Regions of 16S Ribosomal RNA Proximal to Transfer RNA Bound at the P-Site of *Escherichia coli* Ribosomes†

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ABSTRACT: Unmodified uridines have been randomly replaced by 4-thiouridines in transfer RNA^{Phe} (tRNA^{Phe}) transcribed in a T7 RNA polymerase system. These 4-thiouridines serve as conjugation sites for attachment of the cleavage reagent 5-iodoacetamido-1,10-*o*-phenanthroline (IoP). In a reducing environment, when complexed with Cu^{2+}, 1,10-*o*-phenanthroline causes cleavage of nearby nucleic acids. We show here that tRNA−phenanthroline (tRNA−oP) conjugates, when bound at the P-site of 70S ribosomes and 30S ribosomal subunits, caused cleavage of ribosomal RNA (rRNA) mainly in domains I and II of 16S rRNA. Some positions were cleaved only when tRNA−oP was bound to 70S ribosomes or to 30S ribosomal subunits. In domain I, most cleavage sites occurred in or near the 530 pseudoknot region. In domain II, most nucleotides cleaved were near the 690 region and the 790 region. The only positions cleaved in domain III were near the 1050 region. There were no discernible nucleotides cleaved near the 1400 (decoding) region. Our results corroborated results of others, which have shown these sites to be protected from chemical modification by tRNA binding or to be cross-linked to P-site-bound tRNA. Use of cleavage reagents tethered to tRNA provides evidence for additional regions of rRNA that may be proximal to bound tRNA.

Transfer RNA interactions with the ribosome are central to the translational process. The tRNAs are positioned on the ribosome in such a way as to allow for the transfer of the amino acid carried by the incoming aminoacyl-tRNA to a growing peptide chain. The deacylated tRNA is then systematically released from the ribosome. For this to be accomplished, functional regions of the rRNA interact with tRNA as it is positioned on the ribosome. There is evidence that direct contact between 16S rRNA and tRNA occurs (*1*, *2*). Chemical protection assays indicate that 16S rRNA nucleotides G693, A794, C795, G926, and G1401 are strongly protected and nucleotides A532, G966, G1338, and A1339 are weakly protected from chemical modification when tRNA is bound at the P-site (*3*). The anticodon regions of various tRNAs, when bound to the P-site, have been cross-linked to various areas on the 16S rRNA: tRNA^{Arg} from position 32 to 16S rRNA near nucleotide 966 (*4*) and at nucleotide 693 (*5*), tRNA^{Val} from position 34 to 16S rRNA nucleotide 1400 (*2*), and tRNA^{Met} from position 20 to between 16S rRNA nucleotides 1302 and 1398 (*6*). These data taken together indicate that portions of the 16S rRNA apparently far removed from the decoding region may be constrained in the tertiary structure to interact with the tRNA during the translational process.

The 1,10-*o*-phenanthroline−copper complex has been shown to cause scission of nucleic acid (*7*−*9*), due to the formation of an oxidative species formed when the 1,10-*o*-phenanthroline−copper complex is subjected to a reducing environment (*10*, *11*).

We have taken advantage of the activity of 1,10-*o*-phenanthroline by conjugating it to various sites on tRNA^{Phe}. Eighteen resident uridines are present in our transcribed tRNA^{Phe}, which have been randomly substituted with 4-thiouridines (Figure 1). We have attached phenanthroline through a 9 Å linker to these modified uridines. Phenanthroline, when conjugated to tRNA, complexed with copper (II), and bound to various sites on the ribosome has the ability to cleave any rRNA in an approximate 10−15 Å radius from the site of attachment. By this approach, rRNA that is in a position to interact with bound tRNA may be identified.

MATERIALS AND METHODS

Materials and Reagents. All materials were obtained from sources previously noted, and reagents such as 5-iodoacetamido-1,10-*o*-phenanthroline (IoP),[1] 4-thiouridine triphosphate, and [(*N*-acryloylamino)phenyl]mercuric chloride (APM) were synthesized as previously described (*12*).

Ribosome and tRNA Preparation. The preparation of ribosomes and transcribed tRNAs was performed as previ-

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[1] Abbreviations: IoP, 5-iodoacetamido-1,10-*o*-phenanthroline; tRNA−oP, tRNA−1,10-phenanthroline conjugate; APM, [(*N*-acryloylamino)-phenyl]mercuric chloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MPA, 3-mercaptopropionic acid; DMS, dimethylsulfate; 4-SUTP, 4-thiouridine triphosphate.

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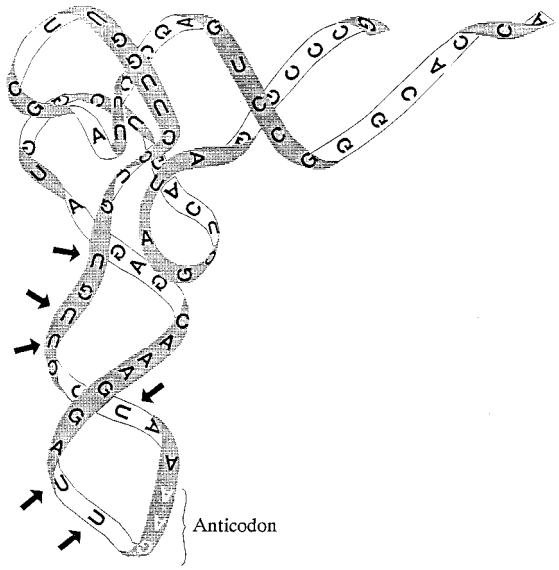

FIGURE 1: Tertiary structure model of the transcribed tRNA. The uridine residues were randomly replaced by 4-thiouridine residues to which 1,10-*o*-phenanthroline was conjugated. Arrows indicate uridine residues that would most likely be in positions to interact with the 30S ribosomal subunit. The ribbon structure of the tRNA is based on a drawing by Irving Geis (*38*).

ously outlined (*12*). The IoP was attached to the 4-thiouridine-modified tRNA following the method of Sigman (*13*) but modified by us as previously outlined (*12*). The structure of the transcribed tRNA was checked using lead cleavage (*14*). To check the positioning of tRNA on the ribosome, chemical modification using DMS was utilized according to the method outlined by Moazed and Noller (*1*).

tRNA−oP Cleavage Reactions. A modified version of cleavage reactions used by David Sigman was implemented (*13*), as outlined previously (*12*). Briefly, cleavage reactions were carried out in 50 μL of cleavage buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM $MgCl_2$), in which 1 μM 70S ribosomes or 30S ribosomal subunits, 4 μM tRNA−oP, and 40 μM $CuSO_4$ were incubated for 20 min at 37 °C. In poly(U)-directed binding of tRNA, the final concentration of poly(U) was 0.2 μg/μL. To induce cleavage, mercaptopropionic acid (MPA) was added to a final concentration of 2 mM and incubated for an additional 30 min at 37 °C. The reaction was stopped by adding 2.5 volumes of ice-cold 95% EtOH and the rRNA was isolated as described previously (*12*). Reactions were carried out in triplicate, using three different samples of tRNA−oP conjugates.

To further ensure that cleavage events were not a result of random binding by the tRNA−oP, competition for the binding site was carried out using a saturating amount (40 μM) of *Escherichia coli* $tRNA^{Phe}$.

To ascertain the ability of yeast $tRNA^{Phe}$ to displace tRNA−oP bound at the P-site of 30S subunits during cleavage reactions, a 2.5:1 ratio of yeast $tRNA^{Phe}$ to tRNA−oP was used.

To identify fragments due to nondirected cleavage, 300 pmol of IoP (6:1 IoP to 70S ribosomes molar ratio) was added to the reaction mixture containing 70S ribosome, poly(U), and *E. coli* $tRNA^{Phe}$.

Cleavage sites were identified using primer extension techniques as previously described (*12*). Intensity of cleavages was scored by visually comparing cleavage bands with sequencing bands on autoradiographs. Those cleavage bands that were less intense than sequencing bands on autoradiographs were scored as low-intensity cleavage sites, those approximately equal in intensity to sequencing bands were scored as moderate intensity, and bands more intense than sequencing bands were scored as high intensity. All primer extension reactions were carried out three times, using three different cleavage templates. Only those bands that were reproduced through all three primer extension reactions were counted.

RESULTS

Synthesis of tRNA−oP Conjugate. Transcribed tRNA contains none of the naturally occurring modified bases of native *E. coli* tRNA, but previous studies (*15*−*17*) have shown that although unmodified tRNAs are somewhat less efficient than native tRNAs in their interactions with the ribosome, they are fitting substitutes. Filter-binding assays showed that the transcribed $tRNA^{Phe}$ binds ribosomes as well as *E. coli* $tRNA^{Phe}$. At a 2:1 ratio of tRNA to 70S ribosomes, in various experiments, from 50% to 70% of the ribosomes are bound by tRNA (data not shown). Ribosome-bound transcribed tRNA was displaced by native *E. coli* tRNA quantitatively as the concentration of native tRNA was increased (data not shown). In cleavage reactions containing 10:1 *E. coli* $tRNA^{Phe}$/transcribed tRNA, there was a marked decrease in the intensity of cleavage events. However, tRNA−oP-induced cleavage events were not diminished in the presence of yeast $tRNA^{Phe}$.

We used two methods to determine incorporation of 4-thiouridine in the tRNA transcripts. Absorbance measurements at A_{260} and A_{330} (ϵ_{330} of 4-thiouridine = 21 000 mol^{-1} cm^{-1}) (*18*) indicated that the typical yield was from three to five 4-thiouridines in the tRNA transcripts. On 6% polyacrylamide gels containing 10 μg/mL [(*N*-acryloylamino)-phenyl]mercuric chloride (APM), the electrophoretic mobility of the transcripts containing thiol groups was retarded by specific interactions with the organomercuric derivative proportional to the number of thiol groups per transcript (*19*). Although we could not quantify the number of substitutions using this approach, samples of various transcription products containing 4-thiouridine had slower migration patterns compared to those of transcribed tRNA without 4-thiouridine present. When IoP was attached to the available thiol groups on the transcribed tRNA, thus protecting the thiol from reaction with the APM matrix, the migration of the tRNA−oP returned to that of unthiolated $tRNA^{Phe}$. Lead cleavage assays (*14*) indicated that the modified tRNA was conformationally equivalent to the unmodified $tRNA^{Phe}$ (data not shown).

Although we have the potential of incorporating 4-thiouridine randomly into 18 different positions in transcribed tRNA, some positions may not be as available for conjugation to phenanthroline molecules as others. In addition, not all of these 4-thiouridines may be equivalently accessible to phenanthroline. However, there should be sufficient numbers of the modified uridines present on the periphery of the tRNA transcript to allow detection of those regions of 16S rRNA in the vicinity of most portions of the tRNA. As can be seen from Figure 1, the aminoacyl acceptor stem has no such modifications, so this region of the tRNA, if proximal to the 30S ribosomal subunit, would not be mapped.

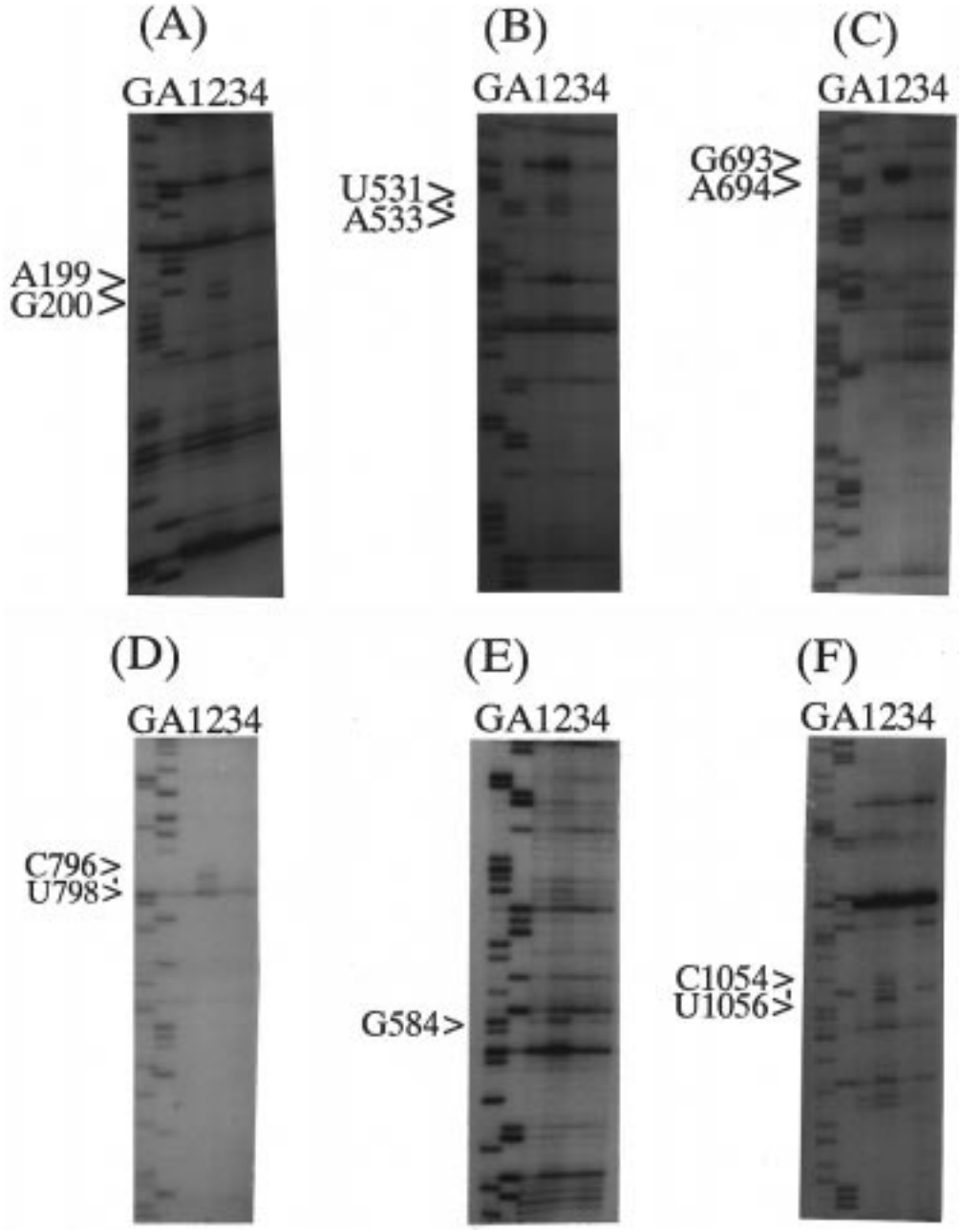

FIGURE 2: Autoradiographs showing regions of cleavage sites on the 16S rRNA in the presence and absence of messenger RNA. G and A are dideoxy sequencing lanes of the 16S rRNA. Lanes 1−4 are all reaction lanes as described in Materials and Methods. Lanes 1, 70S ribosomes; lanes 2, 70S ribosomes + $tRNA^{Phe}$-oP + poly(U); lanes 3, 70S ribosomes + $tRNA^{Phe}$-oP ; lanes 4, 70S ribosomes + poly(U) + $tRNA^{Phe}$ + 300 pmol of free IoP. Primer extension was performed using 16S primers complementary to nucleotides 324−340 (A), 684−700 (B), 906−923 (C), 906−923 (D), 684−700 (E), or 1200−1216 (F).

Positioning the tRNA−oP at the P Site. All of our cleavage reactions were carried out in 10 mM Mg^{2+} and in the presence of 0.2 μg/μL poly(U). When tRNA−oP was bound under these conditions, protection from chemical modification by DMS showed that 16S nucleotide A532 (a P-site protected nucleotide) was protected. This suggested binding of tRNA−oP at the P-site. Previous studies by others have shown that P-site binding of $tRNA^{Phe}$ at Mg^{2+} concentrations less than 20 mM is poly(U)-dependent (*1*). In addition, it has been reported that in poly(U)-programmed ribosomes, the affinity of deacylated tRNA is 200-fold stronger for the P-site than for the A-site under the conditions used in our reactions (*20*). Our results corroborated these findings, since we found that when poly(U) was eliminated from the reactions, all cleavage bands disappeared from the primer extension assays (Figure 2).

Competition studies using 10:1 ratios of native *E. coli* $tRNA^{Phe}$ to phenanthroline-modified tRNA transcripts showed a substantial decrease in the intensity of cleavage bands (data not shown), indicating that native tRNA was competing for binding sites with tRNA−oP conjugates. Since primer extension results are quite nonlinear with respect to concentration, elimination of cleavages entirely cannot be expected. These competition results provided further substantiation that the tRNA−oP was bound at the P-site of the 30S subunit.

tRNA−oP-Induced Cleavage Sites on the 16S rRNA. Primer extension assays identified 12 regions, containing either single or multiple cleavage sites, on the 16S rRNA

Table 1[a]

intensity	cleavage sites on 70S ribosomes by tRNA−oP	cleavage sites on 30S subunits by tRNA−oP
low	A279, C418, G722−U723	G198−A199, G362−A364, A411−G413, G530−A532, A583, A572, C795−C797, G1053−A1055
moderate	C334, G402, G530−A532, C795−C797, G1053−A1055	U692−G693
high	G198−A199, A411−G413, A583, U692−G693	

[a] Nucleotides at these positions on 16S rRNA were cleaved by $tRNA^{Phe}$−oP. The intensity of the cleavage sites was determined by visual comparison of cleavage bands with bands in the sequencing lanes of the primer extension autoradiographs. The bands in the sequencing lanes were used as standards to which bands indicating cleavage sites were visually scored. Low intensities were less intense than bands in sequencing lanes; moderate intensities were approximately equal to bands in sequencing lanes; high intensities were more intense than bands in sequencing lanes. Underlined nucleotides are positions of cleavage that are unique to either 70S ribosomes or 30S subunits.

when tRNA−oP was bound at the P-site on poly(U)-programmed 70S ribosomes and nine regions when tRNA−oP was bound at the P-site on poly(U)-programmed 30S ribosomal subunits (Table 1). Seven regions containing cleavage events were found whether tRNA−oP was bound to the poly(U)-programmed 70S ribosomes or the poly(U)-programmed 30S ribosomal subunits, but the cleavage intensity was not always the same. There were six additional cleavage sites identified, in five regions, only when tRNA−oP was bound to poly(U)-programmed 70S ribosomes: A279, C334, G402, C418, G722, and U723. Four additional cleavage sites were found, in two regions, only when tRNA−oP was bound to poly(U)-programmed 30S ribosomal subunits: G362, A363, A364, and A572 (Figure 2). All cleavage sites, regardless if tRNA−oP was bound to 70S ribosomes or to 30S ribosomal subunits, disappeared in the absence of mRNA. Control experiments in which free IoP was present in concentrations at least 10-fold greater than ribosome concentrations showed no cleavage in the regions cleaved by tRNA−oP.

In domain I, with tRNA−oP bound to poly(U)-programmed 70S ribosomes, cleavage at nucleotides A279 and C418 occurred at low intensities. Cleavage events occurring at nucleotides C334, G402, G530, U531, and A532 were of moderate intensity, and cleavage events at nucleotides G198, A199, A411, A412, and G413 were of high intensity. With tRNA−oP bound to poly(U)-programmed 30S subunits, all nucleotides cleaved, G198, A199, G362, A363, A364, A411, A412, G413, G530, U531, and A532, were at low intensities. In domain II, when tRNA−oP was bound to poly(U)-programmed 70S ribosomes, cleavage of nucleotides G722 and U723 was of low intensity. Cleavage of nucleotides C795, C796, and C797 was as intense, and cleavage of nucleotides A583, U692, and G693 was more intense, than sequencing bands on autoradiographs. When tRNA−oP was bound to poly(U)-programmed 30S subunits, the cleavage at nucleotides A572 and A583 was less intense, while cleavage of nucleotides C795, C796, and C797 was as intense, and cleavage of nucleotides U692 and G693 was more intense, than sequencing bands. The only cleavage events occurring in domain III were at positions G1053, C1054 and A1055. These cleavage events all occurred at an intensity equal to the sequencing bands when tRNA−oP was bound to either poly(U)-programmed 70S ribosomes or 30S ribosomal subunits.

DISCUSSION

We have attached the cleavage reagent 1,10-*o*-phenanthroline (IoP) randomly to various 4-thiouridine residues on tRNA and bound this conjugate to the P-site of 30S ribosomal subunits or tight-couple (TC) 70S ribosomes, to better identify regions of 16S rRNA that may be in a position to interact with tRNA during translation. We identified 17 cleavage sites, in seven regions, when tRNA−oP was bound to either 70S ribosomes or 30S ribosomal subunits. Six additional cleavage events, in five regions, occurred with tRNA−oP bound only to 70S ribosomes, and four additional cleavage sites, in two regions, were unique to tRNA−oP bound only to 30S ribosomal subunits. The intensity of cleavage varied considerably from site to site. This is possibly due to variation in phenanthroline coupled to the various thiouridines in the tRNA itself, or perhaps due to a variation in the proximity of the rRNA to the phenanthroline moiety.

Previous studies by others using cross-linking techniques (*21, 22*), chemical protection assays (*3*), and covariation studies (*23*) have implicated some nucleotides that may be involved in tertiary interactions between different regions of the ribosome, as well as interactions between various ligands and the ribosome. Our results substantiate many of these studies and suggest that these nucleotides are proximal to tRNA bound at the P-site. In addition, our cleavage results identified additional regions of 16S rRNA proximal to P-site-bound tRNA.

There are 18 possible attachment sites to which phenanthroline may have been conjugated to our transcribed tRNA, although, as noted above, it was expected that not all would be equivalently modified. Only a portion of these, especially those located in the anticodon stem and loop, would be expected to be located in positions to interact with the 30S subunit (see Figure 1). Since incorporation of 4-thiouridine was random, it was not possible to identify which conjugation site may have been responsible for a particular cleavage event.

The cleavage events occurred in specific regions of all three major domains of 16S rRNA. Although some of the identified cleavage positions were found within well-defined regions of current 30S models (*24*), several were within poorly defined regions and may aid in better placement of these regions in the model.

The 5′ Domain. In the 5′ domain, the most actively cleaved region was the 530 loop. Cleavage at positions G530−A532 place this region close to bound tRNA and corroborate the results of protection studies (*3*) in which G530 and A532 were protected by A-site- and P-site-bound tRNA, respectively (see Figure 3). Although subsequent analysis from the Noller laboratory has indicated that these protections may be allosteric (*25*), our results suggest that the 530 loop is proximal to some part of the tRNA.

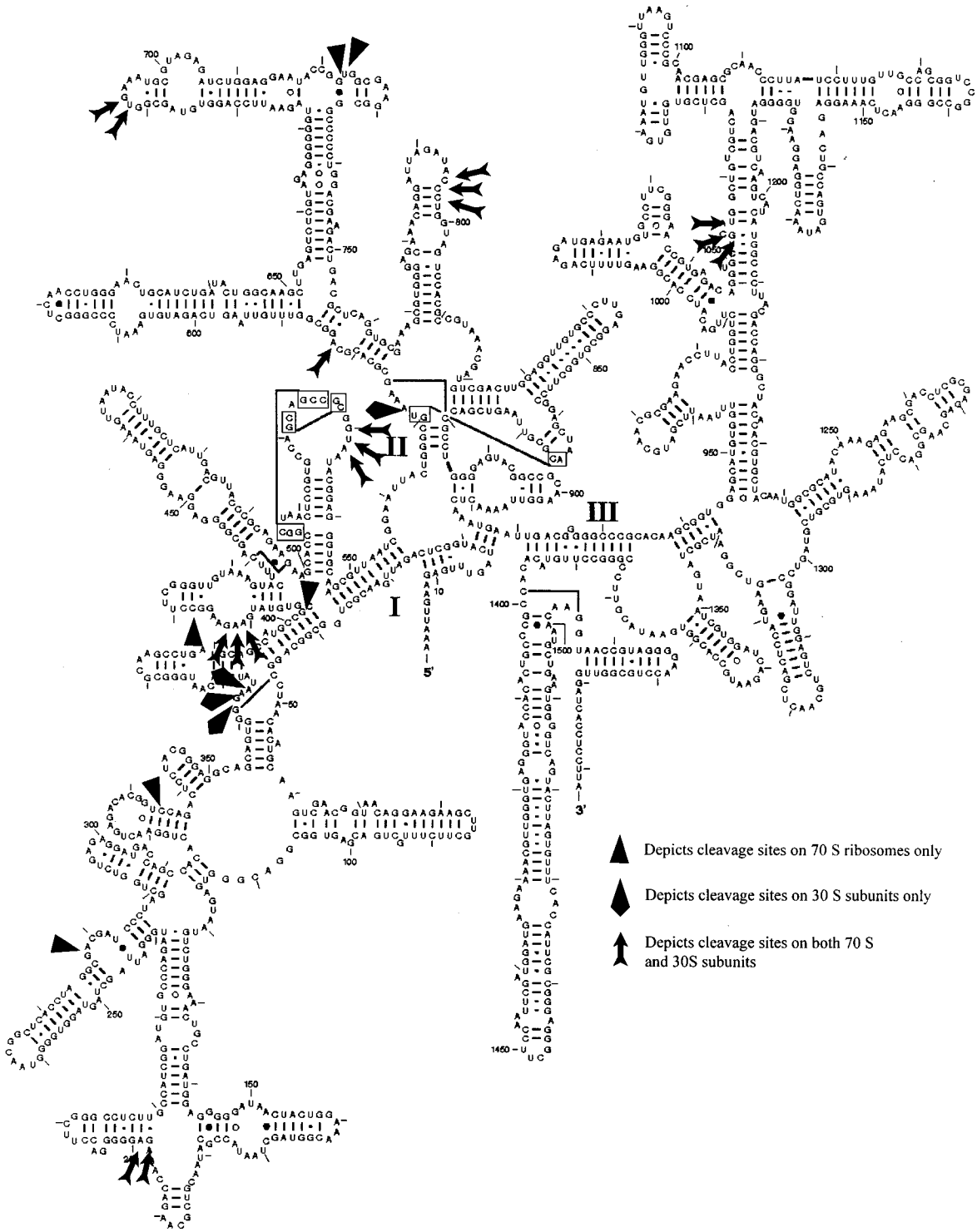

FIGURE 3: Model of the secondary structure of the 16S rRNA. Solid lines connecting boxes enclosing bases indicate regions of covariance. Solid lines between unenclosed bases indicate sites of cross-linking. Dotted lines indicate that the exact base is unknown. Cleavage sites are marked by arrows. (Secondary structure was downloaded from the WWW site of the Ribosomal Database Project at the University of Illinois at Urbana−Champaign.

Additional evidence for this proximity was provided by the finding that this region is also cleaved by IoP attached to the +5 position of mRNA (*26*).

The 530 stem and loop form a pseudoknot as a result of the base pairing of rRNA nucleotides 524−526 with nucleotides 505−507 (*27*). Studies emanating from Noller's laboratory indicated that this region plays an important role in translation and may interact with the decoding region (*28, 29*). Our results provide additional evidence that the 530 pseudoknot could be functional in translation and may have tertiary interactions with other regions of 16S rRNA.

When in the pseudoknot conformation, the G530−A532 cleavage sites are proximal to other 5′ domain cleavage sites, specifically G402, A411−G413, and C418. Since there is covariance between C47 and G361 (*30*), it can be argued that cleavage sites at positions G362−A364 and C334 also may be proximal to the 530 pseudoknot through the proximity of these sites to the 505 region of the pseudoknot.

Nucleotide C334 was cleaved only with tRNA−oP bound to 70S ribosomes, which was not surprising since G337, three nucleotides away, was reported to be protected from chemical modification due to direct contact with or conformational shift caused by association with the 50S subunit (*31*). Nucleotide A279 also was only cleaved when the 50S subunits were present, which agrees with the placement of this region of 16S rRNA on the 50S interface of the 30S subunit in the model proposed by Malhotra and Harvey (*24*).

Information on the placement of the other cleavage sites in domain I, specifically G198−A199, is lacking from other studies. These nucleotides are located in poorly defined regions of the Malhotra and Harvey 30S model (*24*). Although these cleavages uniformly occurred in five preparations, in one preparation a cleavage was seen when poly(U) was not present to program the tRNA. Nonetheless, we have designated these cleavages as being induced by programmed tRNA. Cross-links between A306 and G31 (directly across from U552) (*21*) have been established, which suggest that these nucleotides may at least transiently move closer to the 530 pseudoknot. At this time, however, we can only conclude that they are proximal to tRNA bound to programmed ribosomes.

The Central Domain. In the central domain (see Figure 3), 16S nucleotides U692−G693 were major cleavage sites. Brimacombe's group has previously identified G693 cross-links to position 32 of $tRNA^{Arg}$ when bound at the P-site (*5*). Nucleotide G693 has also been shown to be protected from chemical modification with tRNA bound at the P-site (*3*). These nucleotides are in close proximity to cleaved nucleotides C795−C797, as cross-links between the 690 and 790 loops indicated (*32*). One of these cleaved sites, nucleotide C795, was protected from chemical modification by P-site-bound tRNA (*3*).

The presence of the 50S subunit caused additional cleavage at nucleotides G722−U723, possibly induced by a conformational change of the 16S rRNA. Nucleotide A583, a major cleavage site in the presence of the 50S subunit, was only weakly cleaved when tRNA−oP was bound only to the 30S subunit, and cleavage of nearby A572 occurred only in the absence of the 50S subunit. These last two cleavage sites cannot be placed close to the other cleaved nucleotides in domain II by other results, but they are located within poorly defined regions of the 30S subunit (*24*) and may be drawn near the P-site-bound tRNA−oP through tertiary folding not yet discovered.

The 3′ Domain. The only positions cleaved in the 3′ major domain occurred in helix 34 at nucleotides G1053−A1055 (see Figure 3). These cleavage events occurred with tRNA−oP bound to both 70S ribosomes and 30S ribosomal subunits. In an effort to understand the paucity of cleavage sites in this area, we note that cross-links between the 1090 loop and 1160 region (*21*) indicated that the region 3′ to G1053−A1055 may be folded together. Other cross-links occurring between the 1125 bulge and 1280 bulge (*21*) suggest this entire area may be pulled away from the G1053−A1055 region. This being the case, the G1053−A1055 region, putatively located in the cleft of the 30S subunit, may be more available to interactions with incoming ligands. For instance, nucleotide 1052 has been cross-linked to position +6 of mRNA in the presence of tRNA (*33*). We have also found some evidence from cleavage studies that this region is proximal to position +5 of mRNA (*26*). There have been no protein cross-links to this portion of helix 34 (*24*), suggesting that it may not be as shielded by proteins as other regions in the 3′ domain.

The lack of cleavage events in the decoding region was somewhat surprising. P-site-bound $tRNA^{Val}$ has been shown to be cross-linked from position U34 to 16S rRNA nucleotide C1400 (*2*). Messenger RNA (mRNA) with a photoreactive group located 5′ of the codons bound with appropriate tRNAs to 70S ribosomes was cross-linked near the 3′-terminal end of 16S rRNA (*34*). But tRNA−oP with 4-thiouridine residues at positions 32 and 33 to which IoP should be attached, when bound at the P-site, did not cleave rRNA in this region. In work reported by others, $tRNA^{Arg}$ bound at the P-site with a photoreactive derivative at position 32 also did not cross-link to the decoding region but instead cross-linked to the 960 and 1340 region (*5*). The 960 and 1340 regions of 16S rRNA also include nucleotides weakly protected from chemical modification by P-site-bound tRNA (*3*). However, no cleavage sites were observed in either the 960 or 1340 regions of 16S rRNA.

Perhaps this is due to the positioning of residues 32 and 33 on tRNA−oP in such a way that tRNA itself may obstruct cleavage of these rRNA regions. It is also possible that these regions may be shielded from cleavage by the presence of mRNA. But it may just be a matter of proximity. The tether plus the phenanthroline place the copper ion but 9 Å from the thiol group on the uracil. It is possible that this tether is too short to allow the phenanthroline to stretch into a cleavage position on rRNA that is not directly adjacent. The significance of tether lengths has already been noted by Heléne's group, in which cleavage of DNA by an oligomer bound to the DNA was shown to vary with tether length (*35*). Studies are presently underway to quantify this further.

General Structural Considerations. From cross-linking, chemical protection, and covariation studies, a spatial closeness of most cleavage sites within the separate domains of the 16S rRNA can be inferred. Although we cannot position these distinct regions of the different domains relative to each other or to the tRNA with exactness, the cleavage results emanating from this study suggest that they all must be within 10−15 Å of some part of the modified tRNA.

In a previous study focusing on 23S rRNA−tRNA proximity (*12*), we found the addition of yeast $tRNA^{Phe}$ did not affect most tRNA−oP-induced cleavage events. There were, however, a small number of cleavage events that occurred only in the absence or presence of yeast $tRNA^{Phe}$. In this study, since the cleavage events were not altered noticeably by the addition of yeast $tRNA^{Phe}$, we can only surmise that the binding affinity for tRNA−oP is greater than that of heterologous yeast tRNA for the P-site of the small subunit of *E. coli* ribosomes, as was suggested by others previously (*20*).

Cleavage Approach Considerations. Although the results of this study provide compelling evidence for the proximity of the sites reported, cleavage of rRNA could be induced from several sources, each of which must be properly controlled. For instance, certain divalent metal ions are known to interact with RNA in a variety of ways (*14*, *36*, *37*). Previous work in our laboratory has focused on the possibility of Cu^{2+} alone interacting with ribosomes and causing scission of rRNA under reducing conditions, which

could be interpreted as cleavage sites induced by ligand−oP conjugates. Sequencing rRNA from Cu^{2+}-induced cleavage reactions, using primer extension techniques, we have identified nucleotide G324 in 16S rRNA as a target for cleavage by copper ions (Burrington et al., unpublished data). In the present study, controls using free Cu^{2+} and free IoP were used to monitor for any such cleavage sites.

It is also possible that self-cleavage of the tRNA−oP may have occurred to some extent and in doing so caused nonspecific cleavage. However, we saw no evidence that this occurred with tRNA−oP (data not shown). Also, the tRNA−oP was bound to the ribosome before the introduction of a reducing reagent, and the half-time of dissociation for deacylated tRNA under our conditions is more than 10 h (*20*). We would expect minimal cleavage by any tRNA fragments, which was controlled for by repetition of reactions.

Perhaps the greatest potential for spurious cleavage is from untethered IoP. We have found in independent titration experiments that approximately a 1:1 phenanthroline/rRNA molar ratio is sufficient to cause cleavage of rRNA at susceptible sites. In the controls used in these experiments (see lane 4 of Figure 2) we used a 6:1 molar ratio of untethered phenanthroline to rRNA. If such cleavage was observed, any cleavage from the tethered phenanthroline was discounted. As can be seen in Figure 2, all cleavages reported are induced only by the phenanthroline tethered to tRNA appropriately positioned by mRNA.

Conclusion. In conclusion, we have identified an extended set of nucleotides in the 16S rRNA that may interact with tRNA as it is bound at the P-site. Using this same approach, we previously identified nucleotides in the 23S rRNA that are in close proximity to tRNA−oP bound at the P- and E-sites (*12*). These studies together provide information which allows a "shell" of rRNA that surrounds and possibly interacts with bound tRNA to be identified.

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