METHOD

Fluorescent labeling of tRNAs for dynamics experiments

THU BETTERIDGE,^{1,4} HANQING LIU,^{2,4} HOWARD GAMPER,¹ STANISLAV KIRILLOV,³
BARRY S. COOPERMAN,² and YA-MING HOU¹

¹Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

²Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, USA

³Petersburg Nuclear Physics Institute, RAS, 188300 Gatchina, Russia

ABSTRACT

Transfer RNAs (tRNAs) are substrates for complex enzymes, such as aminoacyl-tRNA synthetases and ribosomes, and play an essential role in translation of genetic information into protein sequences. Here we describe a general method for labeling tRNAs with fluorescent dyes, so that the activities and dynamics of the labeled tRNAs can be directly monitored by fluorescence during the ribosomal decoding process. This method makes use of the previously reported fluorescent labeling of natural tRNAs at dihydrouridine (D) positions, but extends the previous method to synthetic tRNAs by preparing tRNA transcripts and introducing D residues into transcripts with the yeast enzyme Dus1p dihydrouridine synthase. Using the unmodified transcript of *Escherichia coli* tRNA^{Pro} as an example, which has U17 and U17a in the D loop, we show that Dus1p catalyzes conversion of one of these Us (mostly U17a) to D, and that the modified tRNA can be labeled with the fluorophores proflavin and rhodamine 110, with overall labeling yields comparable to those obtained with the native yeast tRNA^{Phe}. Further, the transcript of yeast tRNA^{Phe}, modified by Dus1p and labeled with proflavin, translocates on the ribosome at a rate similar to that of the proflavin-labeled native yeast tRNA^{Phe}. These results demonstrate that synthetic tRNA transcripts, which may be designed to contain mutations not found in nature, can be labeled and studied. Such labeled tRNAs should have broad utility in research that involves studies of tRNA maturation, aminoacylation, and tRNA-ribosome interactions.

Keywords: dihydrouridine synthase; dihydrouridine; rhodamine 110; fluorescent tRNA; Dus1p

INTRODUCTION

Decoding of genetic information into protein sequences is a multistep process that requires specific charging of tRNA molecules with their cognate amino acids by their cognate aminoacyl-tRNA synthetases (aaRSs) to form aminoacyl-tRNA (aa-tRNA), specific binding of aa-tRNAs to the ribosomal decoding center programmed with their cognate triplet codons, and large-scale movements of tRNAs within the ribosome as they shift progressively from the tRNA entry site (the A-site), to the peptidyl-tRNA site (the P-site), and finally to the tRNA exit site (the E-site), from which tRNA dissociation takes place.

Extensive kinetic studies of reactions on the ribosome (Pape et al. 1998, 1999, 2000; Rodnina and Wintermeyer

2001) have elucidated the elementary steps of the elongation cycle of protein synthesis. These studies, combined with recent high-resolution X-ray crystal structures of the bacterial ribosome in complexes with tRNA substrates (Korostelev et al. 2006; Selmer et al. 2006), have established a solid framework for understanding the mechanism of the elongation cycle. This cycle begins with the binding of aa-tRNA having a triplet base anticodon complementary (cognate) to the codon triplet of the mRNA in the A-site. The cognate aa-tRNA arrives in a ternary complex with elongation factor-Tu (EF-Tu) and GTP. Discrimination against non-cognate aa-tRNA is kinetically controlled and occurs in two stages: initial selection and proofreading (Rodnina and Wintermeyer 2001), that are irreversibly separated by GTP hydrolysis. Release of EF-Tu-GDP, peptide bond formation between fMet and the new amino acid, translocation of the mRNA and two tRNAs into the E- and P-sites, respectively, and finally, exit of the discharged tRNA from the E-site completes the elongation cycle. Translocation requires elongation factor EF-G-GTP and results in hydrolysis of another GTP molecule, and the movement of a new mRNA codon into the A-site, awaiting the entry of a new aa-tRNA ternary complex cognate to the codon at the A-site.

⁴These authors contributed equally to this work.

Reprint requests to: Barry S. Cooperman, University of Pennsylvania, Department of Chemistry, Philadelphia, PA 19104-6323, USA; e-mail: coopman@pobox.upenn.edu; or Ya-Ming Hou, Thomas Jefferson University, Department of Biochemistry and Molecular Biology, 233 South 10th Street, Philadelphia, PA 19107, USA; e-mail: ya-ming.hou@jefferson.edu; fax: (215) 503-4954.

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While the elementary steps in elongation have been identified, additional studies will be required to achieve a full understanding of the molecular mechanisms of each step. Of particular interest is the role of tRNAs, which, rather than being passive and rigid substrates for the ribosome, have been more recently implicated as being “active” players in the decoding process (Westhof 2006), interacting strongly with different sites on the ribosome and undergoing substantial conformational changes in migration from the A- to P- to E-sites (Stark et al. 2002; Valle et al. 2002; Konevega et al. 2007). For example, a mutation in the D stem of tRNA^{Trp} (known as the Hirsh suppressor mutation [Hirsh, 1971]) has been shown to promote miscoding at the anticodon. Recent kinetic studies show that this mutation specifically accelerates two forward steps on the ribosome: (i) Tu-dependent GTP hydrolysis that precedes accommodation of aa-tRNA into the A site, and (ii) peptide bond formation (Cochella and Green 2005). Because this mutation is distal from the codon–anticodon interaction, its ability to promote tRNA accommodation and peptide bond synthesis suggests that the tRNA body is in direct communication with both the decoding center of the 30S subunit and the GTPase center of the 50S subunit. Another example is provided by the tertiary core “elbow” region of tRNA, which is formed by extensive interactions between the D- and T-loops. We have demonstrated, using single-turnover rapid kinetics measurements (Pan et al. 2006, 2007), that mutations in the conserved G18:U55 base pair interfere with the ribosomal translocation step, particularly for tRNA moving from the P- to the E-site, consistent with X-ray crystallography results showing that position 55 in E-site tRNA is in direct contact with the L1 stalk of the 50S subunit (Korostelev et al. 2006).

In our studies, an important assay for the translocation rate is based on fluorescent changes of modified natural

tRNAs, isolated from *Escherichia coli* or yeast cells, whose D residues have been replaced with proflavin (Wintermeyer and Zachau, 1979; Robertson and Wintermeyer, 1981; Paulsen and Wintermeyer, 1986; Robertson et al. 1986; Rodnina et al. 1994a, 1997). However, this approach, as so far applied, suffers from two significant limitations. First, it does not allow direct monitoring of the movements of tRNA mutants on the ribosome. This is because mutant tRNAs, which are prepared by runoff in vitro transcription with T7 RNA polymerase (Sampson et al. 1989), lack D residues. Second, proflavin is rapidly photobleached, rendering proflavin-labeled tRNA unsuitable for single-molecule experiments in which fluorescent probes are subject to high light fluxes. Interest in overcoming this limitation is high, because recent work has clearly demonstrated the potential of the single-molecule approach to yield more-detailed mechanistic information about protein synthesis than is available from ensemble single-turnover experiments (Blanchard et al. 2004a,b).

Here we present approaches for fluorescent labeling of tRNA that remove these limitations and will be useful in constructing future experiments. Specifically, we use the yeast enzyme Dus1p (Xing et al. 2004), expressed in *E. coli* as a His-tag fusion, to introduce a D residue into an in vitro tRNA transcript. Dus1p catalyzes U → D conversion specifically at positions 16 and 17 of tRNAs, utilizing FAD as a cofactor and NADH and NADPH as electron donors (Xing et al. 2004). Because virtually all tRNA genes in databases encode U16, U17, or both, this approach allows introduction of D residues at these positions, thus creating potential sites for fluorescent labeling. The heterocyclic ring of D is subject to reductive cleavage by sodium borohydride, yielding 3-ureidopropanol bound to the ribose C-1' position (Cerutti and Miller 1967) (see Fig. 1), which is a facile leaving group that is readily replaced by fluorophores bearing a primary amino group (Wintermeyer and Zachau

FIGURE 1. Reaction scheme for two-step fluorescent labeling of tRNA. In step 1, a tRNA transcript is synthesized by transcription and modified by Dus1p. In step 2, the Dus1p-modified tRNA transcript is reduced by NaBH₄ in alkali to open the nonaromatic dihydrouracil ring, following by replacement of the ureidopropanal group with a primary amine-bearing fluorophore, such as rhodamine 110.

1974). Second, we demonstrate that rhodamine 110, a photobleaching-resistant fluorophore commonly used in single-molecule studies, which also has a higher emission intensity than the dyes, such as proflavin and ethidium, used by Wintermeyer and Zachau (1974), is competent for such replacement, generating a fluorescent-labeled tRNA that is active in protein synthesis.

RESULTS AND DISCUSSION

Modification of an *E. coli* tRNA^{Pro/UGG} transcript by yeast Dus1p

The transcript of *E. coli* tRNA^{Pro/UGG}, prepared by in vitro run-off transcription and containing U residues at positions 17, 17a, and 20 (Fig. 2A), was subjected to modification by Dus1p. The amount of U→D conversion was determined by the previously established colorimetric method (Jacobson and Hedgcoth 1970), which quantifies the amount of acyclic ureido group formed by alkaline cleavage of the D ring. This colorimetric method specifically monitors the ureido group that derives from the U→D conversion and thus is more accurate than simply monitoring the loss of U residues by the decrease of absorbance at 235 nm, although both methods have yielded

identical results in other studies (Xing et al. 2002). The colorimetric assay was calibrated by using dihydrouracil as a positive control, which established a linear relationship between absorption at 550 nm and the concentration of ureido group. Based on this calibration, *E. coli* bulk tRNA was tested by the colorimetric assay over a range of concentrations and A_{550} was converted to the number of D residues (Fig. 3A). The slope of the linear relationship yielded an average of 1.5 ± 0.5 residues of D per tRNA (Fig. 3B), similar to the previously determined value of 1.4 ± 0.1 by the same method (Jacobson and Hedgcoth 1970). The number of D residues per transcript of *E. coli* tRNA^{Pro/UGG} modified with Dus1p was determined by the colorimetric assay to be 1.0 ± 0.2 (Fig. 3A,B), indicating that only one of the three U residues at 17, 17a, and 20 was converted to D.

To map the site of modification within the transcript, D was converted to a ureido group, which blocks primer extension (Xing et al. 2004). An oligonucleotide primer was designed to complement G42 to G22 in *E. coli* tRNA^{Pro/UGG} (Fig. 2A), and the products of primer extension were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 2B). This analysis identifies position G18 in the Dus1p-modified transcript as a major stop site, not seen with the unmodified transcript, indicating that the U→D modification catalyzed by Dus1p occurs at U17a

rather than at U17 (Fig. 2A). Although the mechanism of how Dus1p recognizes tRNA is unknown at the present, the demonstrated specificity at U17a, which is immediately adjacent to the conserved G18–G19 sequence common to all tRNA species, suggests that this enzyme may recognize G18–G19 as the determinant for modification. We denote the product of Dus1p modification as *E. coli* tRNA^{Pro/UGG}(D-17a).

Fluorescent labeling of D-containing tRNAs

In vitro transcribed *E. coli* tRNA^{Pro/UGG}(D-17a) was labeled with either proflavin or rhodamine 110, using standard reductive cleavage conditions (see Materials and Methods in Wintermeyer and Zachau, 1974), leading to incorporation values of 1.0 and ~0.5, respectively, and giving rise to labeled tRNAs denoted tRNA^{Pro}(prf) and tRNA^{Pro}(rhd), respectively. Labeling of native yeast tRNA^{Phe}, which contains D residues at positions 16 and 17, under the same conditions led to incorporation values of 2.0 and 1.0 for proflavin or rhodamine 110, respectively,

FIGURE 2. (A) Sequence and cloverleaf structure of *E. coli* tRNA^{Pro/UGG}. The primer binding site for reverse transcription (residues G22–G42) complementary is indicated by the curved line with an arrow. (B) Primer extension products analyzed by 12% PAGE/7 M urea. M indicates the position of the starting primer, which was 5' end labeled. Reactions 1 and 2 contained 0.6 and 1.2 μ g of *E. coli* tRNA^{Pro/UGG} modified by Dus1p, respectively, while reactions 3 and 4 contained the unmodified transcript.

FIGURE 3. Determination of the D content in tRNA by the colorimetric assay. (A) Proportionality of A₅₅₀ to increasing concentration of *E. coli* bulk tRNA or the transcript of *E. coli* tRNA^{Pro}. The slope of each linear relationship is converted to D/tRNA according to the calibration of A₅₅₀ from titration of increasing concentration of dihydrouracil. (B) Average of D/tRNA value from five independent determinations as shown in (A), graphed for the bulk *E. coli* tRNA and the transcript of *E. coli* tRNA^{Pro}.

and giving rise to labeled tRNAs denoted tRNA^{Phe}(prf) and tRNA^{Phe}(rhd), respectively. Labeling stoichiometries were determined from the ratio of absorption in the visible (462 nm for proflavin, 512 nm for rhodamine 110) to that at 260 nm for tRNA (corrected for a contribution from the dye). The lower stoichiometry in the case of rhodamine may be due to its expected lower nucleophilicity as compared with proflavin, given its much lower pK_a value (rhodamine 110, 4.3 [Boonacker and Van Noorden, 2001]; proflavin, 9.6 [Horobin et al. 2006]) and its more hindered primary amine.

Biochemical analysis of fluorescent-labeled tRNAs

To characterize biochemical properties of fluorescent-labeled tRNAs, the transcripts of *E. coli* tRNA^{Pro} and yeast tRNA^{Phe} were studied. These transcripts were labeled with rhodamine or proflavin, and the labeled fraction was purified away from the unlabeled fraction according to a recently developed method (Hou et al. 2006). In this method, the reaction mixture after labeling is incubated with an oligonucleotide complementary to the site of the label under an annealing condition. Because the unlabeled transcript is accessible to hybridization with the oligonucleotide while the labeled transcript is not, subsequent RNase H digestion allows selective cleavage of the unmodified transcript. Using this method, the rhodamine-labeled transcripts of both yeast tRNA^{Phe} and *E. coli* tRNA^{Pro} were well separated from the cleaved unlabeled transcripts on a denaturing PAGE (Fig. 4A,B) and were purified. Analysis of the denaturing gel revealed that the labeled fraction in tRNA^{Phe} and tRNA^{Pro} represented 30%–40% and ~50% of the total reaction, respectively, similar to measurements determined from absorption. The proflavin-labeled transcripts were similarly purified. Characteristic emission spectra for the transcript of *E. coli* tRNA^{Pro}(rhd) and native

yeast tRNA^{Phe}(rhd) are shown in Figure 5A. The emission intensity of yeast tRNA^{Phe}(rhd) is twofold higher than that of *E. coli* tRNA^{Pro}(rhd). The twofold difference reflects the relative D content (1 versus 2, respectively) of the two tRNAs and possibly also differences in local structure.

Under standard conditions (see Materials and Methods), the unlabeled transcript of yeast tRNA^{Phe} was aminoacylated to the extent of 1300 pmol/A₂₆₀. Compared to this value, the relative aminoacylations of transcribed yeast tRNA^{Phe}(prf), native yeast tRNA^{Phe}(prf), and native yeast tRNA^{Phe}(rhd) were 70%, 85%, and 77%, respectively. The unlabeled transcript of *E. coli* tRNA^{Pro} was only labeled to the extent of 330 pmol/A₂₆₀. Relative to this value, the

purified transcript of *E. coli* tRNA^{Pro}(prf) was aminoacylated to 75%. However, the purified transcript of *E. coli* tRNA^{Pro}(rhd) was aminoacylated to 10% only (33 pmol/A₂₆₀). The low level of aminoacylation activity suggests that the charging enzyme prolyl-tRNA synthetase (ProRS) is inhibited by the presence of the bulkier rhodamine group in the tRNA tertiary core to a much greater extent than when the tRNA is proflavin-labeled. These results are consistent with a previous finding that this enzyme is sensitive to structural alterations in the core (Liu and Musier-Forsyth 1994) and support the notion that the structure of the tRNA tertiary core is a determinant for aminoacylation by ProRS. Thus, while PheRS can easily accommodate rhodamine in the tRNA tertiary core, ProRS cannot. The contrast between these two enzymes illustrates the idiosyncratic nature of aminoacyl-tRNA synthetases with respect to their sensitivity to the structure of the tRNA tertiary core.

To evaluate the ability of a labeled transcript to function in a specific step on the ribosome, the transcript of yeast Phe-tRNA^{Phe}(prf) was tested for the EF-G catalyzed translocation step, which is sensitive to tRNA movement on the ribosome. The proflavin label was chosen to provide a comparison with results of previous studies of translocation of native yeast tRNA^{Phe}(prf16/17) (Savelsbergh et al. 2003; Pan et al. 2006). (The transcript of *E. coli* tRNA^{Pro}[prf] was not tested because of its low levels of aminoacylation.) Here ³H-Phe-tRNA^{Phe}(prf16/17) was synthesized and purified as a single peak by chromatography through an FPLC Mono-Q column to eliminate uncharged or unlabeled species as described previously (Rodnina et al. 1994b). This peak was identified by its A₂₆₀ absorbance, fluorescence emission, and radioactivity arising from the labeled phenylalanyl group. The pretranslocation complex was prepared by loading EF-Tu-GTP-Phe-tRNA^{Phe}(prf16/17) onto a 70S

FIGURE 4. Separation of fluorescent-labeled tRNA from RNase H-mediated cleavage of unlabeled tRNA by gel electrophoresis. Transcripts of (A) yeast tRNA^{Phe} and (B) *E. coli* tRNA^{Pro} were prepared, with and without the rhodamine label, and were hybridized to an appropriate oligonucleotide complementary to the region of the label. After hybridization, these transcripts were subjected to cleavage by RNase H according to (Hou et al. 2006) and were analyzed by a 12% PAGE/7 M urea. The unlabeled transcript of yeast tRNA^{Phe} was cleaved by RNase H to generate fragments of 61-mer and 15-mer, respectively, while the unlabeled transcript of *E. coli* tRNA^{Pro} was cleaved to generate fragments of 60-mer and 17-mer, respectively. The labeled transcript of each tRNA was resistant to the cleavage and was isolated as a full-length tRNA from the gel.

initiation complex containing fMet-tRNA^{fMet} at the P-site. After peptide bond formation, translocation of the pre-translocation complex, containing tRNA^{fMet} at the P-site and fMet-Phe-tRNA^{Phe} (prf16/17) at the A-site, was monitored by a stopped-flow apparatus upon rapid mixing with EF-G-GTP. The transcript of yeast Phe-tRNA^{Phe} (prf) exhibited a single-exponential time course of translocation, with a rate constant of 3.5 s^{-1} similar to the 4.6 s^{-1} rate constant of the native yeast Phe-tRNA^{Phe}(prf) (Fig. 5B). These results demonstrate the functionality of the tRNA^{Phe} transcript in the translocation assay.

Conclusion

We have described here a general method for fluorescent labeling of tRNA that utilizes one of the Dus enzymes to introduce D residues into a tRNA transcript and subsequently replaces the D residues with a primary amine-containing fluorophore, such as proflavin or rhodamine 110. The method is general for tRNA species, because all

but a small number of tRNAs have U residues in the D loop that are substrates for the Dus enzymes (Sprinzl et al. 1998). Even in the rare cases where U is absent from a tRNA (e.g., *Streptomyces lividans* tRNA^{Cys}), it may be possible to introduce a U into the D loop by site-directed mutagenesis, either as a replacement or as an insertion, as long as the introduced U does not alter the normal functions of the tRNA. Here we focus on yeast Dus1p, which is specific for U16 and U17. Other yeast enzymes are specific for U20 (Dus2p) and for U20a and U20b (Dus4p) (Xing et al. 2004). Although less characterized, *E. coli* also encodes several Dus enzymes (Bishop et al. 2002), which offer additional options for introducing D residues. Thus, the potential to use the described method for fluorescent labeling of tRNA transcripts is quite high.

This labeling method should prove useful in a wide variety of research involving tRNAs. Our initial goal is to use this method to directly monitor the dynamic interaction of mutant tRNAs with the ribosome (Pan et al. 2006, 2007), but we expect that other tRNA–enzyme reactions will prove amenable to this approach as well. Since tRNA^{Pro}(prf), tRNA^{Phe}(prf), and tRNA^{Phe}(rhd) are good substrates for their respective synthetases, such labeled tRNAs can be used to yield insights into tRNA conformational rearrangements upon interaction with its cognate tRNA synthetase. Also, because eukaryotic tRNAs are believed to be channeled through a multisynthetase complex (Negrutskii and Deutscher 1991; Han et al. 2006), the technique could be used to study the dynamics of the multisynthetase complex assembly. Other possibilities include enzymatic reactions that occur during maturation of tRNA, such as 5' processing, intron splicing, anticodon modification, and CCA end addition. Finally, the described method will be also useful for studying the kinetics and thermodynamics of tRNA folding, which provides the basis for understanding the folding of larger RNA molecules such as ribozymes.

MATERIALS AND METHODS

Enzyme and tRNA

The bacterial expression clone of yeast Dus1p with a C-terminal His tag was a gift of Dr. Eric Phiziky (University of Rochester, Rochester, NY, USA). The fusion protein was purified from *E. coli* BL21(DE3) by sonication (in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM MgCl₂, and 10% glycerol), binding to the His-link metal-affinity resin (in the sonication buffer), and elution by 200 mM imidazole. Protein concentration was determined by the Bradford assay. The purified Dus1p enzyme was stored in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 50 mM NaCl, and 50% glycerol at -20°C . The gene for *E. coli* tRNA^{Pro}/UGG was cloned into the pTFMa vector. Restriction of the gene with BstN1 provided a template for in vitro transcription by T7 RNA polymerase (Hou 1993). The transcript was purified by a denaturing PAGE, visualized by UV shadowing, and extracted from the gel into TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

FIGURE 5. (A) Emission spectra of native yeast tRNA^{Phe} modified with rhodamine at positions 16 and 17 and the purified transcript of *E. coli* tRNA^{Pro/UGG} modified with rhodamine at position 17a. (B) Single-turnover kinetics of translocation with k_{app} of $4.61 \pm 0.04 \text{ s}^{-1}$ for the native yeast tRNA^{Phe}(prf) and k_{app} of $3.50 \pm 0.04 \text{ s}^{-1}$ for the transcript of yeast tRNA^{Phe}(prf), monitored by stopped-flow fluorescence.

The concentration of the transcript was determined by absorption at 260 nm, based on 1 OD=40 $\mu\text{g/mL}$ tRNA.

Modification of tRNA transcript by purified Dus1p

The tRNA transcript (in 20 μM concentrations) was heat-cooled before use in a typical Dus1p reaction containing 100 mM Tris-HCl, pH 8.0, 100 mM NH_4Ac , 5 mM MgCl_2 , 2 mM dithiothreitol (DTT), 0.1 mM EDTA, 1 mM β -NADH (Sigma N1161), 1 mM

NADPH (Sigma N6505), 250 μM FAD (Sigma F6625), and purified Dus1p ($\sim 5 \mu\text{M}$) in a total volume of 50 μL . After incubation at 30°C for at least 40 min, the tRNA transcript was purified by phenol extraction and ethanol precipitation.

Primer extension

Indicated amounts of tRNA were denatured in 0.1 M KOH at 37°C for 20 min and then neutralized with an equal volume of 5 $\times$ annealing buffer (250 mM Tris-HCl, pH 8.3, 150 mM NaCl, 50 mM DTT). The tRNA (0.6 or 1.2 μg) was subjected to primer extension with AMV reverse transcriptase (2 units, Roche) at 42°C for 30 min. The primer (6 pmol) was 5' labeled with γ - ^{32}P -ATP by T4 polynucleotide kinase and hybridized to the tRNA substrate. The reaction was stopped by phenol/chloroform extraction, ethanol-precipitated, and analyzed on a 12% PAGE/7 M urea gel.

Fluorescent labeling of tRNA

Fluorescent labeling of tRNA with fluorescence dyes was based on the method developed previously (Wintermeyer and Zachau 1979). Rhodamine 110 (Fluka #83695) was dissolved in 8 mg/mL in methanol and stored in the dark at -20°C . In a 20- μL reaction, the D-containing tRNA transcript (1800 pmol in 40 mM Tris-HCl, pH 7.5) was treated with NaBH_4 (100 mg/mL in 10 mM KOH) at a final concentration of 10 mg/mL for 60 min at room temperature on a shaker. The reaction was stopped by lowering the pH to 4–5 by gradually adding 6 M acetic acid. After the tRNA was precipitated and washed, it was resuspended in $\sim 5 \mu\text{L}$ water, 85 μL of 0.1 M NaCO_2H (pH 3.0), and 10 μL rhodamine 110 (0.022 M) and incubated at 37°C for 90 min. The NaCO_2H buffer was chosen on the basis of results of a series of experiments that measured the fluorescent labeling yield as a function of pH. The reaction was then adjusted to pH 7.5 by addition of 2 M Tris-HCl, pH 8.5. The tRNA was phenol-extracted (pH 4.3), ethanol-precipitated, and resuspended in water. Absorption was determined at 260 and 512 nm.

Fluorescent labeling of tRNA with proflavin was performed in an essentially identical manner, following Wintermeyer and Zachau (1979), except that incubation at 37°C was for 45 min.

Colorimetric assay for dihydrouridine

The assay was performed as previously described (Jacobson and Hedgcoth 1970) with some modifications. The D-containing tRNA (at various concentrations in 100 μL of water) was

hydrolyzed by adding 10 μ L of 1 M KOH and incubating at 37°C for 30 min. The reaction was then neutralized by adding 50 μ L of concentrated H₂SO₄, followed by 100 μ L of a 1:1 mixture (v/v) of 3% diacetyl monoxime (2,3-butanedione 2-oxime, Fluka #31550) and saturated *N*-phenyl-*p*-phenylenediamine-HCl (200 mg [Fluka #07920] in 100 mL 10% ethanol). Ureido groups were exposed by heating the reaction at 95°C for 5 min and then at 50°C for 5 min. A solution of 100 μ L of 1 mM FeCl₃ in concentrated H₂SO₄ was added to react with the ureido group. After the reaction cooled to room temperature, absorption was read at 550 nm. A control sample without tRNA was prepared in parallel and used as a blank.

Purification of rhodamine-labeled tRNA

The transcript of *E. coli* tRNA^{Pro/UGG} labeled with rhodamine (880 pmol) was hybridized to a complementary chimeric oligonucleotide (1000 pmol) 5'-mU-mG-mC-mG-mC-mU-mA-mC-CAAG-mC-mU-mG-mC-mG-3', where "m" designates a 2'-O-methyl backbone (Hou et al. 2006). After annealing, the mixture (in 40 μ L) was digested with purified *E. coli* RNase H (50 μ M) at 37°C for 1 h. The RNase H-resistant labeled transcript was separated from the RNase H-cleaved unlabeled transcript by 12% PAGE/7 M urea on a Bio-Rad Minigel apparatus. The labeled transcript was extracted from the gel, ethanol-precipitated, and resuspended in TE.

Fluorescence measurement of rhodamine-labeled tRNA

The emission spectrum of purified rhodamine-labeled *E. coli* tRNA^{Pro/UGG} (0.012 μ M in water) was recorded from 510 to 600 nm using an excitation wavelength of 498 nm in a model QM-4 fluorimeter (Photon Technology International). The rhodamine-labeled native yeast tRNA^{Phe} was run as a control.

Aminoacylation of tRNA

Aminoacylation with proline was carried out as described (Lipman et al. 2002), using the purified *Deinococcus radiodurans* ProRS (Zhang and Hou 2004) at 1.0 μ M for the unlabeled transcript of *E. coli* tRNA^{Pro/UGG} (2.0 μ M) and at 10.0 μ M for the rhodamine-labeled transcript (2.0 μ M). Reactions were incubated at 37°C, and aliquots were removed at various time intervals and precipitated in 5% TCA. Aminoacylation with phenylalanine was performed in similar conditions, but with 30 μ M [¹⁴C]-phenylalanine (870.3 dpm/pmol), 100 mM Tris-HCl (pH 8.0), 10 mM ATP, 50 mM Mg(OAc)₂, 2.5 mM EDTA (pH 8.0), 3 mM β -Me, and 7 mg/mL crude synthetase ($\sim$ 100 μ M) at 37°C for 20 min. The aminoacylated Phe-tRNA^{Phe} was then loaded onto an FPLC Mono-Q column in 50 mM sodium acetate, pH 5.0, washed with the same buffer, and eluted by a gradient of 1 M NaCl in the same buffer. The peak that contained [³H]-Phe-tRNA^{Phe} was identified by absorption, fluorescence, and radioactivity.

Translocation assay

The initiation complex was formed in buffer A (50 mM Tris-HCl, pH 7.5, 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM DTT) at 37°C by incubating the 70S ribosome (2 μ M) with mRNA022 (La Teana et al. 1993) (8 μ M), IF1 (3 μ M), IF2 (3 μ M), IF (3 μ M),

GTP (3 mM), and [³⁵S]fMet-tRNA^{fMet} (3 μ M) for 25 min. The ternary complex was formed by incubating EF-Tu (4 μ M) with [³H]-Phe-tRNA^{Phe} (prf16/17) (2 μ M), GTP (2 mM), phosphoenolpyruvate (Roche) (1 mM), pyruvate kinase (Roche, 0.01 mg/mL) for 15 min. Pretranslocation complex was formed by mixing initiation complex (2 μ M) with ternary complex (2 μ M) for 30 sec. The pretranslocation complex was rapidly mixed with EF-G (2 μ M) and GTP (1 mM) and the kinetics of translocation was monitored by the SX.18MV stopped-flow spectrofluorometer (Applied Photophysics). Proflavin was excited at 462 nm and monitored using a 495-nm long-pass filter. Apparent rate constants were obtained by fitting the data to a single-exponential equation using Igor-Pro (Wavemetrics).

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REFERENCES

- Bishop, A.C., Xu, J., Johnson, R.C., Schimmel, P., and de Crecy-Lagard, V. 2002. Identification of the tRNA-dihydrouridine synthase family. *J. Biol. Chem.* **277**: 25090–25095.
- Blanchard, S.C., Gonzalez, R.L., Kim, H.D., Chu, S., and Puglisi, J.D. 2004a. tRNA selection and kinetic proofreading in translation. *Nat. Struct. Mol. Biol.* **11**: 1008–1014.
- Blanchard, S.C., Kim, H.D., Gonzalez Jr., R.L., Puglisi, J.D., and Chu, S. 2004b. tRNA dynamics on the ribosome during translation. *Proc. Natl. Acad. Sci.* **101**: 12893–12898.
- Boonacker, E. and Van Noorden, C. 2001. Enzyme cytochemical techniques for metabolic mapping in living cells with special reference to proteolysis. *J. Histochem. Cytochem.* **49**: 1473–1486.
- Cerutti, P. and Miller, N. 1967. Selective reduction of yeast transfer ribonucleic acid with sodium borohydride. *J. Mol. Biol.* **26**: 55–66.
- Cochella, L. and Green, R. 2005. An active role for tRNA in decoding beyond codon:anticodon pairing. *Science* **308**: 1178–1180.
- Han, J.M., Lee, M.J., Park, S.G., Lee, S.H., Razin, E., Choi, E.C., and Kim, S. 2006. Hierarchical network between the components of the multi-tRNA synthetase complex: Implications for complex formation. *J. Biol. Chem.* **281**: 38663–38667.
- Hirsh, D. 1971. Tryptophan transfer RNA as the UGA suppressor. *J. Mol. Biol.* **58**: 439–458.
- Horobin, R.W., Stockert, J.C., and Rashid-Doubell, F. 2006. Fluorescent cationic probes for nuclei of living cells: Why are they selective? A quantitative structure-activity relations analysis. *Histochem. Cell Biol.* **126**: 165–175.
- Hou, Y.M. 1993. The tertiary structure of tRNA and the development of the genetic code. *Trends Biochem. Sci.* **18**: 362–364.
- Hou, Y.M., Li, Z., and Gamper, H. 2006. Isolation of a site-specifically modified RNA from an unmodified transcript. *Nucleic Acids Res.* **34**: e21.
- Jacobson, M. and Hedgcock, C. 1970. Determination of 5,6-dihydrouridine in ribonucleic acid. *Anal. Biochem.* **34**: 459–469.
- Konevega, A.L., Fischer, N., Semenov, Y.P., Stark, H., Wintermeyer, W., and Rodnina, M.V. 2007. Spontaneous reverse movement of mRNA-bound tRNA through the ribosome. *Nat. Struct. Mol. Biol.* **14**: 318–324.
- Korostelev, A., Trakhanov, S., Laurberg, M., and Noller, H.F. 2006. Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell* **126**: 1065–1077.

- La Teana, A., Pon, C.L., and Gualerzi, C.O. 1993. Translation of mRNAs with degenerate initiation triplet AUU displays high initiation factor 2 dependence and is subject to initiation factor 3 repression. *Proc. Natl. Acad. Sci.* **90**: 4161–4165.
- Lipman, R.S., Wang, J., Sowers, K.R., and Hou, Y.M. 2002. Prevention of mis-aminoacylation of a dual-specificity aminoacyl-tRNA synthetase. *J. Mol. Biol.* **315**: 943–949.
- Liu, H. and Musier-Forsyth, K. 1994. *Escherichia coli* proline tRNA synthetase is sensitive to changes in the core region of tRNA^{Pro}. *Biochemistry* **33**: 12708–12714.
- Negrutskii, B.S. and Deutscher, M.P. 1991. Channeling of aminoacyl-tRNA for protein synthesis in vivo. *Proc. Natl. Acad. Sci.* **88**: 4991–4995.
- Pan, D., Kirillov, S.V., and Cooperman, B.S. 2007. Kinetically competent intermediates in the translocation step of protein synthesis. *Mol. Cell* **25**: 519–529.
- Pan, D., Kirillov, S., Zhang, C.M., Hou, Y.M., and Cooperman, B.S. 2006. Rapid ribosomal translocation depends on the conserved 18–55 base pair in P-site transfer RNA. *Nat. Struct. Mol. Biol.* **13**: 354–359.
- Pape, T., Wintermeyer, W., and Rodnina, M.V. 1998. Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. *EMBO J.* **17**: 7490–7497.
- Pape, T., Wintermeyer, W., and Rodnina, M. 1999. Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome. *EMBO J.* **18**: 3800–3807.
- Pape, T., Wintermeyer, W., and Rodnina, M.V. 2000. Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome. *Nat. Struct. Biol.* **7**: 104–107.
- Paulsen, H. and Wintermeyer, W. 1986. tRNA topography during translocation: Steady-state and kinetic fluorescence energy-transfer studies. *Biochemistry* **25**: 2749–2756.
- Robertson, J.M. and Wintermeyer, W. 1981. Effect of translocation on topology and conformation of anticodon and D loops of tRNA^{Phe}. *J. Mol. Biol.* **151**: 57–79.
- Robertson, J.M., Paulsen, H., and Wintermeyer, W. 1986. Pre-steady-state kinetics of ribosomal translocation. *J. Mol. Biol.* **192**: 351–360.
- Rodnina, M.V. and Wintermeyer, W. 2001. Fidelity of aminoacyl-tRNA selection on the ribosome: Kinetic and structural mechanisms. *Annu. Rev. Biochem.* **70**: 415–435.
- Rodnina, M.V., Fricke, R., and Wintermeyer, W. 1994a. Transient conformational states of aminoacyl-tRNA during ribosome binding catalyzed by elongation factor Tu. *Biochemistry* **33**: 12267–12275.
- Rodnina, M.V., Savelsbergh, A., Katunin, V.I., and Wintermeyer, W. 1997. Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. *Nature* **385**: 37–41.
- Rodnina, M.V., Semenov, Y.P., and Wintermeyer, W. 1994b. Purification of fMet-tRNA^{fMet} by fast protein liquid chromatography. *Anal. Biochem.* **219**: 380–381.
- Sampson, J.R., DiRenzo, A.B., Behlen, L.S., and Uhlenbeck, O.C. 1989. Nucleotides in yeast tRNA^{Phe} required for the specific recognition by its cognate synthetase. *Science* **243**: 1363–1366.
- Savelsbergh, A., Katunin, V.I., Mohr, D., Peske, F., Rodnina, M.V., and Wintermeyer, W. 2003. An elongation factor G-induced ribosome rearrangement precedes tRNA–mRNA translocation. *Mol. Cell* **11**: 1517–1523.
- Selmer, M., Dunham, C.M., Murphy IV, F.V., Weixlbaumer, A., Petry, S., Kelley, A.C., Weir, J.R., and Ramakrishnan, V. 2006. Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* **313**: 1935–1942.
- Sprinzel, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. 1998. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.* **26**: 148–153.
- Stark, H., Rodnina, M.V., Wieden, H.J., Zemlin, F., Wintermeyer, W., and van Heel, M. 2002. Ribosome interactions of aminoacyl-tRNA and elongation factor Tu in the codon-recognition complex. *Nat. Struct. Biol.* **9**: 849–854.
- Valle, M., Sengupta, J., Swami, N.K., Grassucci, R.A., Burkhardt, N., Nierhaus, K.H., Agrawal, R.K., and Frank, J. 2002. Cryo-EM reveals an active role for aminoacyl-tRNA in the accommodation process. *EMBO J.* **21**: 3557–3567.
- Westhof, E. 2006. The ribosomal decoding site and antibiotics. *Biochimie* **88**: 931–933.
- Wintermeyer, W. and Zachau, H.G. 1974. Replacement of odd bases in tRNA by fluorescent dyes. *Methods Enzymol.* **29**: 667–673.
- Wintermeyer, W. and Zachau, H.G. 1979. Fluorescent derivatives of yeast tRNA^{Phe}. *Eur. J. Biochem.* **98**: 465–475.
- Xing, F., Hiley, S.L., Hughes, T.R., and Phizicky, E.M. 2004. The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. *J. Biol. Chem.* **279**: 17850–17860.
- Xing, F., Martzen, M.R., and Phizicky, E.M. 2002. A conserved family of *Saccharomyces cerevisiae* synthases effects dihydrouridine modification of tRNA. *RNA* **8**: 370–381.
- Zhang, C.-M. and Hou, Y.-M. 2004. Synthesis of cysteinyl-tRNA^{Cys} by a prolyl-tRNA synthetase. *RNA Biol.* **1**: 35–41.

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