Defining a Smaller RNA Substrate for Elongation Factor Tu†

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ABSTRACT: A nuclease protection assay was used to obtain equilibrium dissociation constants of *Thermus thermophilus* EF-Tu with two well-characterized internal deletions of *Escherichia coli* Ala-tRNA^{Ala} and yeast Phe-tRNA^{Phe}. Aminoacylated tRNAs with the anticodon hairpin substituted by a tetranucleotide bind to EF-Tu as well as the corresponding full-sized tRNAs. However, the Ala minihelix, where residue A7 is joined directly to A49, binds to EF-Tu less well than the full-sized Ala-tRNA^{Ala}. Similar data were obtained for *Escherichia coli* EF-Tu. An *in vitro* selection strategy was used to isolate a substrate for EF-Tu from an RNA library where nine random nucleotides inserted between A7 and A49 in the Ala minihelix. After six rounds of enrichment, two groups of RNA were obtained that bound *T. thermophilus* EF-Tu as well as Ala-tRNA^{Ala}. Group I molecules have the consensus sequence UNDUGACUY (N = U, C, A, G; D = U, G; Y = U, C) in the randomized region, and Group II molecules generally have 5′-terminal GUG, but are more variable in the remaining six nucleotides. The selected RNAs bind EF-Tu better than the minihelix either because they provide additional function groups for protein binding or because they have a structure more similar to the aminoacyl acceptor branch of tRNA.

A stable ternary complex of elongation factor Tu (EF-Tu),[1] GTP, and aminoacyl-tRNA (aa-tRNA) is a critical intermediate in protein synthesis. Although high resolution crystal structures of *Escherichia coli* and *Thermus thermophilus* EF-Tu complexes with GTP analogues and GDP are available (Jurnak, 1985; Lippman et al., 1988; Berchtold et al., 1993; Kjeldgaard et al., 1993), less is known about how aa-tRNA binds the protein. The presence of an amino acid is required for ternary complex formation (Jonak et al., 1980), and the stability of the complex depends on the identity of the amino acid (Louie & Jurnak, 1985). Several experiments clearly show that the acceptor stem and T-arm of the tRNA are also important for the interaction [reviewed in Reinboldt et al. (1993)]. For example, the inability of tRNA$^{Met}_f$ to bind EF-Tu is mostly due to the lack of a 1−72 base pair (Seong & RajBhandary, 1987). Similarly, an additional base pair in the acceptor stem of the selenocysteine-inserting tRNA prevents its binding to EF-Tu (Baron & Bock, 1991). Recent experiments with derivatives of Asp-tRNA^{Asp} showed that a minihelix RNA with A7 connected directly to C49 bound to EF-Tu with a K_d only 3.4-fold greater than that of intact tRNA^{Asp}, but shorter derivatives bound poorly (Rudinger et al., 1994).

Several experiments suggest that more of the tRNA molecule may be needed for ternary complex formation. Footprinting experiments (Jekowsky et al., 1977; Wikman et al., 1982) indicate that EF-Tu contacts portions of the variable loop in addition to the acceptor stem and T-arm, while the anticodon hairpin and D-arm are exposed. Truncation experiments with a tRNA-like fragment of viral RNA also suggest that variable loop nucleotides contribute to the interaction (Joshi et al., 1984). However, it is unlikely that EF-Tu binds to nucleoside functional groups outside the acceptor stem since mutations in conserved nucleotides in the body of tRNA did not alter EF-Tu affinity (Nazarenko et al., 1994).

The overall goal of this work was to identify a smaller derivative of tRNA that bound EF-Tu with an affinity equal to that of intact tRNA. Such a substrate should not only contain all the necessary elements for EF-Tu binding, but it should be able to arrange them in a correct, stable conformation. Since the acceptor stem must remain intact, preparing a smaller EF-Tu substrate requires (i) identifying two locations in the molecule where the chain could be cleaved and removing the residues inessential for EF-Tu binding and (ii) joining the resulting two fragments with an appropriate linker sequence which can provide the proper conformation and necessary binding elements. Although the tertiary structure of tRNA is expected to be a useful guide to these steps, the process is not always straightforward because of the possibility that the deleted tRNA does not fold like the corresponding region of intact tRNA. Making a break at certain locations of tRNA or introducing incorrect linker sequences can lead to misfolding of the RNA (Pan et al., 1991; Dichtl et al., 1993; Long & Uhlenbeck, 1994). For this reason, we first measured EF-Tu binding to two well-characterized internal deletions of tRNA^{Phe} and then used an *in vitro* selection strategy to obtain a smaller substrate for EF-Tu.

MATERIALS AND METHODS

RNA Preparation. Transcription templates derived from PCR-amplified DNA or *Bst*N1-digested plasmid DNA were used to generate runoff transcription products *in vitro* with T7 RNA polymerase as described previously (Sampson & Uhlenbeck, 1988). An 8-fold excess of 5′-GMP over GTP in the transcription mixture was used to ensure 5′-monophosphate termini of RNA. Transcripts were purified on denaturing polyacrylamide gels and stored in deionized water.

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[1] Abbreviations: aa-tRNA, amino acyl-tRNA; EF-Tu, elongation factor Tu.

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Aminoacylation of RNAs. Prior to aminoacylation, all the RNAs, except for yeast $tRNA^{Phe}$ with the deleted anticodon hairpin, were heated at 70 °C for 5 min in 30 mM Hepes (pH 7.45) and 25 mM KCl. $MgCl_2$ was added to a final concentration of 10 mM, and the mixture was cooled to 25 °C at a rate of 1 °C/min. Aminoacylation reactions were carried out in 60 μL reaction mixtures containing 30 mM Hepes (pH 7.45), 25 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 8 mM dithiothreitol, 20 μM [^{3}H]-amino acid (45−72 Ci/mmol, Amersham), and 50−100 nM yeast phenylalanyl-tRNA synthetase or *E. coli* alanyl-tRNA synthetase (a gift of Dr. P. Schimmel, Massachusetts Institute of Technology) for 15 min at 37 °C.

In order to permit subsequent aminoacylation, the yeast $tRNA^{Phe}$ with the deleted anticodon hairpin was denatured at pH 7.0, and $MgCl_2$ was added to a final concentration of 20 mM prior to cooling. Aminoacylation was carried out in 60 μL reaction mixtures, containing 30 mM Hepes (pH 7.0), 25 mM KCl, 20 mM $MgCl_2$, 0.5 mM ATP, 8 mM dithiothreitol, 20 μM [^{3}H]phenylalanine (50−70 Ci/mmol, Amersham), and 100 nM *T. thermophilus* phenylalanyl-tRNA synthetase (a gift of Dr. O. Lavrik, Institute of Bioorganik Chemistry of Novosibirsk, Russia) for 25 min at 37 °C.

Aminoacylated RNAs were purified on 40 μL TSK Fractagel DEAE columns as described previously (Nazarenko et al., 1994). The aminoacylated RNA library for *in vitro* selection experiments was additionally desalted on G-25 micro-spin columns (Life Science Product Inc.), equilibrated with 10 mM Hepes, pH 7.0.

Binding of Aminoacylated RNAs to EF-Tu. The affinity of aminoacylated RNA to EF-Tu was determined by an RNase protection assay (Louie & Jurnak, 1985). To prepare the ternary complex for these experiments, a series of dilutions of *T. thermophilus* EF-Tu (a gift of Dr. M. Sprinzl, University of Bayreuth, Germany) in 200 μL of 50 mM Hepes, pH 7.45, 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 3 mM phosphoenolpyruvate, 20 μg/mL pyruvate kinase (470 units/mg; Sigma), and 20 μM GTP (buffer A), or the same as buffer A, but with 50 mM NH_4Cl (buffer B), were made as described previously (Nazarenko et al., 1994) and preincubated at 37 °C for 3 h to convert EF-Tu•GDP to EF-Tu•GTP. The reactions were then cooled on ice, 10 μL of 20 nM [^{3}H]aa-tRNA (50−70 Ci/mmol) was added to each EF-Tu concentration, and the incubation was continued for 15 min at 4 °C to allow the complex to form. The RNase protection assay was performed as described previously (Nazarenko et al., 1994). The experiments comparing different RNAs were always performed side by side with the same preparation of EF-Tu.

When *E. coli* EF-Tu (a gift of Dr. F. Jurnak, University of California—Riverside) was used, it was purified from GDP bound to the protein as previously described (Thompson et al., 1981) and activated in the presence of GTP and regeneration system for 15 min at 37 °C (Nazarenko et al., 1994).

Construction of RNA Library. DNA template, 5′-GAGCTAAGCGGGGTCGAACCGCT(N)$_9$TAG-CCCCTATAG, where the randomized nucleotides are indicated by N, represents 2.6 × 10^5 sequences. Template (2 nM; 10^{10} molecules) was combined with 0.2 μM reverse transcriptase (RVT) primer, 5′-TGGTGGAGCTAAGC-GGGGTCG, and 0.2 μM T7-primer, 5′-GAATTAATAC-GACTCACTATAGGGGCTA, in 80 μL of PCR reaction buffer (Boehringer Mannheim Biochem). PCR was performed for 18 cycles using the protocol: 95 °C, 30 s; 50 °C, 30 s; 72 °C, 90 s. The entire reaction mixture was then used directly in a 200 μL transcription reaction, as described above.

In Vitro Selection Procedure. For these experiments, *T. thermophilus* EF-Tu (0.03−10 μM depending on the round of selection) in 50 mM Hepes (pH 6.9), 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 3 mM phosphoenolpyruvate, 2 μg/mL pyruvate kinase (470 units/mg; Sigma), and 20 μM GTP (buffer C) was incubated at 37 °C for 3 h to convert EF-Tu•GDP to EF-Tu•GTP. To form the ternary complex, 4 μM Ala-RNA library was incubated with 1 μM (rounds 1 and 2), 0.1 μM (rounds 3 and 4), or 0.03 μM (rounds 5 and 6) EF-Tu•GTP in 200−500 μL of buffer C for 15 min at 4 °C to allow the complex to form. The binding reaction was filtered through the pure nitrocellulose membranes "NC", with 0.45 μm pore size (Schleicher & Schuell), presoaked in 50 mM Hepes, pH 6.9, 150 mM NH_4Cl, 10 mM $MgCl_2$, and 5 mM dithiothreitol for at least 1 h. The filters were then washed to remove the unbound RNA with 5 mL of the same buffer. The bound RNA was eluted by soaking the filter in the mixture of 400 μL of phenol and 300 μL of 7 M urea for 1 h at room temperature. The eluted RNA was ethanol precipitated in 0.3 M ammonium acetate and 100 μg/mL glycogen (Boehringer Mannheim Biochem), dried-, and dissolved in 20 μL of water. RVT-primer (5 μL of 4 μM) was added, and the mixture was heated at 70 °C for 10 min and cooled on ice. The complementary DNA was synthesized with 200 units of reverse transcriptase in 40 μL of the buffer supplied by manufacturer (Gibco BRL). Ten microliters of the reaction mixture containing cDNA was then amplified in an 80 μL PCR reaction, transcribed, and purified as described for the starting library.

Two types of negative selections were done. First, the aminoacylated RNA library after the second and the third rounds was incubated in 500 μL of buffer A without EF-Tu and filtered through the three nitrocellulose membranes successively. EF-Tu•GTP was then added to the filtrate, and the positive selection was performed as described above. Second, after the fourth round, nonacylated RNA library was incubated with EF-Tu•GTP and filtered through three membranes successively. RNA that did not bind to the filter was purified on DEAE-cellulose, desalted, and aminoacylated as described above, and after that the positive round of selection was performed.

After six rounds of selection, cDNA was amplified by PCR, using 5′-phosphorylated primers, and purified on a native 15% polyacrylamide gel. The double-stranded DNA was then cloned into the *Sma*I site of pUC18 to allow X-Gal selection. Sixty white colonies were sequenced using Sequenase-2 and the protocol provided by U.S. Biochemicals. Selected DNA plasmids were linearized with *Bst*N1 and transcribed as described above.

RESULTS

Ef-Tu Binding to Previously Characterized tRNA Internal Deletions. Since the acceptor stem and the T-arm are the parts of tRNA recognized by EF-Tu, a good way to decrease the size of this EF-Tu substrate would be to prepare internal deletions of tRNA missing either just the anticodon hairpin or both the anticodon hairpin and the D-arm. An *in vitro*

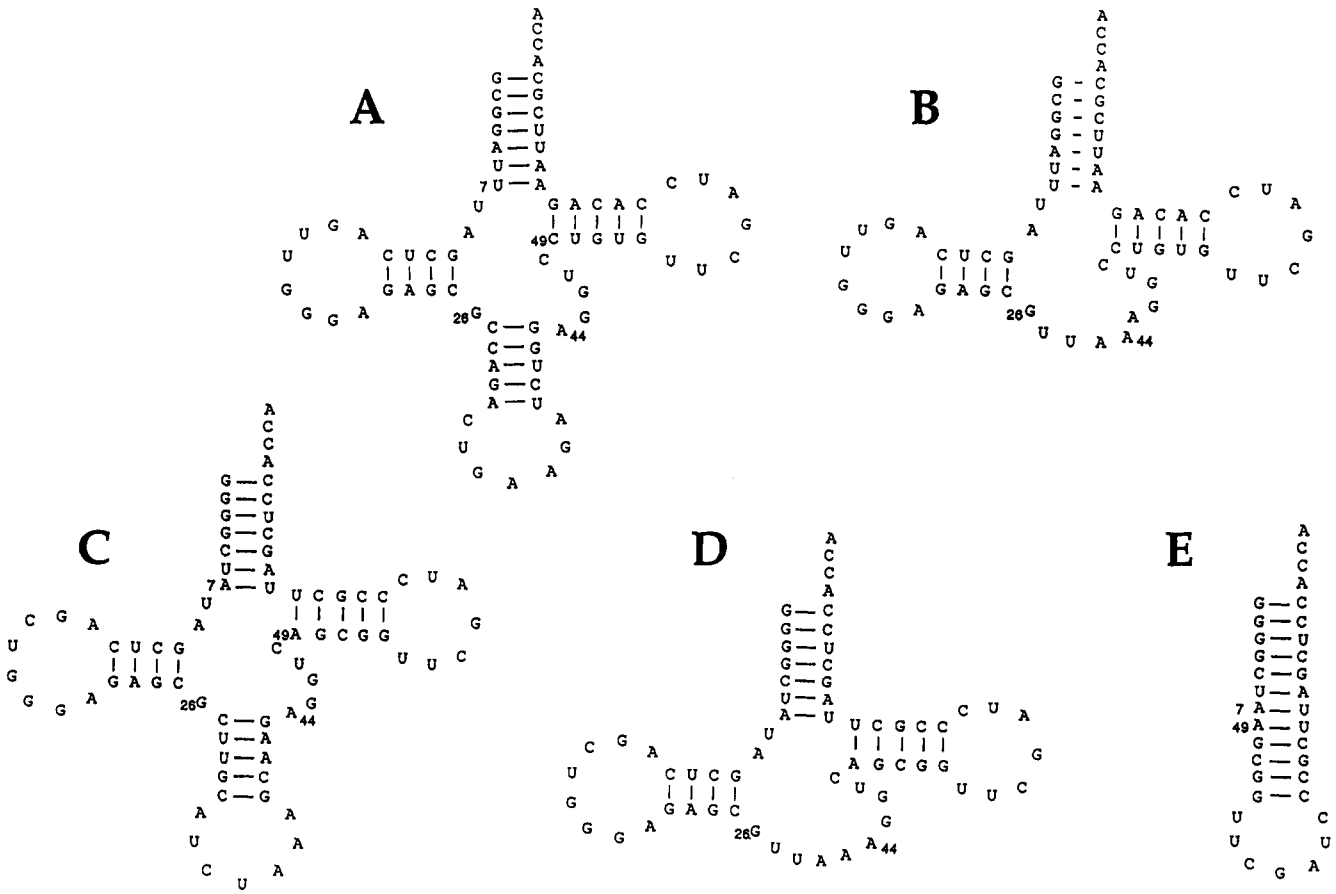

FIGURE 1: The secondary structure of the yeast $tRNA^{Phe}$ (A), yeast $tRNA^{Phe}$ without the anticodon hairpin (B), *E. coli* $tRNA^{Ala}$ (C), *E. coli* $tRNA^{Ala}$ without the anticodon hairpin (D), and *E. coli* $tRNA^{Ala}$ minihelix (E).

selection experiment was previously used to identify properly folded derivatives of $tRNA^{Phe}$ in which the anticodon hairpin had been replaced by a tetranucleotide (Dichtl et al., 1993). One example of such a molecule is shown in Figure 1B, where the replacement tetramer 5′-UUAA was found to permit proper folding of the molecule since the rates of Pb^{2+} cleavage and UV cross-linking were similar to the corresponding rates for intact yeast $tRNA^{Phe}$ (Figure 1A). The corresponding derivative of *E. coli* $tRNA^{Ala}$ (Figure 1C) was made using the same UUAA replacement tetramer (Figure 1D). An RNA molecule which deletes both the anticodon hairpin and the D-arm of $tRNA^{Ala}$ is the minihelix (Figure 1E), where nucleotide residue A7 was joined directly to A49 to form a 12 base pair hairpin (McClain et al., 1987; Francklyn & Schimmel, 1989).

To form a complex with EF-Tu, all the derivatives of tRNA have to be aminoacylated. Since the major determinants of *E. coli* alanyl-tRNA synthetase recognition are located in the acceptor stem (Hou & Schimmel, 1988), both internal deletion variants of $tRNA^{Ala}$ were efficiently aminoacylated. On the other hand, all known phenylalanyl-tRNA synthetases require tRNA anticodon nucleotides for the recognition (Sampson & Uhlenbeck, 1988; Moor et al., 1992; Nazarenko et al., 1992; Peterson & Uhlenbeck, 1992), so it is not surprising that yeast $tRNA^{Phe}$ missing the anticodon hairpin aminoacylates poorly with yeast phenylalanyl-$tRNA^{Phe}$ synthetase. However, as described in Materials and Methods, this derivative can be aminoacylated almost quantitatively with *T. thermophilus* phenylalanyl-tRNA synthetase in the presence of 20% dimethyl sulfoxide under optimized concentrations of Mg^{2+} and ATP.

Table 1: Dissociation Constants for Ternary Complex Formaton with the Wild Type tRNA Transcripts and tRNA Internal Deletion Mutants Determined by the RNase Protection Assay

	Buffer A (150 mM NH_4Cl)		Buffer B (50 mM NH_4Cl)	
RNA transcript	K_d (nM)	fraction of RNA bound (%)	K_d (nM)	fraction of RNA bound (%)
yeast Phe-$tRNA^{Phe}$	2.7	98	6.0	96
yeast Phe-$tRNA^{Phe}$ without anticodon stem	3.0	95	7.0	96
E. coli Ala-$tRNA^{Ala}$	3.0	95	9.0	94
E. coli Ala-$tRNA^{Ala}$ without anticodon stem	2.5	32	8.5	30
Ala minihelix	38.0	92	19	34

The ribonuclease protection assay (Louie & Jurnak, 1985) was used to obtain equilibrium constants for EF-Tu binding to the aminoacylated deletion variants and their corresponding intact tRNAs in a buffer containing either 50 or 150 mM NH_4Cl. Data obtained with *T. thermophilus* EF-Tu are shown in Table 1, and quite similar results were observed with *E. coli* EF-Tu. Both anticodon deletion variants bind EF-Tu just as well as the corresponding intact tRNAs in both buffers. The slightly tighter binding of the $tRNA^{Phe}$ derivatives is consistent with the previous observation that phenylalanine is better than alanine at stabilizing ternary complex (Louie & Jurnak, 1985). These results clearly show that the anticodon hairpin does not contribute to the affinity of EF-Tu for aa-tRNA.

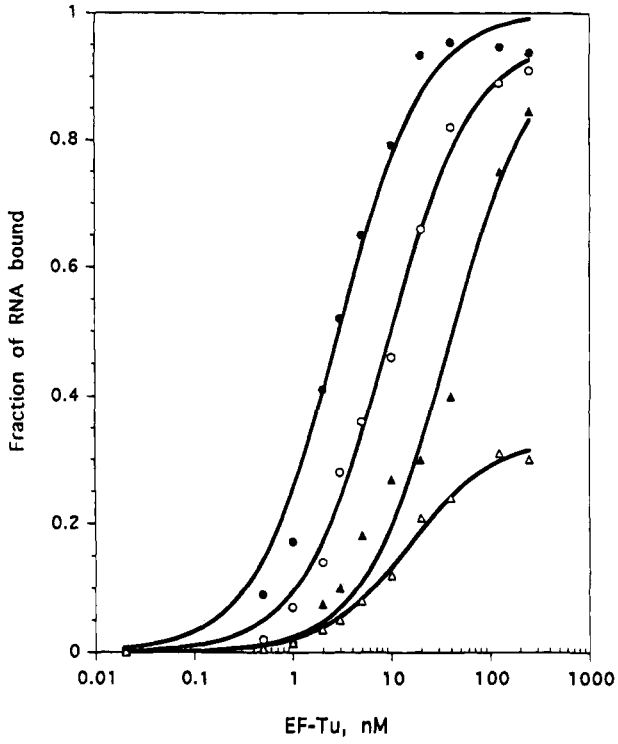

FIGURE 2: Binding of Ala minihelix (triangles) and Ala-tRNA^{Ala} (circles) to *T. thermophilus* EF-Tu in buffer A (closed symbols) or buffer B (open symbols).

One interesting difference between the two anticodon deleted tRNAs is that while virtually all (85−95%) of the anticodon deleted tRNA^{Phe} derivative bound to EF-Tu at the saturating protein concentration, a much smaller fraction (30−35%) of the anticodon deleted Ala-tRNA^{Ala} bound to EF-Tu at saturation in either buffer (Table 1). These results suggest that the remainder of the tRNA^{Ala} molecules were in an inactive conformation despite the fact that they were fully aminoacylated by AlaRS and care was taken to omit RNA denaturants during the subsequent purification (see Materials and Methods). Due to the lability of the aminoacyl linkage, it was not possible to renature the inactive aa-RNA fraction by protocols involving heating and cooling.

Figure 2 compares the equilibrium binding curves for Ala-tRNA^{Ala} and the aminoacylated minihelix of tRNA^{Ala} in the two buffers. In the lower salt buffer, the minihelix binds EF-Tu with a K_d only 3-fold greater than that of Ala-tRNA^{Ala}. However, in contrast to tRNA^{Ala}, only 30% of the minihelix molecules bind EF-Tu at saturating protein concentration. In the higher salt buffer, both molecules are fully active, but the minihelix binds 15-fold less well than tRNA^{Ala}. Unlike Ala-tRNA^{Ala} and several other aa-tRNAs (Antonsson & Leberman, 1982; Harrington et al., 1993) which bind EF-Tu tighter at higher ionic strength, it appears that the Ala minihelix binds protein slightly weaker at higher ionic strength. This suggests that the interaction of the minihelix with the protein is altered in some way.

The low fraction of Ala minihelix binding to EF-Tu at saturating concentration in the low salt buffer is interesting. While conformational heterogeneity is a possibility, this would seem unlikely considering the well-defined structure of the Ala minihelix and its full activity for EF-Tu binding at the higher ionic strength. Another possibility is that, in the low salt buffer, the bound form of the Ala minihelix is unnaturally sensitive to ribonuclease digestion. In order to

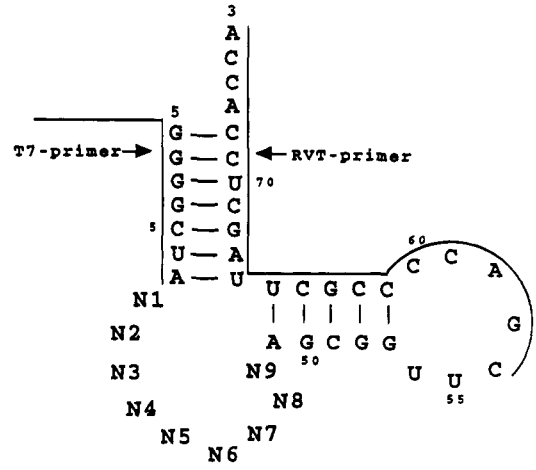

FIGURE 3: The RNA library with reverse transcriptase (RVT) primer and the primer containing T7 RNA polymerase promotor. Numbers in the acceptor stem and T-arm indicate positions corresponding to those in the intact tRNA. The randomized nucleotides are indicated by N1−N9.

test this, the binding of RNAs to EF-Tu was determined using an independent filter-binding protocol. Because standard Millipore filters do not bind ternary complex in typical binding buffers (Miller & Weissbach, 1974), the assay had to be modified in two ways. First, the pH of the buffer was reduced from 7.5 to 6.9 to increase the positive charge on the protein, and second, pure nitrocellulose membranes were used instead of Millipore filters which are composed of a mixture of cellulose acetate and cellulose nitrate. Under these revised conditions, a saturating concentration of *T. thermophilus* EF-Tu (0.5 μM), that protected 85% of *E. coli* Phe-tRNA^{Phe} from RNase digestion, was able to bind 32% of Phe-tRNA^{Phe} to the filters. While this is not a high retention efficiency, it is reproducible for a given lot of filters and very specific since less than 1% of aa-tRNA is bound to the filters if EF-Tu·GDP was used, and no binding of non-aminoacylated [^{32}P]-labeled tRNA to EF-Tu·GTP was detected.

Using this filter binding assay, the binding of the tRNA^{Ala} minihelix was compared to tRNA^{Ala}. At saturating EF-Tu concentration, 34% of the Ala-tRNA^{Ala} binds to the filter while only 14% of the Ala minihelix was found to bind (data not shown). This suggests that at low ionic strength a substantial fraction of the Ala minihelix does not bind to EF-Tu. While it is unclear whether this is a property of all minihelices or just the minihelix derived from *E. coli* tRNA^{Ala}, it clearly indicates that the minihelix does not have binding properties similar to those of tRNA^{Ala}.

In Vitro Selection of Internal Deletion Mutants of tRNA^{Ala} That Bind T. Thermophilus Ef-Tu. A library of RNA molecules was prepared which contained nine random nucleotides inserted between residues 7 and 49 (Figure 3). The choice of a library of this size was based on the fact that footprinting (Jekowsky et al., 1977; Wikman et al., 1982) and terminal truncation (Joshi et al., 1984) experiments suggested that one or more of the five variable loop nucleotides (residues 44−48) could potentially interact with EF-Tu. Therefore, five random positions were inserted. Then, since position 44 in yeast tRNA^{Phe} structure is 27 Å from position 7, an additional four random nucleotides were included to span this distance. Thus, the library was designed to contain sequences that could provide nucleotide-specific

contacts in the variable loop, but entirely delete the D-arm and anticodon arm. Since the library contained the G3–U70 pair needed for $tRNA^{Ala}$ recognition, it is not surprising that it could be aminoacylated to greater than 80% by alanyl-tRNA synthetase.

In order to carry out *in vitro* selection of the aminoacylated library, the SELEX technique was used (Tuerk & Gold, 1990). The filter-binding conditions described above involving pure nitrocellulose filters and pH 6.9 buffer were used throughout. In addition, a lower concentration of pyruvate kinase was used in preparation of ternary complex. Pyruvate kinase appears to be an effective RNA binding protein whose presence at standard concentrations increases the background to unacceptable levels. At the lower concentrations of pyruvate kinase, EF-Tu•GTP formation is unaffected and the tRNA binding in the absence of EF-Tu is less than 1%.

Six rounds of *in vitro* selection were performed. In general, the procedure involved the following: (1) aminoacylation of the RNA library with alanyl-tRNA synthetase under conditions where the minihelix is rapidly aminoacylated, (2) purification of aa-RNA by DEAE-cellulose and desalting by Sephadex G-25, (3) formation of ternary complex with *T. thermophilus* EF-Tu•GTP, and (4) passing the reaction mixture through the filter and eluting bound RNAs from the filter. After each round the RNA was converted to cDNA with reverse transcriptase primer and subjected to PCR amplification with the reverse transcriptase primer and primer containing T7 promotor (Figure 3). In every round of selection, aminoacylated RNA was in excess over the EF-Tu•GTP concentration. The concentration of EF-Tu•GTP was 1 μM for the first two rounds, 0.1 μM for rounds 3 and 4, and 30 nM for rounds 5 and 6. Before rounds 3 and 4, aa-RNA was preincubated in the reaction mixture without EF-Tu and passed through the filter in order to remove those RNAs that bound pyruvate kinase or the filter itself. Before the fifth round, the non-aminoacylated RNA library was incubated with EF-Tu•GTP and passed through the filter. This negative selection step served to remove those RNAs that bound EF-Tu•GTP in a manner that did not require aminoacylation.

Binding properties of the aminoacylated RNA after six rounds of selection are compared to the aminoacylated starting library and Ala-$tRNA^{Ala}$ transcripts using the nuclease protection assay (Figure 4). The affinity of the selected species for EF-Tu increases as the selection proceeds and by the sixth round is comparable to that of the intact tRNA. However, at this stage the binding curve is considerably broader than the binding curve for $tRNA^{Ala}$, suggesting the presence of multiple species with differing affinities for EF-Tu. These RNAs shown no measurable affinity to EF-Tu•GDP (data not shown), indicating the formation of a specific ternary complex.

Analysis of in Vitro Selected RNAs. RNA from the sixth round of selection was converted to cDNA, subjected to PCR, and cloned. Out of 60 clones, only 33 different sequences were obtained, suggesting that the complexity of the library after the sixth round was rather low. Two sequences did not contain a 3′-terminal CCA and therefore could not be aminoacylated and would not bind EF-Tu. Presumably these molecules arose during the cloning of the PCR fragment into the vector. The remaining sequences can be categorized into two groups based on the sequence of the nine randomized positions (Table 2). Group I consists of 70% of the clones

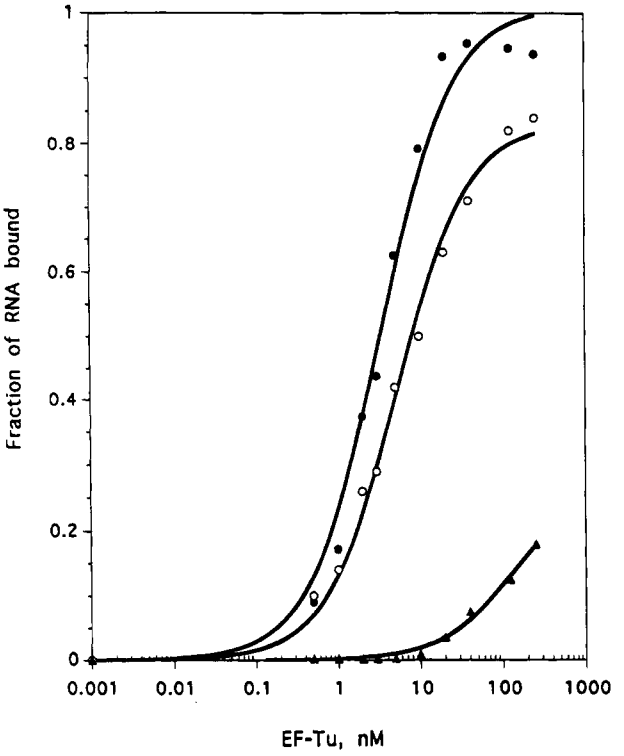

FIGURE 4: Binding of Ala $tRNA^{Ala}$ transcript (●), RNA library before selection (▲), and RNA library after six rounds of selection (○) to *T. thermophilus* EF-Tu. The RNase protection assays were performed in buffer C.

and is characterized with a completely conserved 5′-U and a consensus sequence 5′-UNDUGACUY (N = U, C, A, G; D = U, G; Y = U, C). Group II showed strong preference for GUG in first three 5′-termini positions, but was more variable in the remaining six nucleotides, and therefore may represent few subclasses. For example, five variants in this group have selected 5′-G and variable nucleotides in the next two positions, but a conserved U6C7A8A9. Although most of the cloned sequences are unchanged in the regions outside the nine variable nucleotides, mutations in the T-arm are occasionally found. Most common (in eight clones) is a mutation at position 55, which is probably due to misincorporation of the nucleotide adjacent to the primer in the reverse transcriptase reaction. Four clones have mutations at positions 52 and one at position 54. These mutations are unlikely to affect EF-Tu binding since RNAs that have wild type nucleotides at these positions and an identical selected sequence bind EF-Tu with the same affinity (see below).

RNAs were prepared from a subset of the clones and aminoacylated, and the affinity to EF-Tu was determined by the nuclease protection assay. The data are presented in Table 3. Aminoacylated RNAs from both groups bind EF-Tu•GTP with an affinity comparable to that of the Ala-$tRNA^{Ala}$ transcript. Equilibrium dissociation constants for most of the variants are in the range of 9–40 nM. In group I, **12** had the highest affinity and had the sequence 5′-UUGUGACUC in the selected region. Point mutations of **12** at positions 2 (**53**), 3 (**38**), and 9 (**19**) cause quite small decreases in the affinity to EF-Tu•GTP, and the multiple mutant (**1**) does not bind significantly less well. It is likely that the U8C mutation also has a small effect on binding (**26**). So, nucleotides in positions 2 and 3 of the selected sequences in Group I are not conserved, and in positions 8 and 9, uridine and cytidine can provide equal affinity of the

Table 2: RNA Sequences After Six Rounds of *in Vitro* Selection

Variant number	Sequence in randomized region 1 2 3 4 5 6 7 8 9	Mutations in nonrandomized regions
Group I		
1, 44, 50	U G U U G A C U U	-
20, 35	U G U U G A C U U	U55A
58	U G U U G A C U C	-
19, 46, 60	U U G U G A C U U	-
3	U U G U G A C U U	U55A
12, 14, 30, 57, 63	U U G U G A C U C	-
38, 39, 52	U U A U G A C U C	-
46	U G A U G A C U C	U55A
26	U G A U G A C C C	-
36	U A A U G A C U C	-
53,55	U G G U G A C U C	-
41	U G G U G A C U C	U55A
2	U C U U G A C U C	-
43	U U G G A C U A C	G52A
51	U G U U U A C C	U55C
54	U G U A U A C C	U55C
Group II		
8	G U A U C A C U U	U55C
4	G U G A C A C U U	-
5	G U G C A G A G A	U55C
45	G U G C A G A G A	G52C
6	G U G C G C U G U	-
33	G U G C G C U G C	G52A
24	G U G C G C G A C	U55C
47	G U G U A C U U C	-
56	G U G U A U U C U	U54A, U55C
61	G U G U A C C U U	-
31	G U G A U C U U A	G52C
22	G U A A U U C A A	-
28, 37	G A G U A U C A A	-
40	G U C A A U C A A	-
59	G G G G U U C A A	-

Table 3: Equilibrium Dissociation Constants for Ternary Complexes Formed with Aminoacylated Selected RNAs in Buffer B

Variant number	Selected Sequence 1 2 3 4 5 6 7 8 9	Kd (nM)
Ala-tRNA^{Ala} transcript		10
Group I		
12	U U G U G A C U C	4.5
19	U U G U G A C U U	13
53	U G G U G A C U C	9
38	U U A U G A C U C	10
1	U G U U G A C U U	15
26	U G A U G A C C C	20
46	U G A U G A C U C	10
3	U U G U G A C U U	12
54	U G U A U A C C	50
43	U U G G A C U A C	2500
Group II		
8	G U A U C A C U U	19
4	G U G A C A C U U	11
5	G U G C A G A G A	7
33	G U G C G C U G C	8
47	G U G U A C U U C	8
31	G U G A U C U U A	10
28	G A G U A U C A A	9

Table 4: Dissociation and Association Rate Constants for EF-Tu Binding in Buffer A

RNA transcript	k_{-1} (s^{-1})	k_1, calcd ($M^{-1}\cdot s^{-1}\cdot 10^{-5}$)
E. coli Ala-tRNA^{Ala}	0.0004	1.3
12	0.0005	3.3
Ala minihelix	0.0010	0.26

RNA toward EF-Tu. Two representatives of Group I, **54** and **43**, bind EF-Tu 10 and 500 times weaker than **12**, presumably due to multiple mutations of the conserved residues.

All of the members of Group II bind EF-Tu less well than **12**, but in the same range as most of the members of Group I. Since there is much more variability in the randomized regions of the Group II RNAs, and the consensus sequence is not so pronounced as in Group I, the data are insufficient to make firm conclusions about its sequence requirements. It is clear, though, that this group represents quite a different way to achieve tight EF-Tu binding.

Table 4 compares the dissociation rate constants of aminoacylated **12** with those of the Ala minihelix, anticodon deleted Ala-tRNA^{Ala}, and intact Ala-tRNA^{Ala} in the 150 mM NH_4Cl buffer. By using the binding constants, the association rate was calculated. It is clear from these data that the kinetic properties of **12** closely resemble tRNA^{Ala}, providing additional evidence that it is an appropriate internal deletion for EF-Tu binding. Interestingly, the Ala minihelix has an almost similar k_{-1}, but a smaller k_1 than the other RNAs.

DISCUSSION

While the construction of internal deletions of RNA is conceptually straightforward, the process can be quite difficult experimentally. Even in the case of tRNA, where the known tertiary structure permits intelligent choice of locations for the cleavage sites, the identification of proper linker sequence may depend upon the tRNA molecule used. This is well illustrated by the experiments measuring EF-Tu binding to tRNAs missing anticodon hairpin, a part of the molecule not expected to interact with the protein. Earlier *in vitro* selection experiments with yeast tRNA^{Phe} had identified a subset of 256 tetramers that could be used to replace the anticodon hairpin and not disrupt folding (Dichtl et al., 1993). Since at least one of these tetramers (UUAA) could replace the anticodon arm of *E. coli* tRNA^{Phe} and the acceptor arm of yeast tRNA^{Phe}, it was possible that the same sequence could be used to replace any RNA helix. However, while the UUAA variant of yeast Phe-tRNA^{Phe} bound EF-Tu very well, the corresponding variant of *E. coli* tRNA^{Ala} appeared to partition into two forms, one which bound EF-Tu normally and one that did not bind at all. Since *E. coli* tRNA^{Ala} is expected to have a structure quite similar to that of *E. coli* and yeast tRNA^{Phe}, UUAA should be able to span the distance between residues 26 and 44 in a similar way. Thus, in the context of the tRNA^{Ala} sequence, it appears that the UUAA sequence participates in an alternative RNA secondary or tertiary structure that is not able to bind EF-Tu. Presumably, another tetramer sequence could serve in tRNA^{Ala}, but in turn may not work with tRNA^{Phe}. Thus, the linker sequence used to replace internal deletions will have

to be identified by testing individual sequences or performing selection experiments.

The tRNA minihelix is an almost perfect internal deletion of tRNA (McClain et al., 1987). In the X-ray structure of $tRNA^{Phe}$, the A7−U66 pair is stacked on the A49−U65 pair, so that the 3′-oxygen of ribose 7 is within 5.5 Å of phosphate 49. Thus, the formation of a 2 Å covalent bond between these atoms would only result in a slight local adjustment of the stacking interaction and a straightening of the 14° bend between the acceptor and T-stems (Holbrook et al., 1978). It is also likely that the structure of the T-loop will be different in the minihelix because of the absence of the D-loop tertiary interactions. Recent experiments by J. Rudinger et al. (1994) have shown that aminoacylated minihelix of yeast $tRNA^{Asp}$ binds to EF-Tu nearly as well as intact Asp-$tRNA^{Asp}$ in a low ionic strength buffer. However, they found that the G53C−C61G mutation previously shown to decrease the affinity of yeast Phe-$tRNA^{Phe}$ for EF-Tu (Nazarenko et al., 1994) did not alter the minihelix affinity. This suggested that the minihelix was a good, but not perfect, model for EF-Tu binding. We have reached a similar conclusion in experiments with the $tRNA^{Ala}$ minihelix. In a low salt buffer, the Ala minihelix also binds nearly as well as intact tRNA. However, at higher salt, where Tu binds tRNA better, the minihelix binds considerably worse. It is striking that its 15-fold higher binding constant is mostly the result of a slower association rate rather than the faster dissociation rate. It has previously been noticed that EF-Tu even binds intact aa-tRNAs with an abnormally slow association rate (Louie & Jurnak, 1985), perhaps reflecting a rate limiting conformational rearrangement. Similar decrease of the association rate was also caused by the substitution of GTP by GTP analogues in ternary complex and by some mutations of the conserved nucleotides of tRNA (Nazarenko et al., 1994). This suggests that the minihelix may by unable to carry out such a rearrangement step normally.

An *in vitro* selection strategy was adopted to find an alternative internal deletion of $tRNA^{Ala}$ that bound EF-Tu better than the minihelix. The choice of a library containing 9 randomized nucleotides between residues 7 and 49 was influenced by some data suggesting participation of variable loop nucleotides in ternary complex formation (Jekowsky et al., 1977; Wikman et al., 1982; Joshi et al., 1984). Since the major recognition element of alanine tRNA-synthetase is in a region that was not randomized, the preliminary aminoacylation step with excess enzyme did not introduce a strong selective pressure. Although the mixture of 2.6×10^5 different molecules binds EF-Tu much less well than the minihelix, a small number of sequences emerged at the end of the selection that bound the protein just as well as the intact $tRNA^{Ala}$ and about 15 times better than the minihelix. An analysis of the kinetics of binding of one of the selected variants indicated that it had an association rate constant similar to that of $tRNA^{Ala}$ instead of the abnormally slow value observed for the minihelix. As is often the case in the selection experiments, two distinct classes of sequences were found in the 9 nucleotide linker that resulted in tight protein binding.

What structural features of the selected variants made them superior to the minihelix and the large number of other sequences present in the library? One possibility is that the selected molecules make additional contacts with EF-Tu that increase the affinity. Even though U1, U8, and C9 in the class I selected sequences can be thought to correspond to U8, U47, and C48 in $tRNA^{Ala}$, it is unlikely that they reflect contacts with nucleotide functional groups. Previous mutagenesis experiments in $tRNA^{Phe}$ (Nazarenko et al., 1994) have eliminated the conserved U8 and the semiconserved U47 as important determinants in EF-Tu binding. In addition, the very different class II sequences show no obvious resemblance to either class I selected sequences or $tRNA^{Ala}$. It is possible, however, that the selected sequences form a specific structure that contacts the protein through the ribose−phosphate backbone. Such backbone contacts may involve the same amino acids that are used with tRNA, or they may be adventitious and unique to each selected sequence. It is also possible that the selected 9 nucleotide sequences do not contact the protein directly, but provide the necessary structural or dynamic properties so that the rest of the molecule can bind optimally. For example, the selected residues may maintain the 14° tilt between the acceptor stem and T-stem or the backbone distortion around the A7−U66 and A49−U65 pairs. Alternatively, the selected residues may permit a structural change upon protein binding that is more difficult with the minihelix.

A number of experiments can be proposed that could distinguish between the two classes of models described above. If the selected 9 nucleotides are forming backbone contacts, these contacts can be identified by phosphorothioate or deoxynucleotide modification interference experiments (Milligan & Uhlenbeck, 1989; Gaur & Krupp, 1993; Kleineidam et al., 1993). If the selected sequences only provide certain structural or dynamic properties to the rest of the molecule, it should be possible to achieve these properties with shorter inserted sequences that can be identified by another selection experiment.

The availability of an internal deletion of tRNA that is fully active in EF-Tu binding should be useful in a number of experiments studying the details of EF-Tu−tRNA interaction. The smaller size of the substrate should simplify the location of important backbone contacts by modification interference experiments and the synthesis of RNAs with unique chemical modifications. Studying the RNA conformation when bound to the protein by NMR and other spectroscopic methods would be greatly simplified with a smaller substrate. Finally, the removal of the parts of tRNA that are not contacted by EF-Tu may aid in cocrystallization by providing new opportunities for crystal packing contacts.

It is likely that the ternary complex with the selected RNA substrate will bind the ribosomal A-site with an affinity similar to or perhaps higher than that of noncognate tRNA (Thompson & Dix, 1982). Evaluating its function in dipeptide synthesis and subsequent translocation will complement previous studies with the isolated anticodon loop (Rose et al., 1983).

Finally, it will be interesting to evaluate how well the RNA selected for EF-Tu binding will function with other enzymes that interact with tRNA. For example, the minihelix is a good but not excellent substrate for *E. coli* RNase P (McClain et al., 1987). Recent experiments by G. Tocchini-Valentini et al. (personal communication) suggest that the variants selected here are also better than the minihelix for *Xenopus* RNase P. This suggests that the substrate recognition properties of these two very different enzymes may be similar.

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