Interactions between 23S rRNA and tRNA in the ribosomal E site

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ABSTRACT

Interactions between tRNA or its analogs and 23S rRNA in the large ribosomal subunit were analyzed by RNA footprinting and by modification–interference selection. In the E site, tRNA protected bases G2112, A2392, and C2394 of 23S rRNA. Truncated tRNA, lacking the anticodon stem-loop, protected A2392 and C2394, but not G2112, and tRNA derivatives with a shortened 3' end protected only G2112, but not A2392 or C2394. Modification interference revealed C2394 as the only accessible nucleotide in 23S rRNA whose modification interferes with binding of tRNA in the large ribosomal subunit E site. The results suggest a direct contact between A76 of tRNA A76 and C2394 of 23S rRNA. Protections at G2112 may reflect interaction of this 23S rRNA region with the tRNA central fold.

Keywords: E site; footprinting modification interference; ribosome; translation; tRNA

INTRODUCTION

In the course of translation, tRNA interacts with the ribosome at three different sites. Prior to formation of a new peptide bond, aminoacyl- and peptidyl-tRNA are positioned in the acceptor (A) and donor (P) sites, respectively. After peptidyl transfer and translocation, newly deacylated tRNA is transferred to the exit (E) site. Existence of the E site was realized considerably after the A and P sites were described (Rheinberger et al., 1981; Grajevskaja et al., 1982; Kirillov et al., 1983). Significantly less is known about E site location, composition, interaction with tRNA, and function compared to the other two tRNA-binding sites. Nevertheless, the E site is thought to play an important role in translation. Binding of tRNA in the E site prior to its release from the ribosome may promote translocation (Lill et al., 1986, 1989) and/or improve accuracy of translation (Geigenmuller & Nierhaus, 1990).

The critical characteristic of the E site is its strong preference for a deacylated tRNA 3' end (Rheinberger et al., 1981; Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984; Parfenov & Saminsky, 1985). Modifications of the 3' terminal tRNA residue or its removal dramatically affect E site binding of tRNA, indicating the importance of the tRNA 3' end for binding in

the E site (Lill et al., 1988). The recently deduced X-ray crystallographic structure of the ribosome–tRNA functional complexes showed that in the E site, tRNA contacts the ribosome in three locations: the 3' end and TΨC loop of tRNA make contacts with the large ribosomal subunit in the cleft between the central protuberance and the L1 stalk, and the anticodon loop interacts with the small subunit between the platform and the head (Cate et al., 1999). Uncharged tRNA bound to the E site of *Escherichia coli* 70S ribosomes protects from chemical modification a specific set of bases in 23S rRNA, namely, U2111, G2112, G2116, A2169, and C2394 (Moazed & Noller, 1989). This evidence suggested a close proximity of tRNA to rRNA in the E site. From this, and from the observation that modifications of the 3' terminal adenine of tRNA decreased tRNA binding to the E site, a base pairing between the 3' terminal A of tRNA and U2111 of 23S rRNA was proposed (Lill et al., 1988). However, tRNA–protein interactions can contribute to tRNA binding in the E site as well. tRNA probes carrying 2-azidoadenosine near their 3' ends could be crosslinked to the protein L33 in the ribosomal E site, suggesting that L33 may facilitate tRNA binding (Wower et al., 1993). At the same time, the anticodon loop of the E site-bound tRNA was crosslinked to protein S11 and 16S rRNA, revealing the contacts between the tRNA anticodon arm and the small ribosomal subunit. Consensus has not yet been reached on the extent of contribution of codon–anticodon interaction to the tRNA binding in the E site (Rheinberger et al., 1986, 1990; Lill et al., 1986; Lill & Wintermeyer, 1987; Wower et al., 2000).

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In spite of the contacts that E site-bound tRNA makes with the small subunit, most of the free energy of tRNA binding in the E site comes from its interaction with the large subunit. Even the isolated 50S subunit retains its ability to bind (though fairly weakly) deacylated tRNA in the E site (Kirillov et al., 1983; Gnirke & Nierhaus, 1986). tRNA binding to the isolated 50S subunit can be enhanced significantly by low concentrations of methanol; addition of 20% methanol increases the association constant from $62 \mu\text{M}^{-1}$ to $2,000 \mu\text{M}^{-1}$ (Parfenov & Saminskii, 1985). In addition to the E site, the second tRNA binding site, which has all the characteristics of the P site, is also revealed on the isolated 50S subunit in the presence of methanol (Parfenov & Saminskii, 1985).

Although the general location of the E site on the ribosome has been described rather convincingly, the details of interaction between tRNA and rRNA in the E site remain obscure. In this article we present the results of studies of interaction between tRNA or its truncated or modified derivatives and 23S rRNA in the E site of isolated large ribosomal subunit. RNA footprinting and modification–interference selection allowed us to identify regions of 23S rRNA which make contacts with different parts of E site-bound tRNA. This information will be useful for defining E site configuration in X-ray crystallographic models of the ribosome and its subunits.

RESULTS

Interaction of large ribosomal subunits with deacylated tRNA

In the absence of mRNA, deacylated tRNA binds to P and E sites in the 70S ribosome (Grajevskaja et al.,

1982; Gnirke & Nierhaus, 1986; Lill et al., 1986). tRNA binding results in protection of characteristic sets of nucleotides in 23S rRNA specific for P or E site-bound tRNAs (Moazed & Noller, 1989; Table 1). Binding of tRNA to isolated 50S subunits is weak (Gnirke & Nierhaus, 1986), but can be stabilized by low concentrations of alcohol (Parfenov & Saminskii, 1985). The presence of limited amounts of methanol does not alter the conformation of tRNA or of the large ribosomal subunit (Moazed & Noller, 1991; Bocchetta et al., 1998), and is presumed to stabilize tRNA binding by decreasing dielectric permeability of the medium (Parfenov & Saminskii, 1985). Furthermore, the 50S ribosomal subunit retains its peptidyl transferase activity in the presence of 33% methanol (Monro & Marcker, 1967; Noller et al., 1992). Therefore, in all the experiments described below, binding of tRNA and tRNA derivatives was performed in the presence of 33% methanol. Both P and E sites of isolated 50S subunits are accessible for binding of deacylated tRNA under these conditions (Parfenov & Saminskii, 1985), and peptidyl tRNA binds preferentially to the P site (Bocchetta et al., 1998).

Binding of deacylated tRNA to the 50S ribosomal subunit resulted in protection of positions G2112, G2251, G2252, G2253, A2392, C2394, U2506, U2584, and U2585 (Table 1). When deacylated tRNA was replaced by a peptidyl-tRNA analog, N-Ac-Tyr-tRNA^{Tyr}, only positions G2251, G2252, G2253, A2439, A2451, U2506, U2584, and U2585 were protected, and no protection of positions A2392, C2394, and G2112 was observed. When a preformed peptidyl-tRNA–50S subunit complex was supplemented with deacylated tRNA^{Tyr}, three additional protections appeared, namely G2112, A2392, and C2394 (Table 1). Because peptidyl-tRNA binds preferentially to the P site of 50S subunits, we assigned the protections afforded by 50S subunit-bound peptidyl

TABLE 1. Protection of bases in 23S rRNA by tRNA and its derivatives bound to the 70S ribosome or 50S ribosomal subunit.

Ribosomal site	tRNA substrate	Ribosome or subunit	Bases protected ^a	Reference
P site	tRNA ^{Phe}	70S ^b	U1926, G2252, G2253, G2505, U2506, U2584, U2585	Moazed & Noller (1989)
E site	tRNA ^{Phe}	70S	U2111, G2112, G2116, A2169, C2394	Moazed & Noller (1989)
P site	Ac-Tyr-tRNA ^{Tyr}	50S	G2251, G2252, G2253, A2439, A2451, U2506, U2584, U2585	Bocchetta et al. (1998)
P site	tRNA ^{Tyr} , tRNA ^{Phe}	50S	G2251, G2252, G2253, U2506, U2584, U2585	This report
E site	tRNA ^{Tyr} , tRNA ^{Phe}	50S	G2112, A2392, C2394	This report
E site ^c	tRNA ^{C75}	50S	G2112	This report
E site ^c	tRNA ^{A73}	50S	G2112	This report
E site ^c	tRNA ^{F76}	50S	G2112, A2392, C2394	This report
E site ^c	tRNA ^{ox-red}	50S	G2112, A2392, C2394	This report
E site ^c	minihelix	50S	A2392, C2394	This report
E site	microhelix	50S	none	This report
E site	RNA16-CCA	50S	none	This report
E site	pCCA	50S	none	This report

^aRefers to relative reactivity of individual 23S rRNA bases to chemical modification.

^bBinding to the 70S ribosome was performed in the absence of methanol; binding to the 50S ribosomal subunit was performed in the presence of 33% ethanol.

^cProtections in the P site were only partially verified.

In subsequent experiments, we followed protection of G2112, A2392, and C2394 to monitor interaction of tRNA derivatives with the E site on the large ribosomal subunit.

All the truncated tRNA derivatives with an intact CCA end could bind to the P site, as followed from protection of U2506 and U2585 (data not shown). Neither pCCA.

The results described in the previous section established the importance of the tRNA 3' end for interaction with the 2392–2394 region of 23S rRNA. Therefore, two tRNA derivatives bearing modification at the 3' terminal nucleotide residue were prepared to characterize this interaction further. In one derivative, tRNA_{F76}, the 3'-terminal adenine was replaced with formycin, an adenosine analog with an altered structure of the nitrogen base (Fig. 3A). In another derivative, tRNA_{ox-red}, the ribose ring of tRNA^{Tyr} 3'-terminal adenosine was disrupted by periodate oxidation followed by NaBH₄ reduction (Fig. 3B). Both tRNA_{F76} and tRNA_{ox-red} protect the same positions (G2112, A2392, and C2394) as

FIGURE 2. Chemical footprinting of bases in 23S rRNA caused by binding of deacylated tRNA or truncated tRNA substrates to 50S subunits. Different amounts (20 pmol of tRNA^{Tyr}, and tRNA^{A73}; 20 pmol of minihelix; 40 pmol of microhelix; 60 pmol of RNA16-CCA; 2 nmol of pCCA) of tRNA derivatives were bound to 10 pmol of 50S subunits in a total of 50 μ L of reaction mixture (see Materials and Methods) and 23S rRNA was probed as described in Materials and Methods. A, C, and G are dideoxy sequencing lanes: Refer to the nucleotide sequence of 23S rRNA. Lanes 1: unmodified 50S subunits; all the other lanes contain 50S subunits modified with DMS (A and C) or kethoxal (B and D). 50S subunits were preincubated with the following: lane 2: no tRNA; lane 3: tRNA^{Tyr}; lane 4: minihelix; lane 5: microhelix; lane 6: RNA16-CCA; lane 7: pCCA; lane 8: tRNA^{A73}. Arrowheads indicate positions that are protected from chemical modification.

natural tRNA^{Tyr}. The extent of protections afforded by tRNA^{ox-red} is comparable to those caused by natural tRNA. Therefore, the ribose moiety of the tRNA 3' terminal nucleotide does not appear to be directly responsible for protection of any of the E site-specific positions. In contrast, tRNA_{F76} protects the E site-specific positions notably less efficiently than natural tRNA, which indicates weaker binding of tRNA_{F76}. However, when comparing the footprint patterns of tRNA_{C75} and tRNA_{F76}, one can notice that although both tRNA protect G2112, only tRNA_{F76} produces partial protection in the 2390 region, indicating proximity of the formycin

base to the 2390 region of 23S rRNA. These results are compatible with direct involvement of 3' terminal adenine of native tRNA in interaction with the 2390 region.

23S rRNA residues essential for binding of deacylated tRNA to the 50S subunit

To determine which 23S rRNA bases are critical for binding of deacylated tRNA to the E site of the 50S subunit, a modification–interference selection was employed. tRNA^{Tyr} was biotinylated at the Q base in the

FIGURE 3. **A:** Chemical structures of the 3' terminal nucleotide of native tRNA^{Tyr}, tRNA_{F76}, and tRNA_{ox-red}. **B** and **C:** Protections from chemical modification of bases in 23S rRNA caused by binding of deacylated tRNA or tRNA derivatives with modified 3'-end to 50S subunits. C and G are dideoxy-sequencing lanes and refer to the nucleotide sequence of 23S rRNA. Lane 1: unmodified 50S subunits; all the other lanes contain 50S subunits modified with DMS (**B**) or kethoxal (**C**). 50S subunits were preincubated with the following: Lane 2: no tRNA; lane 3: tRNA^{Tyr}; lane 4: tRNA_{ox-red}; lane 5: tRNA_{F76}; lane 6: tRNA_{C75}. Arrowheads indicate positions that are protected from chemical modification.

anticodon loop (Bocchetta et al., 1998) (Fig. 1). This modification does not interfere with tRNA binding in the P or E sites of the large ribosomal subunit as verified by identical footprinting patterns produced by natural or biotinylated tRNAs (data not shown). tRNA_{Biot} was complexed with 50S subunits that contained 23S rRNA randomly modified with dimethyl sulfate, kethoxal, or carbodiimide, and the tRNA–50S subunit complex was captured on the avidin resin. 23S rRNA was extracted from the subunits retained on the resin and distribution of modified bases was analyzed by primer extension (Von Ahsen & Noller, 1995; Bocchetta et al., 1998). 50S subunits carrying modifications at the 23S rRNA positions essential for the tRNA binding were expected to be underrepresented in the captured complexes.

Because deacylated tRNA interacts with both the P and E sites of the 50S subunit, it was anticipated that modification–interference selection would reveal residues essential for tRNA binding in both sites. Four positions, G2252, C2394, U2506, and U2585, were found undermodified in the 23S rRNA isolated from the resin-captured 50S subunit–tRNA complexes (Fig. 4). Three of these positions (G2252, U2506, and U2585) are known to be required for peptidyl-tRNA binding in the P site (Bocchetta et al., 1998). Only C2394 remained as

a residue essential for binding of tRNA in the E site. Thus, C2394 is the only residue in 23S rRNA (among those accessible to dimethylsulfate (DMS), kethoxal or carbodiimide) whose modification interferes with tRNA binding in the E site of the large ribosomal subunit.

DISCUSSION

Binding studies and functional tests showed that, in the presence of 33% methanol, peptidyl-tRNA analogs bind preferentially to the P site of the isolated 50S subunit (Monro & Marcker, 1967; Parfenov & Saminskii, 1985; Moazed & Noller, 1991; Bocchetta et al., 1998). Under these conditions, deacylated tRNA binds to two sites whose biochemical characteristics are reminiscent of those of P and E sites (Parfenov & Saminskii, 1985). In agreement with these findings, our footprinting experiments showed that while peptidyl-tRNA bound to 50S subunit in the presence of 33% methanol protects exclusively P site-specific positions in 23S rRNA, deacylated tRNA protects three additional positions, G2112, A2392, and C2394. The same three positions become protected when deacylated tRNA is bound to a 50S subunit–peptidyl-tRNA complex, indicating that these positions belong to the E site. Two of these positions,

FIGURE 4. Analysis of base modifications of 23S rRNA in randomly modified 50S subunits before and after selection on the avidin resin- $tRNA_{Biot}$. **A:** Modification with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide; **B:** modification with DMS; **C:** modification with kethoxal. Lane 1: unmodified 50S subunits; lane 2: modified, unselected 50S subunits; lane 3: modified 50S subunits retained on the avidin- $tRNA_{Biot}$ column. Dideoxy-sequencing lanes corresponding to RNA bases U, A, C, and G are shown. Arrowheads indicate positions found undermodified in selected 50S subunits.

G2112 and C2394, were assigned previously to the E site based on footprinting of tRNA on the 70S ribosome (Moazed & Noller, 1989). Protection of A2392 has not been described previously. It appears, however, that the A2392 footprint is not a result of subunit dissociation or the presence of methanol because, in our hands, A2392 was also protected if deacylated tRNA (either $tRNA^{Tyr}$ or $tRNA^{Phe}$) was bound to 70S ribosomes in the absence of methanol (not shown).

Footprinting of tRNA derivatives in the E site of the 50S subunit revealed intimate details of tRNA–rRNA contacts. Interaction of deacylated tRNA with the E site depends critically on the presence of 3′-terminal A76. In the absence of methanol, removal of A76 decreases tRNA binding to the ribosomal E site at least 100-fold (Grajevskaja et al., 1982; Lill et al., 1988). In the presence of methanol, both $tRNA_{C75}$ and $tRNA_{A73}$ can still bind to the E site of isolated 50S subunits, as follows from protection of E site-specific G2112 (Figs. 2 and 3). However, in spite of the apparent binding to the E site, $tRNA_{C75}$ and $tRNA_{A73}$ do not protect A2392 and C2394.

The most straightforward explanation of this result is that A2392 and C2394 are in immediate proximity to the tRNA 3′ terminal nucleotide. This conclusion is in good agreement with the observation that the Fe(II)-EDTA complex, tethered to the end of the E site-bound tRNA, cleaves preferentially the 2390–2440 region of 23S rRNA (Joseph & Noller, 1996). It is further supported by a recently reported crosslink between the E site-bound tRNA containing 2-azidoadenosine at the 3′ terminus and C2422 of 23S rRNA (Wower et al., 2000); in the ribosome tertiary structure the 23S rRNA position 2422 is located in a close proximity from C2394 (Ban et al., 2000).

Although removal of the tRNA 3′ terminal nucleotide completely eliminates protections at A2392 and C2394, $tRNA_{ox-red}$ with the disrupted 3′ terminal ribose still strongly protects these positions, showing that the ribose moiety is not essential for the interaction. This conclusion is supported by the binding studies, in which disruption of the 3′ terminal ribose only marginally affected binding of tRNA to the E site (Lill et al., 1988). In

contrast to the ribose modification, the nature of the nitrogen base of the tRNA 3' terminal nucleotide is critically important for tRNA binding and its interaction with 23S rRNA. Replacement of adenine 76 with C, G, or etheno-A reduced tRNA binding more than 100-fold (Lill et al., 1988). Replacement of adenine 76 with formycin also notably lessens tRNA binding to the E site of 50S subunits, even in the presence of methanol, as can be judged by reduced protection of all E site-specific positions (Fig. 3). At the same time, quantification of the footprints produced by tRNA_{F76} at G2112 and C2394 showed a more significant loss of protection at C2394 and A2392 than at G2112, compatible with the direct participation of the 3' terminal base in interaction with the 2390 region.

The importance of interaction of the tRNA 3' end with the 2390 region is further strengthened by the results of modification–interference selection. Among E site-specific nucleotides in 23S rRNA, only modification of C2394 interfered with binding of uncharged tRNA to the 50S subunit E site. Therefore, C2394 is apparently the primary 23S rRNA residue responsible for establishing contacts with A76 of tRNA in the E site. Besides C2394, modification of three other residues, G2252, U2506, and U2585, also destabilized the complex between the 50S subunit and deacylated tRNA. These three residues were among those that were found to be essential for binding of peptidyl-tRNA in the P site in the previous modification–interference experiments (Bocchetta et al., 1998) and thus, in the current experiments their modification apparently interfered with binding of deacylated tRNA to the large subunit P site. Though modification of A2451 with DMS was found previously to interfere with binding of peptidyl-tRNA to the P site of the 50S subunit, in experiments with deacylated tRNA, modification of A2451 did not preclude tRNA binding. Thus, modification–interference results confirm the conclusion of Noller et al. (1990), made on the bases of tRNA footprinting, that A2451 may interact with the aminoacyl moiety of the P site-bound tRNA.

Crosslinking experiments utilizing tRNA azido-derivatives placed the 3' end of the E site-bound tRNA near ribosomal protein L33 (Wower et al., 1993, 2000). At the same time, L33 was mapped close to C2394 (Osswald et al., 1990). Therefore, the interaction between tRNA A76 and 23S rRNA C2394 could be mediated by L33. This model seems unlikely, however, because deacylated tRNA binds efficiently to the so-called KSP particles and protects E site-specific C2394 (our unpubl. results). KSP particles, which are prepared from *Thermus aquaticus* 50S subunits by treatment with proteinase K, SDS, and phenol, retain only a subset of large ribosomal subunit proteins and apparently lack L33 (Noller et al., 1992; Khaitovich et al., 1999). Thus, protein L33 appears to be dispensable for the interaction of the tRNA 3' end with C2394.

Footprinting and modification–interference results are compatible with a direct interaction of A76 of E site-bound tRNA and C2394 of 23S rRNA. Because tRNA protects C2394 from DMS modification at N3 of the cytidine base and, furthermore, because binding of tRNA to the E site is abolished when C2394 is modified by DMS, it is reasonable to assume that N3 of C2394 participates directly in bond formation with A76 of tRNA. Reduced binding of tRNA containing etheno A or formycin as a 3' terminal base suggests that N6 and N7 of A76 may be involved in bond formation. These considerations can be accommodated by a model in which A76 of tRNA forms a reverse Hoogsteen pair with C2394 of 23S rRNA (Fig. 5B), though other types of interaction between tRNA 3'-terminal residue and C2394 are also possible. Hoogsteen-type base pairing between A76 of tRNA and U2506 or U2585 in ribosomal A- and P-sites, respectively, was proposed previously (Porse et al., 1996; Wower et al., 1999).

It was suggested previously that A76 may form a Watson–Crick base pair with U2111 (Lill et al., 1988, 1989). This hypothesis appears to contradict our footprinting data with truncated tRNA derivatives. tRNA_{A73} and tRNA_{C75} lacking the 3'-terminal CCA sequence, or lacking A76 alone, retain the ability to protect G2112, but fail to protect nucleotides in the 2390 region. This situation is reversed with minihelix: the TΨC-acceptor arm of tRNA protects C2394 and A2392, but not G2112. Because biochemical data as well as X-ray and cryo-EM reconstructions place the anticodon arm of tRNA in contact with the small ribosomal subunit (Frank, 1998; Cate et al., 1999), these data are consistent with the model in which the central fold of the E site-bound tRNA (which is missing in minihelix) is engaged in interaction with the 2112 region. This proposal is consistent with the crosslinking data because E site-bound tRNA derivatized at positions within or close to the central fold could be crosslinked to the 2112 region of 23S rRNA (Rinke-Appel et al., 1995). These crosslinks were originally attributed to both E and P site-bound tRNA; however, recent X-ray reconstructions showed the location of the P site-bound tRNA distant from the corresponding rRNA region. Therefore, crosslinks of the tRNA central fold to the 2112 region probably occurred primarily from the E site-bound tRNA.

In agreement with the X-ray reconstructions, footprinting of tRNA derivatives and modification–interference selection are collectively consistent with the model in which E site-bound tRNA interacts with the large ribosomal subunit at two major sites (Fig. 5A). The 3' terminal nucleotide of tRNA forms a bond with C2394 and protects C2394 and A2392 from DMS modification, while the tRNA central fold forms close contacts with the G2112 region of 23S rRNA.

The footprinting experiments presented have another implication. In contrast with the 50S subunit P site, in which the only tRNA component required for

FIGURE 5. A: Model of interaction between E site-bound tRNA and 23S rRNA. 23S rRNA line drawing was acquired originally from R.R. Gutell web site (<http://www.rna.icmb.utexas.edu/>). **B:** Reverse Hoogsteen A-C base pair.

proper interaction is the 3'-terminal CCA (Monro & Marcker, 1967; Moazed & Noller, 1991; Samaha et al., 1995), a more extended portion of tRNA is required for its placement in the E site. This was evidenced by the inability of microhelix, RNA16-CCA, or pCCA to protect any E site-specific residues. Because A76 accounts for a significant part of tRNA binding free energy in the E site (Lill et al., 1988), we suggest that the requirement for a complete T Ψ C-acceptor stem-loop is related to proper steric presentation of A76 to the 2394 region.

MATERIALS AND METHODS

Ribosomes and subunits

Tight-couple 70S ribosomes from *E. coli* strain MRE 600 and ribosomal subunits were prepared according to Spedding (1990).

Synthesis of tRNA derivatives

E. coli tRNA^{Tyr} (type I) was from Subriden (Rollingbay, Washington). It was converted into N-Ac-[³H]Tyr-tRNA^{Tyr}, HPLC-purified and biotinylated at the unique Q base located at position 34 in the anticodon loop as described in Bocchetta et al. (1998). Biotinylated tRNA was deacylated by incubation for 2 h at 37 °C in 50 mM Tris-HCl, pH 8.0. The resulting tRNA^{Biot} was purified by gel filtration and the efficiency of deacylation was verified by the absence of radioactivity in the tRNA^{Biot} preparation.

For preparation of pCCA, a trinucleotide CCA synthesized by TriLink BioTech (San Diego, California) was 5'-phos-

phorylated by T4 Polynucleotide kinase (Pharmacia) in the presence of ATP and purified using HPLC.

A 19-nt-long single-stranded 3'-terminal fragment of tRNA^{Tyr} (RNA16-CCA) was obtained by digesting 40 μ g of tRNA^{Tyr} in 100 μ l of 50 mM Na Acetate, pH 5.5, 2 mM EDTA with 10 U of ribonuclease T1 (Sigma) for 1 h at 37 °C. RNA16-CCA was purified from 15% gel, and the integrity of the 3'-terminal adenosine was verified as described above.

Microhelix, comprising the tRNA^{Ala} acceptor stem, and mini-helix, comprising the T Ψ C/acceptor arms of tRNA^{Ala} (Francklyn & Schimmel, 1989) were generated by in vitro transcription using T7 RNA polymerase and synthetic DNA templates (Milligan et al., 1987). Transcripts were purified in preparative 15% denaturing polyacrylamide gels. RNA bands were eluted from the gel, phenol extracted, and ethanol precipitated. RNA was resuspended in 50 mM K-HEPES, pH 7.0, 100 mM KCl, 10 mM MgCl₂ and renatured by incubation at 65 °C for 2 min and then cooling to room temperature over a period of 2 h. Renatured RNAs were ethanol precipitated, and resuspended in 10 mM Na acetate, pH 5.5. The integrity of the 3'-terminal A in purified transcripts was verified by ligation with [5'-³²P] pCp (England et al., 1980), digestion with RNase T2, and analysis of the nucleoside-3'-[³²P] monophosphates by two-dimensional thin layer chromatography (Keith, 1995). As an additional control, authenticity of the CCA end in the microhelix and minihelix was confirmed by their aminoacylation in vitro with S100 enzymes (Francklyn & Schimmel, 1989).

For preparation of tRNA_{A73} and tRNA_{C75} exonuclease digestion was employed (Zubay & Takanami, 1964). To prepare tRNA_{A73}, 50 μ g of tRNA^{Tyr} were dissolved in 500 μ l of 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂. Snake venom phosphodiesterase I (Pharmacia) was added to a final concentration of 100 μ g/mL, and the digestion was carried out at 20 °C for 6 h. The reaction mixture was phenol extracted, RNA was purified by gel filtration, ethanol precipitated, and resus-

pended in 10 mM Na acetate, pH 5.5. Analytical gel electrophoresis and 3'-terminal analysis of the digested tRNA confirmed the absence of the full-size tRNA; more than 90% of the preparation was represented by tRNA_{A73}. tRNA_{C75} was prepared in a similar way, but the final concentration of phosphodiesterase I was 1 μ g/mL and digestion was carried out for 20 min at 20 °C. From 3'-terminal analysis and analytical gel electrophoresis, it was determined that the tRNA_{C75} preparation was a mixture of approximately 80% tRNA_{C75} and 20% tRNA_{C74}.

tRNA^{Tyr} containing formycin at position 76 (tRNA_{F76}) was prepared by incubating tRNA_{C75} with formycin 5'-triphosphate (gift of Dr. Sprinzl, University of Bayreuth) in the presence of tRNA nucleotidyl transferase (Sprinzl et al., 1977). This enzyme was purified as a C-terminal His₆-tag fusion from an overproducing *E. coli* strain prepared in this laboratory (M. Bocchetta, unpubl.). Full-length tRNA_{F76} was isolated from preparative 15% polyacrylamide gel using partially digested and full-length tRNA^{Tyr} as size markers. Eluted tRNA was purified and reannealed as described above.

tRNA_{ox-red} was prepared according to Wintermeyer et al. (1979).

Modification–interference selection

Modification–interference selection was performed essentially as described previously (Bocchetta et al., 1998). Briefly, 50S subunits were modified randomly with kethoxal (Research Organics, Inc.), DMS (Aldrich), or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (Aldrich). Modified subunits were separated from the excess of modifying agent and equilibrated in binding buffer (B) (50 mM Tris-HCl, pH 7.5, 400 mM NH₄Cl, 20 mM MgCl₂) by gel filtration. One hundred twenty picomoles of modified subunits were mixed with 120 pmol of tRNA_{biot} in 200 μ L binding buffer, followed by addition of 100 μ L methanol (33% final concentration). Twenty picomoles of modified 50S subunits in buffer B containing 33% methanol (buffer BM) were kept on ice during the modification interference experiment and served as “no selection” control for primer extensions. After incubation of 50S subunits with biotinylated tRNA on ice for 10 min, 30 μ L of Tetralink avidin resin (Promega; blocked earlier by incubation in buffer BM containing 0.3 mg/mL bovine serum albumin) were added. Incubation was continued for 15 min with occasional, gentle stirring. The complex was diluted to 1 mL with cold buffer BM, and loaded on a quick-Snap disposable chromatography column (Isolab) preblocked with buffer BM containing 0.3 mg/mL bovine serum albumin. The resin was washed twice with 1 mL of cold buffer BM and retained subunits were eluted by slowly passing 400 μ L of elution buffer (300 mM Na acetate, pH 5.5, 5 mM EDTA, 0.5% SDS). rRNA was extracted and the extent of modification of individual bases was analyzed by primer extension using a set of DNA primers, allowing scanning of the complete 23S rRNA (Stern et al., 1988).

Footprinting of tRNA and tRNA derivatives

Twenty picomoles of 50S subunits were incubated with 60 pmol of tRNA or its truncated derivatives in 50 μ L of buffer BM on ice for 15 min. In the case of pCCA binding, an in-

creased amount of ligand was used (up to 6,000 pmol). RNA modification with DMS, kethoxal, or carbodiimide was performed according to Moazed and Noller (1989, 1991) and Merryman and Noller (1998). The extent of modification of individual 23S rRNA residues was analyzed on a phosphor-imager (Ambis).

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