Transit of tRNA through the Escherichia coli Ribosome

CROSS-LINKING OF THE 3' END OF tRNA TO SPECIFIC NUCLEOTIDES OF THE 23 S RIBOSOMAL RNA AT THE A, P, AND E SITES

Jacek Wower‡§, Stanislav V. Kirillov§§, Iwona K. Wower‡§, Sadel Guven‡, Stephen S. Hixson**, and Robert A. Zimmermann§

From the ‡Department of Animal and Dairy Sciences, Program in Cell and Molecular Biosciences, Auburn University, Auburn, Alabama 36849-5415 and the Departments of §Biochemistry & Molecular Biology and **Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

When bound to Escherichia coli ribosomes and irradiated with near-UV light, various derivatives of yeast tRNA\(^{\text{Phe}}\) containing 2-azidoadenosine at the 3' terminus form cross-links to 23 S rRNA and 50 S subunit proteins in a site-dependent manner. A and P site-bound tRNAs, whose 3' termini reside in the peptidyl transferase center, label primarily nucleotides U2506 and U2585 and protein L27. In contrast, E site-bound tRNA labels nucleotide C2422 and protein L33. The cross-linking patterns confirm the topographical separation of the peptidyl transferase center from the E site domain. The relative amounts of label incorporated into the universally conserved residues U2506 and U2585 depend on the occupancy of the A and P sites by different tRNA ligands and indicates that these nucleotides play a pivotal role in peptide transfer. In particular, the 3'-adenosine of the peptidyl-tRNA analogue, AcPhe-tRNA\(^{\text{Phe}}\), remains in close contact with U2506 regardless of whether its anticodon is located in the A site or P site. Our findings, therefore, modify and extend the hybrid state model of tRNA-ribosome interaction. We show that the 3'-end of the deacylated tRNA that is formed after transpeptidation does not immediately progress to the E site but remains temporarily in the peptidyl transferase center. In addition, we demonstrate that the E site, defined by the labeling of nucleotide C2422 and protein L33, represents an intermediate state of binding that precedes the entry of deacylated tRNA into the F (final) site from which it dissociates into the cytoplasm.

Knowledge of the molecular events that take place during protein synthesis has been greatly influenced by studies demonstrating that aminoacyl-tRNA, peptidyl-tRNA, and deacylated tRNA are accommodated on the ribosome at the A, P, and E sites, respectively (reviewed in Ref. 1). The three-site model of translation has been modified to include three hybrid binding states, designated A/T, A/P, and P/E, which are adopted by tRNA during its passage through the ribosome (2).

Our earlier photoaffinity labeling studies, which focused primarily on the identification of ribosomal proteins contacted by the 3' end and anticodon of tRNA as it transits the ribosome, allowed us to propose a model for the arrangement of the A, P, and E-site tRNAs on the Escherichia coli ribosome (3, 4). In this model, the tRNA molecules are positioned so that their 3' ends are directed toward the peptidyl transferase center of the 50 S ribosomal subunit, while their anticodons point toward the groove between the head and the body of the 30 S ribosomal subunit where they interact with complementary codons in the mRNA. Relative to the 50 S subunit interface, the A-site tRNA is located on the L7/L12 or "right" side, the E site tRNA is placed near the L1 protuberance on the "left" side, and the P-site tRNA occupies the space between them. A similar model was proposed by Noller et al. (5), who investigated the location of tRNA in ribosomal complexes by chemical footprinting.

Recently, cryo-electron microscopy and x-ray crystallography have permitted the visualization of tRNA molecules bound to the ribosome during different stages of protein synthesis at high resolution (6–8). Stark et al. (7) have demonstrated that the arrangement of the A, P, and E site tRNAs in pre- and posttranslocational ribosomes is very close to that predicted by Wower et al. (3, 4) and Noller et al. (5). An alternative arrangement for E site-bound tRNA, designated as the E2 site, has been proposed on the basis of cryo-electron microscopy (6, 9). This tRNA binding state most likely corresponds to the E site tRNA visualized by x-ray crystallography (8).

Many lines of evidence indicate that the 23 S rRNA is involved in essential ribosomal functions (10, 11). Now that the position of tRNA molecules can be visualized relative to the structure of the ribosome, determining the topography of tRNA-23 S rRNA contacts on the level of individual nucleotides is crucial for interpreting the images provided by cryo-electron microscopy and x-ray crystallography as well as for constructing high resolution models of the different functional states of the ribosome. In the present work, we follow the movement of the 3' terminus of tRNA as it transits the E. coli ribosome during the elongation cycle of translation using photoactive tRNA probes and characterize the interactions of tRNA with the 23 S rRNA at the nucleotide level.

EXPERIMENTAL PROCEDURES

Materials—The sources of tRNAs, enzymes, and radioactively labeled compounds were as described previously (4, 12, 13).

tRNA Derivatives—[5\(^-\)\(^32\)P\(]_{2\text{N}}\text{A}_{76}\text{tRNA}\(^{\text{Phe}}\) and [\(^{14}\)C]tRNA\(^{\text{Phe}}\) were prepared according to earlier described procedures (14, 15). Aminoacylation of [5\(^-\)\(^32\)P\(]_{2\text{N}}\text{A}_{76}\text{tRNA}\(^{\text{Phe}}\) with [\(^{14}\)C]Phe or [\(^{35}\)S]Phe and acetylation of the aminoacylated photoactive tRNA derivative were carried out according to Rappaport and Lapidot (16). All Phe- and AcPhe-
Movement of the 3' End of tRNA through the Ribosome

TABLE I

Segments and nucleotides of 23 S rRNA labeled by (2N3A76)tRNAPhe derivatives bound to the A, P, E, and E sites on the E. coli ribosome

| A site | P site | E site | Poly(U) Nucleotides labeled | RNA segments labeled |
|-------|--------|--------|-----------------------------|----------------------|
| (pmol of tRNA/pmol of 70 S) | (pmol of tRNA/pmol of 70 S) | (pmol of tRNA/pmol of 70 S) | (pmol of tRNA/pmol of 70 S) | (pmol of tRNA/pmol of 70 S) |
| (2N3A76)tRNA | (2N3A76)tRNA | (2N3A76)tRNA | (2N3A76)tRNA | (2N3A76)tRNA |
| (0.64) | (0.53) | (0.51) | (1.22) | (0.59) |
| U2585 | U2506 | G2069, | U2506 | C2452, |
| H1 > H2 | H2 > H1 > H4 | F1, F2a, F4 | H1 > H2 | C2452, |
| F1, F2a, | F1 | F1 | F1 | F3 |
| T11, T12 | T11, T12, T18 | T11, T12, T13, T18 | T11, T12, T19, T20 | T14, T15 |
| T11 | T11, T12 | T11, T12, T13, T18 | T11, T12, T19, T20 | T14, T15 |

a Some tRNA also binds to other sites.
b Phe-tRNA bound to ribosomal A/T or R site as ternary complex with EF-Tu and GMPPNP.

tRNAderivatives were purified by benzoylated DEAE-cellulose chromatography (15).

Ribosomes and Poly(U) Templates—Tight-couple 70 S ribosomes, isolated from E. coli MRE 600 as described by Makhno et al. (17), bound 1.8 molecules of AcPhe-tRNA per ribosome at 15 mM MgCl2. Poly(U) templates used in cross-linking experiments were prepared according to Kirillov et al. (18).

Formation of tRNA-Ribosome Complexes—Binding of [5'32P](2N3A76)tRNAPhe derivatives to the ribosomal P, A, R, and E sites was carried out as described earlier (2, 3, 17, 19) and measured by filter retention (20). Occupation of the P site by Ac[14C]Phe-[5'32P](2N3A76)tRNAPhe was verified by its reactivity with puromycin (21). As noted except as noted, tRNA binding and cross-linking experiments were carried out in TNME buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 0.5 mM EDTA).

Photoaffinity Labeling—Cross-linking was accomplished by irradiating non-covalent tRNA-ribosome complexes for 2 min at 0 °C in a Rayonet Model RPR-100 photochemical reactor equipped with six RPR-3000-A lamps (13). On average, 9–13% of the non-covalently bound (2N3A76)tRNAPhe became covalently cross-linked to the ribosomes under our conditions (3, 4, 13). The cross-linked complexes were fractionated as described previously (13, 22).

Isolation and Analysis of Cross-linked Complexes—Sites in the 23 S rRNA labeled by (2N3A76)tRNAPhe were identified by treatment with RNase H in the presence of selected pairs of oligodeoxyribonucleotides (23). RNase T1 protection experiments (24), and primer extension analysis (25, 26). The oligodeoxyribonucleotides used in these assays were complementary to sequences within the 23 S rRNA encompassing nucleotides 776–2790 (oligo 1), 2616–2630 (oligo 2), 2589–2597 (oligo 2a), 2563–2577 (oligo 3), 2532–2546 (oligo 3a), 2492–2506 (oligo 3b), 2438–2452 (oligo 4), 2534–2548 (oligo 4a), 2229–2243 (oligo 5), 2110–2124 (oligo 5a), 2050–2064 (oligo 6a), 2058–2080 (oligo 6b), 1952–1980 (oligo 7a), 1903–1917 (oligo 7b). Replacement of the 3'-terminal nucleotide to verify site occupancy and puromycin reactivity (Table I and “Experimental Procedures”). Identification of the cross-linking sites allowed us to delineate the path followed by the 3’ terminus of the tRNA during its transit through the ribosome.

To simulate P-site complexes containing tRNA in the pre- and post-transpeptidation states of binding, Ac[14C]Phe-[5'-32P](2N3A76)tRNAPhe and [5'-32P](2N3A76)tRNAPhe were individually bound to poly(U)-programmed 70 S ribosomes (Fig. 1, a and b). Occupancy of the P site by Ac[14C]Phe-[5'-32P](2N3A76)tRNAPhe was confirmed by its quantitative reaction with puromycin. For EF-Tu-GTP-dependent binding to the A site, [14C]Phe-[5'-32P](2N3A76)tRNAPhe was bound to poly(U)-programmed 70 S ribosomes in which the P site was blocked by deacylated E. coli [14C]tRNAPhe. To retain [14C]Phe-[5'-32P](2N3A76)tRNAPhe in the R or A/T site (2, 28), GTP was added to the ribosomes.

RESULTS

Binding and Cross-linking of Yeast (2N3A76)tRNAPhe to Ribosomal Complexes Representing Different Stages of the Elongation Cycle

Irradiation of 2-azidoadenosine with near-UV light induces very short cross-links to adjacent molecules and can therefore provide precise information on the molecular environment of the photoreactive base (27). Replacement of the 3’-terminal adenosine of yeast tRNAPhe with 2-azidoadenosine yields a photoreactive derivative, (2N3A76)tRNAPhe, which can be aminoacylated by yeast phenylalanyl-tRNA synthetase and bound to E. coli ribosomes (14). In the present work, we describe the preparation of [32P]-labeled (2N3A76)tRNAPhe derivatives and their incorporation into ribosomal complexes representing different stages in the translation elongation cycle. The photoreactive and non-photoreactive tRNA derivatives usually contained 3H or 14C labels in either the aminoacyl moiety or the 3’-terminal nucleotide to verify site occupancy and puromycin reactivity. Identification of the cross-linking sites allowed us to delineate the path followed by the 3’ terminus of the tRNA during its transit through the ribosome.

The abbreviations used are: AcPhe-tRNAPhe, N-acetylphenylalanyl-tRNAderivatives; PAGE, polyacrylamide gel electrophoresis; EF-Tu, elongation factor Tu; GMPPNP, guanylyl-imidodiphosphate.
Movement of the 3' End of tRNA through the Ribosome

FIG. 1. Localization of the segments in the 23 S rRNA that are cross-linked to [5-32P]2N,A76tRNA^Phe derivatives by RNase H analysis. Upper row, autoradiograms of RNase H digests of [5-32P]2N,A76tRNA^Phe-23 S rRNA complexes. Covalent tRNA-23 S rRNA complexes were isolated from UV-irradiated ribosomal particles containing (a) P site-bound [5-32P]2N,A76, (b) P site-bound Ac[14C]Phe-[5-32P]2N,A76, (c) P site-bound [14C]tRNA^Phe and R site-bound [14C]Phe-[5-32P]2N,A76tRNA^Phe, (d) P site-bound [14C]tRNA^Phe and A site-bound Ac[14C]Phe-[5-32P]2N,A76tRNA^Phe, (e) P site-bound [5-32P]2N,A76tRNA^Phe and A site-bound Ac[14C]Phe-tRNA^Phe, (f) A and P site-bound Ac[14C]Phe-tRNA^Phe and E site-bound [5-32P]2N,A76tRNA^Phe, and (g) three deacylated [5-32P]2N,A76tRNA^Phe and digested with RNase H in the presence of the following pairs of oligodeoxynucleotides: 3/4, 4/5, 5/6, 6/7, 7/8, and 8/9. The resulting 23 S rRNA fragments were resolved by electrophoresis on 8% polyacrylamide gels. After staining with toluidine blue, gels were dried and subjected to autoradiography. Controls: E+, incubation with RNase H in the absence of oligodeoxynucleotides; E−, incubation without RNase H or oligodeoxynucleotides. H1, H2, H3, H4, and H5 denote [5-32P]2N,A76tRNA^Phe-labeled 23 S rRNA fragments encompassing nucleotides 2567–2904, 2446–2568, 2237–2445, 2025–2092, and 1910–2024, respectively. The termini of the 23 S rRNA fragments defined by RNase H digestion are given in each case to the midpoint of the sequence complementary to the oligodeoxynucleotide concerned. Lower row, diagrams depicting the cross-linked tRNA-70 S ribosome complexes. tRNA in red, [5-32P]2N,A76 (RNA^Phe); tRNA in black, deacylated tRNA^Phe; blue square, AcPhe moiety; blue circle, Phe moiety; TC, ternary complex in green. Poly(U) was used as an mRNA analogue in all experiments.

During the elongation phase of protein synthesis, the ribosome oscillates between the pre- and post-translocation states. Pre-translocation complexes were formed by the non-enzymatic binding of Ac[14C]Phe-[5-32P]2N,A76tRNA^Phe to the A site of poly(U)-programmed 70 S ribosomes in which the P site was filled with deacylated E. coli [14C]tRNA^Phe (see Fig. 1d) or of Ac[14C]Phe-tRNA^Phe to the A site of poly(U)-programmed 70 S ribosomes in which the P site was filled with deacylated [5-32P]2N,A76tRNA^Phe (see Fig. 1c). Comparison of the latter state with that depicted in Fig. 1a was expected to show whether the binding of tRNA to the A site influences the arrangement of tRNA in the P site.

E site complexes were formed by binding deacylated [5-32P]2N,A76tRNA^Phe to poly(U)-programmed ribosomes under conditions in which both the A and P sites were blocked with Ac[14C]Phe-tRNA^Phe (see Fig. 1f) (29).

The finding that poly(U)-programmed E. coli ribosomes can bind three molecules of deacylated tRNA^Phe in vitro was crucial for the development of the three-site model of the ribosome, which suggests that tRNAs bind successively to the A, P, and E sites during the elongation cycle (30–33). Therefore, we examined the pattern of cross-linking in the presence of saturating amounts of deacylated [5-32P]2N,A76tRNA^Phe in addition to the complexes described above (Fig. 1g).

Each tRNA-ribosome complex was irradiated with 300-nm lamps to induce cross-linking (3). Separation of the tRNA-ribosome complexes into 30 S and 50 S subunit fractions by centrifugation at low Mg^2+ concentration demonstrated that the tRNA derivatives cross-linked exclusively to the 50 S subunit. Subsequent analysis of the covalent tRNA-50 S subunit complexes on a sucrose gradient containing SDS showed that the cross-links are distributed between the 23 S rRNA and the 50 S subunit proteins. As we have previously reported (3, 4, 34, 35), A and P site-bound (2N,A76)tRNA^Phe and its aminoacylated derivatives primarily label protein L27, whereas E site-bound (2N,A76)tRNA^Phe labels proteins L33 and L1.

**Determination of tRNA Cross-linking Sites on the 23 S rRNA**

The sites to which (2N,A76)tRNA^Phe cross-links on the 23 S rRNA were determined by a combination of three approaches, RNase H cleavage, RNase protection, and primer extension, as outlined in Fig. 2.

**Excision of Cross-linked rRNA Sequences by Cleavage with RNase H**

Covalent [5-32P]2N,A76tRNA^Phe-23 S rRNA complexes were digested with RNase H in the presence of pairs of 15-mer oligodeoxyribonucleotides complementary to sequences located approximately 100–250 nucleotides apart in the primary structure of the 23 S rRNA (for a list of oligonucleotides see “Experimental Procedures”). Cleavage of the rRNA at sites that bracket the cross-link releases a fragment, which is tagged by covalently attached [5-32P]2N,A76tRNA^Phe or its derivatives and can be readily detected by denaturing PAGE (Fig. 1). Such “scans” of the covalent tRNA-23 S rRNA complexes revealed five labeled segments within domains IV and V of the 23 S rRNA, which encompass nucleotides 2567–2904 (segment H1), 2446–2568 (segment H2), 2237–2445 (segment H3), 2025–2092...
rRNA and the resulting heteroduplexes were digested with oxynucleotides were hybridized to the F regions of the 23 S rRNA using RNase protection analysis in which complementary oligodeoxynucleotides were used. This step was followed by denaturing PAGE (Figs. 2 and 3). These digestions, denoted F fragments, were fractionated by further RNase H digestions using pairs of oligodeoxynucleotides that differed from those used in the initial scan. Products of these digestions, denoted T fragments, were fractionated by denaturing PAGE (Figs. 2 and 3). Consequently, cross-linked oligonucleotides were hybridized to the F regions of the 23 S rRNA ranging from 10 to 20 nucleotides in length (Figs. 2 and 3).

**Protection of Cross-linked Sequences from RNase by Oligonucleotide Hybridization**

To delineate the sites of cross-linking more narrowly, [5'-32P](2N3A76)tRNAPhe-23 S rRNA complexes were subjected to RNase H digestions using pairs of oligodeoxynucleotides that differed from those used in the initial scan. Products of these digestions, denoted F fragments, were fractionated by denaturing PAGE (Figs. 2 and 3a). This step was followed by RNase protection analysis in which complementary oligodeoxynucleotides were hybridized to the F regions of the 23 S rRNA and the resulting heteroduplexes were digested with RNase T1. Protected fragments that retained the 32P label were further analyzed by RNase T1 protection experiments utilizing oligonucleotide pairs that spanned the 23 S rRNA. Oligonucleotides designated T fragments, delimited the sites of cross-linking to sequences of the 23 S rRNA ranging from 10 to 20 nucleotides in length (Figs. 2 and 3b).

**Analysis of Cross-linking Sites by Primer Extension Analysis**

Final identification of the cross-linking sites was carried out using the primer extension method (26). For this purpose, fragments of approximately 250 nucleotides spanning each of the five cross-linking sites in the 23 S rRNA were excised from polyacrylamide gel electrophoresis as in Fig. 1 and subjected to primer extension analysis (Fig. 3a).

**Identity of Nucleotides to Which tRNA Is Cross-linked in the 23 S rRNA**

The cross-linked nucleotide in segment H1 was U2585—Covalent tRNA-23 S rRNA complexes in which [5'-32P](2N3A76)tRNAPhe-labeled segment H1, were cleaved with RNase H in the presence of oligonucleotides 3, 3a, 3b, and 4. Inspection of the radioactively labeled sequences released in this assay revealed that the cross-link site is located within fragment F2a, which spans nucleotides 2499–2539 (Fig. 2). RNase T1 protection experiments revealed that subfragment T11, which corresponds to nucleotides 2488–2524, retained the 32P-labeled T14 and T15, which correspond to nucleotides 2446–2486 (Fig. 2). The site of attachment within F2b was determined by primer extension to be C2452.

The cross-linked nucleotide in segment H2 is U2506—Covalent tRNA-23 S rRNA complexes in which [5'-32P](2N3A76)tRNAPhe-labeled segment H2, were cleaved with RNase H in the presence of oligonucleotides 3, 3a, 3b, and 4. Inspection of the radioactively labeled sequences released in this assay revealed that the cross-link site is located within fragment F2a, which spans nucleotides 2499–2539 (Fig. 2). RNase T1 protection experiments revealed that subfragment T11, which corresponds to nucleotides 2488–2524, retained the 32P-labeled T14 and T15, which correspond to nucleotides 2446–2486 (Fig. 2). The site of attachment within F2b was determined by primer extension to be C2452.

The cross-linked nucleotide in segment H3 is C2422—RNase H cleavage of all tRNA-23 S rRNA complexes in which the cross-linking site is located in the presence of oligonucleotide pairs 4/4a and 4a/5 demonstrated that the site of cross-linking is located in fragment F3, which encompasses nucleotides 2360–2445 (Fig. 2). This portion of the complex was further analyzed by RNase T1 protection experiments utilizing oligonucleotides 14, 15, 16, and 17. Because oligonucleotides 14 and 15 protected two overlapping [5'-32P](2N3A76)tRNAPhe-labeled subfragments, denoted T14 and T15 in Fig. 2, the cross-linking must be located between nucleotides 2415 and 2436. The nucleotide covalently attached to the tRNA was determined to be C2452 by primer extension analysis.

The cross-linked nucleotide in segment H4 is G2069—RNase H digestion of tRNA-23 S rRNA complexes containing tRNA cross-linked to segment H4 in the presence of oligonucleotide pairs 5/5a, 5b/5a, 5/5b, and 5b/5a showed that the cross-link is located in fragment F4, between nucleotides 2024 and 2091 (Fig. 2). RNase T1 protection experiments utilizing oligonucleotide 18 localized the cross-linking site to the sequence encompassing nucleotides 2058–2083 (Fig. 2). Primer extension analysis demonstrated that G2069 was the residue covalently linked to tRNA.

**The Nucleotide Sequence Cross-linked to tRNA within Seg-**
ment H5—When oligonucleotide pairs 7/8 and 7a/8a were used to direct RNase H cleavage of tRNA-23 S rRNA complexes containing tRNA cross-linked to segment H5, the site of tRNA attachment was found to be delimited by nucleotides 1910–1990 within fragment F5 (Fig. 2). Hybridization to oligonucleotides 19, 20, and 21 revealed that only oligonucleotide 21 protects the 23 S rRNA region tagged by [5'-32P](2N3A76)tRNAPhe from RNase T1 digestion; the site of cross-linking must therefore be located between nucleotides 1922 and 1929 (see Fig. 2). Unequivocal identification of the cross-linked nucleotide within this sequence proved impossible owing to stops in all lanes at A1927. We suggest that the stop at A1927 obscures the cross-link of tRNA to U1926, a highly conserved nucleotide that is protected from chemical modification by P site tRNA (2).

Cross-linking in the Presence of Excess tRNA—When three deacylated (2N3A76)tRNAPhe molecules were bound simultaneously to poly(U)-programmed ribosomes and then subjected to UV irradiation, cross-links were observed in segments H1, H2, H3, H4, and H5 (Fig. 1B). Subsequent analysis revealed that the tRNA mainly labeled nucleotides U2506, U2585, and C2422, with smaller amounts cross-linked to nucleotides U1926 and G2069. Because these five nucleotides are exactly the same as those labeled by (2N3A76)tRNAPhe molecules, or their derivatives, placed individually at either the A, P, or E sites, we found no evidence for additional, nonspecific cross-links when tRNA is present in excess.

DISCUSSION

Defining the Nucleotides of the 23 S rRNA At the Peptidyl Transferase Center—Incorporation of 2-azidoadenosine into tRNA yields a photoreactive derivative, (2N3A76)tRNAPhe, which can be aminocylated, will bind to ribosomes, is able to participate in peptide bond formation and, when irradiated with near-UV light, can form 2- to 3-Å cross-links to other...
macromolecules in its immediate vicinity (3, 14). In our earlier studies, (2N₆A₇₆)tRNAₚhe was primarily used for the identification of ribosomal proteins that serve as topographical markers of the A, P, and E sites on the E. coli ribosome. When bound to the A and P sites, (2N₆A₇₆)tRNAₚhe labeled mainly protein L27 (14, 34). In contrast, E site-bound (2N₆A₇₆)tRNAₚhe cross-linked exclusively to protein L33 (4).

Various lines of evidence indicate that interactions of the 3'-terminus of tRNA with 23 S rRNA are important both for peptide bond formation and for the movement of tRNA through the ribosome. Footprinting of tRNA-ribosome complexes demonstrated that, as the 3'-terminal adenosine of tRNA transits the E. coli ribosome, it protects from chemical modification a small number of nucleotides in the 3'-half of the 23 S rRNA (36, 37). According to these studies, the 3'-terminal adenosine of A-site tRNAₚhe protects G2553, whereas that of P-site tRNAₚhe protects U2506, U2585, and U2586. All of these nucleotides are located within or near the central loop of domain V. Several lines of evidence indicate that this region of the 23 S rRNA is located in proximity to proteins L2 and L27, which are positioned on the 50 S subunit interface in the valley between the L1 ridge and the central protuberance (3, 38, 39). At the same time, the 3'-terminal adenosine of the E site tRNAₚhe protects G2112, G2116, and C2394. The two E site guanines are located in proximity to the binding site for protein L1 (40), whereas C2394 is close to the site at which L33 cross-links to 23 S rRNA (41).

To learn more about the path taken by the 3'-terminus of tRNA as it moves through the ribosome during protein syntheses, we have analyzed a number of cross-linked (2N₆A₇₆)tRNAₚhe-ribosome complexes representing different stages of the elongation cycle of translation. Inasmuch as the chemistry of photoaffinity labeling and chemical footprinting are different (27, 42), we expected that the use of azidoadenosine-substituted tRNA probes would enable us to further define the sites that accommodate the 3'-terminal adenosine of deacylated, aminoacyl-, and peptidyl-tRNAs during polypeptide synthesis. In this report, we identify five nucleotides of the 23 S rRNA that are labeled by (2N₆A₇₆)tRNAₚhe as it traverses the E. coli ribosome (Fig. 4). The pattern of cross-linking is distinct for each tRNA binding site or state that we have investigated. The site specificity of these cross-links is substantiated by the fact that cross-linking in the presence of excess (2N₆A₇₆)tRNAₚhe leads to the labeling of all five of these nucleotides, but no others. Labeling of a sixth nucleotide, C2452, was observed only in the absence of mRNA.

Patterns of tRNA-rRNA Cross-linking At the Ribosomal P, A, and R Sites—Deacylated (2N₆A₇₆)tRNAₚhe bound at the P site labels mainly U2585 and, to a lesser extent, U2506 (Fig. 1a and Table I). Similar results were obtained by Kirillov et al. (43), although we have not observed the cross-link to nucleotides C2601/A2602 that they reported. In contrast, AcPhe-(2N₆A₇₆)tRNAₚhe at the P site labels U2506, U2585, and G2069, with the cross-link to U2506 predominating (Fig. 1b and Table I). The effect of the AcPhe group on the cross-linking pattern indicates that the AcPhe moiety, an analogue of the peptidyl group, influences either the positioning or the mobility of the 3'-terminal residue of P site tRNA relative to the 23 S rRNA and is consistent with cryo-electron microscopic observations (44). The labeling of U2506 and U2585 by P-site-bound (2N₆A₇₆)tRNAₚhe indicates that these two nucleotides are close to one another and are likely to play an important role at the peptidyl transferase center as suggested by earlier studies (36, 37, 45–48). The proximity of G2069, which is methylated at N7, to the 3'-terminal adenosine of P site-bound tRNA lends weight to an earlier proposal that a highly conserved structural motif consisting of U2438, A2439, m7G2069, and m1A2071 lies close to the catalytic site of peptidyl transferase (49). Furthermore, the labeling of C2452, in addition to U2506 and U2585, by (2N₆A₇₆)tRNAₚhe bound at the P site in the absence of poly(U) suggests that codon-anticodon interaction, which occurs on the 30 S subunit, can also affect the position or orientation of the 3' end of the tRNA on the 50 S subunit.

Cross-linking patterns derived from complexes containing peptidyl-tRNA analogues bound under A site conditions were of particular interest. As indicated above, A site-bound Phe-(2N₆A₇₆)tRNAₚhe labeled both U2585 and U2506, although cross-linking to U2585 predominated (see Table I). In contrast, A site-bound AcPhe-(2N₆A₇₆)tRNAₚhe preferentially labeled U2506 (see Fig. 1c and Table I). More strikingly, P site-bound (2N₆A₇₆)tRNAₚhe labeled only U2506 when the A site was filled with AcPhe-tRNAₚhe (compare Fig. 1e and 1f; see also Table I). The latter observation shows that the position of the 3' end of tRNA at one ribosomal site can be influenced strongly by the tRNA derivatives that occupy other sites. In addition, these data demonstrate that the 3' end of tRNA in AcPhe-tRNAₚhe, a peptidyl-tRNA analogue, is in close contact with U2506 regardless of whether its anticodon is located in the A site or P site (compare Fig. 1d and 1f; see also Table I).

Incoming Phe-tRNAₚhe in a ternary complex with EF-Tu and a non-hydrolyzable GTP derivative, is stabilized in a binding state designated as the A/T state (2), which can be equated with the ribosomal R or recognition site proposed earlier (28). When part of the ternary complex, Phe-(2N₆A₇₆)tRNAₚhe labeled nucleotides U2585, U2506, and U1926 (see Fig. 1c and Table I),
whereas the same tRNA derivative at the A site labeled U2585 and U2506 (see Table I). From these observations, we conclude that the 3' end of Phe-(2N3A76)tRNAPhe in the ternary complex contacts the peptidyl transferase center even though it cannot participate in peptide bond synthesis, perhaps because of restrictions imposed by the presence of EF-Tu (50). Alternatively, if EF-Tu triggers the transition from the post-translocational state to the pre-translocational state, labeling of U1926 may be indicative of a change in the conformation of 23 S rRNA in the neighborhood of the 3'-end of the incoming tRNA.

The cross-linking experiments reported here, together with chemical footprinting data (36), demonstrate the proximity of U2506 and U2585 to the 3'-terminal adenosine of P site-bound deacylated and peptidyl-tRNAs as well as to A site-bound aminoacyl- and peptidyl-tRNAs. These and other potential contacts between the 3' end of tRNA and the 23 S rRNA are depicted in Fig. 5. Taken together, the above results indicate that there is a complex interplay between nucleotides U2585 and U2506 of the 23 S rRNA and nucleotide A76 of the tRNA, which is of potential mechanistic importance in tRNA-ribosome interaction, peptide bond formation, and translocation. We first note that Phe-(2N3A76)tRNAPhe can cross-link to U2585 and U2506 at the R site as well as the A site and that labeling of U2585 is more intense in both cases. Coincident with peptide bond formation, the 3' terminus of the tRNA becomes more closely associated with U2585 and U2506, as evidenced by the preferential labeling of U2506 by the peptidyl-tRNA analogue, AcPhe-(2N3A76)tRNAPhe, and by the simultaneous exclusion of this residue from labeling by deacylated (2N3A76)tRNAPhe at the A site. Under these conditions, AcPhe-(2N3A76)tRNAPhe is in a state that corresponds to the hybrid A/P binding site (2). The 3' end of the tRNA moiety remains in the same position upon subsequent translocation of the tRNA anticodon. With AcPhe-tRNAPhe still in the A/P state, the 3' end of the deacylated tRNA, previously in the P/P state, assumes a position from which it can label only U2585 and protein L27 while its anticodon remains anchored in the P site. This pattern of cross-linking suggests that the 3' end of deacylated tRNA does not immediately leave the peptidyl transferase center. Our findings thus differ from the conclusion that deacylated tRNA "reaches" into the E site to adopt the P/E state of binding after transpeptidation, which was inferred from chemical footprinting of tRNA-ribosome complexes (2). The pattern of labeling manifested by the A and P site-bound (2N3A76)tRNAPhe derivatives suggests that the 3'-terminal adenosine may be "sandwiched" between U2506 and U2585, where it can readily access either nucleotide during peptide bond formation (35).

**Patterns of tRNA-rRNA Cross-linking At the Ribosomal E and F Sites**—In previous studies, we demonstrated that the E site is topographically distinct from the A and P sites, because (2N3A76)tRNAPhe bound to the E site of poly(A)-programmed 70 S ribosomes cross-links exclusively to protein L33 (4). In contrast, when (2N3A76)tRNAPhe is bound to the E site of poly(U)-programmed ribosomes, nucleotide C2422 of the 23 S rRNA is labeled in addition to protein L33 (Fig. 1f and Table I). Concurrent labeling of C2422 and protein L33 agrees well with the earlier finding that a neighboring nucleotide, C2427, cross-links to L33 when ribosomes are irradiated with UV light (38). Finally, deacylated (2N3A76)tRNAPhe bound to the ribosomal E site in the absence of mRNA exclusively labels proteins L1 and L33 (35). These results suggest that the presence of a cognate codon in the E site influences the positioning of tRNA, in accord with the observation that the affinity of tRNAPhe for the E site increases slightly in the presence of poly(U) (30, 51). The cross-linking patterns described above may correspond to the different E-type states of binding that have been visualized by cryo-electron microscopy (6, 7, 9). This conclusion is also consistent with footprinting data on E site tRNA (36), because chemical protection of G2112, G2116, A2169, and C2394 can be correlated with at least two E-type binding states. Because the first three nucleotides are located in the vicinity of the L1 binding site, they are most likely protected by the variant of E site tRNA whose 3' end contacts protein L1. In our experiments, this type of E site binding was observed only in the absence of mRNA. Interestingly, a similar placement of E site tRNA, designated as the E2 site, was inferred from cryo-electron microscopy of poly(U)-programmed ribosomes to which three deacylated tRNA molecules were bound simultaneously (6). On the other hand, C2394 is most likely protected by tRNA in the E-type binding state observed in post-translational complexes (7). As shown in this report, (2N3A76)tRNAPhe in the latter binding state labels C2422, which is adjacent to C2394 in the secondary structure of the 23 S rRNA (Fig. 4).

Our cross-linking data strongly suggest that protein L1, along with nucleotides G2112, G2116, and A2169, constitute markers for a new site, which we propose to call the F (final) site (Fig. 4). We define the F site as the area on the ribosome from which tRNA dissociates (or is ejected) into the cytoplasm. Before reaching the F site, the tRNA is associated with an intermediate site, the E site, for which protein L33 and the C2422 residue serve as markers. According to our observations, the binding of tRNA to the E site is influenced by the presence or absence of a cognate codon and may involve interactions with the 30 S subunit (Ref. 4 and present work). In this light, most of the earlier published data relating to the E site probably reflect the properties of tRNA bound at the F site, which is completely mRNA-independent.

Together, our results require the following modifications to the hybrid-state model of tRNA-ribosome interaction proposed by Moazed and Noller (2). In particular, we show that the 3' end of the deacylated tRNA that is formed after transpeptidation does not immediately progress to the E site but remains temporarily in the peptidyl transferase center. When and how
it moves to the E site is at this time a matter of conjecture. Given that E site binding is influenced by the presence of a cognate codon, we suggest that this event is dependent on or triggered by translocation. Moreover, we propose that the E site does not correspond to the final site of tRNA-ribosome interaction, but that it represents an intermediate state of binding for tRNA moving toward the F site, from which it dissociates into the cytoplasm.

Acknowledgment—We thank Dr. Bruce A. Maguire for critical reading of the manuscript.

REFERENCES

1. Woyer, J., and Zimmermann, R. A. (1991) Biochimie (Paris) 73, 961–969
2. Moazed, D., and Noller, H. F. (1989) Nature 342, 142–148
3. Woyer, J., Hixson, S. S., and Zimmermann, R. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5232–5236
4. Woyer, J., Scheffer, P., Sylvers, L. A., Wintemeyer, W., and Zimmermann, R. A. (1990) EMBO J. 12, 617–623
5. Noller, H. F., Moazed, D., Stern, S., Powers, T., Allen, P. N., Robertson, J. M., Weiser, B., and Triman, K. (1990) in The Ribosome: Structure, Function and Evolution (Hill, W. E., Dahlgren, A., Garrett, R. A., Moore, P. B., Schlesinger, D., and Warner, J. R., eds) pp. 73–92, ASM Press, Washington, DC
6. Agrawal, R. K., Penczek, P., Grassucci, R. A., Li, Y., Leith, A., Nierhaus, K. H., and Frank, J. (1996) Science 271, 1000–1002
7. Stark, H., Orlov, E. V., Rinke-Appe, J., Junke, N., Mueller, F., Rodina, M., Wintemeyer, W., Brimacombe, R., and van Heel, M. (1997) Cell 88, 19–28
8. Cate, J. H., Yusupov, M. M., Yusupova, G. Zh., Earnest, T. N., and Noller, H. F. (1999) Science 285, 2085–2104
9. Frank, J. (1996) J. Struct. Biol. 124, 142–150
10. Noller, H. F. (1991) Annu. Rev. Biochem. 60, 191–122
11. Muth, G. W., Ortoleva-Donnelly, L., and Strobel, S. A. (2000) Science 289, 947–950
12. Boon, K., Vigenboom, E., Madsen, L. V., Talens, A., Krala, B., and Bosch, L. (1992) Eur. J. Biochem. 210, 177–183
13. Woyer, J., Hixson, S. S., and Zimmermann, R. A. (1988) Biochemistry 27, 8114–8121
14. Sylvers, L., Woyer, J., Hixson, S. S., and Zimmermann, R. A. (1989) FEBS Lett. 245, 9–13
15. Semenkov, Yu. P., Makarov, E. M., and Kirillov, S. V. (1985) Biopolym. Cell 1, 183–193
16. Rappaport, S., and Lapidot, Y. (1974) Methods Enzymol. 29B, 685–693
17. Makino, V. I., Pesch, N. N., Semenkov, Yu. P., and Kirillov, S. V. (1988) Mol. Biol. 22, 528–536
18. Kirillov, S. V., Makhno, V. I., and Semenkov, Y. P. (1978) Eur. J. Biochem. 89, 297–304
19. Semenkov, Yu. P., Shapkina, T. G., and Kirillov, S. V. (1992) FEBS Lett. 296, 207–210
20. Nirenberg, M., and Leder, P. (1964) Science 145, 1399–1407
21. Robertson, J. M., and Wintermeyer, W. (1991) J. Mol. Biol. 151, 57–79
22. Donsova, O., Tishkov, V., Dokudovskiaya, S., Bogdanov, A., Diring, T., Rinke-Appe, J., Thanum, S., Greuer, B., and Brimacombe, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4125–4129
23. Brimacombe, R., Greuer, B., Gulie, H., Kosack, M., Mitchell, P., Osowski, M., Stade, K., and Steige, W. (1990) in Ribosomes and Protein Synthesis. A Practical Approach (Spedding, G., ed) pp. 131–159, Oxford University Press, Oxford
24. Woyer, J., Alexander, R. W., Woyer, J., and Zimmermann, R. A. (1991) Biochemistry 30, 12802–12811
25. Doring, T., Mitchell, P., Osowski, M., Bohkarov, D., and Brimacombe, R. (1994) EMBO J. 13, 2677–2685
26. Moazed, D., Stern, S., and Noller, H. F. (1986) J. Mol. Biol. 187, 399–416
27. Sylvers, L. A., and Woyer, J. (1993) Bioconjug. Chem. 4, 411–418
28. Lake, J. A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1903–1907
29. Kirillov, S. V., and Semenkov, Yu. P. (1982) FEBS Lett. 148, 235–238
30. Kirillov, S. V., Makarov, E. M., and Semenkov, Yu. P. (1983) FEBS Lett. 157, 91–94
31. Lill, R., Robertson, J. M., and Wintermeyer, W. (1984) Biochemistry 23, 6710–6717
32. Rheinberger, H. J., Sternbach, H., and Nierhaus, K. H. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5310–5314
33. Grajevskaia, I. A., Ivanov, Y. V., and Saminsky, E. M. (1982) Eur. J. Biochem. 127, 45–52
34. Woyer, J., Woyer, I. K., Kirillov, S., Rosen, K., Hixson, S. S., and Zimmermann, R. A. (1995) Biochem. Cell Biol. 73, 1041–1047
35. Woyer, J., Woyer, I. K., and Zweig, C. W. (1990) Nucleic Acids Symp. 41, 187–191
36. Moazed, D., and Noller, H. F. (1989) Cell 57, 585–597
37. Bocchetta, M., Xiong, L., Shah, S., and Mankin, A. S. (2000) RNA, in press.
38. Stiege, W., Glatz, C., and Brimacombe, R. (1985) Nucleic Acids Res. 11, 1687–1706
39. Beaucel, A. A., and Cundillle, E. (1988) EMBO J. 7, 3589–3594
40. Zimmermann, R. A. (1980) in Ribosomes: Structure, Function and Genetics (Chamblio, G., Craven, G. B., Davies, J., Davis, K., Kahan, L., and Namas, M., eds) pp. 135–169, University Park Press, Baltimore, MD
41. Osowski, M., Greuer, B., and Brimacombe, R. (1990) Nucleic Acids Res. 18, 6755–6760
42. Christiansen, J., Eeberg, J., Larsen, N., and Garrett, R. A. (1990) in Ribosomes and Protein Synthesis. A practical Approach (Spedding, G., ed) pp. 229–252, IRL Press, Oxford
43. Kirillov, S. V., Porse, B. T., and Garrett, R. A. (1999) RNA 5, 1003–1013
44. Agrawal, R. K., Penczek, P., Grassucci, R. A., Burkhartd, N., Nierhaus, K. H., and Frank, J. J. (1999) J. Biol. Chem. 274, 8723–8729
45. Tamura, K. (1994) FEBS Lett. 353, 173–176
46. Porse, B. T., and Garrett, R. A. (1995) J. Mol. Biol. 249, 1–10
47. Porse, B. T., Thi-Ngo H. P., and Garrett, R. A. (1996) J. Mol. Biol. 264, 472–483
48. Green, R., Samaha, R. R., and Noller, H. F. (1997) J. Mol. Biol. 266, 40–50
49. Rodriguez-Poncea, C., Amils, R., and Garrett, R. A. (1995) J. Mol. Biol. 247, 224–235
50. Nielsen, P., Kjelsgaard, M., Thirup, S., Clark, B. F., Nyborg, J. (1995) Biochimie (Paris) 78, 921–933
Transit of tRNA through the *Escherichia coli* Ribosome: CROSS-LINKING OF THE 3′ END OF tRNA TO SPECIFIC NUCLEOTIDES OF THE 23 S RIBOSOMAL RNA AT THE A, P, AND E SITES

Jacek Wower, Stanislav V. Kirillov, Iwona K. Wower, Sadel Guven, Stephen S. Hixson and Robert A. Zimmermann

*J. Biol. Chem.* 2000, 275:37887-37894.
doi: 10.1074/jbc.M005031200 originally published online August 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005031200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 11 of which can be accessed free at
http://www.jbc.org/content/275/48/37887.full.html#ref-list-1