Evidence for a Conformational Change in the Exit Site of the *Escherichia coli* Ribosome upon tRNA Binding†

J. Stephen Lodmell,‡ William E. Tapprich,§ and Walter E. Hill*

Division of Biological Sciences, University of Montana, Missoula, Montana 59812

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ABSTRACT: The exit (E) site of the *Escherichia coli* ribosome was investigated using oligodeoxyribonucleotides complementary to single-stranded regions of ribosomal RNA suggested to be involved in tRNA binding in the E site [Moazed, D., & Noller, H. (1989) *Cell 57*, 585–597]. Radiolabeled DNA oligomers (probes) were hybridized in situ to complementary sites on the ribosomal RNA of ribosomes or ribosomal subunits, and the effects of simultaneous tRNA or antibiotic binding on probe binding were measured using a nitrocellulose filtration binding assay. Site specificity of probe binding was assured using ribonuclease H to cleave the ribosomal RNA at the site of probe binding. When 50S subunits were hybridized with a probe spanning bases 2109–2119 and deacylated tRNA was added incrementally, probe binding decreased, suggesting that the probe and tRNA competed for the same binding site or that tRNA was allosterically affecting the probe binding site. When 70S ribosomes were substituted for 50S subunits, probe binding to this site initially increased and then decreased at higher concentrations of deacylated tRNA. Titrating probe–ribosome complexes with acylated tRNA, *N*-acetyl-acylated tRNA, tetracycline, or chloramphenicol had no effect on probe binding. The data presented provide evidence for tRNA/rRNA interaction at or near the E site of the *E. coli* ribosome and suggest that a conformational change occurs in the E site when deacylated tRNA is bound to the P site. The data suggest that deacylated tRNA in the P site serves as a translocational trigger by causing the E site to change conformations, making it more available for tRNA (and probe) binding and therefore promoting translocation. Upon saturation of the P site, deacylated tRNA then binds to the E site, competing with the probe bound there.

The classical scheme of protein synthesis described first by Watson (1964) contains two tRNA binding sites on the ribosome: an aminoacyl, or A site, and a peptidyl, or P site.[1] According to this scheme, the first step of elongation is the binding of the cognate aminoacyl-tRNA–elongation factor Tu–GTP ternary complex to the A site, followed by hydrolysis and release of the EF-Tu–GDP. Peptidyl transfer ensues, consisting of the transfer of the nascent peptide chain attached to the P site-bound tRNA to the aminoacyl-tRNA in the A site. Next comes translocation, which is accompanied by the hydrolysis of another GTP by elongation factor G. In translocation, according to the classic model, the peptidyl tRNA in the A site is moved then to the P site, and the deacylated tRNA in the P site is ejected from the ribosome. This relatively simple model of protein synthesis has been refined and updated over the years to accommodate new experimental data, and the current models are correspondingly more complex. For example, several models containing more than two tRNA binding sites have been proposed (Wettstein & Noll, 1965; Hardesty et al., 1969; Lake, 1977; Rheinberger & Nierhaus, 1980; Rheinberger et al., 1981; Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984), as have hybrid tRNA binding states of the ribosome (Moazed & Noller, 1989b).

Over the last decade or so, a large body of evidence has accumulated for the existence of a tRNA exit site on the *Escherichia coli* ribosome. This site, termed the E site, binds deacylated tRNA after its release from the ribosomal P site. The function of this nonclassical tRNA binding site has been variously described as a site of intermediate affinity to facilitate release of tRNA from the P site (Lill et al., 1984), as an allosteric effector of the A site (Gnirke et al., 1989), as a mechanism to keep the ribosome in frame with the message (Rheinberger & Nierhaus, 1983), or as an active promoter of translocation (Lill et al., 1989). One of the characteristics of the E site that is widely agreed upon is its specificity for deacylated tRNA, whereas other aspects of E site tRNA binding, such as the contribution of the codon–anticodon interaction in binding stability, have been less widely agreed upon [e.g., Paulsen and Wintermeyer (1986) and Gnirke et al. (1989)].

Gnirke et al. (1989) have proposed an intriguing model for elongation that includes high- and low-affinity binding states for the A and E sites. In this model, the pretranslocational ribosome has high-affinity A and P sites, while the E site is in a low-affinity state. After translocation, sites P and E are high affinity, and the A site has assumed its low-affinity state. On the other hand, Lill et al. (1989) have suggested that the flexible 3′ end of deacylated tRNA in the P site actively promotes translocation by interacting with nucleotides in the E site resulting in an allosteric interaction with the EF-G/GTPase center and the GTPase activity associated therein. In either case, tRNA is encouraged to move from the P site to the E site.

Moazed and Noller (1989a) have shown through chemical modification studies that certain bases on 23S ribosomal RNA are affected by tRNAs bound to the A, P, or E sites. In particular, each tRNA binding site has a set of bases on the

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‡ Current address: Division of Biology and Medicine, Section of Biochemistry, Brown University, Providence, RI 02912.

§ Current address: Department of Biology, University of Nebraska, Omaha, NE 68182.

[1] Abbreviations: A, P, and E sites, aminoacyl, peptidyl, and exit sites; rRNA, ribosomal RNA; tRNA, transfer RNA; mRNA, messenger RNA; Phe, phenylalanine; *N*-Ac-Phe-tRNA, *N*-acetylphenylalanyl-tRNA; cDNA, complementary DNA; DTT, dithiothreitol; RNase H, ribonuclease H; EF-G, elongation factor G.

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rRNA that are protected against chemical modification when tRNA's are bound in those sites. In this paper, we report results of probing the single-stranded regions of 23S rRNA thought to be involved in E site/tRNA interactions using short, complementary DNA oligomers, or "probes".

The use of complementary oligodeoxynucleotides to investigate various properties of nucleic acids in general and ribosomes in particular has a fairly extensive history. Complementary DNA probes have been used in elucidating the structure of 5S rRNA (Lewis & Doty, 1970), determining the general morphology of the 30S ribosomal subunit using cDNA hybridization electron microscopy [reviewed in Oakes et al. (1990)], in cross-linking studies of the peptidyltransferase center (Muralikrishna & Cooperman, 1991), and extensively by this laboratory to investigate the fine structure and function of local environments on the *E. coli* ribosome [see reviews in Hill et al. (1988, 1990)]. In addition, Weller and Hill (1992) have recently presented evidence for conformational changes on the 30S subunit as detected by cDNA probing. Because cDNA probing is reversible, it is an especially useful technique for investigating dynamic systems, such as the ribosome in various phases of its cycle. By examining the extent of probe binding in various experimental conditions, we have been able to gain some insight into the workings of the E site, and we have found evidence for a conformational change occurring in region 2109–2119 of 23S rRNA as a result of tRNA binding to the ribosome.

MATERIALS AND METHODS

Ribosome Preparation. Ribosomes or ribosomal subunits were isolated from *E. coli* strain MRE600 by a procedure modified from Hill et al. (1969). Twenty grams of cells grown to 0.5–0.8A_{600} were ground in alumina in a buffer containing 10 mM Tris-HCl, 15 mM $MgCl_2$, and 0.5 M NH_4Cl in the cold for 30 min. The suspension was centrifuged briefly to remove the alumina, the alumina was washed once again with the same buffer, and the supernatants were pooled. The cell extracts were centrifuged at 50000*g* for 1 h to remove large cellular debris and unlysed cells. The pellets were discarded and the supernatants were centrifuged again at 250000*g* for 3 h to pellet ribosomes. The pellets were resuspended in the same buffer and subjected to another 50000*g* (1-h) and 250000*g* (3-h) centrifugation. The pellets were then resuspended either in tight-couple 70S buffer (10 mM Tris–HCl, pH 7.4, 6 mM $MgCl_2$, 60 mM KCl) to isolate intact 70S ribosomes or in 30–50 buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM $MgCl_2$, 60 mM KCl) to isolate ribosomal subunits. These crude preparations were purified further by zonal centrifugation through at 10–38% gradient of DEPC treated sucrose (Eikenberry et al., 1970). Appropriate fractions were pooled and the ribosomes or subunits were pelletted by centrifugation. The pellets were resuspended in either TC70S buffer or 30–50 buffer and dialyzed against 4 L of the same buffer over 8 h. The ribosomes or subunits were stored in small aliquots at −70 °C.

DNA Oligomer Preparation. DNA oligomers were synthesized on a Biosearch 8600 DNA synthesizer using phosphoramidite chemistry and purified using Du Pont's NENPrep nucleic acid purification cartridges. 5′-End labeling of the DNA probes was accomplished using T4 polynucleotide kinase and [γ-^{32}P]ATP or [γ-^{35}S]ATP according to the protocol of Chaconas and Van de Sande (1980).

tRNA Preparation. Deacylated $tRNA^{phe}$ was dephosphorylated using calf intestinal alkaline phosphatase prior to 5′-end labeling according to the method of Lill et al. (1986). The product of this reaction was then phosphorylated using T4 polynucleotide kinase first using cold ATP and then [γ-^{32}P]-ATP, so that the smaller fragments were "cold-labeled", and only the intact tRNA would carry the ^{32}P label, since smaller fragments undergo phosphorylation more rapidly than intact tRNA. Labeled tRNA was purified using Nensorb 20 nucleic acid purification cartridges (Du Pont). Homogeneity of the labeled product was checked by gel electrophoresis and autoradiography.

Aminoacylation of the tRNA was accomplished by incubation of $tRNA^{phe}$ (1 A_{260} unit) with 3500 pmol of [^{14}C]Phe and 2 units of aminoacyl-tRNA synthetase in a buffer containing 2 mM ATP, 30 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 25 mM KCl, and 4 mM DTT. The mixture was incubated 15 min at 37 °C and then phenol extracted, and the phenol was back-extracted. The aqueous portion was then ethanol precipitated, and the free tRNA was separated from the aminoacyl-tRNA on a 1 × 15 cm benzoylated DEAE-cellulose (Serva) column. Fractions containing the charged tRNA were collected and pooled, ethanol precipitated, dried, and resuspended in 10 mM Tris-HCl, pH 7.4, and stored at −70 °C (procedure modified from Kristi Harrington, personal communication; Rheinberger et al., 1983). Aminoacylated tRNA was acetylated and purified using techniques described in Moazed and Noller (1989a).

Probe Binding. Binding of probes or tRNA to ribosomes was quantified by filter binding assay (Backendorf et al., 1981; Tapprich & Hill, 1986). Radiolabeled probe and/or tRNA (specific activity 500 cpm/pmol) were/was mixed with ribosomes (25 pmol of ribosomes, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 150 mM KCl, 12.5 μg of poly(U) [optional], 50 μL total volume), incubated at 37 °C for 20 min and then at room temperature for 20 min, and then incubated on ice for 1 h. The mixtures were then diluted to 1 mL and immediately filtered through nitrocellulose filters (Millipore HAWP) and washed with 2 mL of the same buffer. Under these conditions, the nitrocellulose filters bind ribosomes and subunits but very little free tRNA or probe. The radioactivity retained on the filters (less the radioactivity retained on control filters) quantified the probe or tRNA bound to the ribosomes or subunits and was measured by scintillation counting.

Effect of tRNA Binding on Probe Binding. The effect of tRNA binding on probe hybridization was assayed on 50S subunits by preincubating a saturating amount of labeled probe 2109–2119 or 2111–2117 with 25 pmol of 50S subunits and then adding increasing amounts of deacylated tRNA. After further incubation on ice, the reactions were filtered through a nitrocellulose filter, washed twice, and subjected to liquid scintillation counting.

RNase H Assays. RNase H cleavage reactions were used to check probe hybridization specificity by incubating 50 pmol of ribosomes, subunits, or rRNA with an excess of probe and 2 units of RNase H in 50 μL of RNase H reaction buffer (40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 60 mM KCl) and incubated at 37 °C for 30 min [procedure modified from Donnis-Keller (1979)]. The reactions were then extracted twice with phenol, ethanol precipitated, dried, resuspended in tracking dye, and subjected to electrophoresis on a 5% polyacrylamide/7 M urea gel. The size of the RNA fragments on the gel was estimated by comparison to RNA size markers (Bethesda Research Laboratories).

RESULTS

Probe Binding to 50S Subunits and 70S Ribosomes. The binding of oligodeoxynucleotide probes to 50S subunits was assayed by nitrocellulose filtration, which under the buffer conditions used retains ribosomes or ribosomal subunits and

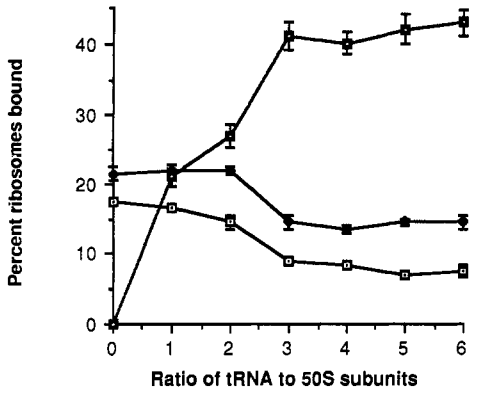

FIGURE 1: Effect of increasing amounts of deacylated tRNA on probe binding to 50S subunits. Increasing amounts of unlabeled deacylated tRNA were added to 25 pmol of 50S subunits in the presence of saturating amounts of ^{32}P-labeled 2109–2119 (open squares) or probe 2111–2117 (closed diamonds). Deacylated tRNA binding from parallel experiments using labeled tRNA is represented by closed squares. The reaction mixtures were incubated and filtered as described in Materials and Methods. Ribosomal subunits and any bound probe and tRNA are retained on the filter, while unbound probe and tRNA is washed through; therefore, radioactivity retained on the filter is indicative of the extent of probe or tRNA binding to subunits. Error bars represent one standard deviation from the mean.

any attached probe but not free probe. The percent binding of probe to subunits or ribosomes, therefore, is determined by the molar ratio of probe retained on the filter (less the control) to ribosomes or subunits. Probe binding to 50S subunits in this study ranged from 13 to 22% at saturation, depending on the probe, and did not bind at all to free 30S subunits (data not shown). None of the probes were cabable if displacing bound tRNA.

Probe binding to 70S ribosomes was assayed in the same way as probe binding to 50S subunits, except that 25 pmol of 70S ribosomes were used instead of free 50S subunits. Probe binding to ribosomes is saturable at a ratio of approximately 20:1 probe to ribosomes under these hybridization and filtration conditions, and maximum probe binding values ranged between 12 and 26% (data not shown). Although there is some variation in probe binding values using different preparations of ribosomes, individual points within an experiment are highly reproducible. The data presented are the average of triplicate trials with error bars spanning one standard deviation.

Effect of Deacylated tRNA Binding on Probe Binding to Subunits. Figure 1 shows that the binding of probes 2111–2117 and 2109–2119 is attenuated when the ratio of tRNA to 50S subunits is 2:1 or greater. Probe binding is cut roughly in half for probe 2109–2119 and is reduced by about 30% for probe 2111–2117 at tRNA concentrations above about a 3:1 molar ratio. Keeping in mind that these probes span regions of rRNA previously implicated in E site tRNA binding (i.e., nucleotides G2112 and G2116; Moazed & Noller, 1989a), this strongly suggests that tRNA and probe are competing for the same binding site or that tRNA causes a conformational change in the ribosome that inhibits probe binding to this region. Probe 2165–2171, by contrast, was not displaced in this experiment, although it spans nucleotide A2169, which is also implicated in E site tRNA binding (data not shown). This may indicate that probe 2165–2171 does not bind to the same region as E site-bound tRNA or that the probe binds rRNA with a higher affinity than the tRNA has for this region, and thus cannot be displaced. A probe spanning nucleotides 2382–2394 did not hybridize well enough to allow accurate analysis of binding characteristics. This probe overlaps nucleotide C2394, which is strongly protected against chemical modification by E site-bound tRNA (Moazed & Noller, 1989a).

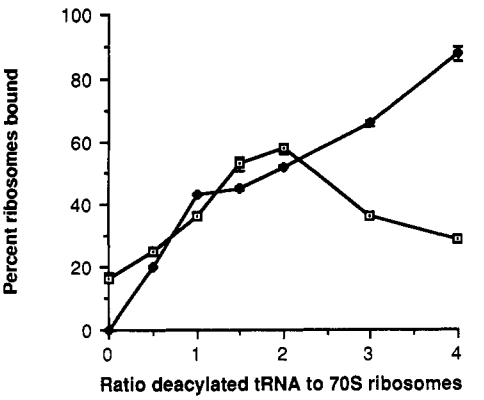

FIGURE 2: Effect of deacylated tRNA on probe 2109–2119 binding to 70S ribosomes. Ribosomes (25 pmol) were preincubated with increasing amounts of ^{32}P-labeled deacylated tRNA (closed diamonds), and then a saturating amount of ^{35}S-labeled probe 2109–2119 (open squares) was added. After incubation, the reaction mixtures were filtered through nitrocellulose disks, and the radioactivity retained was measured for ^{32}P and ^{35}S to determine tRNA and probe binding, respectively.

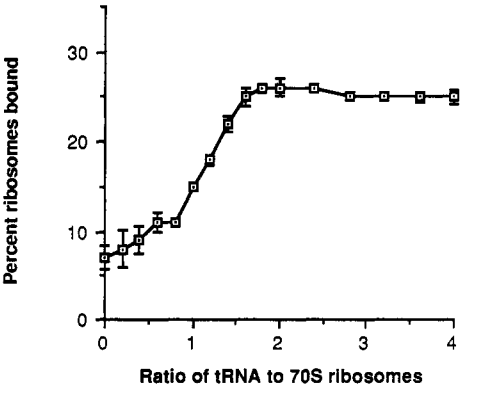

FIGURE 3: Effect of deacylated tRNA on probe 2165–2171 binding. Increasing amounts of unlabeled deacylated tRNA were added to 25 pmol of 70S ribosomes followed by addition of an excess of ^{32}P-labeled probe 2165–2171. Reaction mixtures were incubated and filtered as described in Materials and Methods.

Effect of tRNA Binding on Probe Binding to 70S Ribosomes. Whereas increasing amounts of tRNA simply displace probes spanning the 2109–2119 region on 50S subunits, the effect of increasing amounts of tRNA on 70S ribosomes is more complex. Figure 2 shows the results of a dual-label experiment in which binding of tRNA and probe 2109–2119 were measured simultaneously. Upon addition of the first increments of tRNA (up to 2:1 deacylated tRNA to ribosomes), probe binding is actually enhanced more than 2-fold, but at higher tRNA concentrations, probe binding is attenuated by almost the same amount. Under these experimental conditions, tRNA binds first the P and then the E sites using conventional operational definitions of tRNA binding (Rheinberger et al., 1981; Lill et al., 1984). The results of the dual-label experiment were identical to those seen in single-label experiments in which either the tRNA or the probe (not both) was labeled.

Interstingly, probe 2165–2171 showed enhanced binding at lower ratios of tRNA to ribosomes (as with probe 2109–2119), but it was not displaced at higher concentrations of tRNA (Figure 3). Here again, the presence of a tRNA in the P site appears to enhance the availability of the E site for probe binding. However, as was the case using 50S subunits, probe 2165–2171 was not displaced, even at high tRNA concentrations favoring E site tRNA binding.

Effect of Yeast Deacylated tRNA on Probe Binding to Ribosomes. The effect of binding deacylated tRNA^{phe} isolated

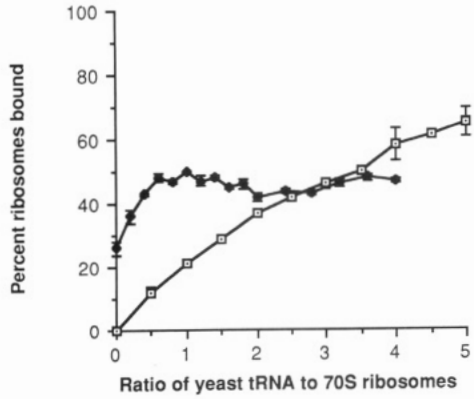

FIGURE 4: Effect of yeast tRNA binding on probe 2109–2119 binding to 70S ribosomes. Increasing amounts of deacylated tRNA isolated from *S. cerevisiae* (open squares) were prebound to 25 pmol of 70S ribosomes, and then an excess of probe 2109–2119 (solid symbols) was added and incubated for an additional hour on ice. Binding curves were determined for tRNA using ^{32}P-labeled yeast tRNA and unlabeled probe and for probe 2109–2119 using ^{32}P-labeled probe and unlabeled yeast tRNA in parallel experiments.

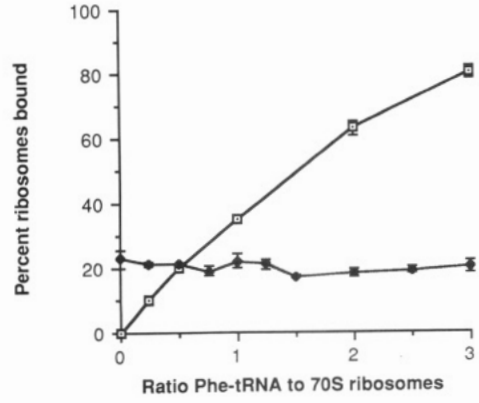

FIGURE 5: Effect of acylated tRNA binding to ribosomes on probe 2109–2119 binding. Increasing amounts of [^{14}C]Phe-tRNA^{phe} (open squares) were preincubated with 25 pmol of ribosomes, and then a saturating amount of ^{32}P-labeled probe 2109–2119 (solid symbols) was added. Samples were incubated and filtered, and ^{14}C and ^{32}P radiaoctivity was measured to determine Phe-tRNA^{phe} and probe binding, respectively.

from *Saccharomyces cerevisiae* on probe 2109–2119 binding was also investigated, and the results are presented in Figure 4. The previous experiments with deacylated tRNA from *E. coli* showed that its binding to the P site has a pronounced effect on probe binding in the E site. tRNA^{phe} isolated from yeast does not bind the E site well (Lill et al., 1986), so it was used to determine what effect, if any, yeast tRNA has on E site probe binding. As with the binding of *E. coli* tRNA to 70S ribosomes, lower concentrations of yeast tRNA increased probe 2109–2119 binding approximately 2-fold. However, probe 2109–2119 was not displaced in the 70S ribosomes at higher yeast tRNA concentrations, unlike the case when *E. coli* tRNA is used. This experiment provides further evidence that the presence of deacylated tRNA in the P site enhances the availability of the E site for cDNA probing.

Effect of Phe-tRNA^{phe} and N-Ac-Phe-tRNA^{phe} Binding on Probe Binding. To determine the effect of binding acylated and acetyl-acetylated tRNAs on probe binding to 70S ribosomes, *E. coli* tRNA^{phe} was acylated with ^{14}C-labeled phenylalanine and then acetylated as needed. In the presence of poly(U), Phe-tRNA^{phe} binds the P site first and then the A site. In the Phe-tRNA^{phe} concentration range shown in Figure 5, where P (and A) site occupancy would be expected, probe 2109–2119 binding is unaffected. Probe 2109–2119 binding was neither enhanced by the presence of acylated tRNA in the P sites (in contrast to deacylated tRNA in the P site) nor was it displaced, since acylated tRNA is excluded from the E site.

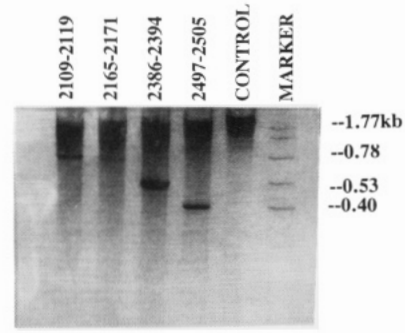

FIGURE 6: Ribonuclease H cleavage of 23S rRNA with several probes. Fifty picomoles of 23S rRNA were incubated with excess probe and 2 units RNase H (conditions described in Materials and Methods). After incubation, reaction mixtures were phenol extracted, ethanol precipitated, resuspended in tracking dye, and subjected to polyacrylamide gel electrophoresis (5% acrylamide/7 M urea). From left to right: probes 2109–2119, 2165–2171, 2386–2394, and 2497–2505, no probe control, size markers.

The effect of titrating 70S ribosomes with *N*-acetyl-Phe-tRNA^{phe} in the absence of poly(U) was also determined. Under these conditions, the *N*-acetyl-Phe-tRNA^{phe} bound only the P site (A site binding requires message). Probe 2109–2119 binding was also unaffected by *N*-Ac-Phe-tRNA^{phe} binding, suggesting that acetyl-acylated tRNA did not enhance E site availability for probe hybridization as did deacylated tRNA (data not shown).

Effect of Tetracycline or Chloramphenicol on Probe Binding. Tetracycline and chloramphenicol were tested for their effects on probe 2109–2119 binding. Tetracycline prevents binding of aminoacyl-tRNA to the A site and thus may act as an A site tRNA analogue (Lucas-Lenard & Haenni, 1968; Kirillov et al., 1983). In this respect, it was of interest to see if binding this analogue to the A site had any effect on probe 2109–2119 binding in the E site, with the P site unoccupied. Results showed that tetracycline had no effect on probe 2109–2119 binding, even at high concentrations of the antibiotic (2000:1 molar ratio of tetracycline to ribosomes; data not shown).

Chloramphenicol inhibits the peptidyltransferase reaction, and thus its site of action may be at or very near the P site. Marconi et al. (1990) showed that chloramphenicol could displace a cDNA probe spanning nucleotides 2497–2505 of 23S rRNA, a region shown by Moazed and Noller (1989a) to be involved in tRNA binding in the P site. Chloramphenicol's effect on probe 2109–2119 binding was investigated to see if it could mimic the effects of P site-bound tRNA. However, even at high concentrations of [^{14}C]chloramphenicol (70:1 molar ratio chloramphenicol to ribosomes), probe 2109–2119 binding was not affected by binding of the antibiotic (data not shown).

Ribonuclease H Cleavage of Probe–rRNA Hybrids. To check specificity of probe binding to rRNA, a RNase H assay was used. Individual probes were incubated with isolated 23S rRNA so that all potential hybridization sites were exposed. The hybrids were then treated with RNase H to cleave the rRNA at the site(s) of DNA probe binding. RNA fragments were subjected to polyacrylamide gel electrophoresis, and the size and the number of fragments were used to determine the sites of hybridization. Figure 6 shows that each probe causes only one major cleavage, and the resulting fragments were of the expected size. Experiments using intact 50S or 70S ribosomes yielded similar results, but the cleavage was somewhat weaker (data not shown).

DISCUSSION

In this study, we have investigated tRNA interactions with the ribosome using DNA probes complementary to single-stranded regions of 23S ribosomal RNA thought to be involved in tRNA binding. The data presented support three general conclusions: (1) The single-stranded region comprising nucleotides 2109–2119 is involved in E site tRNA binding, consistent with the findings of Moazed and Noller (1989a); (2) The placement of deacylated tRNA in the P site causes a change in the E site, making it more available for probe binding; (3) The placement of an acylated or acetyl-acylated tRNA in the P site does not have any effect on E site probe binding. These findings suggest that the P site discriminates between species of tRNA and can trigger a conformational change in the E site if deacylated tRNA is located in the P site.

The DNA probes used in this study were complementary to several single-stranded regions of 23S rRNA shown to be involved in E site tRNA binding (nucleotides G2112, G2116, A2169, and C2394; Moazed & Noller, 1989a). The binding properties of the probes were analyzed in the absence and presence of various tRNA species bound to the A, P, or E sites according to conventional operational definitions of tRNA binding. In this study, probe binding was not stoichiometric as assayed by nitrocellulose filtration. The reasons for substoichiometric probe binding are not definitively known, but it is likely that the apparent binding level, after the probe–ribosome complexes on nitrocellulose are washed with buffer, is less than the equilibrium binding level prior to washing. That is, probes across different regions would be expected to have different on/off rates (depending on the steric environment, higher-order RNA structure, etc.), and thus apparent binding values would be lower for probes having shorter half-lives in the ribosome–probe complex since more of these probes would be lost in the washing steps. Unpublished data from this laboratory have shown that certain probes showing nil binding in the nitrocellulose filtration assay can still cause a strong, specific cleavage of the RNA in ribosomes when incubated with ribonuclease H. Binding values should therefore be regarded as relative, rather than quantitative measurements. Variation in probe binding and likely reasons for substoichiometric probe binding are discussed more thoroughly in Weller and Hill (1992).

That the 2109–2119 region is involved directly or indirectly in E site tRNA binding is demonstrated by the displacement of a probe spanning this region by deacylated tRNA on 50S subunits (Figure 1). On 70S ribosomes, deacylated tRNA binds first to the P site and then, above a ratio of 1.5–2 tRNAs per ribosome, to the E site (Rheinberger et al., 1981; Kirillov et al., 1983; Lill et al., 1984). If deacylated tRNA binding to 50S subunits mimics tRNA binding to 70S ribosomes, the probe is displaced here at E site stoichiometries. However, several researchers have suggested that only the E site can be occupied by deacylated tRNA on 50S subunits; i.e. there is no P site binding (Rheinberger et al., 1990; Kirillov & Semenkov, 1986), in which case displacement of the probe could also only be caused by E site tRNA binding. Contrary to our results with the 2109–2119 probe, a probe spanning nucleotides 2165–2171 was not displaced significantly in titration experiments with deacylated tRNA on 50S subunits, but results discussed below suggest that the 2165–2171 region is indeed part of a dynamic environment associated with the E site.

In contrast to the case with 50S subunits, addition of the first two equivalents of tRNA to 70S ribosomes actually enhanced probe 2109–2119 binding more than 2-fold (Figure 2). However, at ratios higher than 2:1 tRNA to 70S ribosomes, probe binding was attenuated, as seen with 50S subunits. Since deacylated tRNA fills the tRNA binding sites of the 70S ribosome sequentially (in the order P, E, A; Rheinberger et al., 1990; Robertson et al., 1986) and, in the absence of mRNA, the A site is not significantly occupied (Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984), these conditions favor P and then E site binding. Given that the E site is not significantly populated until the tRNA to ribosome ratio exceeds 1.5–2:1, this experiment shows that the presence of deacylated tRNA in the P site actually facilitates probe binding in the E site, whereas tRNA in the E site displaces probe 2109–2119. Probe 2165–2171, like probe 2109–2119, displayed binding enhancement at lower ratios of tRNA but was not displaced at higher levels (Figure 3), suggesting that this region is not involved in direct tRNA/rRNA interaction or that simultaneous binding of probe and tRNA is possible here.

To ensure that the enhancement in probe binding seen in the last experiments was indeed caused by P site tRNA binding, a similar experiment was conducted using $tRNA^{phe}$ isolated from *S. cerevisiae*. This tRNA species binds the P site of *E. coli* with about the same affinity as tRNA isolated from *E. coli*, but it has a very low affinity for the E site (Lill et al., 1986). Therefore, in the absence of mRNA, yeast $tRNA^{phe}$ serves as a P site-specific tRNA on the *E. coli* ribosome. Figure 4 shows that probe 2109–2119 binding was enhanced upon yeast $tRNA^{phe}$ binding, but it was not displaced, even at high levels of the yeast tRNA. This experiment provided additional evidence that the presence of deacylated tRNA in the P site caused a change in a local environment shown earlier to be involved in E site tRNA interaction.

Having established that deacylated tRNA in the P site enhanced probe binding in the E site, we next examined the effect of acylated tRNA binding on probe binding. Figure 5 shows simultaneous binding data for [^{14}C]Phe-$tRNA^{phe}$ and probe 2109–2119 on poly(U) programmed 70S ribosomes. Under these conditions, tRNA binding order is P then A, with no E site binding, as the E site is specific for deacylated tRNA. There was neither enhancement nor attenuation of probe binding throughout the range of acylated tRNA concentrations used. Similar results were obtained with *N*-acetyl-Phe-tRNA (data not shown). The lack of attenuation of probe 2109–2119 binding, even at high Phe-$tRNA^{phe}$ binding levels, supports the assertion that 2109–2119 does lie in the exit site, since it can be displaced by deacylated, but not acylated, tRNA. Also, the lack of enhancement of probe binding upon P site binding of Phe-$tRNA^{phe}$ suggests that deacylated tRNA induces a change in the E site that acylated tRNA does not.

The experimental findings presented above suggest a model for active interaction between tRNA binding sites P and E on the *E. coli* ribosome. Since the presence of deacylated tRNA in the P site only occurs immediately following peptidyl transfer and before translocation, the deacylated tRNA in the P site could serve as an indicator that translocation is imminent, and the E site should be readied to receive deacylated tRNA. Conversely, the presence of an aminoacyl or a peptidyl tRNA in the P site is characteristic of a pre-peptidyl transfer ribosome, and translocation would not be appropriate in such a state, so the E site should not be altered. Our experimental results suggest that the P site can discriminate between species of tRNA and alter the E site's availability for probe binding (and by extension, tRNA binding) accordingly.

Lill et al. (1989) have proposed that the 3′ end of deacylated tRNA in the P site is involved in actively promoting translocation. They found that only deacylated tRNAs containing intact 3′ ends were capable of promoting translocation and suggested that base pairing between nucleotides

at the 3′ end of the tRNA and nucleotides in the exit site helped to destabilize deacylated tRNA binding in the P site. They proposed that an acylated or modified 3′ end could not come in contact with the E site nucleotides and thus could not facilitate translocation. Although we did not find direct evidence for the base pairing they described, we did find that a free 3′ end of tRNA was necessary to cause a change in the E site which could promote translocation.

The allosteric three-site model proposed by Gnirke et al. (1989) describes high- and low-affinity states for the A and E sites. Consistent with the allosteric three-site model, this study demonstrates the existence of two states of the E site, with the high-affinity (or "high-availability") state occurring when a deacylated tRNA is located in the P site. This could provide thermodynamic encouragement for translocation of a deacylated tRNA in the P site to the E site. The present study proposes an allosteric link or a conformational switching mechanism between the P and E sites, a feature not described for these two sites in the allosteric three site model.

The data presented here are in general agreement with the chemical protection data of Moazed and Noller (1989a) identifying E site nucleotides in 23S rRNA. Our results support the idea that the 2109–2119 region is involved in E site binding, since probes spanning that region are displaced in conditions favoring E site tRNA binding on both 50S subunits and ribosomes. Of course, it is possible that the displacement of probes is caused by a change in conformation of the rRNA upon E site tRNA binding. The 2165–2171 probe was not displaced by deacylated tRNA in the E site, but it did show enhanced binding with deacylated tRNA in the P site, like the 2109–2119 E site probe, so its precise role in the E site is still unclear. Nucleotide C2394's role in E site tRNA binding likewise is unclear in the context of this study, since our DNA probes did not hybridize efficiently to this region.

In discussing a refinement of tRNA binding states to the ribosome, Moazed and Noller (1989b) provided evidence for a hybrid P/E state, which is occupied primarily by deacylated tRNA. Deacylated tRNA showed usual 16S rRNA P-site protection with additional protection of C2394, which had been shown to be in the E site (Moazed & Noller, 1989a,b). From these data, they postulated that the tRNA was in a hybrid P/E state. Since we were unable to probe the 2394 region, we could not distinguish between the putative P/E site or the P/P site interaction as defined by them. Deacylated tRNA under our conditions enhanced the availability of regions 2109–2119 and 2165–2171, but nucleotides in these regions were not mentioned in the discussion of the P/E hybrid site interaction with deacylated tRNA (Moazed & Noller, 1989b).

In summary, we have used DNA probes complementary to portions of 23S ribosomal RNA to study the ribosomal exit site and found the exit site to be a dynamic environment. We have shown that the P and E sites are conformationally linked, in that the presence of deacylated tRNA in the P site makes the E site more available for probe hybridization. On the basis of these results, we have proposed a control mechanism in the elongation cycle which is distinct but compatible with several other current models of the ribosomal elongation cycle.

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