identifies the 3'-proximal (A-site) side of bound ribosomes (23). As anticipated, the primer extension reaction for ribosomal complexes with an SD sequence containing mRNA and fMet-tRNA_{fMet} at the P-site revealed a single prominent “toeprint” at the appropriate position (Fig. 1C, lane 6). By contrast, when no SD sequence is present in the mRNA, initiation complexes (with...
only fMet-tRNA\textsubscript{Met} at the P-site) reveal two nearly equivalent ribosome footprints on the mRNA (Fig. 1C, lane 10). Indeed, inspection of the mRNA sequence utilized here (and previously (1)) reveals the presence of two distinct AUG codons, one upstream and typically associated with the SD sequence and another downstream and not positioned next to an SD sequence (this latter AUG simply represents an internal methionine in the coding sequence). The presence of two equivalent toeprints is thus not surprising in the context of a mutated upstream SD sequence. Strikingly, the downstream AUG codon sits just upstream of an intact cognate aspartate codon (GAC).

Toeprinting experiments were also performed on initiation complexes that had been elongated with either Val-tRNA\textsuperscript{Val} or a mix of Val-tRNA\textsuperscript{Val} and Asp-tRNA\textsuperscript{Asp}. The addition of Val-tRNA\textsuperscript{Val} to the complexes results in a characteristic three-nucleotide shift of the upstream ribosome on complexes with or without the SD sequence (and MV dipeptide is produced by this elongation step). By contrast, the downstream toeprinting pattern was shifted three nucleotides only in the presence of Asp-tRNA\textsuperscript{Asp}. These results are strikingly consistent with the observations from the peptidyl transfer reactions in Fig. 1B. The miscoding by Asp-tRNA\textsuperscript{Asp} (to form MD dipeptide) apparently resulted from ribosomal initiation on the downstream AUG codon (with no SD sequence), whereas normal decoding (to form MV dipeptide) resulted from ribosomal initiation on the upstream AUG codon. We subsequently confirmed that the MD dipeptide is produced from the second AUG codon by mutating the second AUG codon to CCC and showing that no MD dipeptide product is then formed (Fig. 1B, lanes 5 and 10).

Although the earlier results of Di Giacco et al. (1) were confirmed in our own reconstituted system, the explanation that the quality of the SD-aSD interaction affects the fidelity of tRNA selection is flawed. The miscoding effects that were previously documented can be understood in terms of a second, downstream AUG codon with a proximal aspartate (GAC) codon and a normal cognate tRNA selection event.

Bound E-site tRNA Fails to Impact Fidelity of tRNA Selection in A-Site—Previous experiments that argued for allosteric interactions between the E- and A-sites were performed using ribosomes programmed either with poly(U) (2, 3) or with specific heteropolymeric mRNAs (1). Here we extended these studies using a number of distinct homo- and heteropolymeric mRNAs to examine the impact of E-site tRNA binding on the fidelity of tRNA selection in the A-site.

We began by programming ribosomes with poly(U) mRNA, N-acetyl-[\textsuperscript{14}C]Phe-tRNA\textsuperscript{Phe} in the P-site, and deacylated tRNA\textsuperscript{Phe} in the E-site (or not). Ribosomal complexes without an E-site tRNA were prepared by simply adding N-Ac-Phe-tRNA\textsuperscript{Phe} to the poly(U)-programmed ribosomes; binding of the deacylated tRNA should be to the classical P-site. Ribosomal complexes loaded with E-site tRNA were prepared as described previously (25, 26) by first binding deacylated tRNA\textsuperscript{Phe} (which binds in either the P/P- or the P/E-state) and then by subsequently adding N-Ac-Phe-tRNA\textsuperscript{Phe} (which binds in the A/P-state, forcing the deacylated tRNA into the P/E-state). Following this so-called nonenzymatic loading, EFG was added to translocate the complex from the pre- to post-translocation state. Ribosomal complexes with or without E-site tRNA were next purified over a sucrose cushion and were subsequently reacted with various ternary complexes (EFTu-GTP with Phe-tRNA\textsuperscript{Phe}, Leu-tRNA\textsuperscript{Leu}, Asp-tRNA\textsuperscript{Asp}, or Cys-tRNA\textsuperscript{Cys}). We note that these experiments were performed in the presence of polyamines, which have been previously shown to stabilize any E-site-bound tRNA approaching 100% occupancy (5, 27). First, when Phe-tRNA\textsuperscript{Phe} is added, we see the production of dipeptide (N-AcFF) (Fig. 2A, lane 1) using our previously described electrophoretic TLC system (22) and even subsequent additions of Phe in complexes that are likely contaminated with EFG (e.g. N-AcFF in Fig. 2A, lane 5). Contrary to earlier studies (1), we observe that the presence of E-site tRNA modestly increases the amount of miscoding as reported on by the reactivity of Leu-tRNA\textsuperscript{Leu} to form N-AcFL dipeptide product (Fig. 2A, lane 6) (Leu on Phe has a codon-anticodon interaction with a mismatch in the first position in this case). We see that both the absolute amount of product and the fraction of N-Ac-Phe converted to dipeptide are increased by the presence of the bound E-site tRNA (from 4.7 to 9.8%). In the case of reactions with other tRNAs including Asp-tRNA\textsuperscript{Asp} (noncognate) or Cys-tRNA\textsuperscript{Cys} (near cognate), we see no evidence of dipeptide formation in the electrophoretic system, suggesting that if miscoding occurs in this case, the reaction is very inefficient.

Although initial experiments by Nierhaus and colleagues (2) used homopolymeric mRNAs, the group switched to heteropolymeric mRNAs and again reported that bound E-site tRNA increases the fidelity of tRNA selection in the A-site (1). As a result, we used the heteropolymeric message MFK to prepare complexes with and without E-site-bound tRNA, as described previously (1). With the homopolymeric mRNA, for no E-site tRNA complexes, N-Ac-Phe-tRNA\textsuperscript{Phe} was simply added to the MFK mRNA-programmed ribosomes; for E-site tRNA containing complexes, deacylated tRNA\textsuperscript{Met} was first added, then N-Ac-Phe-tRNA\textsuperscript{Phe}, and then EFG for translocation from the pre- to the post-translocation state. Again, these ribosome complexes were purified over a sucrose cushion and used for subsequent elongation reactions. Ternary complexes (EFTu-GTP carrying Lys-tRNA\textsuperscript{Lys} or Leu-tRNA\textsuperscript{Leu}) were next added as indicated, and the products were resolved by electrophoretic TLC. Although the cognate dipeptide N-Ac-Phe-Lys was readily formed with and without E-site-bound tRNA, we were unable to detect the formation of noncognate dipeptide N-Ac-[\textsuperscript{14}C]Phe-Leu in this system (<7% relative to N-Ac-Phe-Lys) (data not shown). In equivalent experiments performed by Di Giacco et al. (1), the levels of miscoding detected were on the order of 4%.

Resolution of Products by HPLC Analysis Yields Same Results—We further explored the incorporation of near cognate or noncognate aminoacyl-tRNAs during tRNA selection using the HPLC system to increase the sensitivity of the experiment. Moreover, we increased the overall concentration of ternary complex in the experiments to favor the production of dipeptide product (concentration of 20 \textmu M, well above the $K_d$ of the interaction between the ribosome and ternary complex). In an extensive set of experiments, we used a related group of mRNA sequences encoding MFL\textsuperscript{LUC}, MFL\textsuperscript{LUC}, MFL\textsuperscript{LUG}, MFL\textsuperscript{LUA}, MFK\textsuperscript{AAA}, and MFM\textsuperscript{AUG} to evaluate potential tRNA selection of
[\textsuperscript{3}H]Leu-tRNA on cognate, near cognate (first or third position mismatch between the tRNA–mRNA codon anticodon interaction), and noncognate (more than one mismatch) codons at the A-site (Fig. 2B). The dipeptide (N-Ac-[\textsuperscript{14}C]Phe-[\textsuperscript{3}H]Leu) formed was resolved from reactants via HPLC and quantified based on the amount of \textsuperscript{14}C and \textsuperscript{3}H in the product species. These complexes were prepared as described previously with and without E-site tRNA on the described heteropolymeric mRNA sequences.

As a positive control, we used the mRNA sequence encoding MF(CUC), CUC being a cognate leucine codon that should be readily decoded by Leu-tRNA\textsubscript{2}Leu ternary complex. The products of the reaction (on ribosome complexes with and without E-site tRNA) were resolved by HPLC (see “Experimental Procedures”) and quantified by measuring the incorporated radio-labeled amino acids N-Ac-[\textsuperscript{14}C]Phe and [\textsuperscript{3}H]Leu. We see the formation of substantial amounts (50 and 80% fractional amount) of dipeptide (N-Ac-[\textsuperscript{14}C]Phe-[\textsuperscript{3}H]Leu) in all cases, the E-site codon is AUG (or M for methionine), the P-site is filled with N-Ac-[\textsuperscript{14}C]Phe-tRNAPhe on a Phe (F) codon, whereas the A-site codon is denoted by its full nucleotide sequence. The reaction yield was determined by HPLC separation of the reaction products followed by scintillation counting of the fractions.

FIGURE 2. \textit{In vitro} translation reactions fail to support allosteric model. A, schematic of the poly(U)-programmed complex and the A-site aa-tRNA substrates (showing their anticodons). We used four different aa-tRNAs to test A-site reactivity with or without E-site deacylated tRNA. Mismatched nucleotides are indicated in red. The bottom panel shows an autoradiograph of an electrophoretic TLC used to follow the reactivity of a complex carrying N-Ac-[\textsuperscript{14}C]Phe-tRNA\textsuperscript{Phm} in the P-site with the indicated aa-tRNA (+ Phe, + Leu, + Asp, + Cys) in the absence and presence of E-site tRNA. Products of the reaction are indicated as N-Ac-Phe (\textsuperscript{1}AcP), N-Ac-Phe-Phe (\textsuperscript{1}AcPFF), N-Ac-Phe-Phe-Phe-Phe (\textsuperscript{1}AcFFFF), and N-Ac-Phe-Leu (\textsuperscript{1}AcFL). B, bar graph depicting the yield of dipeptide product (N-AcFL) for complexes programmed with the indicated heteropolymeric mRNA and reacted with Leu-tRNA\textsubscript{2}Leu. In all cases, the E-site codon is AUG (or M for methionine), the P-site is filled with N-Ac-[\textsuperscript{14}C]Phe-tRNAPhe on a Phe (F) codon, whereas the A-site codon is denoted by its full nucleotide sequence. The reaction yield was determined by HPLC separation of the reaction products followed by scintillation counting of the fractions. C, bar graph depicting yield of dipeptide product (N-AcFL) for the YF CUG complex reacting with Leu-tRNA\textsubscript{2}Leu in the presence of the indicated tRNAs. In this complex, the E-site codon is UAC (or Y for tyrosine), the P-site is filled with N-Ac-[\textsuperscript{14}C]Phe-tRNAPhe on a Phe (F) codon, whereas the A-site codon is CUG.
No Role for E-site in tRNA Selection

In this study, we investigated the influence of the Shine-Dalgarno sequence and of the E-site tRNA on the fidelity of tRNA selection during translation elongation. In the case of the SD sequence, the earlier studies are readily rationalized by the presence of a downstream previously unnoticed AUG codon that led to the surprising results. In the case of the E-site tRNA, we are broadly unable to recapitulate the unusual miscoding results reported in several earlier studies despite the fact that the system we used was designed to mimic the particular features of those studies. Our data thus fail to support earlier models arguing for allosteric interactions between the E- and the A-site that modulate the fidelity of tRNA selection. We continue to try to understand at a mechanistic level how interactions in the E-site can impact retrospective editing during translation elongation as characterized in the *E. coli* system (17, 28).

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