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# Topography of the E site on the *Escherichia coli* ribosome

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**Three photoreactive tRNA probes have been utilized in order to identify ribosomal components that are in contact with the aminoacyl acceptor end and the anticodon loop of tRNA bound to the E site of *Escherichia coli* ribosomes. Two of the probes were derivatives of *E.coli* $tRNA^{Phe}$ in which adenosines at positions 73 and 76 were replaced by 2-azidoadenosine. The third probe was derived from yeast $tRNA^{Phe}$ by substituting wyosine at position 37 with 2-azidoadenosine. Despite the modifications, all of the photoreactive tRNA species were able to bind to the E site of *E.coli* ribosomes programmed with poly(A) and, upon irradiation, formed covalent adducts with the ribosomal subunits. The $tRNA^{Phe}$ probes modified at or near the 3′ terminus exclusively labeled protein L33 in the 50S subunit. The $tRNA^{Phe}$ derivative containing 2-azidoadenosine within the anticodon loop became cross-linked to protein S11 as well as to a segment of the 16S rRNA encompassing the 3′-terminal 30 nucleotides. We have located the two extremities of the E site-bound tRNA on the ribosomal subunits according to the positions of L33, S11 and the 3′ end of 16S rRNA defined by immune electron microscopy. Our results demonstrate conclusively that the E site is topographically distinct from either the P site or the A site, and that it is located alongside the P site as expected for the tRNA exit site.**

*Key words:* azidoadenosine/E site/photoaffinity labeling/ribosome model/tRNA–ribosome cross-link

## Introduction

During the elongation phase of protein synthesis, peptidyl-tRNA is translocated from the A site to the P site of the *Escherichia coli* ribosome. At the same time deacylated tRNA is transferred from the P site to the E or exit site prior to its dissociation from the ribosomal complex (Rheinberger *et al.*, 1981; Grajevskaya *et al.*, 1982; Kirillov *et al.*, 1983; Lill *et al.*, 1984). It has been shown that the transient association of deacylated tRNA with the E site is essential for efficient translocation in the presence of EF-G (Lill *et al.*, 1989). This result indicates that the main function of the E site is to promote the release of discharged tRNA from the P site (Lill *et al.*, 1986; Wintermeyer *et al.*, 1990). Alternatively, it has been suggested that occupancy of the E site maintains the ribosome in a post-translocation state, influencing the interaction of aminoacyl-tRNA with the A site and thereby enhancing translational accuracy (Geigenmüller and Nierhaus, 1990). In both views, the presence of tRNA in the E site is envisioned as being allosterically linked with the ribosomal binding sites for either elongation factors (Wintermeyer *et al.*, 1990) or aminoacyl-tRNA (Rheinberger *et al.*, 1990). However, the role of codon–anticodon interaction in the E site is considered marginal by one group (Lill and Wintermeyer, 1987), while it is seen as having an important stabilizing function by the other (Rheinberger *et al.*, 1990). The contribution of codon–anticodon interaction to tRNA binding in the E site has been determined to be ~500- to 1000-fold less than in the P site at 10 mM $Mg^{2+}$ and even lower at 15–20 mM $Mg^{2+}$ (Lill and Wintermeyer, 1987). On the other hand, a specific association of the 3′-terminal A residue of $tRNA^{Phe}$ with the ribosome has been found to be indispensable for the binding of tRNA to the E site (Lill *et al.*, 1988).

Little is known about the location of the E site on the ribosome despite the importance of such information for an understanding of its role in tRNA release. Nonetheless, to carry out its function, the E site is expected to be adjacent to the P site. The 3′ end of E site-bound tRNA has been shown to reduce sharply the reactivity of several nucleotides within the 23S rRNA to chemical modification (Moazed and Noller, 1989a). These residues are in the vicinity of the binding site for protein L1 and a nucleotide that has been cross-linked to protein L33 (Branlant *et al.*, 1981; Mitchell *et al.*, 1990). Both L1 and L33 are close neighbors in the 50S subunit as demonstrated by protein–protein cross-linking experiments (Walleczek *et al.*, 1989a). In addition, fluorescence energy transfer experiments have demonstrated that the distances between the anticodon loops of tRNAs bound either to the P and E sites or to the A and E sites, are ~35 and 42 Å, respectively (Paulsen and Wintermeyer, 1986). These results indicate that, in the 30S ribosomal subunit, the E site is closer to the P site than to the A site, and suggest further that the anticodon of the E site-bound tRNA may not be immediately adjacent to that of the tRNA in the P site.

Affinity labeling has proven to be a useful technique for defining the location of binding sites for functionally important ligands on the ribosome (Cooperman, 1988). In particular, photoreactive tRNA derivatives have played a major role in elucidating the topography of the decoding site on the 30S subunit and the peptidyltransferase center on the 50S subunit. Over the past few years we have exploited for this purpose tRNA probes in which photolabile azidonucleotides are substituted for naturally occurring bases at specific

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positions within the RNA molecule. Because azidonucleotides yield very short cross-links, on the order of 2–4 Å, upon irradiation, they provide precise information on the molecular environment of the photoreactive base. We have already used azidoadenosine-containing tRNAs to study the topography of the A and P sites on the *E.coli* ribosome and, on the basis of these data, we have advanced a model of the tRNA–ribosome complex during polypeptide chain elongation (Wower and Zimmermann, 1991). The relative arrangement of A and P site-bound tRNA proposed in the model is consistent with the orientation of mRNA on the 30S subunit and the location of the binding sites for elongation factors on the 50S subunit. In the present work we have used the azidoadenosine-containing tRNA derivatives to probe the topography of the E site on the *E.coli* ribosome and have extended our model to encompass this new information.

## Results

### Cross-linking of tRNA derivatives containing $2N_3A$ at positions 73 and 76 to the ribosomal E site

To define the neighborhood of the 3′ terminus of tRNA bound to the ribosomal E site, we prepared two derivatives of *E.coli* $tRNA^{Phe}$ in which adenosines at either position 76 or 73 were replaced with the photoreactive analog, 2-azidoadenosine ($2N_3A$). The introduction of $2N_3A$ was achieved by ligating [5′-$^{32}$P]p$2N_3$Ap to tRNA molecules chemically truncated by one or four nucleotides at the 3′ terminus (Wower *et al.*, 1988). In the latter case, the -C-C-A terminus was reconstructed with tRNA nucleotidyl transferase.

The functional activity of the $tRNA^{Phe}$ derivatives was assessed by their ability to bind specifically to the E site of ribosomes programmed with poly(A). The use of poly(A) in this study minimized the spillover of $tRNA^{Phe}$ derivatives from the E site to the P site without compromising the efficiency of cross-linking. The photoreactive $tRNA^{Phe}$ probes were directed to the E site by prefilling the P site of poly(A)·70S ribosome complexes with $tRNA^{Lys}$. We found that under these conditions both $2N_3$A76- and $2N_3$A73-containing tRNAs bound to the E site almost as well as unmodified $tRNA^{Phe}$. Thus, although the 3′-terminal adenosine has been shown to play a crucial role in the interaction of tRNA with the E site (Lill *et al.*, 1988), the substitution of A76 by $2N_3A$ does not significantly affect binding of the modified $tRNA^{Phe}$ to this site. In these experiments, noncovalent binding of [$2N_3$A76]$tRNA^{Phe}$ and [$2N_3$A73]$tRNA^{Phe}$ was 45–50% of the total added to the incubation mixture.

Irradiation of tRNA·poly(A)·70S ribosome complexes with 300 nm light led to the cross-linking of 15–18% of the noncovalently bound $2N_3$A-containing tRNAs to the ribosomes. Covalent attachment was strictly dependent upon irradiation. Moreover, unmodified $tRNA^{Phe}$ did not cross-link to ribosomes under our conditions. Dissociation of the 70S ribosomes into subunits demonstrated that only the 50S subunit fraction was labeled (Figure 1a), while the separation of 50S ribosomal proteins from 23S rRNA revealed that all of the label co-migrated with the 3–5S protein fraction (Figure 1b). 5S rRNA, which co-sediments with the protein fraction, was isolated by phenol extraction and resolved on a denaturing polyacrylamide gel. Autoradiography of the gel showed that no $tRNA^{Phe}$–5S rRNA cross-links were formed (data not shown).

$^{32}$P-labeled protein–[$2N_3$A76]$tRNA^{Phe}$ complexes, isolated as in Figure 1b, were digested with RNase T1 and

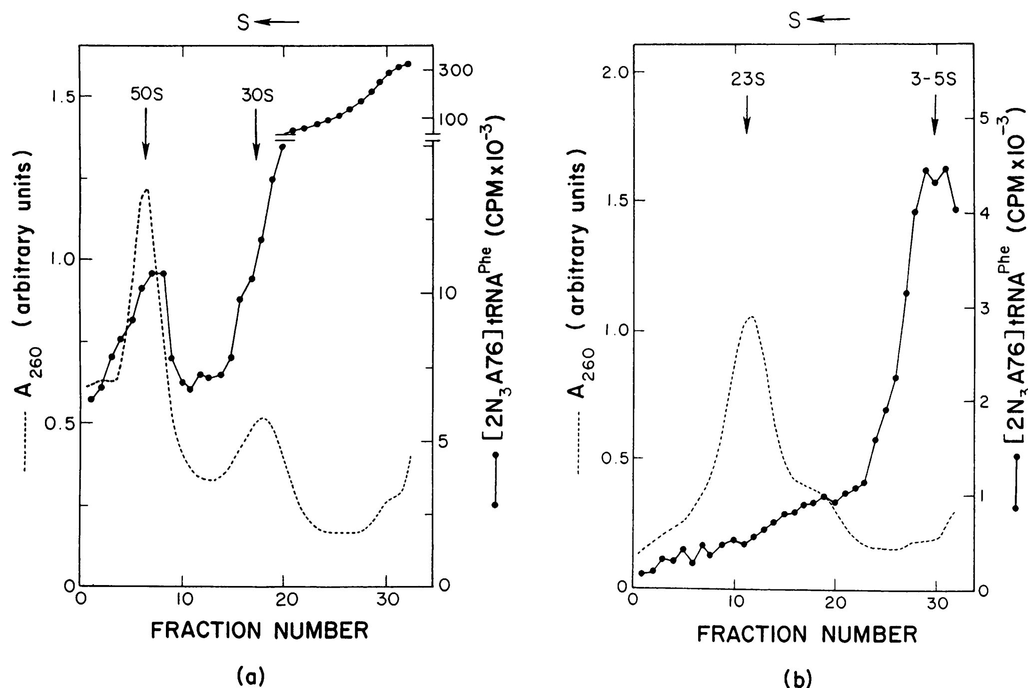


**Fig. 1.** Distribution of [$2N_3$A76]$tRNA^{Phe}$ among the ribosomal subunits and ribosomal components after cross-linking to the E site. Noncovalent [$2N_3$A76]$tRNA^{Phe}$–ribosome complexes were formed under E site conditions in the presence of poly(A) and $tRNA^{Lys}$, and irradiated at 300 nm. **(a)** Ribosomal subunits were resolved by centrifugation through a 10–30% sucrose gradient in 10 mM Tris–HCl, pH 7.6, 50 mM KCl, 0.25 mM $MgCl_2$ and 0.05% (v/v) 2-mercaptoethanol at 40 000 r.p.m. for 105 min at 4°C in a Beckman VTi50 rotor. $^{32}$P radioactivity in fractions 15–20 represents spillover of free tRNA and was not covalently attached to the 30S subunits. **(b)** The 50S subunit fraction was then pooled, concentrated and centrifuged through a 5–20% sucrose gradient in 10 mM Tris–HCl, pH 7.6, 100 mM LiCl, 0.25 mM EDTA and 0.5% (w/v) SDS at 40 000 r.p.m. for 145 min at 10°C in a Beckman VTi50 rotor. ----, $A_{260}$; ●, [$2N_3$A76]$tRNA^{Phe}$.

subjected to one- and two-dimensional PAGE. Autoradiographs revealed the presence of a single radioactive component in each gel (Figure 2a and b). The labeled protein was identified as L33 by the agarose immunological test (Figure 2c). Moreover, L33 was also the only ribosomal component labeled if yeast $[2N_3A76]tRNA^{Phe}$ was used instead of *E.coli* $[2N_3A76]tRNA^{Phe}$. When $[2N_3A73]$ tRNA–protein complexes were analyzed in the same way, the results were identical. Only one $^{32}P$-labeled complex was observed on the polyacrylamide gels, and the immunological analysis showed that L33 was the sole protein labeled.

### *Cross-linking of a $tRNA^{Phe}$ derivative containing $2N_3A$ at position 37 to the ribosomal E site*

The region of the ribosome in proximity to the anticodon loop of E site-bound tRNA was investigated with a derivative

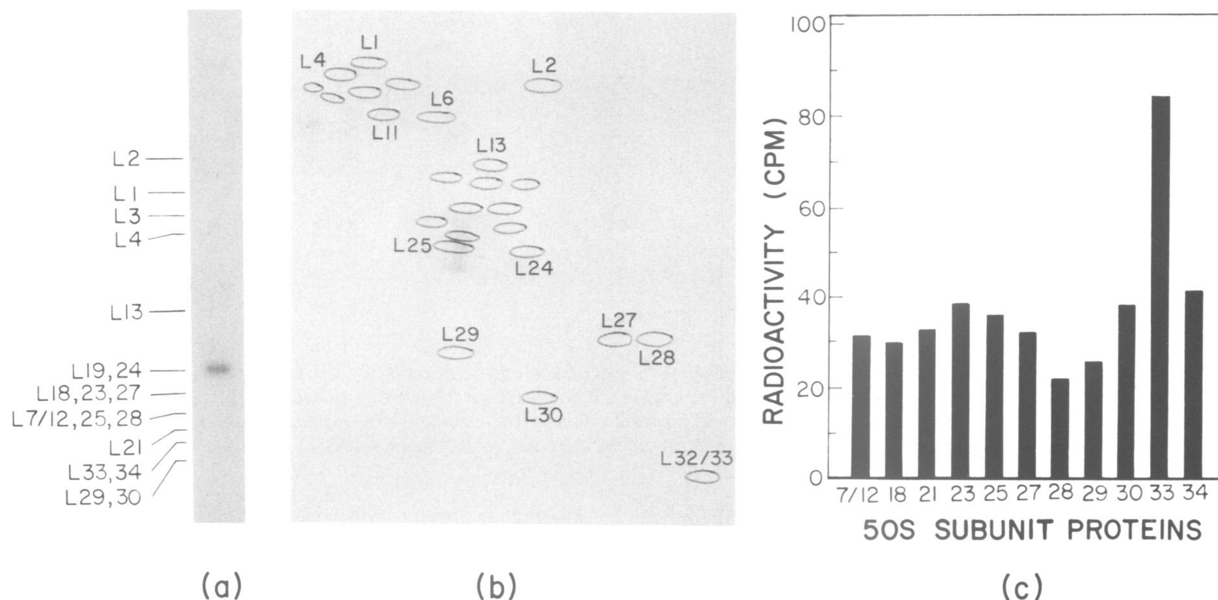


**Fig. 2.** Analysis of covalent $[2N_3A76]tRNA^{Phe}$–protein complexes. The 50S subunit protein fraction was isolated by sucrose gradient centrifugation as described in Figure 1b, digested with RNase T1 and subjected to either (**a**) SDS–PAGE or (**b**) two-dimensional PAGE. (**c**) RNase-treated 50S subunits were screened for reactivity with antibodies to 50S subunit proteins. The mobility of the labeled L33 band is consistent with the presence of the cross-linked pentanucleotide derived from digestion of the tRNA moiety with RNase T1.

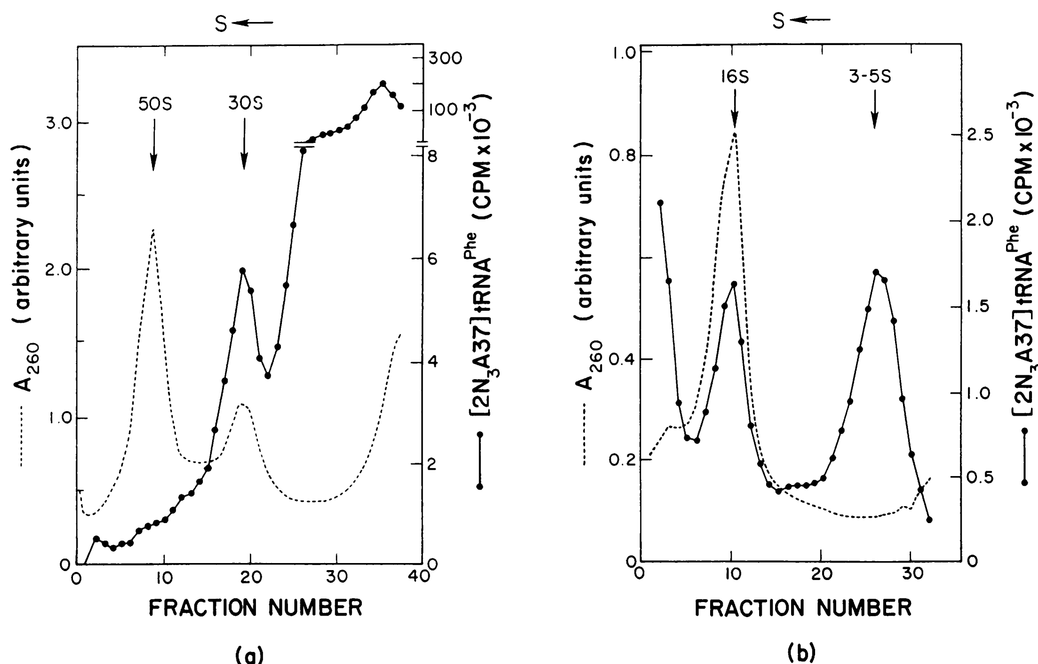


**Fig. 3.** Distribution of $[2N_3A37]tRNA^{Phe}$ among ribosomal subunits and ribosomal components after cross-linking to the E site. Noncovalent $[2N_3A37]tRNA^{Phe}$–ribosome complexes were prepared under E site conditions as described in the legend to Figure 1. (**a**) Ribosomal subunits were resolved by centrifugation through a 10–30% sucrose gradient in 10 mM Tris–HCl, pH 7.6, 50 mM KCl, 0.25 mM $MgCl_2$ and 0.05% (v/v) 2-mercaptoethanol at 40 000 r.p.m. for 135 min at 4°C in a Beckman VTi50 rotor. (**b**) The 30S subunit fraction was then pooled, concentrated and centrifuged through a 5–20% sucrose gradient in 10 mM Tris–HCl, pH 7.6, 100 mM LiCl, 0.25 mM EDTA and 0.5% (w/v) SDS at 40 000 r.p.m. for 220 min at 10°C in a Beckman VTi50 rotor. ----, $A_{260}$; ●, $[2N_3A37]tRNA^{Phe}$.

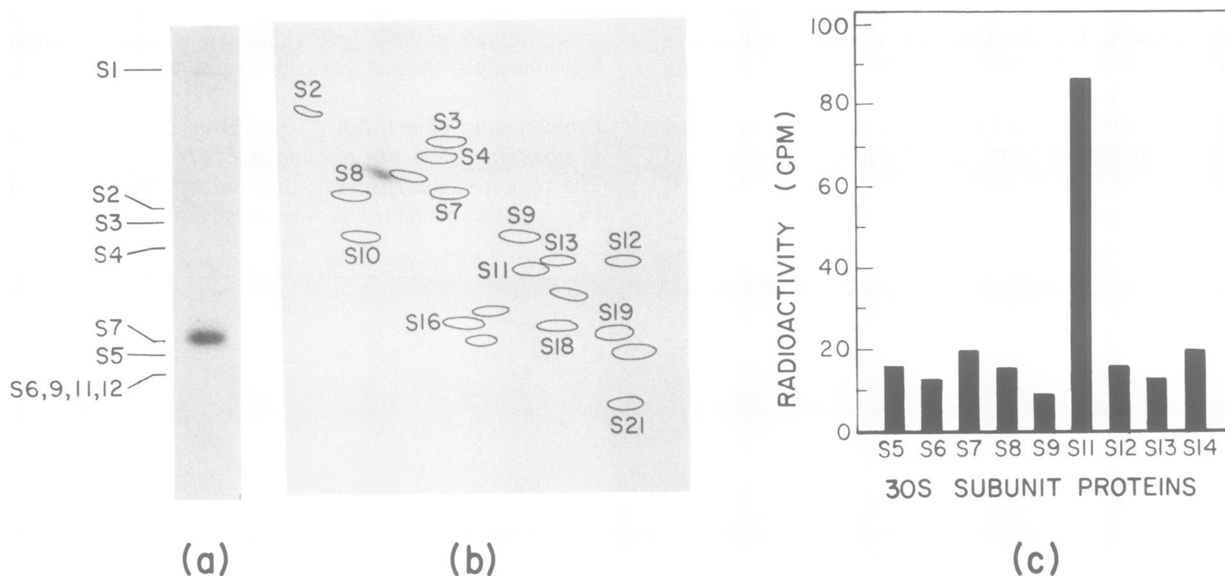


**Fig. 4.** Analysis of covalent $[2N_3A37]tRNA^{Phe}$ – protein complexes. The 30S subunit protein fraction was isolated by sucrose gradient centrifugation as in Figure 3b, digested wih RNases A and T1 and subjected to either (**a**) SDS–PAGE or (**b**) two-dimensional PAGE. (**c**) RNase-treated 30S subunit proteins were screened for reactivity with antibodies to 30S subunit proteins. The mobility of the labeled S11 band is consistent with the presence of the cross-linked pentanucleotide derived from digestion of the tRNA moiety with RNases A and T1.

of yeast $tRNA^{Phe}$ containing $2N_3A$ in place of wyosine at position 37. The biological activity of the modified tRNA was not affected by the substitution as shown by its normal amino acid acceptance and its ability to bind to both the P and A sites (Sylvers *et al.*, 1992).

In the present study, $[2N_3A37]tRNA$ was bound to the E site of poly(A)-programmed ribosomes after prefilling the P site with *E.coli* $tRNA^{Lys}$. On average, 35% of the $[2N_3A37]tRNA$ was bound to the E site. When the $[2N_3A37]tRNA$ – ribosome complexes were irradiated, ~8% of the noncovalently bound tRNA probe became covalently attached to the ribosome. Covalent attachment was dependent upon both UV irradiation and the presence of $2N_3A$. Sucrose gradient centrifugation of the cross-linked $[2N_3A37]tRNA$ – ribosome complexes revealed that the label was incorporated only into 30S subunits (Figure 3a). Further analysis of the 30S subunit fraction showed that the cross-links were almost equally divided between protein and 16S rRNA (Figure 3b).

After treatment with RNases A and T1, the 30S subunit protein fraction yielded a single $^{32}P$-labeled band upon one-dimensional SDS – PAGE (Figure 4a). Similarly, only one $^{32}P$-labeled protein was observed when the same digest was resolved by two-dimensional PAGE (Figure 4b). Immunological analysis revealed that the sole protein labeled by E site-bound $[2N_3A37]tRNA^{Phe}$ was S11 (Figure 4c).

To identify the segment of 16S rRNA to which $[2N_3A37]tRNA$ became cross-linked, $^{32}P$-labeled tRNA – 16S rRNA complexes, isolated as shown in Figure 3b, were subjected to oligonucleotide-directed digestion with RNase H. Eight oligodeoxyribonucleotides complementary to sequences between positions 500 and 1542, the 3′ terminus of the 16S rRNA, were used to direct cleavage. The RNase H digestion mixtures were resolved on a denaturing polyacrylamide gel and visualized by autoradiography. Figure 5a shows the products that arise in the presence of five of the oligonucleotides that hybridized to sequences within the 3′ one-third of the 16S rRNA. The smallest $^{32}P$-labeled fragment was obtained with an oligonucleotide complementary to G1511 – G1525 (Figure 5a, lane 1). Thus, the site of $[2N_3A37]tRNA$ attachment must be within the 3′-terminal 30 nucleotides of the 16S rRNA. When the small fragment was isolated from lane 1 of the gel shown in Figure 5a and digested with RNase T1, several labeled oligonucleotides were produced (data not shown). This result indicates that the tRNA cross-linked to more than one site within the 3′-terminal 30 nucleotides. To determine the proximity of the label to the 3′ end of 16S rRNA, tRNA – 16S rRNA complexes were cleaved with RNase H in the presence of an oligonucleotide complementary to nucleotides 1533 – 1542. As this digestion produced a short $^{32}P$-labeled oligonucleotide (Figure 5b), we conclude that at least a portion of the $tRNA^{Phe}$ was cross-linked within the ten 3′-terminal nucleotides of 16S rRNA.

## Discussion

When bound to the ribosomal E site, photoreactive $tRNA^{Phe}$ derivatives containing a single $2N_3A$ residue at or near the 3′ end exclusively labeled 50S subunit protein L33 upon irradiation with UV light. As photolysis of $[2N_3A]tRNA$ – ribosome complexes leads to the formation of 2 – 4 Å bonds, only ribosomal components that are in close contact with tRNA can be cross-linked. The labeling of protein L33 by E site-bound $[2N_3A73]tRNA^{Phe}$ and $[2N_3A76]tRNA^{Phe}$, as described here, and that of protein L27 by the same $tRNA^{Phe}$ derivatives at the P and A sites, as reported previously (Wower *et al.*, 1989; L.A.Sylvers, J.Wower and R.A.Zimmermann, unpublished results), demonstrate a clear demarcation between the position of the 3′ end of tRNA in the E site and at the peptidyltransferase center.

Our results are consistent with a considerable body of topographical information relating to the 50S ribosomal subunit. Protein – protein cross-linking experiments, for instance, have shown that L33 and L27 are close neighbors

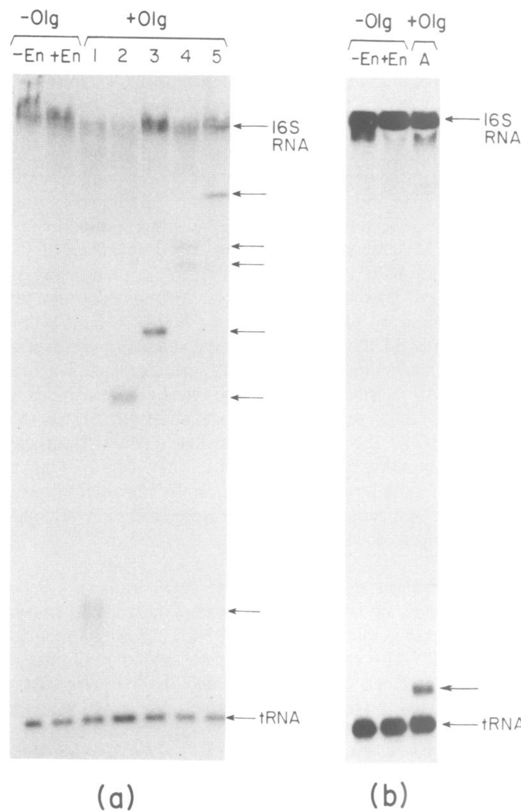


**Fig. 5.** Analysis of the [$2N_3A37$]tRNA$^{Phe}$ − 16S rRNA complex by directed cleavage with RNase H. The 16S rRNA fraction isolated as described in Figure 3b was annealed to complementary oligonucleotides and the hybrid duplexes digested with RNase H. The products were then electrophoresed through a denaturing 5% polyacrylamide gel. In the control reactions, oligonucleotide was omitted (−Olg) and the complex was incubated in the absence or presence of the enzyme (−En and +En, respectively).
(**a**) Oligonucleotides were complementary to positions 1511−1525 (1), 1443−1462 (2), 1399−1415 (3), 1297−1306 (4) and 1107−1116 (5). The second cleavage product in lane 4 results from the partial complementarity of this oligonucleotide to positions 1341−1349.
(**b**) Oligonucleotide A was complementary to positions 1533−1542 in the 16S rRNA.

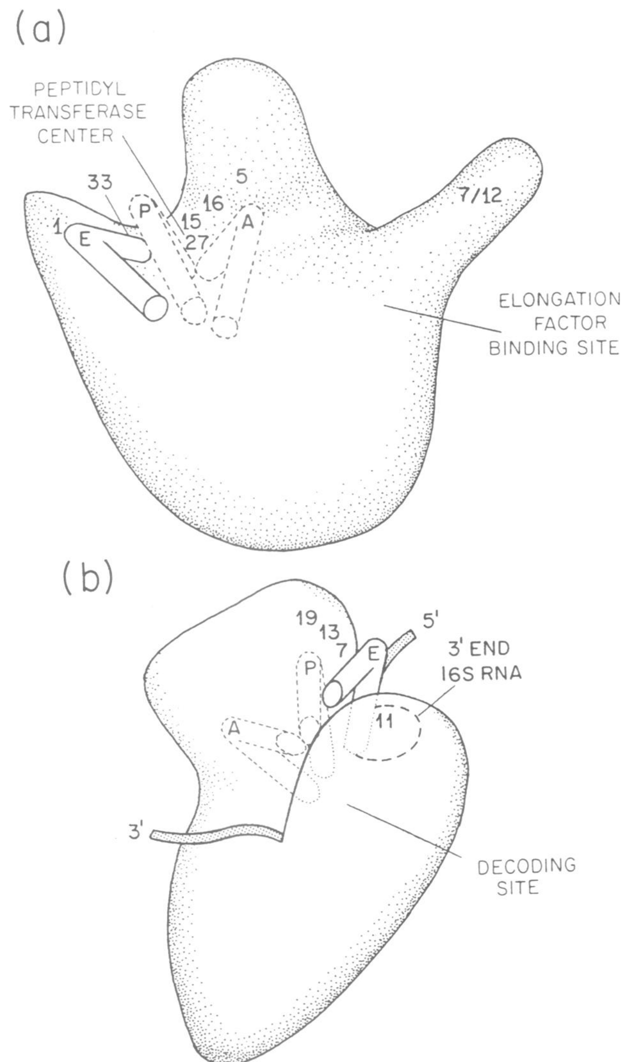


**Fig. 6.** Models of the 50S and 30S ribosomal subunits depicting tRNAs at the ribosomal A, P and E sites. (**a**) 50S ribosomal subunit. The locations of proteins L1, L5, L7/12, L15, L16 and L27 were mapped by immune electron microscopy (Oakes *et al.*, 1986; Stöffler and Stöffler-Meilicke, 1986). The position of L33 was interpolated from protein−protein cross-linking data (Walleczek *et al.*, 1989a,b). (**b**) 30S ribosomal subunit. The locations of proteins S7, S11, S13 and S19 (Oakes *et al.*, 1986; Stöffler and Stöffler-Meilicke, 1986) and the 3′ end of 16S rRNA (Olson and Glitz, 1979; Shatsky *et al.*, 1979) were determined by immune electron microscopy. The mRNA is oriented according to Olson *et al.* (1988). Subunit models are as depicted by Lake and co-workers (Oakes *et al.*, 1986). Although a different orientation of the A- and P-site tRNAs has been proposed by Lim *et al.* (1992), our cross-linking results suggest that anticodon stem and variable loop are in contact with the 30S subunit as shown (Wower *et al.*, 1990; Sylvers *et al.*, 1992; K.Rosen, J.Wower and R.A.Zimmermann, unpublished).

within the 50S ribosomal particle (Redl *et al.*, 1989). In addition, L33 has been cross-linked to protein L1 (Walleczek *et al.*, 1989a). The protein−protein cross-links, together with the localization of proteins L1 and L27 by immune electron microscopy (Lake and Strycharz, 1981), indicate that protein L33, and by inference the 3′ end of E-site tRNA, is located between protein L1, which occupies the ridge, and protein L27, which is located below the central protuberance, on the interface side of the 50S ribosomal subunit (Figure 6a). Placement of the E site on the 50S subunit at or near proteins L1 and L33 is also supported by chemical modification studies which revealed that the accessibility of nucleotides G2112, G2116 and C2393 of the 23S rRNA is reduced by E site-bound tRNA (Moazed and Noller, 1989a). Protection of G2112 and G2116, which lie within the L1 binding site of the 23S rRNA (Branlant *et al.*, 1981), by E site-bound tRNA led Noller and his collaborators to propose a position for the E site similar to that shown in Figure 6a (Noller *et al.*, 1990). The cross-linking of protein L33 to U2423 (Mitchell *et al.*, 1990), which is located close to C2393 in the secondary structure of the 23S rRNA, also connects the cross-linking data to the chemical modification results.

Cross-linking of the tRNA$^{Phe}$ containing $2N_3A$ in the anticodon loop to the E site results in a roughly equal distribution of the label between the 30S subunit protein S11 and the 3′-terminal 30 nucleotides of the 16S rRNA. This observation provides the first evidence for the existence of a direct contact between E site-bound tRNA and the 30S subunit. The E site is clearly differentiated from the decoding site because the same tRNA derivative, when bound to the A or P sites, labels protein S7 (Sylvers *et al.*, 1992). Moazed and Noller (1989b) reported that there were no differences in the chemical reactivity of nucleotides within the 16S rRNA

when tRNA was positioned at the E site. This observation can be attributed to the fact that nucleotides 1503–1542, which encompass the cross-links reported here, were not scanned by primer extension in their experiments (see Moazed and Noller, 1986).

Our cross-linking results indicate that the anticodon loop of E site tRNA contacts the 30S ribosomal subunit at the top of the platform where both S11 and the 3′ end of the 16S rRNA have been mapped by immune electron microscopy (Olson and Glitz, 1979; Shatsky *et al.*, 1979; Oakes *et al.*, 1986; Stöffler and Stöffler-Meilicke, 1986; see Figure 6b). Given that the anticodon of P site-bound $tRNA_1^{Val}$ can be photochemically attached to C1400 of the 16S rRNA (Prince *et al.*, 1982), a residue that was mapped in the cleft between the head and the platform of the 30S subunit (Górnicki *et al.*, 1984), the E site must be located to the right of the P site on the 30S subunit, as depicted in Figure 6b. This arrangmenent is consistent with the relative arrangement of the P and E sites on the 50S subunit.

Our predictions about the location of the E site on the ribosome are based largely on the positions of proteins S11 and L33, and of the 3′ end of 16S rRNA, determined by immune electron microscopy. The fact that $[2N_3A37]tRNA^{Phe}$ can be cross-linked to the 3′-end of 16S rRNA is of considerable interest as this region of 16S rRNA contains two elements important for ribosome function, the anti-Shine–Dalgarno sequence which interacts with a complementary segment in mRNA during the initiation of protein synthesis (Shine and Dalgarno, 1974; Steitz and Jakes, 1975) and a pair of adjacent $N^6$,$N^6$-dimethyladenosine residues which mediate the sensitivity of ribosomes to the antibiotic kasugamycin (Helser *et al.*, 1971). Both of these features, as well as the 3′ end of 16S rRNA, map close to protein S11 on the platform of the 30S subunit (Olson *et al.*, 1988; Politz and Glitz, 1977; Olson and Glitz, 1979; Shatsky *et al.*, 1979). The heterogeneity of the cross-links between $[2N_3A37]tRNA^{Phe}$ and the 3′-terminal sequence of 16S rRNA may be ascribable to the mobility of either the tRNA anticodon or the 3′-terminus of the 16S rRNA. Indeed, at least two states of the E site complex were revealed by kinetic experiments in which fluorescent $tRNA^{Phe}$ derivatives were used in conjunction with poly(U)-programmed ribosomes (Robertson *et al.*, 1986).

## Materials and methods

### Materials

Phenylalanine-specific tRNA from yeast (specific amino acid acceptance, 1100 pmol/$A_{260}$) and poly(A) were purchased from Boehringer Mannheim. *E.coli* $tRNA^{Phe}$ and $tRNA^{Lys}$ (specific amino acid acceptances, 1400 and 1200 pmol/$A_{260}$) were from Subriden RNA. *E.coli* RNase H was obtained from Epicentre Technologies. Oligonucleotides were synthesized by either Genosys or the DNA Synthesis Facility at the University of Massachusetts (Amherst). The sources of enzymes, radioactively labeled compounds and other biological materials were as described previously (Wower *et al.*, 1988; Sylvers *et al.*, 1989). Published procedures were used for preparing $[5'-^{32}P]p2N_3Ap$ (Sylvers *et al.*, 1989), $tRNA^{Phe}$ derivatives containing $2N_3A$ (Wower *et al.*, 1988; Sylvers *et al.*, 1992) and tight-couple 70S ribosomes from *E.coli* MRE 600 (Robertson and Wintermeyer, 1981). The concentration of active ribosomes was determined by the indicator binding assay (Lill *et al.*, 1988) and, in the present experiments, was 13.8 pmol/$A_{260}$ unit.

### Formation and cross-linking of tRNA–ribosome complexes

All experiments were performed in buffer A (50 mM Tris–HCl, pH 7.4, 100 mM $NH_4Cl$, 20 mM $MgCl_2$). Ribosomal complexes containing *E.coli* $tRNA^{Phe}$ substituted with $2N_3A$ at positions 73 or 76 at the E site were prepared as follows. A 3 $\mu$M solution of tight-coupled ribosomes was first incubated with poly(A) at a final concentration of 1.5 $A_{260}$ units/ml for 5 min at 37°C. The P site was then blocked by adding 1.3 mol of $tRNA^{Lys}$ per mol of ribosomes and incubating for 30 min at 25°C. Finally, 0.7 mol of $^{32}P$-labeled $[2N_3A73]tRNA^{Phe}$ or $[2N_3A76]tRNA^{Phe}$ was added per mol of ribosomes and the incubation at 25°C was continued for 2 min. E-site complexes containing $^{32}P$-labeled $[2N_3A37]tRNA^{Phe}$ derived from yeast were prepared in the same way except that the ribosome concentration was 6 $\mu$M, and the molar ratio of labeled $tRNA^{Phe}$ to ribosomes was 0.1.

Cross-linking was accomplished by irradiating the complexes at 4°C for either 30 s on a UV transilluminator using a 300 nm filter, or 10 min in a Rayonet Model RPR-100 photochemical reactor equipped with six RPR-3000A lamps. Irradiation under these conditions does not affect the tRNA binding capacity of the ribosomes (Wower *et al.*, 1988). After irradiation, 10 mM DTT was added and the samples were incubated for an additional 10 min to quench unreacted 2-azido groups. The binding of $^{32}P$-labeled $tRNA^{Phe}$ derivatives to the ribosomes was determined from the amount of radioactivity adsorbed to nitrocellulose membranes (Schwartz and Ofengand, 1974). The fraction of cross-linked $tRNA^{Phe}$ was calculated as the ratio of the radioactivity retained at 0.1 mM $Mg^{2+}$ to that retained at 20 mM $Mg^{2+}$; the amount of labeled tRNA remaining bound to the membranes when unirradiated complexes were filtered at 0.1 mM $Mg^{2+}$ was subtracted from all values.

### Analysis of covalent tRNA–ribosome complexes

Following irradiation, ribosomes were dissociated into subunits by centrifugation through sucrose gradients in 0.25 mM $Mg^{2+}$. Separation of ribosomal proteins from rRNAs was achieved by centrifuging ribosomal subunits through sucrose gradients containing LiCl and SDS (Wower *et al.*, 1988). Proteins cross-linked to photoreactive $tRNA^{Phe}$ derivatives were identified by SDS–PAGE (Laemmli and Favre, 1973), two-dimensional PAGE (Wower *et al.*, 1988) and the 'agarose' immunological method (Gulle *et al.*, 1988). The segment of the rRNA labeled by $2N_3A$-containing tRNA derivatives was determined by oligonucleotide-directed RNase H digestion according to Hayase *et al.* (1990). Complementary oligodeoxyribonucleotides were annealed to 16S rRNA in 8 $\mu$l of 10 mM Tris–HCl, pH 7.9, 60 mM KCl, 1 mM DTT, 10 $\mu$g/ml BSA and 4% glycerol by heating the reaction mixtures for 3 min at 65°C and then cooling slowly to 30°C. After the addition of $MgCl_2$ to a final concentration of 4 mM, digestion was initiated with 1 U of RNase H. Incubation was continued for 1 h at 30°C. The digestion products were resolved by electrophoresis in gels containing 5% polyacrylamide in 100 mM Tris–100 mM $H_3BO_4$, pH 8.3, 2.5 mM EDTA and 8 M urea, and visualized by autoradiography (Wower *et al.*, 1988).

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