

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 C₈₆₆A₈₆₅ 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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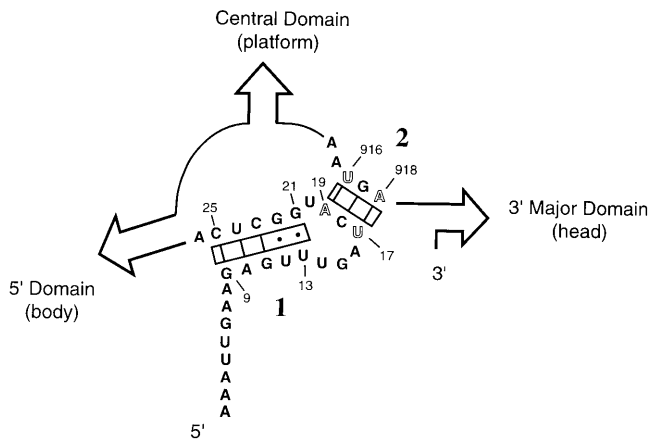


Figure 1. Scheme of the central pseudoknot structure in *E. coli*, connecting the three major domains in 16S rRNA. The secondary structure is according to Stern *et al.* (3). The central pseudoknot consists of helix 1 (nt 9–13/21–25) and helix 2 (nt 17–19/916–918). The arrows indicate the relative orientation of the three major domains protruding from this structure. The first base pair U₁₇–A₉₁₈ and third base pair A₁₉–U₉₁₆ 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 pP_LASDX-Spc^R-CATX we used K5637 (11). This strain contains a thermolabile P_L 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 phage M13 (18). A 1.9 kb *KpnI*–*XbaI* fragment from pP_LASDX-Spc^R-CATX (11) was cloned into the polylinker of M13mp18 (17). This fragment contains the complete specialized 16S rRNA gene and part of the tRNA₂^{Glu} gene. Mutagenesis was performed as described by Kunkel (18). A shorter *KpnI*–*ApaI* fragment of the 16S rRNA gene containing the mutation(s) was recloned into pP_LASDX-Spc^R-CATX. Mutations were checked by dideoxynucleotide plasmid sequencing (20).

Measurement of ribosomal activity by CAT assay

K5637 cells harboring pP_LASDX-Spc^R-CATX were grown overnight at 28°C in LC medium containing 100 mg/l ampicillin. Cultures were diluted 100 times, grown for 1 h at 28°C and then induced at 42°C. Samples of 1 ml were taken at $t = 0, 30, 60, 90$ and 120 min after induction. CAT assays were performed essentially as described by Brink *et al.* (11) except that volumes were decreased 10 times to 20 μ l. After reaction 180 μ l water were added to the mixture. [³H]Diacetylchloramphenicol was extracted with 1 ml CARBO LUMA™ scintillation fluid (LUMAC*LSC Inc.) and counted as a two phase system. The CAT activity calculated is amount of [³H]diacetylchloramphenicol (c.p.m.)

synthesized by CAT protein divided by optical cell density (OD₆₅₀) at the time of sampling.

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. CAT 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 P_L promoter. Expression of the *rmB* operon on plasmid pP_LASDX-Spc^R-CATX, 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 pP_LASDX-Spc^R-CATX 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 pP_LASDX-Spc^R-CATX Δ *KpnI*–*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 or 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.

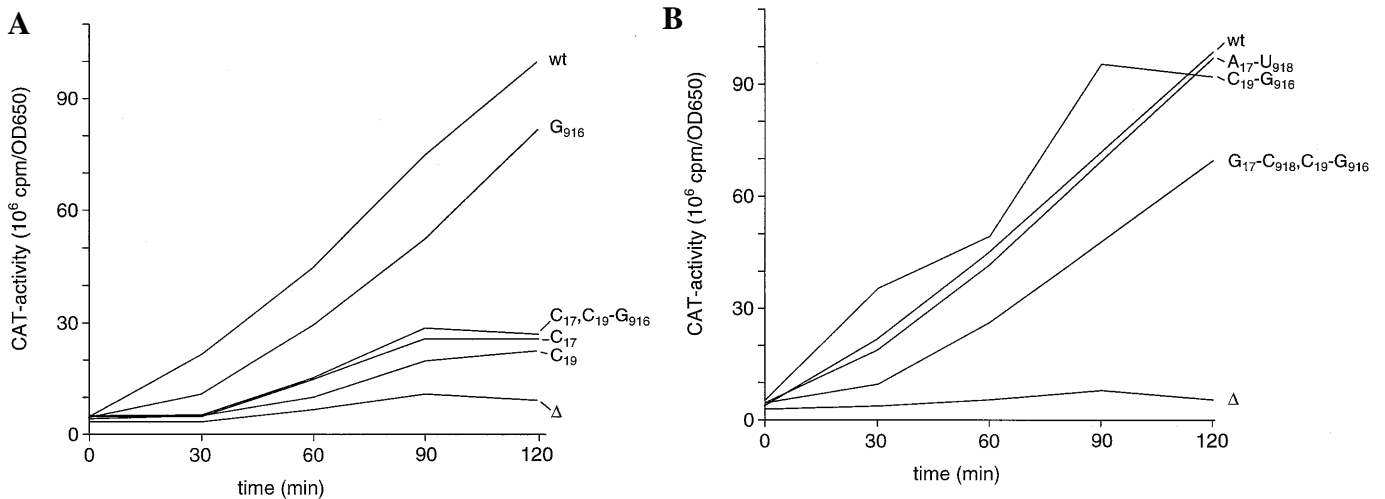


Figure 2. Ribosomal activity at 42°C of specialized ribosomes having a wild-type or mutant helix 2. Activity was assessed by measuring CAT activity in cell lysates. Samples were taken at intervals of 30 min after temperature induction of specialized ribosome synthesis ($t = 0$). CAT activity was determined by measuring the amount of [³H]diacetylchloramphenicol formed. Cells harboring specialized ribosomes with a wild-type helix 2 (wt) were compared with cells harboring the indicated helix 2 mutations. The negative control is represented by cells not expressing specialized ribosomes (Δ). Each point represents the average of CAT values obtained in two different experiments. The accuracy of the values indicated is $\pm 15\%$.

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, C₁₉-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 $\Delta H^\circ - T\Delta S^\circ$. 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 λ P_L promoter. As the cultures continue to grow the already synthesized specialized ribosomes will be diluted over an increasing

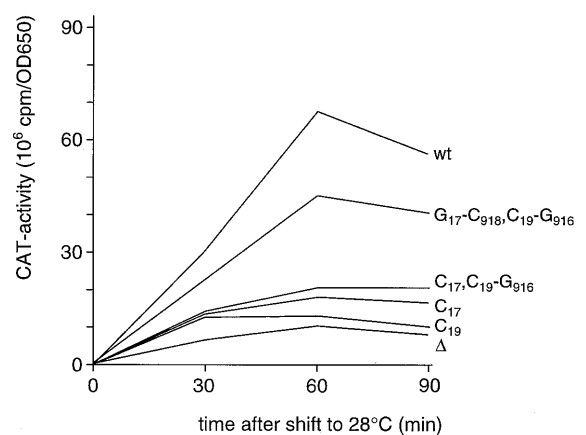


Figure 3. Ribosomal activity at 28°C of specialized ribosomes having a wild-type or mutant helix 2. Activity was assessed by measuring CAT activity in cell lysates. Specialized ribosome synthesis was induced by growing at 42°C for 1 h. Samples were taken at intervals of 30 min after shifting the growth temperature to 28°C ($t = 0$). To determine the activity of CAT protein synthesized at 28°C we subtracted the activity of the sample at $t = 0$. See legend to Figure 2 for further details.

number of cells and, at some point, become limiting for maximal translation of the CAT messenger.

The first mutant tested contained the substitutions G₁₇-C₉₁₈ and C₁₉-G₉₁₆. 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 C₁₇ the first base pair is changed into a C-A mismatch, while in mutant C₁₉ 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 between the two remaining base pairs in the C₁₇ and C₁₉ mutant helices by 0.4 kcal/mol. Nevertheless, mutants C₁₇ and C₁₉ have a residual activity at 28°C that is comparable with activity at 42°C (compare Figs 2A and 3)

Mutant C₁₇,C₁₉-G₉₁₆ 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 C₁₇ 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 G₉₁₆, 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 A₁₉-G₉₁₆ 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 ¹⁹AAU₁₇₋₉₁₆UUA₉₁₈ and ¹⁹AUU₁₇₋₉₁₆UAA₉₁₈ (11) contribute a stacking energy of -1.8 kcal/mol at 42°C, while for the fully active mutant ¹⁹CCU₁₇₋₉₁₆GGA₉₁₈ 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 ¹⁹CCC₁₇₋₉₁₆GGA₉₁₈ has a higher stacking energy than ¹⁹AAU₁₇₋₉₁₆UUA₉₁₈. 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 A₁₈ in the central base pair of helix 2, which probably destroys the central pseudoknot. A₁₈ mutant 30S subunits were shown to be functionally unstable due to loss of ribosomal proteins (13). The A₁₈ 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 pairing proposed by Kössel and co-workers. Base pair substitutions A₁₇-U₉₁₈ and G₁₇-C₉₁₈ change basepair U₁₇-A₁₅₃₁ 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

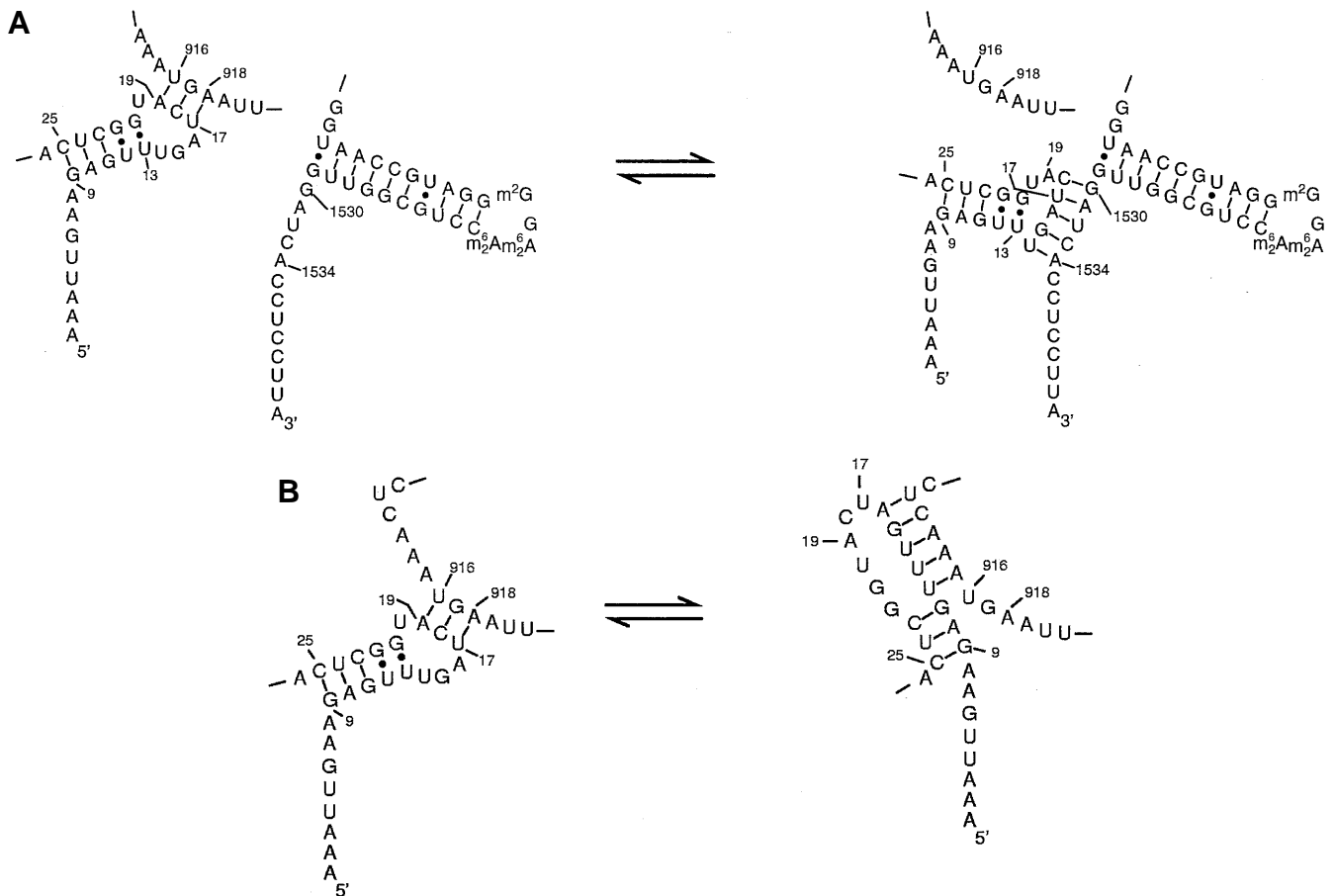


Figure 4. Models for alternative conformations of the central pseudoknot structure. (A) Equilibrium between the central pseudoknot and the alternative structure proposed by Kössel *et al.* (15). (B) Equilibrium between the central pseudoknot and the alternative structure proposed by LeClerc and Brakier-Gingras (16).

the classical conformation and the alternative structure. However, as we find no correlation between stability and ribosomal activity, the existence of this alternative structure is not supported by our results. In a more recent paper by the group of Brakier-Gingras (27) it was shown that mutations that introduce mismatches in the alternative helical structure do not affect cell growth. It therefore seems that the central pseudoknot is not in equilibrium with the proposed alternative structures. Recently a subtle conformational switch during mRNA decoding was reported involving nt 910–912 (28).

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