Binding and cross-linking of tmRNA to ribosomal protein S1, on and off the *Escherichia coli* ribosome

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UV irradiation of an *in vitro* translation mixture induced cross-linking of 4-thioU-substituted tmRNA to *Escherichia coli* ribosomes by forming covalent complexes with ribosomal protein S1 and 16S rRNA. In the absence of S1, tmRNA was unable to bind and label ribosomal components. Mobility assays on native gels demonstrated that protein S1 bound to tmRNA with an apparent binding constant of $1 \times 10^8 \text{ M}^{-1}$. A mutant tmRNA, lacking the tag coding region and pseudoknots pk2, pk3 and pk4, did not compete with full-length tmRNA, indicating that this region is required for S1 binding. This was confirmed by identification of eight cross-linked nucleotides: U85, located before the resume codon of tmRNA; U105, in the mRNA portion of tmRNA; U172 in pk2; U198, U212, U230 and U240 in pk3; and U246, in the junction between pk3 and pk4. We concluded that ribosomal protein S1, in concert with the previously identified elongation factor EF-Tu and protein SmpB, plays an important role in tmRNA-mediated *trans*-translation by facilitating the binding of tmRNA to ribosomes and forming complexes with free tmRNA.

Keywords: photoaffinity labeling/ribosomal protein S1/ ribosome/tmRNA/*trans*-translation

Introduction

The serendipitous discovery of protein tagging, a *trans*-translational process that adds a short peptide to incompletely synthesized proteins, led to a breakthrough in the two-decade-long study of a small stable *Escherichia coli* RNA molecule known as 10Sa RNA (Ray and Apirion, 1979) or SsrA RNA (Keiler *et al.*, 1996). Since the termini of this RNA resemble tRNA (Komine *et al.*, 1994) and its internal segment is bracketed by the 'resume' and 'stop' codons to form an open reading frame encoding a peptide tag, recent publications refer to 10Sa RNA as 'transfer-messenger RNA' or 'tmRNA'. To date, tmRNA genes have been identified in bacterial and some chloroplast genomes (Felden *et al.*, 1999).

Comparative analyses of tmRNA sequences demonstrated that the tRNA- and mRNA-like portions are connected by pseudoknots (Williams and Bartel, 1996; Felden *et al.*, 1999; Zwieb *et al.*, 1999a,b). Of the four

predicted pseudoknots in *E. coli* tmRNA, pk1 precedes the tag-peptide coding region, while pk2–pk4 are located closer to the 3' terminus. Mutational analysis indicates that pk1 is indispensable for proper folding and function of the *E. coli* tmRNA (Nameki *et al.*, 1999). To date, the function of the other pseudoknots has not been determined.

According to the current model of *trans*-translational protein tagging, aminoacyl-tmRNA binds to the A site of stalled 70S ribosomes and accepts an incomplete protein from the P site-bound tRNA during transpeptidation (Keiler *et al.*, 1996). After translocation, an aminoacyl-tmRNA binds to the resume codon of tmRNA to continue with another transpeptidation reaction until the tmRNA stop codon enters the ribosomal A site.

In the course of *trans*-translational peptide tagging of incomplete proteins, tmRNA forms stable complexes with ribosomes (Komine *et al.*, 1996; Tadaki *et al.*, 1996). Extrapolating from protein synthesis, it can be expected that this process is facilitated by a variety of protein factors. Two of these factors, elongation factor Tu (EF-Tu) and a small protein SmpB (Karzai *et al.*, 1999; Rudinger-Thirion *et al.*, 1999; Barends *et al.*, 2000), were shown to bind tmRNA while the research reported here was in progress.

To detect additional cytoplasmic components that might bind tmRNA, we used a photoaffinity labeling approach in which 4-thiouridine (^{s4}U) is incorporated randomly during *in vitro* transcription to form photoreactive ^{s4}U-substituted RNA. Although the hydrogen bonding properties of ^{s4}U and U differ (Kyogoku *et al.*, 1967), in general, such substitutions do not affect either the three-dimensional structure or the biological activity of [^{s4}U]RNA probes (Rosen *et al.*, 1993). Irradiation of ^{s4}U with near-UV light (300–360 nm) leads to its photo-oxidation and the formation of unstable intermediates that react with neighboring nucleophiles (Frischauf and Scheit, 1973). Because ^{s4}U yields very short cross-links upon UV irradiation, it provides precise information about the molecular environment of the photoreactive base.

In the work reported here, we use an *E. coli in vitro* translation system to demonstrate that tmRNA binds and, upon UV irradiation, cross-links to ribosomal protein S1. This interaction is essential for binding of tmRNA to ribosomes, does not require EF-Tu and is independent of tmRNA aminoacylation, as in the case of SmpB protein (Karzai *et al.*, 1999). Protein S1 binds to the mRNA-like portion and 3' terminal pseudoknot-rich domain of tmRNA, leaving pk1 and the tRNA-like portion available to bind EF-Tu and protein SmpB.

Results

Preparation of photoreactive tmRNA derivatives

Escherichia coli [^{s4}U]tmRNA containing a single U216C mutation, and its truncated derivative [^{s4}U]tmRNA

$\Delta 90$ –299 lacking both the mRNA-like segment and pseudoknots pk2–pk4, were prepared by *in vitro* transcription of the *Bst*NI-digested plasmids ptmR and ptm $\Delta 90$ –299 in the presence of s^4 UTP, as described in Materials and methods. These photoreactive transcripts co-migrated with unmodified tmRNAs during electrophoresis on both the denaturing and non-denaturing polyacrylamide gels, thus indicating that our procedure yields monomeric [s^4 U]tmRNA probes with conformations similar to the unmodified molecules (not shown). Approximately 6% of both the photoreactive and unmodified tmRNA transcripts lacked the 3' terminal adenosine, as judged by the incorporation of [α - 32 P]ATP by yeast ATP/CTP tRNA nucleotidyl transferase, which yields 3'- 32 P-labeled tmRNA species (Figure 1).

Random incorporation of up to four s^4 U moieties per tmRNA molecule had no visible effect on its aminoacylation. As illustrated in Figure 1, typically 52% of tmRNAs and 98% of tmRNA $\Delta 90$ –299 could be charged with alanine. However, because digestion of Ala-tmRNA $\Delta 90$ –299 and aminoacylated tRNA species with RNase T₁ deaminoacylates them by 16–20%, we assumed that the level of tmRNA aminoacylation indicated by gel-shift assay was too low, and the actual value is likely to approach 70%. Observed differences in aminoacylation may be attributed to structural instability of the tmRNA, as suggested by its UV-absorbance melting profiles (Nameki *et al.*, 1999; Barends *et al.*, 2000). Consistent with this suggestion, the aminoacylation of tmRNA $\Delta 90$ –299 was always very efficient, indicating that removal of the mRNA-like portion in addition to the segment containing pk2–pk4 does not affect the structural integrity of the tRNA-like portion of tmRNA. *In vitro* assays, carried out similarly to the procedure described by Himeno *et al.* (1997), showed that only aminoacylated tmRNA derivatives, both unmodified and photoreactive, were able to stimulate incorporation of [14 C]alanine into a tag peptide (not shown).

Cross-links between tmRNA and cell components

Because s^4 U cross-links to its immediate neighbors upon UV irradiation, we used photoreactive tmRNA derivatives to search for those cellular components that might interact with tmRNA during *trans*-translation. Exploratory cross-linking experiments were carried out in a translation system supplemented with internally 32 P-labeled [s^4 U]tmRNA derivatives. To avoid cross-linking between the message and ribosomes, poly(U), which is usually used in *trans*-translation assays (Himeno *et al.*, 1997; Nameki *et al.*, 2000), was replaced with rpsH/*Xmn*I mRNA, which encodes a truncated version of ribosomal protein S8 (Wu *et al.*, 1993). Cross-linking was induced by irradiation of translation mixtures with near-UV light as described earlier (Rosen *et al.*, 1993). Although under these conditions cross-linking reaches its maximum after 30 min, UV irradiations were limited to 10 min to avoid any significant inactivation of ribosomes (Wower *et al.*, 1989). Centrifugation of irradiated translation mixtures through a 14–40% sucrose gradient at 10 mM Mg²⁺ revealed that ~11% of the [s^4 U]tmRNA was cross-linked to 70S ribosomes (Figure 2A). No cross-linking was observed

Fig. 1. Aminoacylation of s^4 U-substituted tmRNA derivatives. (A) The 3'- 32 P-labeled [s^4 U]tmRNA and (B) [s^4 U]tmRNA $\Delta 90$ –299 were charged with alanine in the presence of partially purified alanyl-tRNA synthetase. The extent of aminoacylation was analyzed by PAGE according to Varshney *et al.* (1991). Prior to PAGE, Ala-[s^4 U]tmRNA and [s^4 U]tmRNA were digested with RNase T₁ to yield CUCCACCA-Ala and CUCCACCA, respectively. (+) and (–) indicate the presence and the absence of the alanyl-tRNA synthetase. Solid and open arrows show the positions of Ala-[32 P]RNA and [32 P]RNA on the gel, respectively.

with [s^4 U]tmRNA $\Delta 90$ –299 (not shown). Analysis of the 3–5S fractions (Figure 2A) by Tricine–SDS–PAGE revealed that an additional 6% of the [s^4 U]tmRNA was cross-linked to protein(s) (Figure 2C, lane 4). The total yield of tmRNA cross-linking to 70S ribosomes was ~15% in the absence of the rpsH/*Xmn*I RNA, probably due to the lack of competition between tmRNA and truncated mRNA derivative for a binding site on the ribosomes.

The 70S ribosome fraction was subjected to a second round of sucrose-gradient centrifugation at 0.25 mM Mg²⁺ to separate 30S from 50S ribosomal subunits. This analysis demonstrated that cross-linked tmRNA was associated exclusively with 30S ribosomal subunits (Figure 2B). Tricine–SDS–PAGE of [s^4 U]tmRNA-labeled 30S ribosomal subunits showed that ~58% of cross-linked [s^4 U]tmRNA was with ribosomal protein and the remainder with 16S rRNA.

Unfractionated cross-linking mixtures, 3–5S (Figure 2A) and 30S fractions (Figure 2B) were digested with RNase T₁ and resolved by Tricine–SDS–PAGE. Autoradiography revealed that the majority of the label migrated as a single species corresponding to a 66 kDa protein–oligonucleotide complex (Figure 2C, lanes 2, 3 and 4). This material, unlike the molecules with mobilities of ~32 and 45 kDa, could be digested with proteinase K. After RNase T₁ digestion, a 32 P-labeled tmRNA fragment would be expected to remain cross-linked to the protein. As a result, the mobility of the complex would undergo a shift relative to its unlabeled counterpart and reliable identification of the labeled protein could not be made by PAGE alone. To determine conclusively the identity of cross-linked protein(s), an 'agarose gel' immunological assay was used. It showed that ribosomal protein S1 was labeled by both free and ribosome-bound [s^4 U]tmRNA. This identification is consistent with the earlier determined molecular weight of *E. coli* ribosomal protein S1 (61 159 Da; Kimura *et al.*, 1982) and the mobility of covalent complexes formed between [s^4 U]tmRNA and pure protein S1 (Figure 3, lane 2).

Fig. 2. Fractionation of the components of the *in vitro* translation system cross-linked to ^{32}P -labeled $[\text{s}^4\text{U}]\text{tmRNA}$. (A) Isolation of $[\text{s}^4\text{U}]\text{tmRNA}$ –70S ribosome complexes from the UV-irradiated *in vitro* translation system by centrifugation through the 14–40% sucrose gradient. (B) Separation of the $[\text{s}^4\text{U}]\text{tmRNA}$ –70S ribosome complexes into 30 and 50S ribosomal subunits by centrifugation through the 10–30% sucrose gradient. Dashed lines represent UV absorbance and closed squares trace the radioactivity profile. (C) Electrophoretic analysis of proteins cross-linked to $[\text{s}^4\text{P}][\text{s}^4\text{U}]\text{tmRNA}$. The *in vitro* translation system containing $[\text{s}^4\text{P}][\text{s}^4\text{U}]\text{tmRNA}$ before irradiation (lane 1) and after irradiation (lane 2), as well as 70S (lane 3) and 3–5S (lane 4) fractions from the sucrose gradient (A), were treated by RNase T_1 and subjected to Tricine–SDS–PAGE. The positions of protein S1 labeled by a fragment of tmRNA (solid arrow) and molecular weight markers are indicated.

Binding tmRNA to 70S ribosomes

Photolabeling of protein S1 by ribosome-bound $[\text{s}^4\text{U}]\text{tmRNA}$ suggested that the protein may facilitate tmRNA

Fig. 3. Cross-linking of ^{32}P -labeled $[\text{s}^4\text{U}]\text{tmRNA}$ to wild-type and protein S1-depleted ribosomes. Aliquots of UV-irradiated $[\text{s}^4\text{U}]\text{tmRNA}$ (lane 1), complexes of $[\text{s}^4\text{U}]\text{tmRNA}$ and protein S1 (lanes 2 and 5), complexes of $[\text{s}^4\text{U}]\text{tmRNA}$ and wild-type 70S ribosomes (lanes 3 and 6), and complexes of $[\text{s}^4\text{U}]\text{tmRNA}$ and 70S ribosomes lacking protein S1 (lanes 4 and 7), as well as non-irradiated $[\text{s}^4\text{U}]\text{tmRNA}$ (Co), were digested with RNase T_1 and subjected to Tricine–SDS–PAGE. Complexes indicated by a solid arrow were identified as $[\text{s}^4\text{U}]\text{tmRNA}$ –protein complexes by their susceptibility to proteinase K (lanes 5, 6 and 7). The positions of molecular weight markers are indicated.

binding to ribosomes. This possibility was explored *in vitro* using both S1-free 70S and 30S ribosomal particles prepared by affinity chromatography on poly(U)–Sephacrose as described by Subramanian (1983). The selective removal of protein S1 was confirmed by PAGE (see Materials and methods). At least 90% of the ribosomal particles were free of protein S1, assuming a detection limit by Coomassie Blue of $\sim 1.0 \mu\text{g}$ protein per spot (Copeland, 1994). The S1-free 70S ribosomes displayed a very low activity (~ 5 –8%) in polymerization of $[\text{s}^4\text{C}]\text{phenylalanine}$ at very low concentrations of poly(U). Consistent with earlier studies (Linde *et al.*, 1979), the inactivation caused by the removal of protein S1 was reversed completely upon addition of the isolated protein (not shown).

When $1 \mu\text{M}$ tmRNA was incubated with either $1 \mu\text{M}$ 70S ribosomes or $1 \mu\text{M}$ 30S ribosomal subunits under the standard conditions used for binding tRNA to ribosomes (Wower *et al.*, 1993), ~ 0.2 pmol of tmRNA per picomole of ribosomal particles was bound non-covalently. In contrast, under the same conditions, <0.03 pmol of tmRNA was bound per picomole of either S1-free 70S ribosomes or S1-free 30S ribosomal subunits. The binding was not affected either by alanylation or random incorporation of s^4U into tmRNA.

This observation allowed us to use affinity labeling to investigate whether protein S1 is essential for the binding of tmRNA to ribosomes. Upon UV irradiation of non-covalent complexes of $[\text{s}^4\text{U}]\text{tmRNA}$ and wild-type 70S ribosomes, $[\text{s}^4\text{U}]\text{tmRNA}$ labeled both protein S1 (Figure 3, lane 3) and 16S rRNA (not shown). In contrast, consistent with the binding assay, no labeling was detected when S1-free 70S ribosomes were used (Figure 3, lane 4).

Binding tmRNA to purified protein S1

Earlier studies showed that the C-terminal segment of protein S1 binds strongly to various natural RNAs as well as to numerous homopolymers such as poly(A), poly(C) and poly(U) (for a review see Subramanian, 1984). To investigate tmRNA interactions with free protein S1, we used primarily S1_{His}, a His-tagged recombinant version of protein S1. This protein displayed the same affinity for

Fig. 4. Gel-shift analysis of binding between ³²P-labeled tmRNA and protein S1_{His}. (A) Titration of 3'-³²P-labeled tmRNA (0.5 nM) with protein S1. Aliquots of binding mixtures were analyzed by electrophoresis on a 5% polyacrylamide gel (40:1) in TGE buffer. (B) Graphical representation of the PhosphorImager-derived data from the gel in (A). (C) Binding of [³²P]tmRNA (1 nM) to protein S1 (100 nM) in the presence of unlabeled competitor RNAs: (1) tmRNA transcript; (2) poly(U); (3) tmRNAΔ90–299; and (4) *E. coli* tRNA.

poly(U) as the wild-type protein S1 isolated from ribosomes by chromatography on poly(U)–Sepharose (Subramanian, 1983).

Interactions between tmRNA and protein S1 were tested by gel mobility shift assay (Figure 4A). Increasing amounts of protein S1_{His} were added to [³²P]tmRNA, held at a constant low concentration of 0.5×10^{-9} M, until binding was saturated. The apparent association constant (K'_a) was calculated by determining the concentration of unbound S1 that led to the formation of equal amounts of complexed and free tmRNA molecules. The value of K'_a for the tmRNA–protein S1 interaction was determined to be $\sim 1 \times 10^8$ M⁻¹ (Figure 4B). This calculation assumes that one molecule of protein S1 binds one tmRNA molecule.

The specificity of the interaction between tmRNA and protein S1 was investigated by several competition assays.

Increasing concentrations of unlabeled competitor RNAs, either a mixture of all *E. coli* tRNAs, short poly(U) fragments or tmRNAΔ90–299, were added to the binding reaction, which contained 10^{-9} M [³²P]tmRNA and 10^{-7} M protein S1_{His} (Figure 4C). Competition effects were compared at the half-saturation point of the tmRNA–S1 complex. The concentration of tRNA required to reduce tmRNA binding by 50% was ~ 600 -fold greater than competitor tmRNA. Truncated tmRNA behaved similarly to tRNA, with an ~ 160 -fold higher concentration needed to reduce the tmRNA binding by 50%. In contrast, a 20-fold higher concentration of poly(U), a strong binder of protein S1, achieved the same degree of competition. These observations indicated that protein S1 binds tmRNA specifically, with a binding affinity comparable to or higher than those of many other ribosomal proteins for rRNAs (Draper *et al.*, 1988). Moreover, since truncated tmRNA was a poor competitor, these experiments suggest that protein S1 recognizes primarily a region encompassing the mRNA-like domain and pseudoknots pk2, pk3 and pk4.

Topography of covalent complexes formed by free tmRNA and protein S1

To gain further insight into the role of protein S1, we analyzed the topography of the covalent complexes formed between [⁴S]tmRNA and protein S1_{His}. After a 10 min irradiation with near-UV light, $\sim 20\%$ of [³²P][⁴S]tmRNA cross-linked to protein S1 (Figure 5A, lane 2). Such treatment also induced intramolecular cross-links in 2.5% of [⁴S]tmRNA (Figure 5A, lane 1). When fractionated by Tricine–SDS–PAGE, internally cross-linked tmRNA migrated somewhat slower than tmRNA–protein S1 complexes. Both types of cross-linking were mediated by ⁴S, as no cross-linking was observed in control experiments with unmodified tmRNA.

The sites to which protein S1 cross-linked were determined in three steps using RNase H cleavage, primer extension and RNase T₁ protection analyses. RNase H cleaves the RNA portion of DNA–RNA hybrids exclusively (Berkower *et al.*, 1973). Thus, tmRNA can be digested at specific sites by hybridizing synthetic oligodeoxyribonucleotides complementary to these sites. We used this method to determine the 3' and 5' boundaries of the tmRNA segment containing cross-linked nucleotides. The RNase H analysis shown in Figure 5B was carried out with an unpurified cross-linking reaction mixture containing both covalent complexes formed by the [³-³²P][⁴S]tmRNA and protein S1_{His}, and free photolyzed [³-³²P][⁴S]tmRNA. Seven oligodeoxyribonucleotides complementary to sequences located ~ 30 –50 nucleotides apart in the primary structure of tmRNA were used to shorten tmRNA progressively at its 3' end (see Materials and methods). The ³²P-labeled products of RNase H digestion, when resolved by PAGE, formed two diagonals (Figure 5B). The diagonal with faster migrating fragments originated from non-cross-linked [⁴S]tmRNA, while the diagonal with slower migrating fragments was derived from cross-linked [⁴S]tmRNA–protein S1_{His} complexes. Digestion of [⁴S]tmRNA–protein S1 complexes in the presence of oligodeoxyribonucleotides TM4 and TM3 (see Materials and methods) did not produce tmRNA fragments cross-linked to protein S1 (Figure 5B,

Fig. 5. Cross-linking tmRNA to ribosomal protein S1 and RNase H analysis of covalent tmRNA–protein S1 complexes. (A) The 3′-³²P-labeled [s⁴U]tmRNA (lane 1) and non-covalent [s⁴U]tmRNA–protein S1 complexes (lane 2) were irradiated with UV light for 10 min and analyzed on a 6% Tricine–SDS–polyacrylamide gel. (Co) Non-irradiated 3′-³²P-labeled [s⁴U]tmRNA. UV irradiation yields internally cross-linked tmRNA, which, after cleaving with RNase H in the presence of oligodeoxyribonucleotide TM3, co-migrates with non-irradiated [s⁴U]tmRNA (not shown). (B) Unpurified UV-irradiated 3′-³²P-labeled [s⁴U]tmRNA–protein S1 complexes (Co) were digested by RNase H in the presence of oligodeoxyribonucleotides TM3 (lane 1), TM4 (lane 2), TM5 (lane 3), TM6 (lane 4), TM7 (lane 5), TM8 (lane 6) and TM9 (lane 7) as listed in Materials and methods, and separated on a 6% Tricine–SDS–polyacrylamide gel. The sites of hybridization of the oligodeoxyribonucleotides (solid bars) in tmRNA are illustrated in the diagram.

lanes 1 and 2), indicating that protein S1 was not cross-linked to the 3′ terminal fragment of tmRNA encompassing nucleotides 247–363. Similar analyses carried out with cross-linked complexes containing [s⁴U]tmRNA ³²P-labeled either on its 5′ end or internally, showed that the shortest fragment not covalently attached to protein S1 was produced by RNase H in the presence of oligodeoxyribonucleotide TM9. This result indicated that protein S1 did not cross-link within the segment encompassing nucleotides 1–60. Thus, RNase H analysis confined the cross-linked site to the region encompassed by nucleotides 60–247, which contains the mRNA-like segment and pseudoknots pk2–pk4.

To identify the nucleotides cross-linked to protein S1, we used primer extension (Moazed *et al.*, 1986). This method takes advantage of the observation that the extension of the complementary DNA strand by reverse transcriptase primed by short oligodeoxyribonucleotides

Fig. 6. Primer extension analysis of nucleotides in [s⁴U]tmRNA molecules that, upon irradiation with near-UV light, are cross-linked to a free protein S1 (A) and a 70S ribosome-bound protein S1 (B). U, C, G and A, sequencing lanes; 1, non-irradiated tmRNA; 2, UV-irradiated tmRNA; 3, tmRNA cross-linked to protein S1. The examples of primer extension reactions depicted were carried out in the presence of primers TM2 and TM6.

complementary to the analyzed RNA template is terminated by a cross-linked nucleotide, yielding characteristic ‘stops’ when reverse transcripts are analyzed by denaturing PAGE. Cross-linked [3′-³²P][s⁴U]tmRNA–protein S1_{His} complexes were separated from free and internally cross-linked [3′-³²P][s⁴U]tmRNA by chromatography on a Ni²⁺-NTA–agarose column and then ‘scanned’ with reverse transcriptase in the presence of either primer TM2 or TM6 (Figure 6A). Five termination sites in [s⁴U]tmRNA cross-linked to protein S1_{His} were identified. These sites corresponded to A86, A174, C199 (not shown) and C241/A242, and, as predicted by RNase H analysis, were located in the tmRNA segment encompassing nucleotides 60–247. Because reverse transcriptase usually aborts one nucleotide before the cross-linked site, nucleotides U85 in the mRNA-like domain, and U198 and U240 in pseudoknot pk3 were identified as the cross-linked sites (Figure 8). However, since all cross-linking was s⁴U dependent, the termination site at A174 is most likely indicative of the cross-linking between U172 in pseudoknot pk2 and protein S1. To address the potential difficulty that intra-RNA cross-links might be interpreted as tmRNA–protein S1 cross-links, purified covalent complexes of internally ³²P-labeled [s⁴U]tmRNA and protein S1_{His} were digested to completion with RNase T₁ in the presence of 30-nucleotide-long oligodeoxyribonucleotides complementary to segments of tmRNA containing the nucleotides identified by primer extension analysis as the sites of covalent attachment of protein S1 to tmRNA. RNase T₁ protection experiments confirmed all the sites of cross-linking found by primer extension. Examples of RNase T₁ digestion profiles obtained in the presence of oligodeoxyribonucleotides that protect segments of tmRNA encompassing cross-linked nucleotides U85 and U240 are shown in Figure 7A (lanes 1 and 2). These reactions yielded protein S1 labeled by a tmRNA fragment

Fig. 7. RNase T_1 protection analysis of tmRNA–S1 cross-links. (A) Covalent complexes formed by UV irradiation of ^{32}P -internally labeled $[\text{s}^4\text{U}]$ tmRNA and free protein S1 were first digested with RNase T_1 , either in the absence (control reaction, Co) or presence of oligodeoxyribonucleotides xl-85 (lane 1), xl-240 (lane 2) and xl-3'-end (lane 3), and then analyzed by electrophoresis on a 6% polyacrylamide gel in Tricine–SDS buffer. Cross-linked segments of tmRNA that are protected by oligodeoxyribonucleotides xl-85 and xl-240 are indicated by a solid arrow. Other tmRNA segments protected only by protein S1 are indicated by an open arrow. (B) The same analysis carried out on cross-linked tmRNA–protein S1 complexes isolated from ribosomal particles using 5'-biotinylated oligodeoxyribonucleotides xl-105B (lane 1), xl-240B-1 (lane 2), xl-212B (lane 3) and xl-240B-2 (lane 4). The sites of hybridization of the oligodeoxyribonucleotides (solid bars) in tmRNA are illustrated in the diagram. Since the analyzed samples were purified on Ni^{2+} -NTA–agarose prior to electrophoresis, cross-linked tmRNA fragments protected only by protein S1 are not present on the gel. The positions of molecular weight markers are indicated.

protected by oligodeoxyribonucleotides (indicated by a solid arrow). Control reactions, carried out either without oligodeoxyribonucleotide or in the presence of oligodeoxyribonucleotide complementary to the segment of tmRNA not cross-linked to protein S1, produced only protein S1 that was labeled by short fragments of tmRNA protected by the protein itself (indicated by an open arrow).

Topography of the tmRNA–protein S1 complexes formed on the ribosome

Covalent tmRNA–protein S1 complexes were isolated from 70S ribosomes that had been irradiated with near-UV light as described above. RNase H analysis showed that, also on the ribosome, protein S1 cross-links to the tmRNA region encompassing nucleotides 60–247 (not shown). When this region was scanned by primer extension, five termination sites corresponding to nucleotides A106, G213, A231, C241 and A247 were found exclusively in cross-linked tmRNA–protein S1 complexes (Figure 6B). Therefore, the cross-linked nucleotides were U105 in the mRNA-like domain, nucleotides U212, U230 and U240 in pk3, and U246 in the junction between pk3 and pk4. These sites were confirmed by RNase T_1 protection experiments. RNase T_1 digestions of covalent tmRNA–protein S1 complexes, carried out in the presence of 5' biotinylated 30-nucleotide-long oligodeoxyribonucleotides comple-

mentary to the sites of cross-linking revealed by primer extension analysis, produced protein S1 labeled by fragments of $[\text{s}^4\text{P}]$ tmRNA (solid arrow in Figure 7B). Because digestion products were purified on avidin beads prior to their electrophoresis on polyacrylamide gel, digestion products other than tmRNA fragments protected by oligodeoxyribonucleotides were not visible in the gel.

Discussion

Recent studies have demonstrated that EF-Tu and protein SmpB facilitate the binding of tmRNA to the *E. coli* ribosome (Rudinger-Thirion *et al.*, 1999; Barends *et al.*, 2000). Given that all presently available data indicate that tmRNA acts both as tRNA and mRNA, one can anticipate that its functions in *trans*-translation are also facilitated by additional molecules. Identifying these cellular components is expected to further our understanding of *trans*-translation, and the structure and function of tmRNA. To determine the nature of these molecules, we chose a photoaffinity labeling approach that utilized tmRNA derivatives randomly substituted with the photo-reactive nucleoside s^4U . Our experiments demonstrated that the photoreactive $[\text{s}^4\text{U}]$ tmRNA derivatives, both intact and truncated, are aminoacylated as efficiently as their unmodified counterparts. This observation is consistent with earlier studies, which show that incorporation of s^4U into tRNAs and mRNAs does not significantly affect their function in protein synthesis (Rosen *et al.*, 1993), in spite of the differing hydrogen bonding properties of s^4U and U (Kyogoku *et al.*, 1967).

When added to an *in vitro* translation system and irradiated with near-UV light, only the intact $[\text{s}^4\text{U}]$ tmRNA bound to ribosomes, where it was cross-linked to 16S rRNA and ribosomal protein S1. This protein was also labeled by free $[\text{s}^4\text{U}]$ tmRNA, consistent with partitioning of protein S1 as free in the cytosol and associated with ribosomes (Subramanian and van Duin, 1977; Subramanian, 1983). Cross-linking of tmRNA to 16S rRNA has been reported previously (Komine *et al.*, 1996; Wower *et al.*, 2000). Here we showed that ribosomes depleted of protein S1 were unable to bind tmRNA, thus suggesting that, at least in *E. coli*, protein S1 plays an essential role in *trans*-translation. Since this interaction did not require EF-Tu, tmRNA was most likely bound to ribosomes in the 'mRNA mode'. Moreover, competition experiments showed that protein S1 binds to tmRNA 600 times better than to tRNA. Only weak interactions were observed between protein S1 and a truncated tmRNA derivative lacking most of its mRNA-like and pseudoknot-rich domains. This indicates that the tRNA-like domain is not part of the S1 binding site. This conclusion is supported by the inability of protein S1 to discriminate between aminoacylated and non-aminoacylated tmRNAs, and the pattern of cross-linking induced by UV irradiation of $[\text{s}^4\text{U}]$ tmRNA–protein S1 complexes. Although all cross-linked sites were in the mRNA-like and pseudoknot-rich domains of tmRNA, only nucleotide U240 of tmRNA cross-linked to both free and ribosome-bound protein S1 (Figure 8). These findings highlight structural differences between tmRNA–protein S1 complexes formed on and off the ribosome.

Fig. 8. Distribution of the sites of cross-linking to protein S1 in the secondary structure of the *E. coli* tmRNA. Squares and circles indicate the nucleotides of [^s4U]tmRNA cross-linked to protein S1 on and off the ribosome, respectively. Letters 't' and 'm' identify tRNA- and mRNA-like domains of tmRNA. Pseudoknots are labeled pk1–pk4. Solid arrows indicate sites of deletion that yielded tmRNA Δ 90–299. Cross-links define the domain of tmRNA that is available for interactions with protein S1.

One of the cross-linked nucleotides, U at position 85, is located within AAAAAAUAGUCG, preceding the 'resume' codon. Interestingly, Williams *et al.* (1999) found that neighboring nucleotide A86 plays an important role in positioning the 'resume' codon of tmRNA on the ribosome. Taking into account the possibility that protein S1 disrupts helical regions of RNAs (Bear *et al.*, 1976), one may speculate that the interaction of protein S1 with tmRNA contributes to the initiation of *trans*-translation by exposing the sequence preceding the 'resume' codon and thus making it available for decoding. This suggestion is consistent with studies that indicate that protein S1 is required for bringing mRNA into the proximity of the ribosomal decoding center, and for an efficient translation of natural and synthetic mRNAs (Sorensen *et al.*, 1998). Furthermore, S1 plays a role in enhancing the efficiency and fidelity of translation of the tag-peptide coding region, as it does in mRNA to optimize pairing with the anticodon of a cognate tRNA (Linde *et al.*, 1979; Potapov and Subramanian, 1992).

Other cross-linking sites are located in pseudoknots pk2 and pk3, which may contain recognition sites for protein S1, as suggested by SELEX experiments in which protein S1 selected pseudoknots from a pool of randomized RNA molecules (Ringquist *et al.*, 1995). These ligands resemble not only pseudoknots in tmRNA (Zwieb *et al.*, 1999a,b), but also translational initiation sites at the autogenously regulated S1 ribosome binding site (Christiansen and

Pedersen, 1981; Skouv *et al.*, 1990) and the S1 binding domain from the bacteriophage Q β genome (Goelz and Steitz, 1977; Barrera *et al.*, 1993).

The ability of protein S1 to facilitate binding of tmRNA to ribosomes and to form complexes with free tmRNA in the cytoplasm has broad implications for our understanding of the mechanistic aspects of *trans*-translation and, possibly, regular translation. The tmRNA binding mode of ribosomes may be similar to that proposed by Odom *et al.* (1984). According to this proposal, protein S1, while attached to the ribosome through its N-terminal domain (Giorginis and Subramanian, 1980), utilizes its extended flexible RNA-binding domain to search for mRNAs that are lacking the strong Shine–Dalgarno sequence usually required to form an efficient mRNA–ribosome complex (Sorensen *et al.*, 1998). Since tmRNA does not have a discernible Shine–Dalgarno sequence preceding its tag-encoding sequence, one can speculate that S1 helps to direct tmRNA to the ribosome. Protein S1-assisted binding of tmRNA to ribosomes may be a general mechanism in Gram-negative bacteria, in the high-G + C group of Gram-positive bacteria and even chloroplasts (Muralikrishna and Suryanarayana, 1985). In the low-G + C group of Gram-positive bacteria, which appear to lack protein S1, tmRNA binding might be facilitated by other RNA-binding proteins, including initiation factor IF1, or cold shock proteins, which contain the S1 binding motif (Bycroft *et al.*, 1997).

According to recent studies, tmRNA binding to ribosomes is also likely to involve EF-Tu (Rudinger-Thirion *et al.*, 1999; Barends *et al.*, 2000) and protein SmpB (Karzai *et al.*, 1999). As EF-Tu recognizes primarily the acceptor arm of tRNA (Hou and Schimmel, 1988), EF-Tu is unlikely to interfere with protein S1. Although the binding domain for protein SmpB is not yet known, all three proteins can bind to tmRNA simultaneously (J. Wower, unpublished data). One can speculate that SmpB binding is mediated by helices 2 and 3, and possibly pk1. Such an arrangement of the SmpB protein on tmRNA is compatible with the suggestion that protein SmpB may stabilize interactions between tmRNA and EF-Tu that are much weaker than the analogous interactions with alanyl-tRNA^{Ala} (Barends *et al.*, 2000). *In vitro* experiments have demonstrated that, unlike EF-Tu, purified SmpB binds with high affinity to both deacylated and alanylated tmRNAs (Karzai *et al.*, 1999).

After binding to ribosomes and fulfilling its function as an amino acid carrier, tmRNA might undergo a conformational change to serve as an mRNA analog. This structural alteration may be facilitated by the RNA-unwinding capacity of protein S1 (Van Dieijen *et al.*, 1975) and could involve destabilization of one or more pseudoknots. At that stage, protein S1 may also play a role in the correct codon-dependent selection of aminoacyl-tRNA. This was suggested earlier by Potapov and Subramanian (1992). In addition, protein S1 could facilitate the departure of tmRNA from the ribosome, as its interactions with ribosomes are relatively weak (Draper and von Hippel, 1979; Goss *et al.*, 1983). Furthermore, it is likely that after dissociation from the ribosome, protein S1 remains associated with tmRNA for some time before it is recycled back to ribosomes. This would provide protection from ribonucleases, contribute to tmRNA stability in the cell and facilitate tmRNA recycling (Wower *et al.*, 2000). As in the case of tRNA, these processes may also involve other proteins such as AlaRS, EF-Tu and SmpB.

Materials and methods

Chemicals and enzymes

The sources of enzymes, radioactively labeled nucleotides and other biological materials are given in Wower *et al.* (1989).

Cloning, synthesis and purification of tmRNA derivatives

Plasmid ptmR with the *ssrA* gene under the control of the T7 promoter was constructed by PCR amplification of *E. coli* genomic DNA with an upstream primer (5'-CGAATTCTAATACGACTCACTATAGGGGCTGATTCTGGATTCGAC-3') containing an *EcoRI* restriction site and the T7 promoter sequence, and a downstream primer (5'-CGGGATCCTTGGAGCTGGCGAGAGTTGAAC-3') containing a *BamHI* restriction site. The amplified DNA was digested with *EcoRI* and *BamHI* and then cloned into phSR vector (Zwieb, 1991). The internal *BstNI* restriction site in the *ssrA* gene was changed to a unique *SmaI* site. As a result of this mutation, U216 of wild-type tmRNA was replaced by a C residue in the tmRNA transcript. Plasmid ptmΔ90–299 was constructed by PCR mutagenesis (Nelson and Long, 1989) with ptmR as a template and a single mutagenic primer to delete a segment corresponding to nucleotides 90–299 of mature *E. coli* tmRNA.

The *E. coli* tmRNA and its truncated derivative tmRNAΔ90–299 were synthesized by *in vitro* transcription of ptmR and ptmΔ90–299 linearized with restriction nuclease *BstNI* (Rosen *et al.*, 1993). [α -³²P]ATP (150 Ci/mmol) was included in the transcription reaction mixture to obtain internally labeled tmRNA derivatives.

To prepare photoreactive tmRNA derivatives randomly substituted with s⁴U, s⁴UTP was added to the transcription reaction mixture at an

s⁴UTP:UTP ratio of 1:3. Under these conditions, one in every 18 uridines was substituted by the s⁴U moiety. tmRNA transcripts were purified by electrophoresis on a 5% polyacrylamide gel (40:1) in native TGE buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA, 2.5% glycerol). Purified tmRNA transcripts, ~6% of which lacked a 3' terminal adenosine, were ³²P-labeled at their 3' ends by incorporation of [5'-³²P]AMP. A 200 μ l labeling reaction mixture consisted of 50 pmol of tmRNA, 10 μ l of [α -³²P]ATP and 1 μ l of a crude preparation of yeast ATP/CTP tRNA nucleotidyl transferase in 20 mM glycine-NaOH pH 9.5, 17 mM KCl, 7 mM MgCl₂, 5 mM dithiothreitol (DTT). The integrity of the 3'-³²P-labeled tmRNA was tested by electrophoresis on a denaturing 5% polyacrylamide gel in 100 mM Tris–100 mM H₃BO₄ pH 8.3, containing 2.5 mM EDTA and 8 M urea.

Aminoacylation

Aminoacylations of 3'-³²P-labeled tmRNA and tmRNAΔ90–299 were carried out as described by Sampson and Uhlenbeck (1988) and analyzed by gel-shift electrophoresis (Varshney *et al.*, 1991). Prior to electrophoresis, alanyl-tmRNA (Ala-tmRNA) was completely digested with RNase T₁ in 10 mM sodium acetate pH 4.5.

Preparation of mRNA templates

rpsH/XmnI RNA, a 3' truncated mRNA encoding the N-terminal portion of ribosomal protein S8, was prepared by run-off transcription of pETrpsH plasmid linearized with the restriction enzyme *XmnI* (Wu *et al.*, 1993). Poly(U), purchased from Boehringer, was subjected to alkaline hydrolysis and fractionated on Sephadex G-100 to isolate 40- to 80-nucleotide-long fragments (Katunin *et al.*, 1980).

Isolation of the wild-type E. coli ribosomal protein S1

The wild-type ribosomal protein S1 was extracted from the *E. coli* ribosomes and purified by chromatography on a poly(U)–Sephacose column (Subramanian, 1983).

Cloning, expression and purification of the His-tagged E. coli ribosomal protein S1

The gene *rpsA* encoding the *E. coli* ribosomal protein S1 was amplified by PCR from the plasmid pJS200 (Schnier *et al.*, 1986) using an upstream primer (5'-TAACTTTAAGAAGGAGATATACATATGACTGAATCTTTTGCTCAAC-3') and a downstream primer (5'-GTGGTGGTGGTGCTCGAGCTCGCCTTTAGCTGCTTT-3'). The resulting DNA fragment was digested with *NdeI* and *XhoI*, and cloned into plasmid pET-23a (Novagen) to yield plasmid pETrpsA. This plasmid encoded ribosomal protein S1 tagged at its C-terminus with six histidine residues, named S1_{His}.

Protein S1_{His} was overexpressed in *E. coli* strain BL21(DE3)pLysS transformed with pETrpsA, purified on Ni²⁺-NTA-agarose (Qiagen) under denaturing conditions and then re-chromatographed on poly(U)–Sephacose (Pharmacia) as described by Subramanian (1983). Purity was determined on a 10% Tricine–SDS–polyacrylamide gel (Schagger and von Jagow, 1987).

Gel mobility shift assay

Reaction mixtures (10 μ l) contained [³²P]tmRNA (0.5 $\times$ 10⁻⁹ M) and increasing amounts of protein S1 (0.5 $\times$ 10⁻¹⁰–0.75 $\times$ 10⁻⁶ M) in binding buffer (10 mM Tris–acetate pH 7.6, 100 mM NH₄Cl, magnesium acetate, 1 mM DTT, 0.02% NP-40 and 100 μ g/ml bovine serum albumin) (Ringquist *et al.*, 1995). After 10–30 min incubation on ice, aliquots were analyzed by electrophoresis on a 5% polyacrylamide gel (40:1) in TGE buffer. The specificity of the interactions between tmRNA and protein S1 was investigated in competition assays with 1 nM [³²P]tmRNA mixed with varying concentrations of competing RNA species and 100 nM protein S1. Unlabeled tmRNA (0.5 $\times$ 10⁻⁹–0.75 $\times$ 10⁻⁶ M), crude *E. coli* tRNA (10⁻⁷–0.75 $\times$ 10⁻³ M), 40- to 80-nucleotide-long poly(U) (0.5 $\times$ 10⁻⁸–1 $\times$ 10⁻⁵ M) and tmRNAΔ90–299 (0.25 $\times$ 10⁻⁸–0.5 $\times$ 10⁻³ M) were used as competitors. Binding of tmRNA to protein S1 was visualized and quantified using PhosphorImager S1 and ImageQuant 1.2 software (Molecular Dynamics).

Preparation of 70S ribosomes and their derivatives lacking protein S1

Ribosomes were isolated from *E. coli* MRE600 (Makhno *et al.*, 1988). To remove protein S1, a ribosome solution was passed through a poly(U)–Sephacose column (Subramanian, 1983). The selective removal of protein S1 was confirmed by both SDS–Tricine and two-dimensional PAGE as described by Schagger and von Jagow (1987) and Subramanian (1974), respectively. The ability of both 70S and S1-free ribosomes to

bind the 40- to 80-nucleotide-long fraction of poly(U) was tested by filter binding assay (Katunin *et al.*, 1980). To minimize poly(U) adsorption, the nitrocellulose filters were first treated with 0.5 M KOH for 30 min. The ability of ribosomes to participate in poly(U)-dependent polymerization of phenylalanine was tested as described by Potapov and Subramanian (1992).

Binding and cross-linking tmRNA to ribosomes

Preliminary cross-linking experiments were carried out in a translation system for linear templates (Promega). The activities of the tmRNA transcripts were tested by measuring the incorporation of [¹⁴C]alanine into a tag peptide as described by Himeno *et al.* (1997) using both poly(U) and rpsH/XmnI RNA as templates. For cross-linking, *in vitro* reaction mixtures were first supplemented with 0.7 pmol/μl [³²P][s⁴U]tmRNA derivatives and 0.2 μg/μl rpsH/XmnI RNA, then incubated for 30 min at 37°C and, after placing on ice, irradiated for 10 min with four 300 nm UV lamps (RPR-3000-Å lamps; Rayonet, Southern New England Ultraviolet Company, CT). Irradiation under these conditions does not inactivate ribosomes (Wower *et al.*, 1989). Irradiated samples were fractionated on a 14–40% sucrose gradient in 25 mM Tris–HCl pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT for 2 h at 40 000 r.p.m. and 4°C in a VTi50 rotor (Beckman). Fractions containing [³²P][s⁴U]tmRNA were analyzed to demonstrate the presence of tmRNA–protein and tmRNA–RNA cross-links (Wower *et al.*, 1989).

Binding of photoreactive tmRNA derivatives to ribosomes was performed by incubating 0.2–2 μM [³²P][s⁴U]tmRNA with either 2 μM 70S ribosomes or 2 μM 70S ribosomes lacking protein S1, in 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 60 mM NH₄Cl for 10 min at 37°C. The amount of [s⁴U]tmRNA bound non-covalently to ribosomes was determined from the retention of ³²P-labeled material on membrane filters (Nirenberg and Leder, 1964). The reactions were chilled on ice and cross-linked by UV light as described above. Cross-linked tmRNA–protein S1 complexes were isolated on a 6% polyacrylamide gel in Tricine–SDS buffer (Schagger and von Jagow, 1987).

Cross-linking tmRNA to purified protein S1

To cross-link tmRNA to protein S1, 1 μM [³²P][s⁴U]tmRNA was mixed with 1–2 μM protein S1 or S1_{His} in 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 60 mM NH₄Cl, incubated for 10 min on ice, and irradiated for 10 min with four 300 nm UV lamps. Covalently bound tmRNA–protein S1_{His} complexes were purified on Ni²⁺-NTA-agarose as described above.

Analysis of covalent tmRNA–protein S1 complexes

Protein(s) cross-linked to [³²P][s⁴U]tmRNA derivatives were identified by the ‘agarose gel’ immunological assay (Gulle *et al.*, 1988).

The sites of covalent attachment of protein S1 to tmRNA were determined by treatment with RNase H (PanVera Corp.) in the presence of selected oligodeoxyribonucleotides (Brimacombe *et al.*, 1990), as well as RNase T₁ protection experiments (Rosen *et al.*, 1993) and primer extension analysis (Moazed *et al.*, 1986; Döring *et al.*, 1994). Oligodeoxyribonucleotides were complementary to tmRNA residues at positions 348–363 (TM1), 304–318 (TM2), 256–270 (TM3), 241–254 (TM4), 214–229 (TM5), 194–208 (TM6), 126–139 (TM7), 95–109 (TM8), 53–67 (TM9), 299–330 (xl-3′-end), 71–103 (xl-85), 160–186 (xl-173) and 229–255 (xl-240).

Some RNase T₁ protection experiments were carried out using 5′-biotinylated oligodeoxyribonucleotides complementary to tmRNA residues at positions 78–112 (xl-105B), 184–221 (xl-212B), 229–255 (xl-240B-1) and 221–257 (xl-240B-2). Segments of tmRNA protected by biotinylated 30-nucleotide-long oligodeoxyribonucleotides were isolated using avidin–magnetic beads according to the procedure provided by the vendor (Pierce) and then analyzed by PAGE.

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