Cleavage of 16S rRNA within the Ribosome by mRNA Modified in the A-Site Codon with Phenanthroline-Cu(II)†

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ABSTRACT: Cleavage of 16S rRNA was obtained through mRNA modified at position +5 with the chemical cleavage agent 1,10-*o*-phenanthroline. In the presence of Cu^{2+}, and after addition of reducing agent to the modified mRNA−70S complex, cleavage of proximal nucleotides within the 16S rRNA occurred. Primer extension analysis of 16S rRNA fragments revealed that nucleotides 528−532, 1196, and 1396−1397 were cleaved. Nucleotides 1053−1055 were also cleaved but did not show the same level of specificity as the former. These results provide evidence that at some point in the translation process these regions are all within 15 Å of position +5, the A-site codon, on the mRNA.

Messenger RNA must be bound correctly, decoded accurately, and translocated properly during the course of translation. Clearly the binding track that it occupies during protein biosynthesis must be specific enough to allow for those functions.

The mRNA binding track has been visualized through electron microscopy, showing the mRNA to be looping through the cleft of the 30S subunit, between the platform and neck (Montesano-Roditis & Glitz, 1994; Shatsky et al., 1991; Olson et al., 1988). Within that track the total amount of mRNA protected by the ribosome from nuclease digestion has been determined to be approximately 49 nucleotides (Kang & Cantor, 1985). Primer extension inhibition (toeprint) assays of mRNA bound to 30S subunits in the presence of cognate tRNA has shown that a specific stop of reverse transcription occurs 15 nucleotides from the first nucleotide of the P-site codon (Hartz et al., 1988). This result fits well with those of Kang and Cantor (1985), who showed that while protection persisted to about 29 nucleotides from the tRNA−mRNA interaction, the limit of strongly protected mRNA falls approximately 16 nucleotides from the P-site codon.

Within the binding track, specific contacts between 16S rRNA and mRNA have been found. It has been established that mRNA interacts directly with rRNA through the Shine−Dalgarno region (Jacob et al., 1987). It is also surmised that mRNA must be proximal to C1400 of 16S rRNA, since this site cross-links with the anticodon loop of tRNA (Prince et al., 1982; Gornicki et al., 1985). In addition, modified mRNA carrying thiouridines in the portion of the molecule 5′ to the decoding site have revealed proximity of this region of mRNA to the 3′ end of 16S rRNA (Rinke-Appel et al., 1994; Stade et al., 1989). Within the coding region, Brimacombe's group and collaborators found cross-links from +4, +6, +7, and +11 of the mRNA to 16S rRNA positions 1402, 1052, 1395, and 532, respectively (Rinke-Appel et al., 1991, 1993; Dontsova et al., 1992). These results provide evidence that the 530 region, helix 34, and the 1400 region may be close to one another. Wollenzien's group has also produced cross-linking data that show the mRNA to be proximal to those regions, but the mRNA modification position sensitivity of particular cross-links is not evident in those studies (Bhangu et al., 1994; Bhangu & Wollenzien, 1992; Wollenzien et al., 1991). Evidence for the proximity of the 530 loop and the 1400 region has also been provided through the work of Alexander et al. (1994), who were able to cross-link photolabile cDNAs annealed to the 530 loop or the 1400 region to similar positions of ribosomal protein S7. A recent study by Heilik and Noller (1996) also demonstrated the proximity of these regions to a single point of ribosomal protein S5. However, demonstration of the proximity of these regions has been controversial because of prior localization of those areas by electron microscopy to disparate regions of the 30S subunit.

Electron microscopy has shown the 1400 region of 16S rRNA, which should contain a portion of the mRNA binding track, to be in the cleft of the 30S ribosomal subunit (Oakes & Lake, 1990; Oakes et al., 1986), while the 530 loop was found on the opposite side of the 30S neck and facing the cytoplasm (Trempe et al., 1982; Oakes et al., 1990; Oakes & Lake, 1990). However, Lasater et al. (1990) found the 530 loop to be somewhat closer to the decoding site using similar techniques.

We undertook this study, utilizing the nucleic acid cleavage proclivity of 1,10-*o*-phenanthroline, to investigate the above outlined differences, and the potential that additional 16S rRNA sites proximal to mRNA binding track have not yet been observed.

If 16S rRNA does play a significant role in the positioning of mRNA, the nucleic acid environment of the binding track can be examined by determination of sites within the 16S rRNA that are not only in contact with the message during

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[1] Abbreviations: IOP, 5-iodoacetamido-1,10-orthophenanthroline; OP, 1,10-*o*-phenanthroline; APM, [(*N*-acryloylamino)phenyl]mercuric chloride; TC70, tight-couple 70S ribosome; OP-5-mRNA, mRNA modified at position +5 with 1,10-*o*-phenanthroline.

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5'-GGACCAGGAGGACACCGCACC AAA GUA GAC GCA ACA CAA CAC AGA GGG-

-AAC ACA *ACA GGG AAC AGA CAC*-3'

FIGURE 1: Sequence of the mRNA used in this study. The position of modification is shown in bold at +5 from the first nucleotide of the P-site codon (Lys, underlined). The mRNA also contains a Shine−Dalgarno sequence (underlined) and a primer binding site (italic) used for the primer extension inhibition assay.

the course of an experimental approach but also in a position to interact with the message during translation. The use of mRNA covalently modified with phenanthroline allows this type of examination.

The mechanism of nucleic acid cleavage by phenanthroline is not fully understood [descriptions of proposed mechanisms can be found in Hermann and Heumann (1995) and Sigman and Chen (1990)], but the result is apparent. Many studies have utilized 5-haloacetamido-1,10-*o*-phenanthroline derivatives to covalently attach OP to a targeting molecule (Chen et al., 1993; Perrin et al., 1994; Sun et al., 1988). This scheme gives a short tether between the attachment point and the cleaving phenanthroline-Cu(I). When the conjugate species is a nucleic acid itself, the complementary target is cleaved within a 4−6 nucleotide range, proximal to the modification position (Chen et al., 1993; Perrin et al., 1994; Sun et al., 1988). The distance between the origin of the tether and the cleavage site then would be estimated to be about 15 Å.

Using mRNA modified with phenanthroline at position +5 from the first nucleotide of the P-site codon, and bound to the ribosome with cognate tRNA to "lock" the message into position, we have observed cleavage at nucleotides 528−532, 1053−1055, 1196, and 1396−1397 of 16S rRNA.

MATERIALS AND METHODS

Ribosome Preparation. Ribosomes were harvested from early log phase *Escherichia coli* strain MRE600 using the protocol outlined by Tam et al. (1980) and Lodmell et al. (1993).

mRNA Design. The mRNA used in this study was designed to be linear and contain a single uridine that could be replaced by thiouridine. This thiouridine was used as the modification point for the covalent attachment of phenanthroline. A Lys codon, AAA, was placed as the first codon, giving the modification point a designed position of +5 (see Figure 1).

RNA Synthesis. The method of Milligan et al. (1987) was used for T7 RNA polymerase transcription of oligodeoxynucleotide templates for the production of the mRNA used in this study. Transcription products were recovered through phenol extraction, gel filtration, and ethanol precipitation. Full-length transcripts were gel-purified on 10% polyacrylamide/7 M urea gels.

Introduction of thiouridine into transcripts occurred by substitution of 90% of the uridine triphosphate with 4-thiouridine triphosphate [obtained by substrate level phosphorylation of 4-thiouridine diphosphate (Sigma) according to Simon et al. (1990)] within the transcription reaction. The only uridine available for substitution was at position +5 from the first nucleotide of the P-site codon (see Figure 1).

Selective Nuclease Sequencing of mRNA Analogs. The sequence of the mRNA analog was confirmed by enzymatic sequencing using the U.S. Biochemical Corp. RNA sequencing kit, using manufacturer's guidelines, with 5′-end-labeled [^{32}P]RNA (data not shown).

5-Iodoacetamido-1,10-o-phenanthroline Synthesis. The phenanthroline derivative was synthesized by the method of Sigman and Chen (1991). The identity of the product was confirmed by ^{1}H NMR (data not shown).

RNA Modification. Transcripts were reacted with IOP in order to covalently attach the cleavage agent to thiol groups incorporated within the RNA. The reaction conditions were 4 mM IOP, 25% acetonitrile, 100 mM TEAA (pH 7.0), 5 mM DTT, and 10 μM RNA. Excess phenanthroline was removed by five consecutive 1:1 *n*-butanol−chloroform extractions and one chloroform extraction (Chen et al., 1993). The products were dried and redissolved in 10 mM Tris-HCl (pH 7.5). In each series of experiments a mock sample was prepared, in addition to the modified sample, by running identical transcripts lacking the thiol groups through the same preparation.

The presence of thiol groups and subsequent reaction with IOP was assessed by electrophoresis of the samples through 25 μM [(*N*-acryloylamino)phenyl]mercuric chloride (APM)/7 M urea/10% polyacrylamide gels, as developed by Igloi (1988). The coupling reaction yield approached 100% (data not shown).

Binding of mRNA to Tight-Couple 70S Ribosomes. Whether for binding assays or in preparation for cleavage, the binding conditions used were identical. Tight-couple 70S (TC70S) ribosomes were "run-off" in 10 mM Tris-HCl (pH 7.5), 60 mM KCl, and 6 mM $MgCl_2$ at 37 °C for 30 min (Staehelin & Falvey, 1971). After this time mRNA, $tRNA^{Lys}$, and $CuSO_4$ were added with adjustment buffer to make the final concentrations of all constituents 1.7 μM TC70S ribosomes, 1.7 μM mRNA, 1.7 μM $tRNA^{Lys}$, 0.1 mM $CuSO_4$, 40 mM Tris-HCl (pH 7.5), 60 mM KCl, and 6 mM $MgCl_2$. Binding to ribosomes was allowed to take place for 30 min at 37 °C. In some cases the concentrations of +5-OP−mRNA, competitor mRNA, and tRNA were varied.

The ligand being traced during binding studies was 5′-end-labeled by first treating the nucleic acid with shrimp alkaline phosphatase (U.S. Biochemical Corp.) following manufacturers guidelines, and then reaction with 6000 Ci/mmol [^{32}P]ATP, catalyzed by T4 polynucleotide kinase (U.S. Biochemical Corp.) under manufacturer's conditions. Undegraded, full-length RNA was gel-purified, and the specific activity was adjusted to 500 cpm/pmol.

The fraction of ribosomes bound by mRNA was assessed by filtering mRNA−ribosome complex through Millipore 0.45 μm HAWP nitrocellulose filters followed by five 1-mL washes of the filters with 40 mM Tris-HCl (pH 7.5), 60 mM KCl, and 6 mM $MgCl_2$. Liquid scintillation counting of the filters was used to determine the percent ribosomes bound by mRNA.

Primer Extension Inhibition (Toeprint) Assay. Analysis of unmodified and modified mRNA binding to tight-couple 70S ribosomes by this method was done using the conditions outlined by Hartz et al. (1988) to verify the proper placement of mRNA analogs.

Directed rRNA Cleavage by +5-OP−mRNA. Once mRNA was bound to the ribosomes, in the presence of $CuSO_4$, 3-mercaptopropionic acid (MPA) was added to a final concentration of 2 mM. Cleavage was allowed to proceed for 2 h at 37 °C. Reactions were quenched by the addition

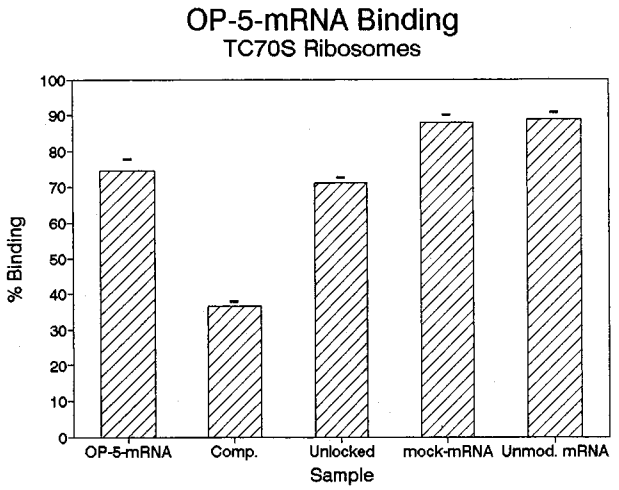

FIGURE 2: Filtration binding assay of +5-OP−mRNA binding versus mock mRNA and unmodified mRNA. The samples contained the standard buffer given in the Materials and Methods, 1.7 μM TC70S ribosomes, and 1.7 μM $tRNA^{Lys}$ (except where noted) and (1) 1.7 μM OP-5-mRNA, (2) 1.7 μM OP-5-mRNA and 5.0 μM unmodified mRNA, (3) 1.7 μM OP-5-mRNA but no tRNA, (4) 1.7 μM mock mRNA, and (5) 1.7 μM unmodified mRNA.

of 2,9-dimethyl-1,10-*o*-phenanthroline (neocuproine) to a final concentration of 1 mM.

Products were ethanol-precipitated and redissolved in 0.3 M NaOAc (pH 5.0), 5 mM EDTA, and 0.1% SDS. RNA was extracted from ribosomal proteins through three vigorous phenol extractions and two chloroform extractions. The final step in RNA template preparation was ethanol precipitation followed by redissolving the RNA to a concentration of 0.2 μM 16S rRNA in H_2O.

Primer Extension Analysis of Cleavage Sites. Primer extension of cleavage products was carried out by the method of Moazed et al. (1986), using the same primer set described therein. Products of the reaction were resolved on 0.25 mm × 60 cm 7 M urea/6% polyacrylamide/Tris−borate gels. The gels were then transferred to Whatman 3MM paper and dried. Visualization of the radioactive products was accomplished by exposing the gel to Kodak X-OMAT film.

RESULTS

Binding of Modified mRNA to Ribosomes. Binding of tight-couple 70S ribosomes by the mRNA−OP complex was about 75%, using a 1:1:1 ratio of mRNA:tRNA:ribosomes. Under identical conditions, the underivatized mRNA and mock mRNA (nonthiolated RNA exposed to the phenanthroline attachment conditions) bound at about 88%. These comparable binding levels suggest that the modification did not affect mRNA−ribosome complex formation negatively (Figure 2).

When the modified mRNA was bound in the absence of cognate tRNA, no change in the percent of mRNA bound to ribosomes was detected, within the margin of error (Figure 2).

A 3-fold excess of unmodified mRNA did cause a decrease in binding of +5-OP−mRNA, but the competition was not linear with respect to the concentrations used (Figure 2). This may be due to intercalation of the planar, hydrophobic phenanthroline derivative with nearby rRNA.

Primer Extension Inhibition (Toeprint) Analysis. When the modified or unmodified mRNA was locked into position

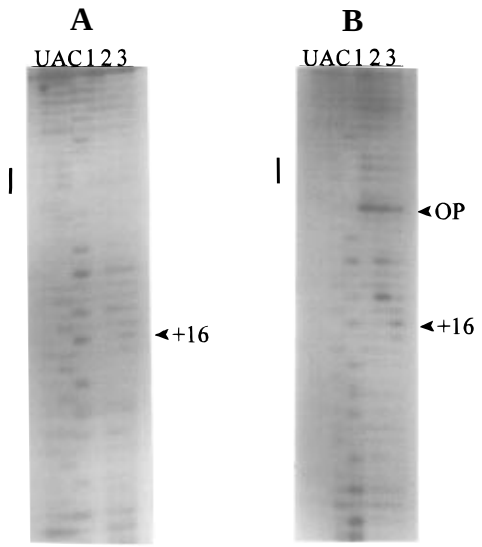

FIGURE 3: Primer extension inhibition (toeprint) assay of mRNA binding. (Panel A) Toeprint of unmodified mRNA. (Panel B) Toeprint of OP modified mRNA. Lanes U−C, dideoxy sequencing lanes with ddATP, ddTTP, and ddGTP, respectively; lanes 1, extension of the mRNA in the presence of $tRNA^{Lys}$; lanes 2, extension of the mRNA in the presence of ribosomes; lanes 3, extension of the mRNA in the presence of $tRNA^{Lys}$ and ribosomes. The vertical bar indicates the Lys codon, +16 indicates the toeprint, and OP indicates the stop unique to phenanthroline modification.

on tight-couple 70S ribosomes by $tRNA^{Lys}$, specific stops in the reverse transcription of the mRNA were observed (Figure 3). These stops found at positions +15, +16, and +17 were unique to mRNA bound to ribosomes in the presence of $tRNA^{Lys}$ and were absent when $tRNA^{Lys}$ was omitted. The intensity of the observed bands was greatest at position +16. These data confirm that the mRNA was binding in the correct manner to the ribosome. That the intensity of the toeprint was greatest at position +16, rather than +15 as observed by Hartz et al. (1988), may be due to binding of the tRNA to AAG rather than AAA, giving a +1 frameshift. If the mRNA was locked one position out of the intended frame then the modification would still be placed at a fixed site upon the ribosome, within the A-site codon of mRNA.

This primer extension experiment also allowed direct observation of the covalently attached phenanthroline. When the toeprints of modified versus unmodified mRNA are compared, a strong stop is present only for the modified samples at position +6. This stop indicates the presence of phenanthroline at position +5.

Cleavage of 16S rRNA. Specificity of cleavage was analyzed in four ways: (1) the reduction or absence of the cleavage when mock-derivatized mRNA was used, (2) competition for the binding of the modified ligand with 3-fold excess unmodified mRNA, (3) titration of the cleavage intensity in response to increasing concentrations of +5-OP−mRNA, and (4) the dependency of the cleavage on the presence of tRNA. In all cases, the results were replicated. All cleavage results are presented within Figure 4.

Cleavage Events C528−A532. G530 was heavily cleaved as can be seen in Figure 4A, lane 4. It can be seen that the intensity of cleavage is significantly greater than that observed with mock-derivatized mRNA (lane 2) or in the presence of a 3-fold excess of competitor unmodified mRNA (lane 3). C528−G529 showed the same response in the

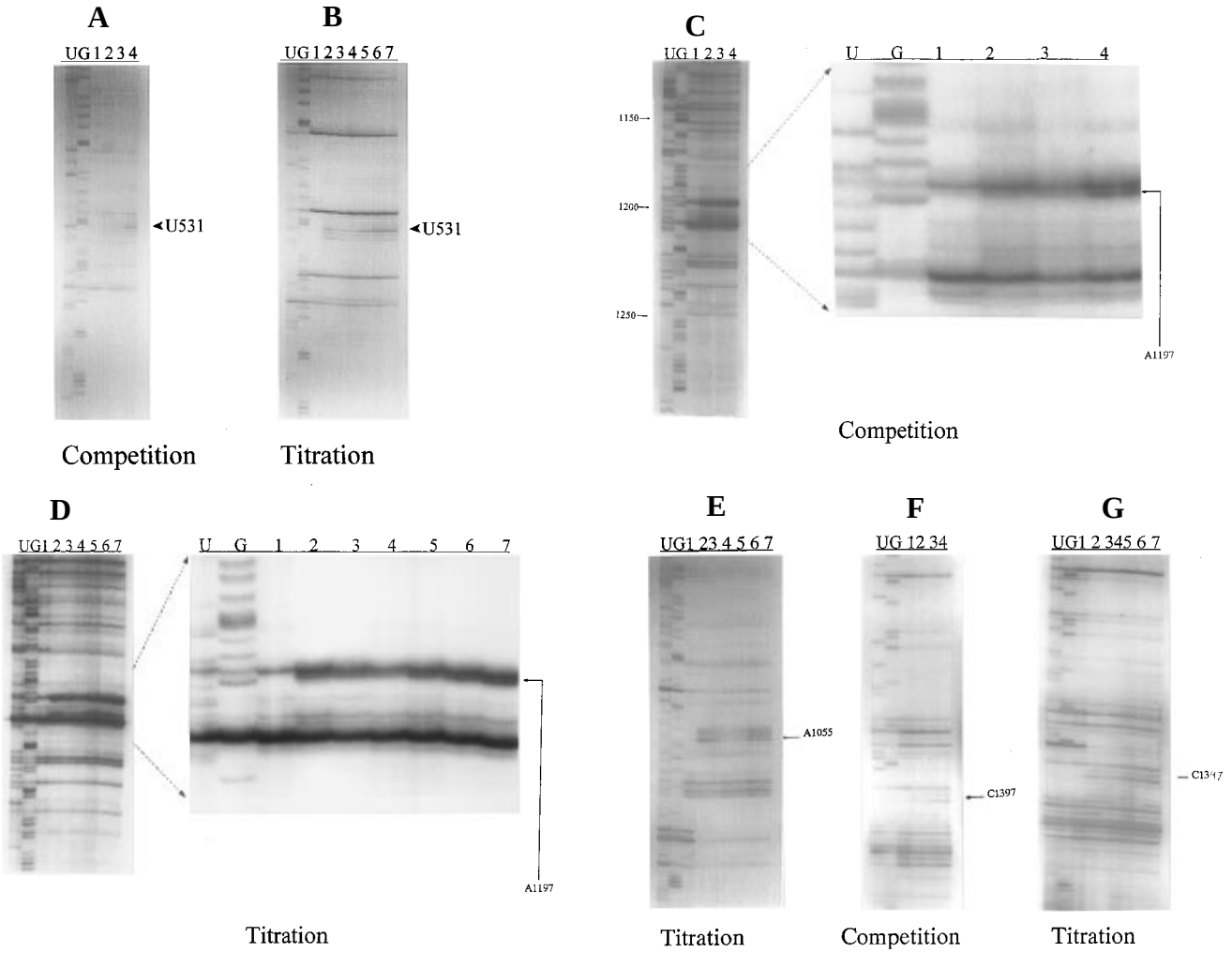

FIGURE 4: Primer extension assays of cleavage products. Arrows indicate the regions of cleavage, numbers indicate an observed stop within the region of cleavage (the stops are one nucleotide 3′ to the cleaved nucleotide). For each experiment there were competition and titration samples used. **Competition gels** have the following lane order: Lanes U−G, Dideoxy sequencing of control samples using ddATP and ddCTP. The remaining lanes are extensions of products from reactions with reagents as indicated plus 1.7 μM ribosomes and 1.7 μM tRNA: Lanes 1, products using RNA from ribosomes incubated with unmodified mRNA without phenanthroline; lanes 2, ribosomes incubated in the presence of 1.7 μM mock mRNA; lanes 3, competition of cleavage by 1.7 μM +5-OP−mRNA with a 3-fold excess (5.0 μM) of unmodified mRNA; lanes 4, directed cleavage by 1.7 μM +5-OP−mRNA. **Titration gels** have the following lane order: Lanes U, G, dideoxy sequencing of control samples using ddATP and ddCTP. The remaining lanes are extension of products from reaction with reagents as indicated below, 1.7 μM ribosomes, and except where noted, 1.7 μM $tRNA^{Lys}$. Lanes 1, products using RNA from ribosomes incubated with unmodified mRNA without phenanthroline; lanes 2, products from ribosomes incubated with 5 μM mock mRNA; lanes 3, directed cleavage with 5 μM +5-OP−mRNA without tRNA; lanes 4, directed cleavage using 0.84 μM +5-OP−mRNA; lanes 5, directed cleavage using 1.7 μM +5-OP−mRNA; lanes 6, directed cleavage using 3.3 μM +5-OP−mRNA; lanes 7, directed cleavage using 5.0 μM +5-OP−mRNA. (Panel A) Competition gel using primer ss683 (small subunit RNA primer 683; the first nucleotide incorporated during polymerization was complementary to 16S nucleotide 683) showing the 530 region of 16S rRNA. (Panel B) Titration gel using primer ss683 showing the 530 region of 16S rRNA. (Panel C) Competition gel using primer ss1490 showing the 1200 region of 16S rRNA. (Panel D) Titration gel using primer ss1490 showing the 1200 region of 16S rRNA. (Panel E) Titration gel using primer ss1199 showing the 1050 region of 16S rRNA. (Panel F) Competition gel using primer ss1490 showing the 1400 region of 16S rRNA. (Panel G) Titration gel using primer ss1490 showing the 1400 region of 16S rRNA.

experiments, but with less intensity of cleavage. U531−A532 are also cleaved, but the intensity of cleavage was low enough that the specificity of the cleavage was difficult to score.

When the concentration of +5-OP−mRNA was titrated from 0.85 to 5.0 μM, holding all other concentrations constant, the intensity of these bands increased as expected (Figure 4B, lanes 4−7). As before, the cleavage at G530 was the focal point of activity, C528−G529 was less intense with a similar pattern, and U531−A532 remained difficult to score because of the lower intensity of the bands.

The level of cleavage in the mock mRNA sample was greater in the titration experiment (Figure 4B, lane 2) than the competition experiment (Figure 4A, lane 2). In each experiment the level of mock mRNA was set to match the level of OP-mRNA: 1:1 in the competition experiment and 3:1 in the titration experiments. At nucleotides 531−532 the intensity of the cleavage band at higher levels of mock mRNA in the titration experiment was as great as the specific cleavage, which is in contrast to the competition experiments.

The cleavage within the 530 loop decreased markedly in the absence of $tRNA^{Lys}$ (Figure 4B, lane 3), suggesting that this rRNA cleavage site was only proximal to mRNA that was "locked" into the mRNA binding track. This corroborates the toeprint data, suggesting that mRNA binds differently in the absence of cognate tRNA (Figure 3).

Cleavage Event at 1196. Cleavage at this position (Figure 4C, lane 4) was observed to be specific. Upon competition

the intensity decreased (Figure 4C, lane 3), as well as when +5-OP−mRNA concentration was decreased (Figure 4D, lanes 4−7). The cleavage was intense only when cognate $tRNA^{Lys}$ was present to "lock" in the mRNA (Figure 4D, lane 3), and mock mRNA cleavage at this position was minimal (Figure 4C, lane 2).

This cleavage is especially interesting, since reproducible cleavage at positions 1053−1055 (Figure 4E), in the vicinity of this site, was present but not observed to be specific. Namely, the cleavage intensity did respond to the concentration of the +5-OP−mRNA (Figure 4E, lanes 4−7), but the intensity of mock cleavage was as intense as the directed cleavage bands (Figure 4E, lane 2). Also, when the binding of +5-OP−mRNA was competed with 3-fold excess unmodified mRNA, the intensity of cleavage did not decrease (data not shown). However, if $tRNA^{Lys}$ was omitted during the binding period the resulting intensity of cleavage did diminish (Figure 4E, lane 3), suggesting that the cleavage is dependent upon properly positioned mRNA or upon the presence of tRNA.

These results taken together suggest that the helix 34 is proximal to position +5 of mRNA, but the reactivity difference observed for the different sides of the helix may be displaying an undetermined dynamic aspect in the placement of mRNA within the region.

Cleavage Events 1396−1397. Although not as robust as the events at other positions, cleavage at positions 1396−1397 was present (Figure 4F, lane 4) and specific. The products of cleavage met the criteria of specificity set forth above. Both nucleotides were cleaved in a manner dependent on cognate tRNA (Figure 4G, lane 3) and concentration of the +5-OP−mRNA (Figure 4G, lanes 4−7). These cleavage events were also competed by the presence of excess unmodified mRNA (Figure 4F, lane 3), and the band intensity observed when mock mRNA was used was lower than that of the directed cleavage by +5-OP−mRNA (Figure 4F, lane 2).

DISCUSSION

Cleavage of 16S rRNA at the 530 loop, helix 34, and the C1400 region caused by 1,10-*o*-phenanthroline covalently attached to a single site on the mRNA demonstrates that these three regions are not only functionally linked but physically proximal, at some time in translation, to mRNA. These results corroborate previous cross-linking results linking these three regions. However, they are in disagreement with some electron microscopy results.

Although phenanthroline cleavage of nucleic acids has been assumed to take place via a hydroxyl radical (Hermann & Heumann, 1995), the involvement of a highly diffusible species has not been demonstrated.

Preliminary modeling studies of the phenanthroline−nucleic acid structure have shown that the tether is flexible and that the distance between the Cu^{2+} and the sulfur in the tether is 8−9 Å (modeling was done through SYBYL software and implemented in SPARTAN 3.1). If contact is required for cleavage, adding in 2 Å for the distance from Cu^{2+} to the target gives a cleavage distance from the tether point of approximately 11 Å. This suggests that rRNA cleavage events emanating from +5-OP−mRNA, locked into position by cognate tRNA, are a result of arrangement of those points within a sphere of no more than about 22 Å in diameter. Experimental results, from studies utilizing phenanthroline tethered to nucleic acids and annealed to complementary targets, and resulting in a limited span of cleavage proximal to the modification point (Chen et al., 1993; Perrin et al., 1994; Sun et al., 1988), suggest that the distance between the tether and the target of cleavage are no more than about 15 Å. Therefore, we conclude that the 530 loop, helix 34, and the 1400 region of 16S rRNA are all within approximately the width of a nucleic acid from each other on the ribosome, if we assume static positions. Another possibility is that these regions lie more distant in relation to each other, but during the process of translation, structural rearrangements occur such that each position would approach position +5 of the mRNA.

Models of the 30S subunit, derived from electron microscopy, place the 1400 region of 16S rRNA in the cleft of the 30S subunit (Oakes & Lake, 1990; Oakes et al., 1986). The 530 loop has been placed on the solvent side of the subunit, well away from the cleft (Trempe et al., 1982; Oakes et al., 1990; Oakes & Lake, 1990). Similarly, helix 34 has been placed in position either high in the head of the subunit (Stern et al., 1988b) or lower in the head toward the neck (Brimacombe et al., 1988). But other studies indicate that the location of these regions must be closer to each other than suggested by the above-mentioned studies.

Cooperman's group showed that a short cDNA oligomer carrying a cross-linking agent and annealed to the 530 loop resulted in cross-linking of the 3′ end of 16S rRNA. Also, cDNAs carrying a cross-linking agent and annealed to the 530 loop or the 1400 region labeled similar portions of ribosomal protein S7 (Alexander et al., 1994). Heilik and Noller (1996) have also shown proximity of these ribosomal landmarks with ribosomal protein S5.

The relative position of mRNA with the ribosomal constituents was probed by Brimacombe's group and collaborators (Rinke-Appel et al., 1991, 1993; Dontsova et al., 1991, 1992). Their findings that the 530 loop, helix 34, and the 1400 region were proximal to the mRNA forced a reexamination of the placement of these functionally important regions. The present study, using phenanthroline cleavage, directly probes the mRNA−ribosome interaction also. Our results provide direct corroboration of Brimacombe's results and additional primary evidence for the proximity of these three regions of rRNA relative to mRNA. It is important to note that the use of cleavage reagents, a distinctly different probing mechanism, provided no evidence for additional mRNA−rRNA interaction.

Clearly mRNA has to be able to move along its binding track. In order to ensure specific placement of the mRNA, we used cognate $tRNA^{Lys}$ to lock it into position followed by the determination of unique binding by use of the toeprint assay as developed by Hartz et al. (1988). Our results showed that the mRNA was specifically bound in its track, placing the modification exclusively at its designed position, and thus all of the cleavage events took place from the designed point upon the ribosome.

Previous cross-linking studies using mRNA modified at position +11 cross-linked to the 530 loop, while placement of the agents at position +4 and +7 labeled the 1400 region (Rinke-Appel et al., 1993; Dontsova et al., 1992). This separation of the modification sites, while small, may have been used to argue that the distances between regions cross-linked were farther apart then they appeared due to two

considerations: (1) The overall conformation of the mRNA may have allowed unexpected arrangements of +4 in relation to +11, and (2) if the binding strength between the ribosome and mRNA varied along the length of the molecule, then position +11 could be expected to be less constrained and possibly randomly reactive. Placing the modification at a defined position in the A-site codon, as was done in this study, allows firm placement of the modification. Locking the mRNA in with tRNA allows little room for the modification position to be moved relative to the ribosome, except for the rotation of phenanthroline upon its tether. Thus, cleavage from position +5 secures the placement of the cleavage target sites close to the decoding region of the 30S ribosomal subunit.

A further advantage in the use of phenanthroline is that of the mock control. During preparation of the modified ligands, following the coupling reaction, excess phenanthroline is extracted from the RNA ligand by extraction with organic solvents. Even though the majority of unreacted cleaving agent is removed, some free phenanthroline remains in bulk solvent and possibly intercalated in the RNA. In this study, the mock sample was RNA that had no thiol groups with which the IOP can react, but the phenanthroline which came through the purification procedure would be at levels identical to those in the actual labeling reaction. In cleavage reactions this excess phenanthroline is fully active as a cleaving agent, and in the presence of Cu^{2+} and reducing agent, cleavage occurs. Regions of rRNA excessively reactive to the cleavage reagent in the solution are cleaved, but only those that are truly proximal to the mRNA will have increased activity toward samples with covalently attached phenanthroline.

Mock cleavage, caused by residual free phenanthroline, was observed at the positions cleaved by phenanthroline tethered to the mRNA (see all panels of Figure 4). However the intensity of mock cleavage was less at those positions. In addition, many other regions were found to be reactive to free phenanthroline cleavage but not to mRNA-directed cleavage (data not shown). These additional cleavage events were not specific since (1) the cleavage induced with mock samples was as intense as the cleavage produced with the mRNA-tethered phenanthroline, (2) the cleavage intensity did not respond predictably to the concentration of mRNA used, and (3) in many cases the cleavage was not tRNA-dependent.

In one case, that of cleavage at nucleotides 1053−1055, the concentration dependence and tRNA-induced dependence of the cleavage was observed while the mock cleavage was as intense or more intense than the mRNA-directed cleavage (Figure 4E). Also, in competition experiments with excess unmodified mRNA the intensity of cleavage was not reduced (data not shown). This cannot be explained, although helix 34 was specifically cleaved nearby at nucleotide 1196. The 1196 cleavage event is difficult to observe because of a reverse transcription strong stop revealed immediately above the event on the autoradiograph (Figure 4C,D), but it does respond in the predicted manner. All of the criteria for specificity set forth above were met.

On the basis of these arguments it is evident that the 530 loop, helix 34, and the 1400 region were all specifically cleaved by phenanthroline covalently tethered to the A-site of appropriately positioned mRNA.

Functional Significance. There are many studies that show that the regions cleaved in this study act together in the process of decoding.

Analogs of the decoding center isolated from the structure of the ribosome do give similar chemical protection patterns in the presence of tRNA as does the actual structure within the ribosome (Stern & Purohit, 1995). Cross-linking studies have connected the anticodon loop of tRNA with the 1400 region of the 16S rRNA (Prince et al., 1982; Gornicki et al., 1985). Hence, the mRNA must be proximal to this region during the process of decoding.

The 530 loop has been heavily implicated in the process of decoding by the nature of phenotypes resulting in the mutation of the loop (O'Connor et al., 1992; Powers & Noller, 1991; Leclerc et al., 1991) or its associated proteins (Stern et al., 1986, 1988a). Also, the EfTu−tRNA−GTP ternary complex has been shown to interact with this region (Powers et al., 1988).

Evidence also has been produced that helix 34 is involved not only in the specialized function of termination (Göringer et al., 1991) but also in standard decoding. Dahlberg's group showed that mutations at several points, 1054, 1057, 1058, 1199, and 1200, caused increased frameshifting and read-through of stop codons and decreased cell growth (Moine & Dahlberg, 1994).

These functional studies all tend to implicate these three regions in the decoding process. This study provides primary evidence for the proximity of each of these 16S rRNA regions with the A-site codon of mRNA, when the message is locked into position with cognate tRNA at the P-site.

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REFERENCES

Alexander, R. W., Muralikrishna, P., & Cooperman, B. S. (1994) *Biochemistry 33*, 12109.
Bhangu, R., & Wollenzien, P. (1992) *Biochemistry 31*, 5937.
Bhangu, R., Jusamiene, D., & Wollenzien, P. (1994) *Biochemistry 33*, 3063.
Brimacombe, R., Atmadja, J., Stiege, W., & Schuler, D (1988) *J. Mol. Biol. 199*, 115.
Chen, C. B., Gorin, M. B., & Sigman, D. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4206.
Dontsova, O., Kopylov, A., & Brimacombe, R. (1991) *EMBO J. 10*, 2613.
Dontsova, O., Dokudovskaya, S., Kopylov, A., Bogdanov, A., Rinke-Appel, J., Jünke, N., & Brimacombe, R. (1992) *EMBO J. 11*, 3105.
Göringer, H. U., Huazi, K. A., Murgola, E. J., & Dahlberg, A. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 6603.
Gornicki, P., Ciesiolka, J., & Ofengand, J. (1985) *Biochemistry 24*, 4924.
Hartz, D., McPheeters, D. S., Traut, R., & Gold, L. (1988) *Methods Enzymol. 164*, 419.
Heilek, G. M., & Noller, H. F. (1996) *Science* 272, 1659.
Hermann, T., & Heumann, H. (1995) *RNA 1*, 1009.
Igloi, G. L. (1988) *Biochemistry 27*, 3842.
Jacob, W. F., Santer, M., & Dahlberg, A. E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4757.
Kang, C., & Cantor, C. R. (1985) *J. Mol. Biol. 181*, 241.
Lasater, L. S., Montesano Roditis, L., Cann, P. A., & Glitz, D. G. (1990) *Nucleic Acids Res. 18*, 477.

Leclerc, D., Melancon, P., & Brakier-Gingras, L. (1991) *Biochimie 73*, 1431.
Lodmell, J. S., Tapprich, W. E., & Hill, W. E. (1993) *Biochemistry 32*, 4067.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. K. (1987) *Nucleic Acids Res. 15*, 8783.
Moazed, M., Stern, S., & Noller, H. F. (1986) *J. Mol. Biol. 187*, 399.
Moine, H., & Dahlberg, A. E. (1994) *J. Mol. Biol. 243*, 402.
Montesano-Roditis, L., & Glitz, D. J. (1994) *J. Biol. Chem. 269*, 6458.
Oakes, M. I., & Lake, J. A. (1990) *J. Mol. Biol. 211*, 897.
Oakes, M. I., Clark, M. W., Henderson, E., & Lake, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 275.
Oakes, M. I., Kahan, L., & Lake, J. A. (1990) *J. Mol. Biol. 211*, 907.
O'Connor, M., Göringer, H. U., & Dahlberg, A. E. (1992) *Nucleic Acids Res. 20*, 4221.
Olson, H. M., Lasater, L. S., Cann, P. A., & Glitz, D. G. (1988) *J. Biol. Chem. 263*, 15196.
Perrin, D. M., Mazumder, A., Farshid, S., & Sigman, D. (1994) *Biochemistry 33*, 3848.
Powers, T., & Noller, H. F. (1991) *EMBO J. 10*, 2203.
Powers, T., Noller, H. F., Stern, S., & Changchien, L. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1364.
Prince, J. B., Taylor, B. H., Thurlow, D. L., Ofengand, J., & Zimmermann, R. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5455.
Rinke-Appel, J., Jünke, N., Stade, K., & Brimacombe, R. (1991) *EMBO J. 10*, 2195.
Rinke-Appel, J., Jünke, N., Brimacombe, R., Dokudovskaya, S., Dontsova, O., & Bogdanov, A. (1993) *Nucleic Acids Res. 21*, 2853.
Rinke-Appel, J., Jünke, N., Brimacombe, R., Lavrik, I., Dokudovskaya, S., Dontsova, O., & Bogdanov, A. (1994) *Nucleic Acids Res. 22*, 3018.
Shatsky, I. N., Bakin, A. V., Bogdanov, A. A., & Vasiliev, V. D. (1991) *Biochimie 73*, 937.
Sigman, D. S., & Chen, C. B. (1990) *Annu. Rev. Biochem. 59*, 207.
Sigman, D. S., Kuwabara, M. D., Chen, C. B., & Bruice, T. C. (1991) *Methods Enzymol. 208*, 414.
Simon, S. S., Grabowski, S., & Whitesides, G. M. (1990) *J. Org. Chem. 55*, 1834.
Stade, K., Rinke-Appel, J., & Brimacombe, R. (1989) *Nucleic Acids Res. 17*, 9889.
Staehelin, T., & Falvey, A. K. (1971) *Methods Enzymol. 20*, 433.
Stern, S., & Purohit, P. (1995) *Biochem. Cell Biol. 73*, 899.
Stern, S., Wilson, R. C., & Noller, H. F. (1986) *J. Mol. Biol. 192*, 101.
Stern, S., Powers, T., Changchien, L.-M., & Noller, H. F. (1988a) *J. Mol. Biol. 201*, 683.
Stern, S., Weiser, B., & Noller, H. F. (1988b) *J. Mol. Biol. 204*, 447.
Sun, J.-s., Francois, J. C., Lavery, R., Saison Behmoaras, T., Montenay Garestier, T., Thuong, N. T., & Hèlene, C. (1988) *Biochemistry 27*, 6039.
Tam, M. F., & Hill, W. E. (1980) *FEBS Lett. 120*, 230.
Trempe, M. R., Ohgi, K., & Glitz, D. G. (1982) *J. Biol. Chem. 257*, 9822.
Wollenzien, P., Expert-Bezancon, A., & Favre, A. (1991) *Biochemistry 30*, 1788.

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