Base complementarity in helix 2 of the central pseudoknot in 16S rRNA is essential for ribosome functioning

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ABSTRACT

Helix 2 of the central pseudoknot structure in Escherichia coli 16S rRNA is formed by a long-distance interaction between nt 17–19 and 918–916, resulting in three base pairs: U17–A918, C18–G917 and A19–U916. Previous work has shown that disruption of the central base pair abolishes ribosomal activity. We have mutated the first and last base pairs and tested the mutants for their translational activity in vivo, using a specialized ribosome system. Mutations that disrupt Watson–Crick base pairing result in strongly impaired translational activity. An exception is the mutation U916→G, creating an A·G pair, which shows almost no decrease in activity. Mutations that maintain base complementarity have little or no impact on translational efficiency. Some of the introduced base pair substitutions substantially alter the stability of helix 2, but this does not influence ribosome functioning, neither at 42 nor at 28°C. Therefore, our results do not support models in which the pseudoknot is periodically disrupted. Rather, the central pseudoknot structure is suggested to function as a permanent structural element necessary for proper organization in the center of the 30S subunit.

INTRODUCTION

The central pseudoknot structure in 16S rRNA, first proposed by Pleij et al. (1), connects the 5′-domain, the central domain and the 3′-domain (2,3). The structure of this pseudoknot is almost universally conserved (4–6). The pseudoknot for Escherichia coli is presented in Figure 1. It consists of a local stem–loop structure, helix 1, formed by base pairing of nt 9–13 and 21–25, and a long distance interaction, helix 2, between nt 17–19 and 916–918.

So far two other pseudoknot structures in small subunit rRNA have been predicted by phylogenetic comparison (7,8). Powers and Noller (9) showed that the pseudoknot, predicted in the 530 hairpin region, is essential for ribosome functioning. Similarly, the pseudoknot formed by a long-distance interaction between nt C3866A3865 and G570U571 was shown to be indispensable for translation by Vila et al. (10).

Functioning of the central pseudoknot was studied by Brink et al. (11), using a specialized ribosome system (12). Mutations that disrupt the central base pair in helix 2, and probably destroy the central pseudoknot, caused loss of translational activity in vivo (11). 30S subunits having such a mutation in their 16S rRNA did not form polysomes, suggesting that the structural element is involved in initiation of translation. Processing of the 5′-end of the 16S rRNA or formation of 30S particles was not affected by the mutations (11). An in vitro analysis showed that the mutant 30S subunits are still capable of forming a 30S initiation complex (13). However, the mutant particles appear physically unstable; they easily lose some of their ribosomal proteins.

Here we describe a mutational analysis of the first and last base pairs in helix 2, U17–A918 and A19–U916 respectively. Mutations that disrupt and restore the base pairing were introduced. Ribosomes containing the mutant 16S rRNA were tested for their translational capacity in vivo, using a specialized ribosome system (11,12,14). In this system E.coli cells contain a plasmid encoding 16S rRNA with an anti-Shine–Dalgarno (ASD) sequence, altered from 5′-CCUCC to 5′-CACAC. These ribosomes recognize a plasmid-encoded CAT mRNA with a corresponding Shine–Dalgarno (SD) sequence, 5′-GUGUG. Chromosomally encoded ribosomes do not translate this CAT mRNA. Therefore, mutations in the specialized 16S rRNA can be tested for their impact on translational activity by measuring in vivo production of chloramphenicol acetyltransferase, the cat gene product. Specialized ribosomes do not interfere with endogenous protein synthesis and debilitating mutations introduced in these ribosomes therefore do not cause growth defects. Furthermore, the mutations do not affect the concentration of wild-type or specialized ribosomes in the cell (unpublished results). Thus translational activities of mutant ribosomes directly reflect their individual efficiency.

The results of our analysis show that complete base pairing in helix 2 is necessary and sufficient for translational activity. Changing the thermodynamic stability of the pseudoknot had no effect on translation, neither at 28 nor at 42°C. Base pair U17–A918 is highly conserved in nature. Therefore, base identity at these positions might be important. However, the pair could be substituted with other pairs without an effect on translation. This suggests that the sequence conservation at these positions does not correlate with an essential role of the bases proper in translational activity.

Our data are also discussed in relation to models that propose a conformational switch in the central pseudoknot during translation (15,16). The results do not favor these conformational rearrangements. We suggest that the central pseudoknot serves as

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Figure 1. Scheme of the central pseudoknot structure in *E. coli*, connecting the three major domains in 16S rRNA. The secondary structure is according to Stern et al. (3). The central pseudoknot consists of helix 1 (nt 9–13/21–25) and helix 2 (nt 17–19/916–918). The arrows indicate the relative orientation of the three major domains protruding from this structure. The first base pair U17–A918 and third base pair A19–G916 of helix 2 are represented with open letters.

a rigid element that is important for structural organization in the center of the 30S subunit.

**MATERIALS AND METHODS**

**Bacterial strains and media**

M13 constructs were grown in *E. coli* strain JM101 (17). As host for the specialized ribosome plasmid pPL ASDX-SpcR-CA TX we used K5637 (11). This strain contains a thermolabile P1 repressor on its chromosome. *Escherichia coli* strain BW313 (18) was used in oligonucleotide-directed mutagenesis. Strains were grown on LC medium (19).

**Construction of the mutants**

Mutants were made by oligonucleotide-directed mutagenesis on plasmid pPL ASDX-SpcR-CA TX, encoding specialized 16S rRNA, can therefore be accomplished by shifting the growth temperature of the cell culture from 28 to 42°C. Ribosomes containing the specialized 16S rRNA recognize a modified CA T mRNA also encoded by the plasmid. Therefore, we can study the effect of mutations in this 16S rRNA on translational activity by CA T assay.

Ribosomal activity at 28°C

Cultures were prepared as described above. After 1 h induction at 42°C an equal volume of LC medium (14°C) was added and growth was continued for 2 h at 28°C. Samples of 1 ml were taken before induction (t = –60 min), at the temperature shift to 28°C (t = 0) and at t = 30, 60, 90 and 120 min after the shift. CA T assays were performed as described above. The amount of [3H]diacetylchloramphenicol synthesized per optical density unit in the sample at t = 0 was subtracted from the total amount to yield synthesis of CAT protein at 28°C.

**RESULTS**

**Determination of the translational activity of mutant ribosomes using a specialized ribosome system**

The specialized ribosome system is incorporated in *E. coli* strain K5637 (11), harboring on its chromosome the thermolabile cI repressor of the phage λ P1 promoter. Expression of the rmb operon on plasmid pPL ASDX-SpcR-CA TX, encoding specialized 16S rRNA, can therefore be accomplished by shifting the growth temperature of the cell culture from 28 to 42°C. Ribosomes containing the specialized 16S rRNA recognize a modified CAT mRNA also encoded by the plasmid. Therefore, we can study the effect of mutations in this 16S rRNA on translational activity by CAT assay.

Cells harboring pPL ASDX-SpcR-CA TX without mutations in helix 2 were used as the wild-type control. The contribution of chromosomally encoded 30S subunits was measured in cells transformed with pPL ASDX-SpcR-CA TXΔKpnI–ApaI. In this plasmid a KpnI–ApaI fragment (900 bp) in the specialized 16S rRNA gene is replaced by a fragment of 300 bp containing murine rDNA. Cells harboring this plasmid do not produce specialized ribosomes. The presence of the various plasmids did not affect the amount of chromosomally encoded ribosomes in the cell (unpublished data; 14).

**Disruptive mutations in helix 2 impair ribosome function**

We introduced mutations that change base pair U17–A918 to A19–U916 in helix 2 of the central pseudoknot into a mismatch. Base pair U17–A918, in this paper referred to as the first base pair (see also Fig. 1), was changed to C17–A918. Figure 2A shows that this mutation causes a decrease in CAT activity to 30% of the wild-type control.

Additional to the C17 mutation, we changed the third base pair of helix 2 from A19–U916 to C19–G916. It seemed possible that the more stable C–G base pair could compensate for the loss in stability caused by the mismatch. However, the activities of the triple mutant and the single mutant were the same (Fig. 2A, compare mutants C17 and C17,C19–G916), suggesting that base complementarity rather than helix stability is essential for function of helix 2. In accordance, a single mutation changing the third pair to C19–U916 also resulted in a residual activity of 30%. Surprisingly, changing the same pair to A19–G916 had very little effect on efficiency of translation. As shown in Figure 2A, activity of the G916 mutant was still 80% of the control. Apparently, an A→G pair at the third position is an allowed non-Watson–Crick base pair.
In conclusion, the results show that disruptions of the first and last base pairs in helix 2 are deleterious for efficient translation. Mutant G916 appears an exception.

**Mutations that maintain complementarity in helix 2 preserve activity**

To further explore whether nucleotide identity and thermodynamic stability play a role in the function of the central pseudoknot, we made mutations in helix 2 that replaced the first and/or the last base pair with another Watson–Crick pair.

In the first mutant we changed the third base pair from A19–U916 to C19–G916. This substitution increases the stacking energy at 42°C from 3.9 to 5.0 kcal/mol (21). Nevertheless, as shown in Figure 2B, the C19–G916 mutant had the same ribosomal activity as the wild-type control. To further stabilize helix 2 we additionally changed base pair U17–A918 to C17–G918. The stacking energy of this mutant helix was 6.0 kcal/mol. Figure 2B shows that these mutations also cause only a slight decrease in activity to 70% of the wild-type. The data above suggest that stability of helix 2 is not an important factor for ribosome function.

The wild-type U17–A918 pair is almost universally conserved (5). The phenotype of mutant G17–C918,G19–G916 showed that the conserved U–A pair can be replaced without a deleterious effect on translation. To further investigate this issue we introduced an A–U pair at the position of the conserved pair. As shown in Figure 2B, this base pair reversal had no effect on translational activity. Thus, despite strong sequence conservation, there appears to be no special requirement for the base composition at the first position in helix 2.

**Lowering the temperature does not affect the activity of the mutants**

The free energy change of helix formation is ΔH° – TΔS°. Since ΔS° is always negative in the case of helix formation, a lower temperature will lead to more negative ΔG° values and therefore to an increase in stability. We tested the effect on translation of a temperature decrease for some of our helix 2 mutants, as it is conceivable that increased stability affects the phenotype of the mutant helices.

In practice we induced synthesis of specialized ribosomes by growing the culture for 1 h at 42°C. After taking a sample (t = 0) the culture was diluted with an equal volume of LC medium at 14°C to reach 28°C. The CAT activity per OD650 at the time of the shift down (t = 0) was subtracted from the CAT activity obtained at 28°C to determine synthesis of CAT protein by the mutant ribosomes at the lower temperature. Figure 3 shows that maximal CAT activity was obtained 60 min after the temperature shift. Beyond this time CAT activity decreases, probably because shifting the temperature to 28°C reinstates repression of the λP L promoter. As the cultures continue to grow the already synthesized specialized ribosomes will be diluted over an increasing
number of cells and, at some point, become limiting for maximal translation of the CAT messenger.

The first mutant tested contained the substitutions G\textsubscript{17}–C\textsubscript{918} and C\textsubscript{19}–G\textsubscript{916}. At 28°C this mutant helix is 2.2 kcal/mol more stable than the wild-type (21). As shown in Figure 3, the decrease in temperature from 42 to 28°C did not affect activity of this mutant, which is still 70% of the wild type.

In mutant C\textsubscript{17} the first base pair is changed into a C–A mismatch, while in mutant C\textsubscript{19} a C–U mismatch occurs at the third position in helix 2. Accordingly, these helices are weaker than the wild-type. The temperature drop increases the stacking energy position in helix 2. Accordingly, these helices are weaker than the wild-type.

Mutant C\textsubscript{17}C\textsubscript{19}–G\textsubscript{916} contains a C–G base pair at the third position instead of the natural A–U pair. At 42°C this helix is therefore 0.7 kcal/mol more stable than the C\textsubscript{17} helix, but still less stable than the wild type. Again, as shown in Figs 2A and 3, the reduction in temperature does not influence translational activity.

The results show that the impact of the mutations on ribosome activity is independent of temperature. Together with the tolerance that we observe in substituting base pairs in helix 2, this suggests that complementarity, rather than stability, is relevant for functioning of helix 2.

**DISCUSSION**

**Base complementarity, rather than sequence or thermodynamic stability, is important for a functional helix 2**

We have measured the translational activity of ribosomes with mutations in the first and last base pairs of helix 2 of the central pseudoknot in 16S rRNA. Our results show that base complementarity in helix 2 is necessary and sufficient for efficient ribosome functioning in *E.coli*. In a previous report Brink et al. (11) showed that disrupting the central pseudoknot, by changing the middle base pair of helix 2 into a mismatch, impaired ribosomal activity. Alternative base pairs at this position maintained function.

The introduction of mismatches in the first and last base pairs of helix 2 may not completely disrupt the pseudoknot. Still, we always find a large decrease in ribosomal activity. The only exception was mutation G\textsubscript{916}, creating an A–G pair at the third position. This mutation has almost no effect on activity. Phylogenetic comparison studies show that A–G pairs often occur in rRNA, especially at the end of a helix (6,22). At some positions in the rRNA secondary structure an A–G pair can only be replaced by a G–A pair, while at other positions an A–G pair was found to be replaced by all kinds of canonical base pairs (6). Because of these variable replacement patterns the authors suggest that not all A–G pairs in rRNA have the same geometry and therefore need different substitutions to maintain the local structure. An A\textsubscript{19}G\textsubscript{916} pair at the third position in helix 2 may have the same geometry as a canonical pair. Such a pair would not distort the structure of helix 2 and would therefore not adversely affect translation.

We show that alternative base pairs can replace the natural pairs at the first and third positions in helix 2 without a negative effect on translation. Brink et al. (11) substituted other pairs in the middle position and also found no effect. Due to the altered base pair composition, the thermodynamic stability of these mutant helices differed from the wild-type. For example, the least stable functional helices 19AAU\textsubscript{17}–916UU\textsubscript{918} and 19AAU\textsubscript{17}–916UAA\textsubscript{918} contribute a stacking energy of –1.8 kcal/mol at 42°C, while for the fully active mutant 19CCU\textsubscript{17}–916GG\textsubscript{918} this value is –5.0 kcal/mol. Efficient functioning of both helices suggests that thermodynamic stability of helix 2 is not an important determinant for ribosomal activity. Nevertheless, mismatches in helix 2 are not allowed. This intolerance of disruptions in base pairing seems therefore unrelated to stacking energy values. For instance, the mutant with sequence 19CCU\textsubscript{17}–916GG\textsubscript{918} has a higher stacking energy than 19AAU\textsubscript{17}–916UU\textsubscript{918}. Still, CCC/GGA is less active. The results above suggest that an undisturbed Watson–Crick-type helical structure is the only condition for helix 2 to be functional.

The central pseudoknot is proposed to be in the topological center of the 30S subunit, from which the 5′-domain, central domain and 3′-domain protrude (3). An undisturbed helix 2 may therefore be important for structural organization of the ribosome center. Indications of such a function were found upon *in vitro* analysis of 30S subunits with mutation A\textsubscript{18} in the central base pair of helix 2, which probably destroys the central pseudoknot. A\textsubscript{18} mutant 30S subunits were shown to be functionally unstable due to loss of ribosomal proteins (13). The A\textsubscript{18} mutation also caused inhibition of N-terminal acetylation of S5 (23). This ribosomal protein binds the 30S subunit close to the central pseudoknot (24), which suggests that inhibition of S5 acetylation was due to a perturbed S5 binding site.

**No support for alternative conformations involving the central pseudoknot during the ribosome cycle**

Two alternative structures were proposed to be in equilibrium with the central pseudoknot. Kössel et al. (15) suggested base pairing of nt 14–18 (*E.coli* numbering) with nt 1530–1534, positioned directly upstream of the anti-Shine–Dalgarno sequence at the 3′-end of the 16S rRNA (Fig. 4A). This alternative conformation disrupts the central pseudoknot, while a new one is created. The alternative structure would represent the conformational state of 16S rRNA in the elongating ribosome, while during initiation the rRNA would be in the ‘classical’ conformation.

Another conformational switch in the central pseudoknot area was postulated by Leclerc and Brakier-Gingras (16). In their proposal a pseudoknot is formed by base pairing of nt 12–16 with nt 911–915 (Fig. 4B). Formation of the alternative structure was suggested to be promoted by the antibiotic streptomycin or mutations in ribosomal protein S4. Streptomycin and mutations in S4 are both known to reduce translational fidelity of the ribosome (25). This effect has been ascribed to a putative error-prone conformation of the ribosome (26). Both proposals for alternative structures are based on the complementarity of sequences in helix 1 to extremely conserved areas in 16S rRNA. Therefore, phylogenetic support is scarce.

Some of our mutations in helix 2 have direct consequences for the alternative base pair proposed by Kössel and co-workers. Base pair substitutions A\textsubscript{19}–G\textsubscript{916} and G\textsubscript{17}–C\textsubscript{918} change basepair U\textsubscript{17}–A\textsubscript{1531} in the alternative helix into a mismatch, thereby reducing the interaction from five to three consecutive base pairs (in *E.coli*). These mutations are expected to disrupt the geometry and to substantially decrease the stability of the alternative helix. However, since they do not cause a severe decrease in translational activity, existence of the alternative structure becomes unlikely.

The base pair substitutions in helix 2 do not alter the base pairing properties of the alternative interaction proposed by Leclerc and Brakier-Gingras (16). However, they do change the thermodynamic stability of helix 2 which, in turn, will affect the equilibrium between
Figure 4. Models for alternative conformations of the central pseudoknot structure. (A) Equilibrium between the central pseudoknot and the alternative structure proposed by Kössel et al. (15). (B) Equilibrium between the central pseudoknot and the alternative structure proposed by LeClerc and Brakier-Gingras (16).

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