In Vitro Selected RNA Molecules That Bind to Elongation Factor Tu†

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ABSTRACT: RNA molecules which bind to elongation factor Tu from *T. thermophilus* were isolated from a pool of ribooligonucleotides with a randomized sequence region. These RNAs interact with elongation factor Tu in both the GTP and the GDP form. A slight preference for the GTP form of the protein was observed. The isolated RNA aptamers compete with each other for a common binding site on elongation factor Tu. This binding site is different from the binding site for aminoacyl-tRNA or the binding site for elongation factor Ts and is located on domain II of elongation factor Tu. The selected RNAs do not bind to elongation factor G. The EF-Tu binding RNAs share a short consensus sequence, 5′-ACCGAAG-3′, which was also found in the α-sarcin domain of *T. thermophilus* 23S rRNA. The isolated RNAs have a hairpin structure with the 5′-ACCGAAG-3′ sequence located in non-base-paired regions. Chemical probing and deletion experiments indicate that the consensus sequence is required for the interaction with elongation factor Tu.

The elongation factor Tu (EF-Tu) is a GTP/GDP binding protein which controls the binding of aminoacyl-tRNA to the ribosomal A-site and codon-reading by the anticodon of aminoacyl-tRNA (*1*). EF-Tu occurs either in GDP or in GTP form which substantially differ with respect to the mutual position of the three domains of the protein and in the conformation of domain I (*2*−*4*). EF-Tu in complex with GTP binds aminoacyl-tRNA with high affinity, regardless of the structure of the aminoacyl moiety (*5, 6*). The structure of the ternary complex formed by yeast Phe-tRNA, *T. thermophilus* EF-Tu, and a slowly hydrolyzing GTP analogue, GppNHp, has been solved (*3*). Surprisingly, only few molecular contacts exist in this ternary complex between tRNA and the protein. The aminoacyladenosine on the 3′-end of aminoacyl-tRNA interacts with domain II of EF-Tu near the interface between domain II and the nucleotide binding domain I. The 5′-phosphate and part of the TΨC stem of tRNA make contacts with domain III of EF-Tu.

The elongation cycle of protein biosynthesis starts with the binding of the aminoacyl-tRNA•EF-Tu•GTP ternary complex, a process which involves several steps (*7, 8*). The initial binding of the ternary complex to the ribosome is followed by codon−anticodon interaction between tRNA and mRNA. Subsequently, GTP is cleaved, and EF-Tu•GDP dissociates from the ribosome. The structural information which is available about the complex between the ribosome and EF-Tu is rather fragmentary. It was demonstrated that the α-sarcin domain, a structural element around position 2670 of *T. thermophilus* 23S rRNA (*9*) which consists of an RNA stem and a hairpin−loop, is important for factor-related functions. This structural element is conserved in all RNAs of the large ribosomal subunits. The bacterial elongation factors Tu and G protect several bases in this region from chemical modification (*10*). The cleavage after G2661 in *E. coli* 23S rRNA with α-sarcin blocks the binding of both elongation factors (*11*). It was also shown that a single-base substitution from guanine to cytosine at position 2661 of *E. coli* 23S rRNA results in the reduction of elongation rate and an altered affinity of the ribosome for the aminoacyl-tRNA•EF-Tu•GTP ternary complex. However, this effect can be compensated by mutations in the ribosomal protein S12 (*12*). The importance of the α-sarcin domain for the interaction of elongation factors with the ribosome was also demonstrated by a C to U mutation at position 2666 in the α-sarcin domain of 23S rRNA of *E. coli* (*13*). In this work, the formation of a standard Watson−Crick base pair extending the stem region led to increased levels of stop codon readthrough and frameshifting. This experiment indicates that nucleotides which are located within the stem region of the α-sarcin domain are important for the interaction with EF-Tu. It has been suggested that the conformation of the α-sarcin loop changes during the elongation cycle (*14, 15*) and that the elongation factors Tu and G interact sequentially with specific conformations of the α-sarcin domain. Accordingly, different conformations for the α-sarcin domain may exist (*15, 16*). The elongation factors are thought to catalyze the conformational change of the 23S rRNA which is required for a particular step (*17*). Which conformation of the α-sarcin loop is required for binding EF-Tu or EF-G, or which parts of this rRNA domain are crucial for the interaction between the ribosome and EF-Tu or EF-G, remains unanswered.

In this work, we used an in vitro selection procedure to find RNA molecules which reflect the state in which rRNA interacts with EF-Tu. The aim was to identify an EF-

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Tu•RNA complex and to describe the structure of the RNA which interacts with EF-Tu.

MATERIALS AND METHODS

Materials. Nitrocellulose sheets (BA 85, 0.45 μm) were obtained from Schleicher & Schuell (Dassel, Germany). C-terminally His-tagged EF-Tu, wild-type EF-Tu, EF-Ts, and EF-G were purified from overproducing *E. coli* cells (*18*−*20*). The isolated domains of EF-Tu were purified as described (*21, 22*). The DNA template for the starting RNA pool was provided by M. Yarus, University of Colorado, Boulder, CO. T7 polymerase was prepared as described by Zawadzki and Gross (*23*). AMV reverse transcriptase was from U.S. Biochemicals Corp. (Cleveland, OH). Taq polymerase was from MBI Fermentas (Vilnius, Lithuania). T4 polynucleotide kinase, alkaline phosphatase, RNase T1, and DNase I were purchased from Boehringer (Mannheim, Germany). The Ni−NTA agarose was from Qiagen (Hilden, Germany). Other reagents used in this work were of the highest quality obtainable. Radioactive nucleotides were purchased from Hartmann Analytik (Braunschweig, Germany).

Nucleic Acids. RNAs were synthesized by in vitro transcription from PCR-amplified DNA oligonucleotides by T7 RNA polymerase. PCR amplification was performed in 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl (pH 9.0), 1.25 mM $MgCl_2$, and dNTPs (present at 0.2 mM each). Taq polymerase was used at 2.5 units per 100 μL reaction. Transcription was performed in 40 mM Tris-HCl (pH 8.1), 22 mM $MgCl_2$, 5 mM DTE, 1 mM spermidine, 0.01% Triton X-100, and 4 mM NTPs. The reaction mixture was incubated at 37 °C for 3 h and then treated with 10 units of DNase I for an additional hour. The RNA transcript was ethanol-precipitated, loaded on a 8% polyacrylamide/8 M urea gel, visualized by UV shadowing, excised from the gel, and eluted with 0.3 M sodium acetate. The transcript was ethanol-precipitated and redissolved in water. Transcription of ^{32}P-labeled RNA was performed as described, except that 10 μCi of [α-^{32}P]GTP was present in the reaction mixture.

Cloning of the nucleic acid repertoire was performed using double-stranded DNA that was generated from the RNA by reverse transcription and PCR amplification. PCR-amplified DNA was digested with the restriction enzymes *Hin*dIII and *Eco*RI, ligated into compatible sites within pUC19, and transformed into the *E. coli* strain DH5α. Plasmids were isolated using a Flexiprep-Kit (Pharmacia), and sequencing of the DNA was performed using a T7-Sequencing-Kit (Pharmacia). *E. coli* $tRNA^{Val}$ was isolated and aminoacylated as described by Nawrot et al. (*24*).

Selection of RNA. The RNA selection was done as described by Tuerk and Gold (*25*) using a DNA template containing a 50-nucleotide variable sequence. The selection was performed by immobilizing 8.5 nmol of His-tagged EF-Tu•GTP on approximately 50 μL of sedimented Ni−NTA agarose. The material was washed with selection buffer which contained 50 mM Tris-HCl (pH 7.5), 1 mM GTP, 50 mM NaCl, and 7 mM $MgCl_2$ to remove excess protein. Prior to each round of selection, 1 nmol of RNA was incubated for 3 min at 85 °C, cooled to ambient temperature, and treated with 100 μL of Ni−NTA agarose in selection buffer to remove the RNA that binds to the Ni^{2+}−agarose. The RNA that did not bind to Ni−NTA was then incubated with Ni−NTA-immobilized EF-Tu•GTP. After removal of the unbound RNA with four washing steps with 200 μL of selection buffer, the protein−RNA complexes were eluted from the Ni−NTA agarose with 250 mM imidazole in the selection buffer. The protein was removed by phenol extraction, and the RNA was ethanol-precipitated. The selected RNA was reverse-transcribed in a total volume of 20 μL with 50 mM Tris-HCl, pH 8.7, 40 mM KCl, 6 mM $MgCl_2$, 0.5 mM dNTPs, and 50 units of AMV reverse transcriptase for 1 h at 42 °C. The cDNA was PCR-amplified and transcribed into RNA as described above. The selection−amplification experiment was repeated 9 times.

Probing of RNA Structure. The minimal length of the RNAs which is sufficient for interacting with EF-Tu was determined according to Ringquist et al. (*26*). The RNA was radioactively labeled on 5′-ends and on 3′-ends, respectively. The labeled RNA was purified by electrophoresis on an 8% denaturing polyacrylamide gel. Partial hydrolysis was performed by incubation of RNA in 10 μL of 50 mM $NaHCO_3$ (pH 9.0) and 1 mM EDTA for 10 min at 90 °C. The reaction was stopped by cooling to −70 °C. Binding to EF-Tu was assayed with immobilized EF-Tu•GTP as described above for the selection procedure. Bound RNA was recovered and loaded on a 12% polyacrylamide gel for electrophoresis. A sequence ladder of the RNA was generated by partial RNase T1 digestion in a 5 μL reaction mixture containing 1 pmol of end-labeled RNA, 5 units of RNase T1, 7 M urea, 1 mM EDTA, and 20 mM sodium citrate (pH 5.0). The reaction mixture was incubated for 5 min at 50 °C and stopped by cooling to −70 °C.

Modification of RNA with dimethyl sulfate was done according to reported methods (*27, 28*).

For Fe^{2+}-EDTA cleavage, 0.8 pmol of 5′-end-labeled RNA and 1 nmol of EF-Tu•GDP were incubated at 0 °C for 5 min in 20 μL of selection buffer, and then 3 μL of a solution of 100 μM $Fe(NH_4)_2(SO_4)_2$•$6H_2O$, 12.5 mM ascorbic acid, and 1 mM EDTA was added. After incubation at 0 °C for a further 4 min, the reaction was stopped by adding 2 μL of 3 M sodium acetate (pH 6.5). The RNA was ethanol-precipitated and loaded on a 12% denaturing polyacrylamide gel.

Limited cleavage with nuclease S1 was achieved by incubation of 0.4 pmol of 5′-end-labeled RNA and 1 μg of *E. coli* $tRNA^{bulk}$ in 4 μL of 50 mM NaCl, 5 mM $ZnCl_2$, 5% glycerol, 30 mM sodium acetate, pH 5.0, and 1 unit of nuclease S1 at 37 °C. The reaction was terminated with 3 μL of gel loading buffer and analyzed by electrophoresis on a 12% polyacrylamide gel.

Filter Binding Assays. Filter binding assays were done according to Carey et al. (*29*). Reaction mixtures contained 10 nM ^{32}P-labeled RNA and protein in concentrations ranging from 0.01 to 10 μM in selection buffer. EF-Tu•GTP or EF-Tu•GDP was serially diluted to the desired concentrations. The reaction was initiated by addition of protein solution to the reaction mixture. After 5 min at 4 °C, 350 μL of the solution was applied to a presoaked nitrocellulose filter. The filtration time was 4 s. The filters were dried and counted. The background, less than 5% of total counts, obtained on the filter with RNA in the absence of protein was subtracted.

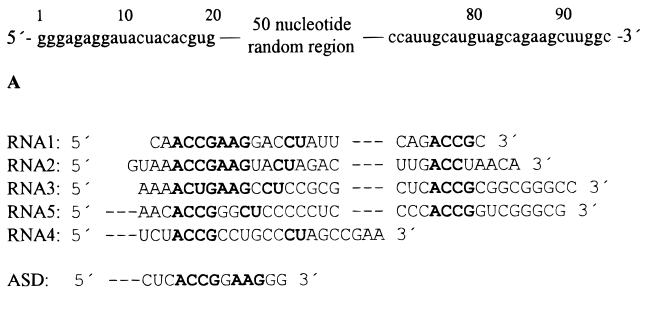

FIGURE 1: (A) Construction of the initial oligoribonucleotide pool for the selection−amplification experiment. (B) Nucleotide sequences of the originally randomized part of the selected RNAs. Hyphens indicate deletions. A part of the sequence of the α-sarcin domain (ASD) of 23S rRNA of *T. thermophilus* is shown for comparison. The consensus sequence elements are in boldface.

Competition assays contained 50 nM ^{32}P-labeled RNA and 10 μM EF-Tu•GDP or EF-Tu•GTP•Val-tRNA^{Val} in selection buffer. Val-tRNA^{Val} and the EF-Tu•GTP•Val-tRNA^{Val} ternary complex were prepared as described (*30*). The complexes between EF-Tu and labeled RNA were formed at 4 °C for 10 min. Unlabeled competitor RNA was diluted to the desired concentrations and added in 5 μL aliquots to the preformed complex to result in final concentrations ranging from 10 nM to 10 μM. After incubation for 5 min on ice, the reaction mixtures were applied to nitrocellulose filters as described above.

Polyacrylamide Gel Electrophoresis. Electrophoresis experiments with EF-G, EF-Tu•GTP, EF-Tu•GTP•Val-tRNA^{Val}, and EF-Tu domains were done on 6% polyacrylamide gels under nondenaturing conditions. The gel buffer contained 25 mM Tris, 25 mM acetic acid, and 5 mM magnesium acetate. The complexes between RNA and protein were preformed in a total volume of 10 μL for 5 min on ice in selection buffer from 200 pmol of unlabeled RNA, 0.1 pmol of 5′-end-labeled RNA, and 200 pmol of protein. After addition of 2 μL of loading buffer (30% glycerol, 0.25% dyes), the gels were run for 1 h at 100 V. Radioactive bands were detected by electronic autoradiography with an Instant Imager (Packard Instruments, Meriden, CT).

RESULTS

In Vitro Selection of RNA. RNA which binds to *Thermus thermophilus* EF-Tu was selected from a RNA pool of approximately 10^{13} RNA molecules consisting of a 50 nucleotide long random region and 2 flanking constant regions (Figure 1). This mixture was submitted first to Ni−NTA agarose to eliminate RNAs binding to the matrix itself. His-tagging of proteins and affinity chromatography on Ni−NTA agarose is widely used for protein purification. Therefore, the procedure described here offers a general procedure for in vitro selection of protein binding RNA ligands. However, it is essential to perform a preselection step and to fully saturate the Ni−NTA with protein in order

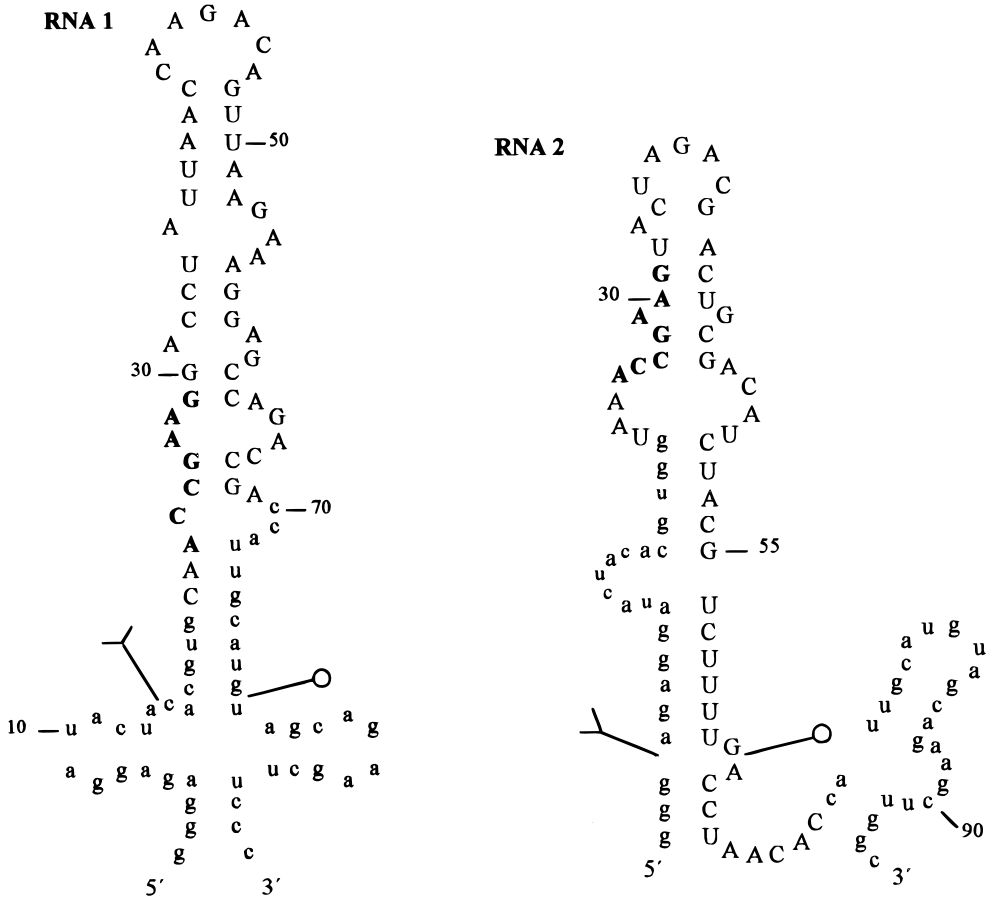

FIGURE 2: Secondary structures of the selected RNA 1 ($\Delta G°$ −31 kcal/mol) and RNA 2 ($\Delta G°$ −25 kcal/mol) as predicted by lowest $\Delta G°$ values using the algorithm of Zucker (*34*). The results of the boundary analyses are indicated with (>—) for 3′-boundaries and (○—) for 5′-boundaries. The consensus sequence of both RNAs is in boldface.

to circumvent the selection of RNA ligands which bind to Ni^{2+} (*31*). To achieve specific binding to Ni−NTA-immobilized EF-Tu, it proved to be advantageous to use a batch procedure. As compared to affinity chromatography with RNA in the mobile phase and His-tagged EF-Tu on the Ni−NTA matrix, the batch procedure allows a better control of volumes for equilibration, washing and elution steps. RNA which bound to EF-Tu was eluted with imidazole and was reverse-transcribed into cDNA. The subsequent, enriched RNA pool was obtained by in vitro transcription (*25*). After nine rounds of selection and amplification, the RNA population was reduced to a pool of RNA molecules that displayed high affinity for EF-Tu (Figure 1B). DNA obtained from this pool was cloned, and 40 clones were sequenced.

Sequence and Secondary Structure of the Selected RNAs. The sequenced clones contained only five different sequences. The most abundant sequences RNA 1, RNA 3, and RNA 4 made up for 32.5%, 30%, and 27.5% of the total cloned sequences, respectively. Some of the sequenced clones had single base exchanges at various positions. Sequences RNA 2 and RNA 5 made up for another 7.5% and 2.5% of the cloned sequences.

Comparison of the five different sequences reveals a short consensus sequence, 5′-ACCG-3′, which is extended to 5′-ACCGAAG-3′ in three of the five sequences (Figure 1B). Moreover, in four of the five sequences, this short sequence element exists twice. All five sequences also contain a 5′-CU-3′ placed 3′ to the 5′-ACCG-3′ element.

The sequences 5′-ACCG-3′ and 5′-ACCGAAG-3′ occur in the α-sarcin domain of *T. thermophilus* 23S rRNA which is believed to be located in the ribosomal binding site for both EF-Tu and EF-G. Recent structural investigations place domain II of EF-Tu also in the vicinity of the 30S subunit of the ribosome (*32*) where the ribosomal proteins S4, S5, and S12 are localized together with the 530 stem-loop domain of the 16S rRNA (*33*).

The predicted secondary structures (*34*) of the isolated RNA molecules shown in Figure 1 have the potential to form relatively stable hairpin structures with structurally rather undefined 3′- and 5′-termini. Structures of RNA 1 and RNA 2 are representatively shown in Figure 2. All sequences also share a tendency to form bulges or internal loops in the middle of their stems. Strikingly, the 5′-ACCG-3′ consensus sequence is always found in these bulges (Figure 2). In accordance with reports that point to the importance of single-stranded bulged or looped sequence elements for RNA function (*35*), the 5′-ACCG-3′ consensus sequence may be involved in EF-Tu binding.

Boundary experiments were performed to determine whether the whole hairpin structure together with the bulged regions is required for binding to EF-Tu. As can be seen in Figure 2, truncation of the stem or removal of the loop is not tolerated. The 5′-ACCG-3′ element is always within the boundaries of the reduced structures.

RNA 1 and RNA 4 were investigated by chemical and enzymatic modification experiments. Figure 3 provides a summary of the experiments performed with RNA 4. They corroborate the prediction of the structure shown in Figure 3. RNA 4 was also treated with hydroxyl radicals in the absence and presence of EF-Tu. The bulged region including the 5′-ACCG-3′element seems to be involved in the formation of the complex between the RNA and EF-Tu. However, the loop region and the G26-A33 base pair are also protected in the presence of EF-Tu (Figure 3).

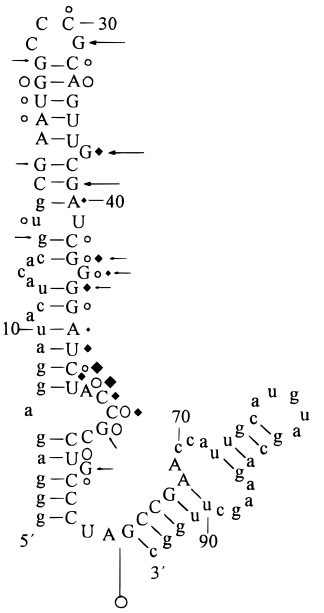

FIGURE 3: Secondary structure of RNA 4. (○) Residues modified by OH radicals. The size of the circle corresponds to the extent of protection with EF-Tu from cleavage. Bases which have been methylated by DMS are indicated with (→). The size of the arrows correlates with the extent of modification. S1 cleavage sites are marked with (◆); the size of the symbol correlates with the extent of cleavage. The 3′-boundary is indicated with (○—). A 5′-boundary could not be detected in this case and is identical with the 5′-end of the RNA.

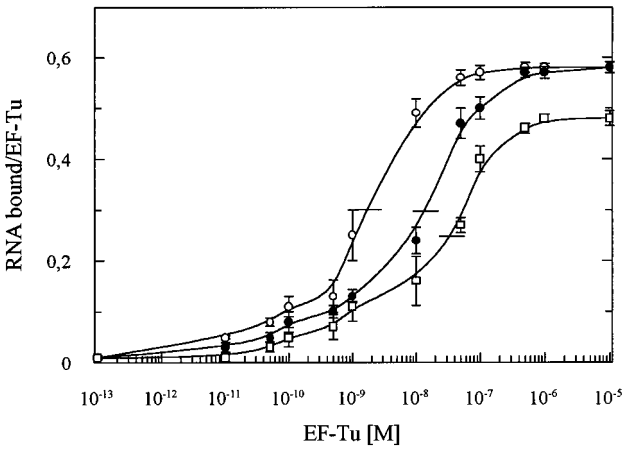

FIGURE 4: Binding of 5′-^{32}P-labeled RNA 1 to EF-Tu•GDP (●) and EF-Tu•GTP (○) and binding of 5′-^{32}P-labeled RNA 4 to EF-Tu•GTP (□). The concentrations at half-saturation providing the K_D values (Table 1) are indicated with a horizontal line.

Interaction of the Aptamers with EF-Tu. The interactions between the RNA aptamers and EF-Tu were investigated by filter binding experiments (*29*). For this, RNA of constant concentration was incubated with increasing amounts of EF-Tu•GDP and EF-Tu•GTP, respectively, and filtered through nitrocellulose membranes (Figure 4). The complex between the selected RNAs and EF-Tu was retained on the filter. The various RNAs show a different affinity for EF-Tu. In the case of RNA 1, the K_D is 10 times lower for EF-Tu•GTP than for EF-Tu•GDP. This difference is smaller in the case of other RNAs (Table 1). In contrast to aminoacyl-tRNA

Table 1: Apparent Dissociation Constants for the Interactions of Selected RNAs with EF-Tu·GTP and EF-Tu·GDP, Respectively

RNA	EF-Tu·GTP K_D (M)	EF-Tu·GDP K_D (M)
RNA 1	2.0×10^{-9}	2.0×10^{-8}
RNA 2	1.0×10^{-7}	1.0×10^{-7}
RNA 3	5.0×10^{-8}	7.0×10^{-8}
RNA 4	3.0×10^{-8}	6.0×10^{-8}

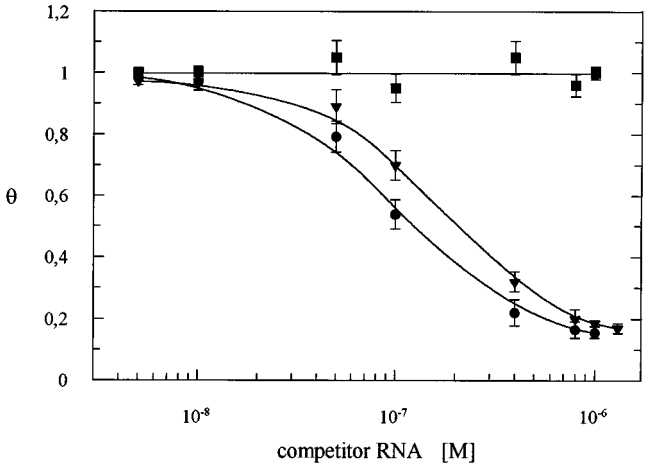

FIGURE 5: Competition for EF-Tu binding between labeled RNA 1 and RNA 4 (▼), RNA 3 (●), and Val-tRNA^{Val} (■), respectively. θ is defined as the ratio of radioactivity bound to the filter in the presence of competitor to the radioactivity in the absence of competitor.

which has at least 10^5-fold higher affinity for EF-Tu•GTP as compared to EF-Tu•GDP (*36*, *6*), the RNAs isolated by in vitro selection do not differ significantly in their affinity for GDP or GTP conformations of EF-Tu. The results summarized in Table 1 therefore imply that RNA binding takes place in a region of EF-Tu which is not affected by the conformational change between the EF-Tu•GTP and EF-Tu•GDP forms. The affinity of the selected RNAs for EF-Tu is nevertheless very high, and the K_D reaches values typical for aminoacyl-tRNA•EF-Tu•GTP interactions (*36*, *6*, *5*).

Figure 4 demonstrates that even at saturating protein concentrations only 50−60% of the RNA is bound to the filter. Since a similar effect was observed in different binding experiments, it does not appear to be due to incomplete retention of the complexes but rather to the presence of an alternative RNA fold which cannot bind EF-Tu.

To test whether all selected RNAs bind to the same region of EF-Tu, competition experiments were performed. For this, unlabeled RNA was added to an equilibrium mixture of labeled RNA at a saturating concentration of EF-Tu•GTP. After a new equilibrium was reached, the residual retained RNA was determined by filtration on nitrocellulose filters. Provided that both RNAs compete for the same binding site, the competition by the unlabeled RNA should result in a reduction of the amount of labeled RNA retained on the filter. Figure 5 depicts the results for the competition of unlabeled RNA 1 with labeled RNA 3 and RNA 4, respectively. Similar results (not shown) were also obtained with RNA 2. This suggests that the selected RNAs compete with each other probably for the same binding site on EF-Tu. However, we cannot exclude the possibility of a non competitive inhibition with these experiments.

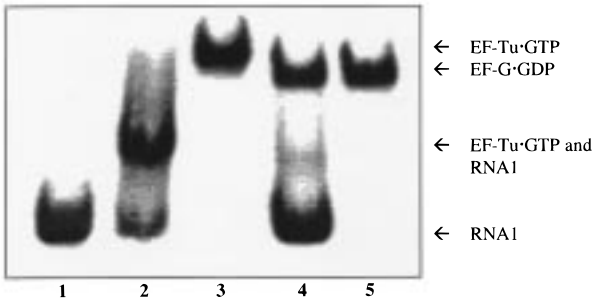

FIGURE 6: Polyacrylamide gel electrophoresis of RNA 1 in the presence of EF-Tu•GTP and EF-G•GDP, respectively. RNA 1 (lane 1), RNA 1 and EF-Tu•GTP (lane 2), EF-Tu•GTP (lane 3), RNA 1 and EF-G•GDP (lane 4), EF-G•GDP (lane 5).

A crucial point of the selection was to exclude that tRNA-like RNAs that bind to EF-Tu had been selected. The described competition assay was therefore used to examine whether there is a competition between Val-tRNA^{Val} and any of the selected RNAs for binding to EF-Tu•GTP. Figure 5 demonstrates that this is not the case. The Val-tRNA^{Val} does not compete with RNA 1. Similar results were obtained for RNA 3 and RNA 4 (not shown).

Domain II of EF-Tu Is the Target for the Selected RNAs. It is believed that EF-Tu and EF-G bind to a similar location on the ribosome (*10*). It was also suggested that the overall shape of the Phe-tRNA^{Phe}•EF-Tu•GppNHp complex is similar to that of EF-G•GDP (*3*). Based on this observation, it was proposed that both elongation factors share a common binding pocket on the ribosome. Therefore, we tested if the RNA selected for EF-Tu binding also binds EF-G. The polyacrylamide gel electrophoresis under native conditions shown in Figure 6 demonstrates that this is not the case. Whereas the ternary complex of EF-Tu•GTP and RNA 1 is readily formed (lane 2), no efficient complex formation can be detected for EF-G•GDP (lane 4). The same results were obtained for EF-G•GTP (not shown). Filter binding assays performed with EF-G instead of EF-Tu confirmed this observation, since no efficient binding of the EF-Tu-selected RNA to EF-G was detected. We therefore conclude that the selected RNA is specific for EF-Tu.

We investigated the location of the binding site of the selected RNAs on EF-Tu in more detail. The binding of the selected RNAs to the isolated domains I, III, II/III, and I/II of EF-Tu from *T. thermophilus* (*21*) was studied by gel retardation experiments (Figure 7). Both RNA 1 and RNA 4 are able to bind tightly to domain II/III (lanes 1−3, panel B). No difference in the stability of the RNA complexes with domain II/III or complete EF-Tu was observed. However, the selected RNAs do not bind to domain III (lanes 6−8, panel A). As visible in lanes 3−5 of panel A, domain I/II is able to bind to both RNA 1 and RNA 4, although the binding is clearly weaker than to domain II/III. On the other hand, domain I by itself is not able to bind to RNA 1 or RNA 4 (lanes 4−6, panel B). In summary, the experiments presented in Figure 7 demonstrate that domain II is crucial for binding the selected RNAs. RNA binding occurs on domain II, even though the presence of domain III seems to stimulate the interaction of the RNA with domain II. As the RNAs do not bind to domain III alone, this fact can perhaps be attributed to the increased stability of domain II in the presence of domain III. It should be noted that the

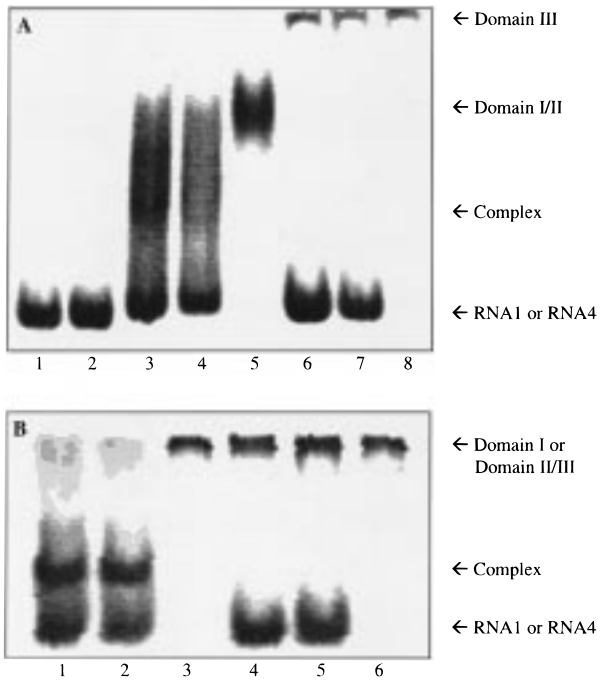

FIGURE 7: Polyacrylamide gel electrophoresis of the complexes between selected RNAs and isolated domains of EF-Tu under nondenaturing conditions. (A) RNA 1 (lane 1), RNA 4 (lane 2), domain I/II and RNA 1 (lane 3), domain I/II and RNA 4 (lane 4), domain I/II (lane 5), domain III and RNA 1 (lane 6), domain III and RNA 4 (lane 7), domain III (lane 8). (B) Domain II/III and RNA 1 (lane 1), domain II/III and RNA 4 (lane 2), domain II/III (lane 3), domain I and RNA 1 (lane 4), domain I and RNA 4 (lane 5), and domain I (lane 6). Coomassie Brilliant Blue and Toluidine Blue were used successively for staining of protein and RNA.

preference of RNA 1 for binding to EF-Tu•GTP as compared to EF-Tu•GDP demands the possibility to sense the alternative conformations of the protein. As domain I is closer to domains II and III in the GTP state of EF-Tu, the higher affinity of RNA1 for EF-Tu•GTP could be due to additional contacts of the RNA to domain I.

The binding of the selected RNAs to domain II raises a question about the possible overlapping of the binding sites of selected RNA and aminoacyl-tRNA. The experiment shown in Figure 8 excludes the possibility of such a common binding site. The gel electrophoresis under native conditions exhibits a band for a quaternary complex between EF-Tu, GTP, aminoacyl-tRNA, and RNA 1 or RNA 4, respectively (lane 8). This result confirms the observations presented in Figure 5, where we showed that Val-tRNA^{Val} does not compete with RNA 1 for EF-Tu binding.

Deletion of the 5′-ACCG-3′ Sequence Element Destroys Binding. To examine the importance of the 5′-ACCG-3′ consensus sequence (Figure 1) for interaction with EF-Tu, this element was deleted from RNA 4. Enzymatic probing of the mutated RNA 4(ΔACCG) with nuclease S1 did not reveal any major changes in the secondary structure compared to the secondary structure of RNA 4 (not shown). Figure 8 demonstrates that the deletion of the 5′-ACCG-3′ element results in loss of binding capacity of RNA 4. In contrast to wild-type RNA 4 (lane 8), RNA 4(ΔACCG) is no longer able to bind to the aminoacyl-tRNA•EF-Tu•GTP ternary complex (lane 4), nor to EF-Tu•GTP alone (lane 2). This result suggests that the 5′-ACCG-3′ element is essential for binding to EF-Tu.

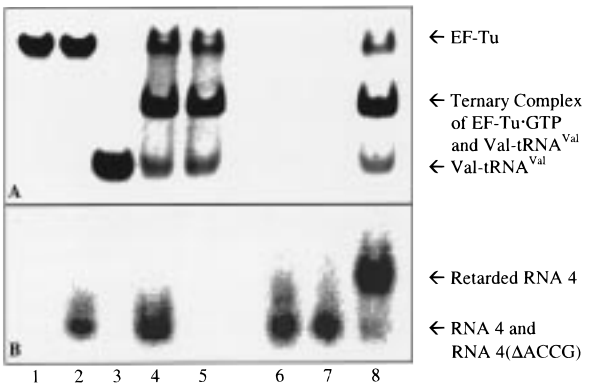

FIGURE 8: Electrophoretic analysis of binding of [^{32}P] RNA 4 and [^{32}P]RNA 4(ΔACCG) to EF-Tu·GDP and the EF-Tu·GTP·Val-tRNA^{Val} ternary complex on a polyacrylamide gel under nondenaturing conditions. (A) shows the gel that was successively stained with Coomassie Brilliant Blue and Toluidine Blue; (B) is the corresponding autoradiography. EF-Tu•GDP (lane 1); EF-Tu•GDP and RNA 4(ΔACCG) (lane 2); Val-tRNA^{Val} (lane 3); Val-tRNA^{Val}, EF-Tu, GTP, and RNA 4(ΔACCG) (lane 4); Val-tRNA^{Val}, EF-Tu, GTP (lane 5); [^{32}P]RNA 4(ΔACCG) (lane 6); [^{32}P]RNA 4 (lane 7); Val-tRNA^{Val}, EF-Tu, GTP, and RNA 4 (lane 8).

DISCUSSION

In vitro selection of RNA which interacts with EF-Tu was used in this work to identify sequences of ribosomal RNA which might be localized in the ribosomal A-site. Short RNAs which resemble ribosomal components provide the possibility to examine features of the interaction between EF-Tu and the ribosome. A similar approach to investigate the ribosomal peptidyl transferase center with the help of in vitro selection has been performed recently (*37*).

However, it has to be kept in mind that in vitro selection cannot precisely reflect a complex in vivo situation. The selected RNAs rather focus on a special aspect of the RNA−protein contact and meet the needs for optimal binding. The contact between the protein and RNA may reflect only one step in a series of events that have to take place during the elongation cycle.

The 5′-ACCG-3′ Consensus Sequence Is Important for Binding to EF-Tu. The five selected RNAs include a short common sequence, 5′-ACCG-3′, which is extended to 5′-ACCGAAG-3′ in three cases. The occurrence of this consensus sequence is statistically significant since the probability to accidentally select from a pool of 10^{13} randomized molecules 5 different species which contain a common 4-mer or 7-mer is low. Furthermore, the removal of this element from one of the selected RNAs (RNA 4) resulted in loss of binding to EF-Tu. However, this may have two reasons. Either the deletion of 5′-ACCG-3′ leads to a loss of the crucial binding element or the deletion causes a conformational change which destroys the binding capacity of the RNA. Chemical modification experiments with RNA 4 and RNA 4(ΔACCG) provide evidence to exclude a major conformational change upon deletion of ACCG from RNA 4. The finding that the ACCG is always located in bulges or internal loops of the RNA molecules is in good agreement with former studies on RNA−protein interactions. In several cases, nucleotides of loops or bulges could be shown to represent the main targets for site-specific recognition of RNA by proteins. This is for example the case with the bulged loop in the HIV TAR RNA, which is the binding

site for the *trans*-activator protein tat (*38*, *39*). It has been proposed that specific recognition of RNA by tat protein is facilitated by bulges or loops because they may open the narrow RNA, major groove and allow recognition of the functional groups on the bases (*35*, *40*). Obviously the location of the 5′-ACCG-3′ element in a bulge is also crucial for binding to EF-Tu. Footprint experiments indicate that these bases and some of the nucleotides in the loop are involved in contacts with EF-Tu. With respect to the obviously small area which is responsible for contacts between EF-Tu and the RNA, it is surprising that it is not possible to delete parts of the RNA structure larger than those determined in boundary studies. A reason for this might be that the ACCG elements of all selected RNAs are situated in structurally flexible and not well-defined parts of the RNA which demand the integration into a larger structural context to be stabilized and correctly presented for the interaction with EF-Tu.

The consensus sequence 5′-ACCG-3′ (5′-ACCGAAG-3′) is present in all selected RNAs and can also be found in the α-sarcin domain of *T. thermophilus* 23S rRNA. The α-sarcin domain was suggested as a binding site for EF-Tu and EF-G. So far the main interest was focused on the loop of the α-sarcin domain whose sequence is one of the most highly conserved sequences known. Studies on depurination by ricin or cleavage by α-sarcin (*41*, *11*) pointed to the importance of the loop region for binding the elongation factors. Recent experiments indicate not only that the loop of the α-sarcin domain is important for proper function of ribosomes but that moreover the stem region of this structure plays a crucial role in recognition of EF-Tu (*13*). Mutations which result in the formation of canonical Watson−Crick base pairs extending the stem of the α-sarcin domain led to increased levels of stop codon readthrough and frameshifting, indicating a weakening of the interaction between EF-Tu and the ribosome. Meyer et al. (*42*) could hybridize an antisense DNA with the 3′-strand of the stem of the α-sarcin domain and block cleavage by α-sarcin, whereas an antisense DNA that only crossed the tetraloop region could not prevent this reaction. The authors conclude that α-sarcin approaches the cleavage site from the 3′-half of the loop. Similar results were obtained by Saxena and Ackermann (*43*), who could show that microinjected oligonucleotides complementary to the α-sarcin domain were more effective as inhibitors of protein synthesis if the oligonucleotides covered not only the α-sarcin and ricin cleavage sites but also part of the stem region. The results of these investigations indicate that the potential binding site for EF-Tu is part of the stem region. In Figure 9 the different secondary structures of the α-sarcin domain are compared. The closed conformation is a structure derived from NMR studies (*16*, *15*). However, the α-sarcin loop is thermodynamically not very stable (*15*) and can undergo a conformational change. This conformational change might trigger a change in ribosomal conformation that is important for elongation (*14*). The possible formation of a tetraloop could then rather be required for tertiary interactions with other parts of the ribosomal RNA, a function that could be shown for other tetraloops (*44*). Cleavage in this tetraloop could lead to the damage of the whole RNA domain and to loss of its function. The implication that loss of elongation factor-dependent functions after cleavage in the tetraloop with α-sarcin is related to the

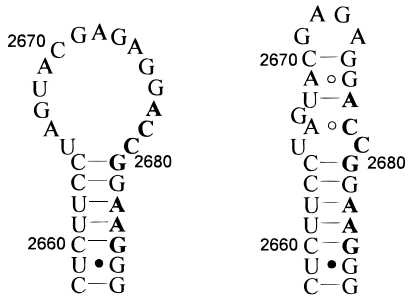

FIGURE 9: Alternative secondary structures of the α-sarcin domain of *T. thermophilus* 23S rRNA. The left structure corresponds to an "open" and the right structure corresponds to a "closed" conformation, based on the NMR-derived structure of the rat cytoplasmic α-sarcin domain (*16*). The symbols (—) and (●) indicate standard Watson−Crick and G−U wobble pairing, respectively; (○) indicates noncanonical pairings.

binding site of EF-Tu and EF-G in the loop of the α-sarcin domain (*11*) is therefore not compulsory.

According to these models, the ACCG element is situated in a sensitive region of the α-sarcin domain that can provide information about the state of this RNA in the complex with the elongation factor.

Domain II of EF-Tu Is Important for Binding RNA. The selected RNAs bind to both EF-Tu•GTP and EF-Tu•GDP with high affinity and form an RNA•aminoacyl-tRNA•EF-Tu•GTP quaternary complex that probably mimics the interaction between the ribosome and EF-Tu. Binding studies with the isolated domains of EF-Tu indicate that domain II is the predominant target for the RNA. This result is in good agreement with studies performed with EF-Tu mutants that suggest a probable binding site for the ribosome on domain II. It was shown that mutation of glycine 222 to aspartate in *E. coli* EF-Tu causes an anomalous interaction with the ribosome both in the absence and in the presence of aminoacyl-tRNA (*45*, *46*), or that the substitution of valine 280 for glycine in *Salmonella typhimurium* EF-Tu causes a severe defect in the interaction of the aminoacyl-tRNA•EF-Tu•GTP ternary complex with the ribosome (*47*). Comparison of the sequences of GTP binding proteins involved in translation reveals that domain II is present in all of these proteins (*48*). If domain II is in fact the domain of EF-Tu which interacts with the ribosome, it is obvious that both EF-Tu•GDP and EF-Tu•GTP will be targets for the selected RNA, as domain II itself does not change upon transition of EF-Tu from the GDP to the GTP form.

It is important to point out that the EF-Tu•RNA complex was selected for optimal stability, whereas in vivo the binding site of EF-Tu on the ribosome has to sustain the dissociation of EF-Tu as well, implicating a dynamic structure of rRNA during translation. We demonstrated in this work that the presentation of the 5′-ACCG-3′ consensus sequence in a single-stranded, rather flexible RNA is required for the formation of a stable complex with EF-Tu. For the α-sarcin domain of the 23S rRNA of the ribosome, this result implies that the binding of EF-Tu demands an open conformation of this RNA domain. Formation of a complex between isolated α-sarcin domain and EF-Tu has not yet been achieved, indicating that the closed structure of the α-sarcin loop in vitro (*16*) might be different from its conformation when it comes to binding EF-Tu in the ribosome. Such a change of the α-sarcin loop conformation during elongation cycles was already proposed (*14*, *15*).

Interestingly, the selected RNA molecules do not bind to EF-G, even though both elongation factors are thought to interact with the same ribosomal binding site. It has already been proposed that EF-Tu and EF-G interact sequentially with the ribosome, recognizing different states, one for each elongation factor, and might even promote the transition to the alternative state (*17*). The results of this work support the mechanism in which both factors interact with the α-sarcin domain of the 23S rRNA. The conformational change in the α-sarcin domain of 23S rRNA regulates in turn the binding of EF-Tu and EF-G. The binding of domain II of EF-Tu requires or induces a conformation of the α-sarcin domain of 23S rRNA in which the flexible stem region is recognized, whereas the binding of EF-G probably requires a closed conformation of the α-sarcin loop (Figure 9).

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Modification experiments with DMS (RNA 4), hydroxyl radicals (RNA 4), and nuclease S1 (RNA 4). Boundary determination for RNA 1 (4 pages). Ordering information is given on any current masthead page.

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