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Evidence for a tRNA/rRNA Interaction Site within the Peptidyltransferase Center of the *Escherichia coli* Ribosome†

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Received February 26, 1988; Revised Manuscript Received July 18, 1988

ABSTRACT: A nine-base oligodeoxyribonucleotide complementary to bases 2497–2505 of 23S rRNA was hybridized to both 50S subunits and 70S ribosomes. The binding of the probe to the ribosome or ribosomal subunits was assayed by nitrocellulose filtration and by sucrose gradient centrifugation techniques. The location of the hybridization site was determined by digestion of the rRNA/cDNA heteroduplex with ribonuclease H and gel electrophoresis of the digestion products, followed by the isolation and sequencing of the smaller digestion fragment. The cDNA probe was found to interact specifically with its rRNA target site. The effects on probe hybridization to both 50S and 70S ribosomes as a result of binding deacylated $tRNA^{Phe}$ were investigated. The binding of deacylated $tRNA^{Phe}$, either with or without the addition of poly(uridylic acid), caused attenuation of probe binding to both 50S and 70S ribosomes. Probe hybridization to 23S rRNA was decreased by about 75% in both 50S subunits and 70S ribosomes. These results suggest that bases within the 2497–2505 site may participate in a deacylated tRNA/rRNA interaction.

In an attempt to elucidate the mechanisms of protein biosynthesis, considerable attention has been focused on the interactions of transfer RNA (tRNA) with the ribosome. Recent evidence suggests that the ribosome possesses three tRNA binding sites (Rheinberger et al., 1981) designated the P-site (for peptidyl), A-site (for aminoacyl), and E-site (for exit). While the stoichiometry of tRNA binding has now been defined, the portions of the ribosome involved in tRNA/ribosome interactions have not yet been delineated.

Evidence is accumulating which suggests that ribosomal RNA (rRNA) may participate in tRNA/ribosome interactions. A-site-bound tRNA derivatives have been cross-linked to 16S rRNA (Schwartz et al., 1975; Gornicki et al., 1985) while P-site-bound tRNA derivatives have been cross-linked to 23S rRNA (Barta & Kuechler, 1983; Sonenberg et al., 1978) and 16S rRNA (Schwartz & Ofengand, 1978; Prince et al., 1982). Shielding of certain rRNA bases from chemical modification has been shown to occur upon the binding of

† This work was sponsored in part by grants from the National Science Foundation (DMB8417297) and the National Institutes of Health (GM35717).

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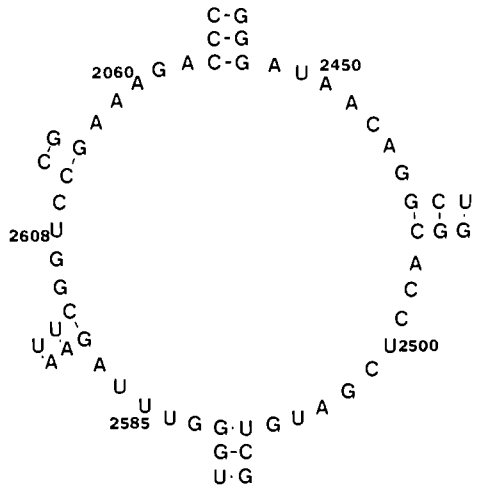

FIGURE 1: Secondary structure map of the central loop of the peptidyltransferase center of *E. coli*.

tRNA (Meier & Wagner, 1984; Moazed & Noller, 1986). While these results do not demonstrate conclusively that a direct interaction occurs, they suggest that tRNA, while bound to the ribosome, may reside in close proximity to the rRNA.

Several sequences of rRNA have been shown by comparative sequence analysis to be phylogenetically highly conserved (Woese et al., 1983). Secondary structure maps of several organisms suggest that these conserved sequences are organized into conserved domains (Branlant et al., 1981; Noller et al., 1981). The peptidyltransferase center of the 50S subunit is comprised of highly conserved rRNA, and its secondary organization varies little among evolutionarily diverse organisms (Figure 1). Such conservation of sequence and secondary structure suggests that these regions may be functionally or structurally required for protein biosynthesis (Woese et al., 1983; Noller, 1980).

We have investigated possible interactions between 23S rRNA and deacylated $tRNA^{Phe}$ in the 50S subunit and 70S ribosome through the use of a complementary DNA (cDNA) probe. The probe, 5'-CATCGAGGT, was synthesized, purified, 5' end labeled with ^{32}P or ^{35}S, and hybridized to a complementary single-stranded, conserved sequence containing bases 2497–2505 of the peptidyltransferase center. This approach allowed us to determine (a) if a site is available for hybridization and thus potentially capable of interacting with tRNA directly and (b) if tRNA and cDNA probes compete for the same binding site. Our results show that the rRNA bases, 2497–2505, are accessible for hybridization with a cDNA probe and that deacylated $tRNA^{Phe}$ will compete for this rRNA binding site.

Materials and Methods

Materials. All reagents employed in the synthesis and purification of cDNA probes were of HPLC grade. All radioisotopes were obtained from New England Nuclear; RNase H, spleen phosphodiesterase II, reverse transcriptase, and all deoxynucleotides and dideoxynucleotides were from Pharmacia; Bal 31, RNA size markers, and NACs columns were from Bethesda Research Labs; poly(ethylenimine)–cellulose thin-layer chromatography sheets were from Brinkmann; alkaline phosphatase, deacylated $tRNA^{Phe}$, and poly(uridylic acid) [poly(U)] were from Boehringer Mannheim, and nitrocellulose filters (HAWP, 0.45 μm) were from Millipore.

Isolation of Ribosomes and Ribosomal Subunits. Ribosomes and ribosomal subunits were isolated during midlog phase from *Escherichia coli* MRE 600, as previously described (Marconi & Hill, 1988). Tight-couple 70S ribosomes were prepared according to Noll et al. (1973). Reassociated 70S ribosomes were obtained by the method of Tapprich and Hill (1986). To verify complete association, aliquots from each preparation were centrifuged on a 5–20% sucrose gradient (in 10 mM $MgCl_2$, 60 mM KCl, 10 mM Tris-HCl, pH 7.4) for 4.5 h at 37 000 rpm, in a Beckman SW41 rotor at 4 °C. Fractions were collected, and the absorbance of each fraction was measured at 260 nm. One A_{260} unit of 70S ribosomes or 50S subunits was taken to equal 25.5 pmol and 39 pmol, respectively.

Subunit preparations were checked for homogeneity by sedimentation velocity centrifugation in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. The integrity of the 23S rRNA was checked by phenol extraction of the 23S rRNA from 50S subunits, ethanol percipitation of the rRNA, electrophoresis [35:1 acrylamide/bis(acrylamide), 7 M urea, 89 mM Tris–borate, 1 mM EDTA, pH 8.3] on a 5% polyacrylamide gel at 12.5 mA for 5 h, and staining with 0.025% methylene blue.

Synthesis, Purification, and Sequencing of cDNA Probes. Complementary DNA probes were synthesized on a Biosearch Model 8600 automated DNA synthesizer utilizing β-cyanoethyl diisopropylamino phosphoramidite chemistry, deprotected according to the manufacturer's protocol, and purified by reverse-phase high-performance liquid chromatography (Marconi & Hill, 1988). Probes were enzymatically sequenced by the method of Black and Gilham (1985). Probes are designated by the numerical base assignment given to the rRNA to which they are complementary. One A_{260} unit of a nine-base oligomer was taken to equal 12 270 pmol.

5' End Labeling of cDNA Probes and Deacylated $tRNA^{Phe}$. Purified probes were 5' end labeled by polynucleotide kinase with either [γ-^{32}P]ATP (Chaconas & Van de Sande, 1980) or [^{35}S]ATPγS. To label with [^{35}S]ATPγS, 30 pmol of probe was incubated with 30 pmol of [^{35}S]ATPγS and 20 units of polynucleotide kinase in 50 μL of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 3 mM DTT, and 0.1 mM EDTA for 2.5 h at 37 °C. After labeling with either isotope, the reactions were phenol extracted and purified on a NACs column according to the manufacturer's protocol. Labeled probes were diluted with unlabeled probe to achieve final specific activities between 500 and 1000 cpm/pmol. Labeled preparations were checked for homogeneity by electrophoresis on a 20% polyacrylamide/7 M urea gel, for 50 min at 40 mA, followed by autoradiography.

Deacylated $tRNA^{Phe}$ was dephosphorylated with calf intestinal phosphatase and 5' end labeled with [γ-^{32}P]ATP by polynucleotide kinase (Lill et al., 1984). Labeled tRNA preparations were checked for homogeneity by electrophoresis on a 10% polyacrylamide/7 M urea gel for 2 h at 12.5 mA.

Hybridization of cDNA Probes to 70S Ribosomes and 50S Subunits. Hybridization of cDNA probes to 50S subunits was carried out as previously described (Marconi & Hill, 1988). Hybridization to 70S ribosomes was conducted in an analogous fashion with an equimolar amount of 70S ribosomes used in place of the 50S subunits.

Probe binding to ribosomal subunits was also monitored qualitatively by sucrose gradient centrifugation. Thirty-seven picomoles of 50S subunits was incubated with radiolabeled probe (500 000 cpm) for 4 h in binding buffer (10 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl, pH 7.4) on ice. The reactions were layered on a 5–20% sucrose gradient (in binding buffer) and centrifuged for 1.75 h at 54 000 rpm (4 °C) in a Beckman SW60 rotor. After centrifugation, the centrifuge tubes were punctured, and 200-μL fractions were collected and

diluted to 500 μL with 300 μL of binding buffer. Migration of subunits was monitored spectrophotometrically while radiolabeled probe migration was followed by liquid scintillation of the fractions.

Determination of Hybridization Sites. The cDNA/subunit complexes were incubated with RNase H according to Tapprich and Hill (1986) with some modifications (Marconi & Hill, 1988). Digestion products were electrophoresed on a 5% polyacrylamide/0.15% bis(acrylamide) gel (7 M urea) for 5 h under a constant current of 12.5 mA, and their size was estimated to within ±10 bases by use of RNA size markers. After the approximate size of the digestion product was obtained, the site of cleavage was determined by a computer search of the 23S rRNA sequence which revealed all potential probe binding sites.

Isolation and Sequencing of the Ribonuclease H Generated 23S rRNA Digestion Product. As a final verification of the identity of the probe/23S rRNA hybridization site, the 400-base fragment generated by RNase H digestion of the rRNA in the presence of the 2497 probe was isolated and sequenced. The portion of the gel containing the 400-base fragment was excised from the gel under UV light, crushed with a siliconized glass rod, resuspended in 600 μL of elution buffer [500 mM NH_4Cl, 10 mM MgOAc, 1 mM EDTA, and 0.1% (w/v) SDS] in a siliconized test tube, incubated for 18 h at 37 °C, and spun in a table-top centrifuge (1 min) to pellet the acrylamide. The supernatant was removed and transferred to another siliconized test tube, and the acrylamide pellet was resuspended in 500 μL of elution buffer and again treated as before, combining the supernatants. Three volumes of ice-cold absolute ethanol was added, followed by centrifugation (1 h, 4 °C) in a Sorvall HB-4 rotor (10000 rpm). The pelleted RNA was recovered and resuspended in 10 mM Tris-HCl, pH 7.4.

The sequence of the isolated 400-base rRNA fragment was determined through the reverse transcriptase primer extension dideoxynucleotide method of Lane et al. (1985) with some modifications (Lane et al., 1988). The primer (5'-GAA-CAG-CCA-TAC-CCT-TG) was complementary to bases 2540–2556, which is approximately 35 bases downstream of the intended cleavage site. The sequencing reactions were loaded onto a 43 × 38 cm, preelectrophoresed (2 h), 0.35 mm thick, 10% polyacrylamide gel [35:1 acrylamide/bis(acrylamide), 89 mM Tris–borate, 1 mM EDTA, pH 8.3, and 7 M urea]. Electrophoresis was carried out for 2 h at a constant voltage of 1600 V followed by autoradiography.

Competition Binding Experiments. Saturating amounts of 5' ^{32}P-labeled cDNA probe (between 700 and 800 pmol as determined from saturation curves) were incubated with 25 pmol of either 50S subunits or tight-couple 70S ribosomes in 40 μL of binding buffer (15 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl, pH 7.4) for 4 h on ice. After this initial incubation, increasing amounts of unlabeled deacylated $tRNA^{Phe}$ (up to a 2:1 ratio of tRNA/subunits) either with or without poly(U) (10 μg) were added to the reactions. Final volumes were brought to 50 μL and the salt conditions adjusted so that all reactions were as in the previously described binding buffer. The reactions were incubated for an additional 0.5–4 h on ice, diluted to 1 mL with binding buffer, and immediately filtered through nitrocellulose filters. The filters were washed twice (flow rates were maintained at 12 mL/min) with 1 mL of ice-cold binding buffer and dried, and the amounts of radiolabeled probe bound to the subunits were determined by liquid scintillation counting. Probe retention by the filters was determined by layering an identical amount of radiolabeled probe in binding buffer on the filters and treating as previously described. This value was then subtracted from the values obtained for each reaction.

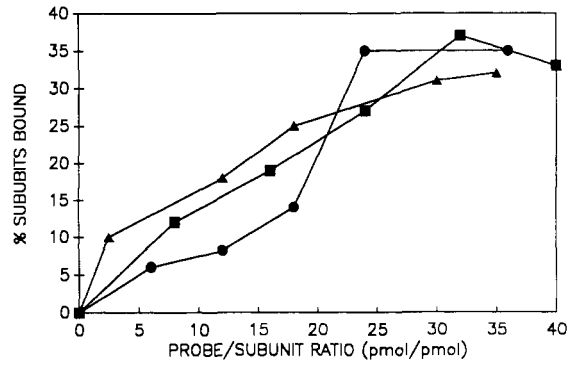

FIGURE 2: Binding curves for 2497 probe to 50S subunits, tight-couple 70S ribosomes, and reassociated 70S ribosomes. Increasing amounts of ^{32}P-labeled 2497 probe were incubated with 25 pmol of either 50S subunits (triangles), tight-couple 70S ribosomes (circles), or reassociated 70S ribosomes (squares) for 18 h in 50 μL of binding buffer (10 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl, pH 7.4) at 0 °C. Reactions were diluted to 1 mL with binding buffer and filtered through nitrocellulose filters at a flow rate of 12 mL/min, washed with two 1-mL washes of binding buffer, and dried, and the retained radioactivity was measured by liquid scintillation counting.

To verify that tRNA and probe were binding to the same population of ribosomes, ^{35}S-labeled probe and ^{32}P-labeled tRNA were coincubated with 50S subunits under the conditions described above, followed by nitrocellulose filtration and liquid scintillation counting. The amount of probe added was maintained at 800 pmol while tRNA was added in increasing amounts up to a 2:1 ratio (50 pmol) of tRNA/50S subunits.

The effects of poly(U) on probe binding to 50S subunits was also assayed. Increasing amounts of poly(U) were added to a series of reactions containing 750 pmol of radiolabeled 2497 probe and 25 pmol of 50S subunits in 50 μL of binding buffer. The reactions were incubated on ice for 6 h and treated as described above.

Transfer RNA Preparation and Binding. Deacylated $tRNA^{Phe}$ was purchased in lyophilized form and redissolved in distilled H_2O. Transfer RNA binding to 50S subunits was assayed by incubating increasing amounts of ^{32}P-labeled deacylated $tRNA^{Phe}$ with 25 pmol of 50S subunits, either with or without poly(U) (10 μg), in 50 μL of binding buffer for 2 h on ice. Reactions were diluted to 1 mL with tRNA binding buffer and treated as described under Competition Binding Experiments.

RESULTS

Hybridization of cDNA Probes to Ribosomes and Ribosomal Subunits. The binding of a probe, designated 2497, to 50S subunits, tight-coupled 70S, and reassociated 70S ribosomes was assayed by nitrocellulose filtration and liquid scintillation of the filters. This probe was complementary to bases 2497–2505 of 23S rRNA. The maximum binding to 50S subunits was 32% with saturation occurring at a ratio of 30:1 (probe/subunits). A slightly higher level (approximately 35%) of binding occurred with tight-couple 70S and reassociated 70S ribosomes (Figure 2).

Probe/subunit complex formation was also assayed by sucrose gradient centrifugation. This approach allows a qualitative analysis of the probe/subunit interaction. Comigration of the radiolabeled probe with 50S subunits was observed (Figure 3).

Hybridization Specificity. The specificity of the probe/rRNA interaction was demonstrated by digestion of the rRNA

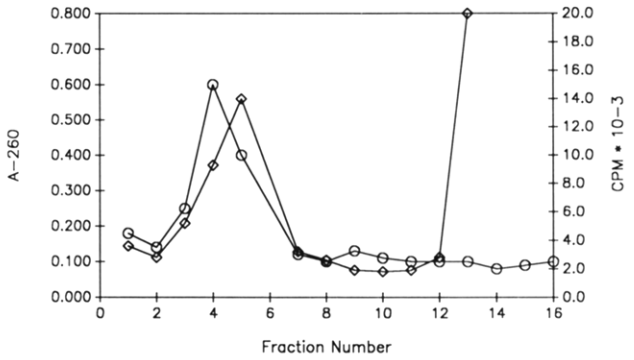

FIGURE 3: Sucrose gradient binding analysis of 2497 probe/50S subunit complex formation. Conditions were as described under Materials and Methods. Circles denote the A_{260} profile of 50S subunit migration while diamonds denote the radioactivity associated with ^{32}P-labeled probe migration.

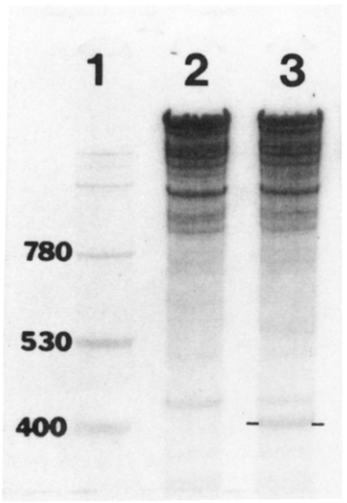

FIGURE 4: Ribonuclease H digestion of 50S subunits with 2497 probe. Fifty picomoles of 50S subunits was incubated with 150 pmol of 2497 probe and 2.5 units of RNase H in 10 mM $MgCl_2$, 60 mM KCl, and 40 mM Tris-HCl (pH 7.9) for 18 h at 4 °C. Reactions were extracted with equilibrated phenol three times, and the rRNA was ethanol percipitated with 3 volumes 95% EtOH, pelleted, washed with 3 volumes of 70% EtOH/40 mM NaCl, pelleted, resuspended, and then run on a 5% polyacrylamide/7 M urea gel (89 mM Tris–borate, pH 8.3, 2 mM EDTA) at 12.5 mA for 5 h, after which it was stained with methylene blue. (Lane 1) RNA size markers. (Lane 2) Control, 50S subunits only, treated as described above. (Lane 3) RNase H reaction: 50S subunits incubated with 2497 probe and RNase H as described above. The marks indicate the 3′ fragment generated upon digestion.

within the rRNA/DNA heteroduplex by RNase H (Figure 4). The digestion products were electrophoresed, and only one cleavage was observed. The smaller fragment generated by this cleavage was estimated to be approximately 400 (±10) bases in length on the basis of comparison with RNA size markers.

Sequencing of a Portion of the 400-Base rRNA Fragment Generated by Digestion with Ribonuclease H in the Presence of the 2497 Probe. The 400-base rRNA fragment generated upon RNase H digestion of the 23S rRNA in the presence of the 2497 probe was isolated and partially sequenced. If RNase H clipping of the 23S rRNA is occurring within the 2497–2505 region, then extension of the 2450–2466 primer should terminate at approximately base 2505. The partial sequence of the rRNA can be inferred from the extended primer DNA sequence up to base position 2505 (Figure 5). The RNA sequence, starting at position 2529, reads (3′ to 5′) G*C-GGG-GCC-C*A-CAC-*AC-*CG-GC*-G, with (*) in-

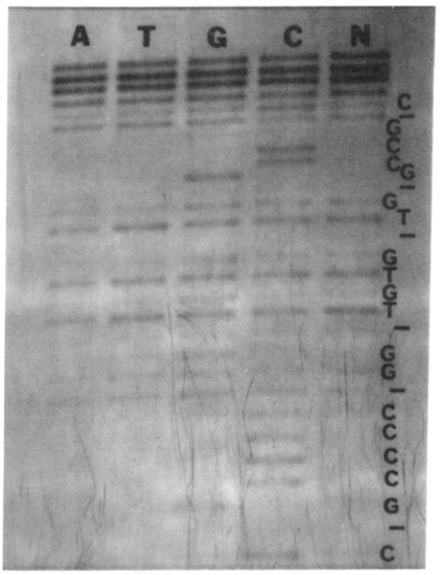

FIGURE 5: Partial sequencing of the 400-base rRNA fragment generated by digestion of the rRNA/DNA heteroduplex with ribonuclease H. The 400-base digestion fragment was isolated and partially sequenced as described in the text. Lanes A, T, G, and C denote the dideoxynucleotide included in the reaction. Lane N contained no dideoxynucleotides. The partial sequence of the extended DNA primer is listed adjacent to the N lane with the inferred rRNA sequence listed in the text. Ambiguous residues are marked by a hash mark.

dicating residues which could not be positively identified. While several positions are ambigous, comparison with the known *E. coli* 23S rRNA sequence reveals that the sequence of the isolated fragment matches that expected for the 2505–2529 region of 23S rRNA, demonstrating that the probe was hybridizing to its intended target site spanning bases 2497–2505. The four bands seen on the top of the autoradiograph (with the top corresponding to the 5′ terminus of the digestion fragment) are most likely a result of the heterogeneity of the 5′ terminus of the fragment which results from the RNase H digestion.

Effects on the Conformation of an Adjacent Double-Stranded Region upon Hybridization of the 2497 Probe. To determine if the hybridization of the 2497 probe induces conformational changes in adjacent double-stranded regions, the accessibility of bases 2489–2496 to an oligomer designated 2489-96 (5′-GCCAAACA) was probed in the presence or absence of the 2497 probe. According to current secondary structure maps, this site exists in a double-stranded conformation with bases 2455–2461. As expected, when the 2489-96 probe was incubated alone with 50S subunits, the maximum binding to this site was low (approximately 5%), and digestion by RNase H was not observed. The binding of ^{32}P-labeled 2489-96 was not affected by the coincubation of ^{35}S-labeled 2497. This suggests that the binding of the 2497 probe to the rRNA does not disrupt the conformation of this adjacent double-stranded stem in such a way as to allow increased hybridization of the 2489-96 probe.

Deacylated Transfer RNA Binding to 50S Subunits. The maximum percent binding of tRNA to 50S subunits obtained under the conditions described here is in general agreement with the percent binding values reported by Gnirke and Nierhaus (1986). The maximum percent binding values for deacylated $tRNA^{Phe}$ were about 45 and 30%, with and without poly(U), respectively (Figure 6).

Effects on the Binding of 2497 Probe upon the Binding of Deacylated $tRNA^{Phe}$. Competition experiments were performed to determine if the 2497 probe and deacylated $tRNA^{Phe}$ would compete for the same binding site on 23S rRNA. The

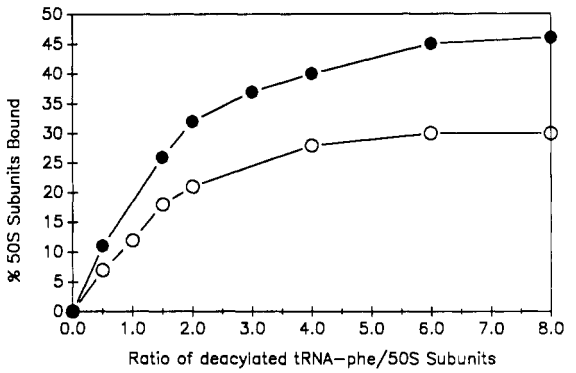

FIGURE 6: Binding of deacylated $tRNA^{Phe}$ with and without poly(U). Twenty-five picomoles of 50S subunits with (filled circles) and without poly(U) (open circles) were incubated with increasing amounts of ^{32}P-labeled deacylated $tRNA^{Phe}$ in 50 μL of tRNA binding buffer (15 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl, pH 7.4) for 2 h on ice. Reactions were diluted to 1 mL with tRNA binding buffer, immediately filtered through nitrocellulose filters (HAWP, 0.45 μm), washed twice with 1 mL of buffer, and dried, and the retained radioactivity was measured by liquid scintillation counting of the filters.

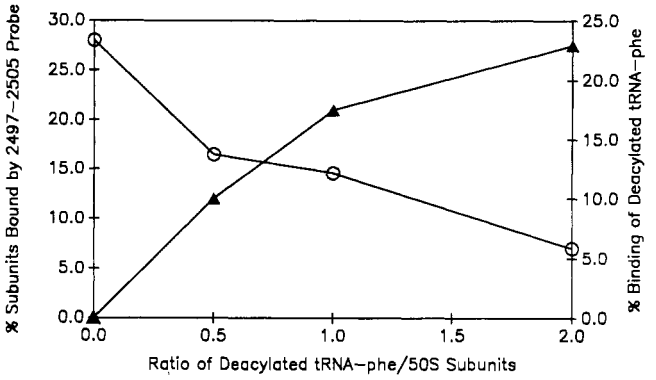

FIGURE 7: Dual-label competitive binding experiment between ^{35}S-labeled 2497 probe and ^{32}P-labeled deacylated $tRNA^{Phe}$ to 50S subunits. Increasing amounts of ^{32}P-labeled deacylated tRNA were added to a series of reactions containing 25 pmol of 50S subunits and 750 pmol of ^{35}S-labeled 2497 probe. Conditions were as described under Materials and Methods. Probe binding is labeled with open circles and tRNA binding with triangles.

binding of ^{35}S-labeled 2497 to 50S subunits decreased in a linear fashion upon the addition and binding of increasing amounts of ^{32}P-labeled deacylated $tRNA^{Phe}$ (Figure 7). At a 2:1 ratio of tRNA/50S subunits a 74% decrease in probe binding was observed. This dual-label experiment suggests that the probe and tRNA may be competing for a single binding site and that they were both binding to the same population of ribosomes.

In a similar experiment, ^{32}P-labeled probe was coincubated with deacylated $tRNA^{Phe}$, poly(U), and 50S subunits. In this case probe binding decreased by 72%. The binding of ^{32}P-labeled probe to tight-couple 70S ribosomes was also assayed in the presence of deacylated $tRNA^{Phe}$ and poly(U). Consistent with the results obtained with the 50S subunits, probe binding was seen to decrease by 76%. In all cases the maximum inhibition of probe binding occurred when the tRNA/ribosome ratio was approximately 2:1 and did not increase upon further addition of deacylated $tRNA^{Phe}$.

The addition of a saturating amount (800 pmol) of 2497 probe to the tRNA binding reactions (under the conditions described under Transfer RNA Preparation and Binding) did not cause an attenuation in the maximum percent binding of ^{32}P-labeled deacylated $tRNA^{Phe}$ to 50S subunits (data not shown). The results of the competition studies are summarized in Table I.

Table I: Competition Data Summary for 2497–2505 Site

ribosome preparation	inhibitor added	percent inhibition of probe binding
(1) 50S	tRNA/poly(U)	74
(2) 50S	tRNA	72
(3) TC70S	tRNA/poly(U)	76
(4) 50S	poly(U)	0

Effects of the Addition of Poly(U) on 2497 Probe Binding to 50S Subunits. These experiments were performed to determine whether poly(U) itself would have an effect on probe binding through either its interaction with the ribosome or its hybridization with the probe. Poly(U) did not cause a decrease in the binding of the probe to the 2497–2505 region.

Discussion

The ribosome must of necessity interact with tRNA during the process of protein biosynthesis. This interaction or binding is probably mediated through several contact points. The results of this study suggest that one of the sites with which tRNA may interact is the 2497–2505 region of 23S rRNA. A cDNA oligonucleotide probe hybridizes specifically and readily to this site in both 50S subunits and 70S ribosomes. However, probe binding is dramatically attenuated upon binding of deacylated $tRNA^{Phe}$, suggesting that tRNA and the probe maybe competing for the same rRNA binding site.

The region probed in this study exhibits a high degree of phylogenetic conservation and is located in the central loop of the peptidyltransferase center. It has been suggested that such highly conserved regions may play functional roles in protein biosynthesis (Woese, 1980).

The attenuation of probe binding upon the addition and binding of tRNA may be interpreted to represent direct competition between the tRNA and the 2497 probe for binding at the 2497–2505 rRNA site. One of the possible interactions is between the TΨC-loop and 23S rRNA. Bases 2500–2503 of the 23S rRNA are complementary to bases 55–58 of the TΨC-loop (or pseudouridine loop) of *E. coli* $tRNA^{Phe}$ and thus are potentially capable of hybridization. Both the tRNA (Singhal & Fallis, 1979) and the rRNA sequences are phylogenetically highly conserved.

The concept of a tRNA TΨC-loop/rRNA interaction has been suggested by several groups [see Noller (1984)]. Some of the most direct evidence has come from studies in which tRNA fragments or oligonucleotides (5′-GTUC) were shown to inhibit the binding of Phe-$tRNA^{Phe}$ to both the P- and A-sites (Ofengand & Henes, 1969; Shimizu et al., 1970; Richter et al., 1973). These tRNA analogues were presumed to act upon the 50S subunits. It was initially suggested that the TΨC-loop interaction may be with 5S rRNA. However, this possibility has now been ruled out (Pace et al., 1982; Zagorska et al., 1984). It seems more likely that the interaction is with 23S rRNA.

Evidence that tRNA binds in close proximity to the central loop of the peptidyltransferase center has also come from the cross-linking of a P-site-bound photoreactive benzophenone derivative of yeast Phe-tRNA to the 23S rRNA bases U-2584 and U-2585 (Barta & Kuechler, 1983). The site probed in this study resides within this central loop. If bases 2500–2503 of 23S rRNA hybridize with bases 55–58 of the TΨC-loop of $tRNA^{Phe}$, this would position the aminoacyl arm such that cross-linking to bases U-2584 and U-2585 could occur.

There may be some questions concerning the availability of the TΨC-loop in solution. The conserved cloverleaf structure and the large number of invariant bases in tRNAs suggest that the tRNAs may possess "tertiary" hydrogen

bonds. Residues 18/55 (G/U), 19/56 (G/C), and 54/58 (T/mA) have been postulated to hydrogen bond to one another (Singhal & Fallis, 1979). These suggestions would argue against a TΨC-loop/rRNA interaction since the TΨC-loop would not be accessible for hybridization. However, Pongs et al. (1973) have reported that residues 53–60 (5′-GTUCGAUC) of yeast $tRNA^{Phe}$ are all accessible, to varying degrees, for hybridization with oligonucleotides. The authors suggest that the accessibility of specific sequences of tRNA molecules in solution may change in more physiological conditions or upon interaction with the ribosome. The postulated TΨC-loop/rRNA interaction presented here is dependent upon such a conformational change occurring in the tRNA.

Other possibilities exist to account for the decrease in probe binding upon the binding of tRNA. Steric exclusion of the probe from its complementary binding site may occur as a result of the binding of tRNA to either ribosomal proteins or other proteins of rRNA in the peptidyltransferase center. Binding competition was not observed between deacylated tRNA and probes complementary to the single-stranded regions spanning bases 2448–2454 or 2468–2482 (data not shown). Therefore, it does not appear likely that the tRNA is sterically preventing the binding of the 2497 probe as a result of an interaction with these nearby rRNA regions. It is possible that tRNA may simply block access of the probe to the 2497–2505 site without directly interacting with the rRNA itself. The data presented here do not allow us to exclude such a possibility.

Alternatively, the binding of deacylated tRNA at a local or distant site may cause a conformational change in the rRNA between bases 2497 and 2505, which is unfavorable for hybridization of the 2497 probe. The enzymatic binding of Phe-$tRNA^{Phe}$ to tight-couple 70S (TC70S) ribosomes has been reported to induce conformational changes in the 50S subunit (Srivastava & Burma, 1985). However, the authors also report that the binding of uncharged tRNA to 50S subunits does not result in conformational changes. These observations suggest that conformational changes are not responsible for the attenuation of probe binding upon the binding of deacylated tRNA.

We have shown in this study that the binding of deacylated tRNA to TC70S ribosomes as well as to 50S subunits alone results in a marked attenuation of probe binding to the 2497–2505 rRNA site. The ratio of deacylated tRNA/TC70S ribosome utilized in the 2497 probe/tRNA competition assay ranged from 0 to 2:1. Under these conditions deacylated tRNA has been shown to bind predominantly into the P-site (Robertson & Wintermeyer, 1981; Lill et al., 1984). The first binding site filled by deacylated tRNA on poly(U)-programmed 70S ribosomes is the P-site (Wantanabe, 1972). Binding into the E-site is not initiated until the deacylated tRNA/TC70S ratio approaches at least (1.5–2):1 (Lill et al., 1984; Nierhaus et al., 1981). The attenuation of probe binding which resulted upon the binding of deacylated tRNA to TC70S reached a maximum when the ratio of deacylated tRNA/TC70S was 2:1 but was initiated prior to a 0.5:1 ratio. Attenuation of 2497 probe binding appears to be initiated and continues as the P-site is filled, reaching a maximum (76%) before E-site binding of the deacylated tRNA becomes significant.

When the 50S particle is used in the 2497 probe/deacylated tRNA competition experiments, virtually identical attenuation of probe binding is observed. Gnirke and Nierhaus (1986) have suggested that the P-site resides on the 30S particle and the E-site on the 50S particle and that the A-site is formed upon association of the subunits. Since identical attenuation is observed on both the 70S and 50S particles, it appears that the interaction of deacylated tRNA with the 70S or 50S particle is identical. If so, then the assignment of the P-site tRNA binding site solely to the 30S subunit should be reevaluated. It appears more likely that at least a portion of the P-site is also found on the 50S subunit and that the portion of the binding site located on the 50S subunit may represent the TΨC-loop interaction site postulated here.

The addition of saturating amounts (800 pmol) of unlabeled 2497 probe to the deacylated $tRNA^{Phe}$ binding reactions did not result in an attenuation of tRNA binding as measured by the nitrocellulose membrane filtration technique described above. If the postulated TΨC-loop interaction with rRNA bases 2500–2503 (a four-base complementarity) were the only contact between the ribosome and deacylated tRNA, one would expect that the nine-base probe, having a greater binding constant than the tRNA, would displace the tRNA. In that tRNA/ribosome interactions are most likely mediated through several contact points, the inability of the probe to inhibit the binding of, or displace, the tRNA is not unexpected.

The ratio of probe/ribosomal particle required to saturate an intended target site, as determined by the nitrocellulose filtration technique, was approximately 30:1 for the 2497–2505 site. This level is consistent with those required to saturate other single-stranded regions of rRNA in the peptidyltransferase center (Marconi & Hill, 1988) as well as those reported for other rRNA sites on both the 30S and 50S subunits (Tapprich & Hill, 1986; Hill & Tassankajohn, 1987). The requirement for ratios between 15:1 and 40:1 is not surprising in that the ribosome contains, in addition to the target site, several other sites of partial complementarity for each probe employed. While the interaction at these secondary sites is certainly characterized by very low binding constants due to the low degree of complementarity, these sites will nonetheless undergo transient weak interactions with the probes, thus requiring a higher effective concentration or ratio of probe/ribosome to saturate. The stability of interactions at secondary sites of partial complementarity is apparently low enough that the binding to these sites cannot be measured by nitrocellulose filtration techniques. These possible interactions are also not detected by RNase H digestion assays.

To determine if the ratios employed to saturate would result in nonspecific interactions, probes to several double-stranded regions in the peptidyltransferase were synthesized, and their interaction with 50S subunits was measured by the nitrocellulose filtration technique described earlier. The target sites chosen were ones with sequences not repeated in other portions of the 23S rRNA molecule. The measured percent binding values for probes complementary to regions 2489–2496, 2589–2594, 2506–2513, and 2455–2461 ranged from 2 to 5% (data not shown). The low percent binding values observed for these double-stranded sites suggest that the ratios employed in this study to saturate an intended target site will not result in a significant amount of nonspecific binding of the probes to the ribosomal subunits.

Additional evidence is required in order to determine the precise location of tRNA/ribosome interaction sites. Chemical protection data on the interior of 23S rRNA upon the binding of tRNA would be of great value. However, to date this approach has been applied only to the last 200 bases from the 3′ terminus of the 23S molecule (Meier & Wagner, 1984).

Probing interactive sites with short cDNA oligomers provides circumstantial evidence for such interactions. When this technique is used, it is critical to ascertain the specificity of

probe binding which is most readily determined through the use of the enzyme RNase H. RNase H will cleave the RNA contained within an RNA/DNA heteroduplex (Donis-Keller, 1979). Upon electrophoresis of the digestion reactions, we have observed that only one cleavage results when the 2497 probe, 50S subunits, and RNase H are incubated together. RNA size markers were used to estimate the size of the resulting fragments to within ±10 bases. The smaller fragment obtained was approximately 400 bases in length. Since there was only one site of cleavage, this fragment must then be derived from either the 3′ or 5′ end of the 23S rRNA molecule.

A computer search of the 23S rRNA sequence revealed three potential sites of hybridization. Two of these sites were only partially complementary and occurred at base positions 47–51 and 1375–1380. The observed 400-base fragment generated by RNase H cleavage could not have resulted from hybridization to these sites. The third site, the intended target site (bases 2497–2505), occurs 400 bases from the 3′ end. Since this is the only site which could have generated a 400-base fragment, the cleavage must have occurred between bases 2497 and 2505. On the basis of this approach, the specificity of the probe/rRNA hybridization can generally be concluded.

Nonetheless, final verification of the specificity of the interaction was obtained through the isolation and partial sequencing of the smaller rRNA fragment generated by digestion of the RNA/DNA heteroduplex with RNase H. Since the sequence of 23S rRNA from *E. coli* is known, only a partial sequence is required to determine the location of the hybridization site. The sequence obtained by this approach revealed unambigously that the probe was hybridizing within its intended target site spanning bases 2497–2505.

Probing with cDNA oligomers provides a valuable approach for defining the precise segments of rRNA involved in the process of protein biosynthesis. Work is continuing in this direction.

Acknowledgments

We thank our colleagues Dr. David Camp, Thomas Gluick, and Gary White for their help and discussion in the preparation of the manuscript.

Registry No. Peptidyltransferase, 9059-29-4.

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