Acetylation of ribosomal protein S5 affected by defects in the central pseudoknot in 16S ribosomal RNA?

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Abstract We have analyzed the ribosomal protein profile of *Escherichia coli* 30S subunits with the mutation $C_{18}A$ in the central pseudoknot of their 16S ribosomal RNA. This mutation was shown to inhibit translational activity in vivo and to affect ribosome stability in vitro. The majority of the mutant 30S particles were present as free subunits in which a reproducible decrease in amount of proteins S1, S2, S18 and S21 was observed. The protein gels also showed the appearance of a satellite band next to S5. This band reacted with anti-S5 antibodies and had a slightly increased positive charge. The simplest interpretation of these findings, also considering published data, is that the satellite band is S5 with a non-acetylated N-terminal alanine. Underacetylation of S5 due to mutations in the 16S rRNA implies that the modification is performed on the ribosome.

Key words: 16S rRNA; Ribosomal protein; Acetylation; Ribosome; Pseudoknot

1. Introduction

The important role of ribosomal RNA for ribosome functioning in translation is nowadays well established (reviewed in reference [1]). Recent experiments showed the direct involvement of rRNA in the translation reactions [2–4]. A universally conserved structural element in 16S rRNA is the central pseudoknot [5–8], shown for *Escherichia coli* in Fig. 1. RNA secondary structure models [9,10] positioned this structure element in the center of the molecule where it connects the 5′ domain, the central domain and the 3′ domain.

The importance of the central pseudoknot structure for translation was shown by Brink et al. [11] using the specialized ribosome system [11–13]. Replacing the central basepair in helix 2 by a mismatch abolished translation to a level less than 10% of the control. Replacement by another base pair restored ribosome functioning. Ribosomes with a disrupted helix 2 did not form polysomes which suggested that these mutant ribosomes do not enter the elongation cycle [11].

Previously, we showed that disrupting the central base pair of helix 2 by mutation A_{18} affected ribosome stability in vitro and correlated with a loss of ribosomal proteins [14]. The mutant ribosomes appeared impaired in the formation of 70S tight couples since they predominantly accumulated as free subunits. Mutant 30S particles derived from 70S tight couples were fully active in 30S initiation complex formation while the free 30S subunits had very little activity [14].

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Here, we show an analysis of the ribosomal proteins from specialized 30S subunits carrying the A_{18} mutation in their 16S rRNA. Proteins from tight-couple-derived particles or from free subunits were separated by 1- and 2-dimensional gel electrophoresis and compared to proteins from wild-type 30S subunits.

2. Materials and methods

2.1. Strains, media and plasmids

E. coli strain K5637, used for the isolation of ribosomes, was constructed by D.H. Miller and has been described [13]. Cells were grown in LC medium [15]. When appropriate, ampicillin (Sigma) was supplied at a final concentration of 100 mg/l. Plasmid $pP_LASDX\text{-}Spc^R$-CATX, in this paper referred to as pASC, encoding the specialized ribosome system and used as a source for specialized 30S subunits, was derived from pASDX-PSDX-hGH [13] and has been described [11,12]. The *16S rRNA* gene on pASC encodes 16S rRNA with an altered anti Shine-Dalgarno (ASD) sequence and its transcription is driven by the thermo-inducible P_L promoter. In addition to the altered ASD sequence, the C residue at position 1192 of the 16S rRNA was changed into U, conferring resistance to spectinomycin [11,12].

2.2. Isolation of 30S subunits

30S subunits were isolated as described [15]. Strain K5637 harboring pASC was used for isolating specialized 30S subunits with a wild-type central pseudoknot in their 16S rRNA. Appropriate mutant derivatives of this plasmid [11] were used for the isolation of specialized 30S subunits containing the single A_{18} or the double C_{1065}, G_{1191} substitution.

2.3. Determination of 30S subunit identity by primer extension on 16S rRNA

The ratio of specialized to chromosomally encoded 30S in the isolated ribosome fractions was determined using primer extension on 16S rRNA [16,17] and exploits a C_{1192} to U_{1192} base substitution in specialized 16S rRNA [11].

2.4. Isolation of ribosomal proteins

Two volumes of glacial acetic acid were added to isolated 30S subunits followed by overnight incubation at −20°C to precipitate the 16S rRNA [18]. RNA was removed from the solution by centrifugation and ribosomal proteins were precipitated by adding 5 volumes of acetone followed by incubation for 2 h at −20°C. Ribosomal proteins were spun down and pellets were washed 2 times with acetone, dried under vacuum and dissolved in the appropriate loading buffer.

2.5. Ribosomal protein analysis

The ribosomal protein content of the different 30S subunit isolates was first analyzed by 1D electrophoresis on a 15% SDS-polyacrylamide gel [19]. Approximately 50 μg of material (150 pmol) was used for each run. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) [20]. To estimate molecular masses, we used Prestained Protein Marker, Broad Range (New England Biolabs).

2D gel electrophoresis was performed as described by Geyl et al. [21]. Per run, 15 μg of 30S ribosomal protein (50 pmol) was used. Electrophoresis in both dimensions was performed at 4°C in a Bio-Rad Mini-Protean II 2D cell system. Electrophoresis in the first di-

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mension was for 1.5 h at 250 V and 0.5 mA per tube gel (the fuchsin tracking dye is then at the end of the tube gel). After running the first dimension, the tube gel was layered on the second dimension slab gel. Fuchsin/urea loading buffer (50 μl) was sprayed on top of the tube gel, followed by electrophoresis for 1.5 h at 250 V and 30 mA per gel (the fuchsin tracking dye has then just run off the gel). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 as described above.

2.6. Western blot analysis

Ribosomal proteins were dissolved in fuchsin/urea loading buffer and separated on a 8% polyacrylamide urea slab gel using the second dimension electrophoresis buffer conditions, as described above. Electrophoresis was for 2 h at 250 V. Then, the gel was soaked for 30 min in blot buffer (25 mM Tris-base, 190 mM glycine, 20% methanol) containing 0.5% SDS and was blotted onto nitrocellulose according to standard techniques. Ribosomal protein S5 and its derivatives were immunodetected by using sheep antibodies against S5 (a gift from Dr. R. Brimacombe and prepared by Dr. G. Stöffler) and goat anti-sheep antibodies, linked to alkaline phosphatase (Sigma).

3. Results

3.1. Distribution of mutant 30S particles over free subunits and 70S tight couples

To obtain 30S subunits with a disrupted pseudoknot, we used the specialized ribosome system [11] encoding 16S rRNA with an altered anti Shine-Dalgarno sequence at its 3′ end. In the present study, this plasmid contained in addition the C_{18} to A_{18} mutation. For simplicity, specialized ribosomes harboring the wild-type or mutant pseudoknot will be mostly referred to as wild-type or mutant ribosomes, respectively. The real wild-type ribosomes, encoded by the chromosome, will be called chromosomally encoded ribosomes.

Cells were harvested 2 h after induction at 42°C. An S30 extract, which contains a mixture of plasmid-derived specialized ribosomes and chromosomally encoded ribosomes, was fractionated on a sucrose gradient at 4.2 mM Mg acetate and the ratio of the two types of ribosomes in each fraction determined [16]. Extracts containing specialized ribosomes with a wild-type central pseudoknot showed a dominant 70S tight-couple peak (wt/70S), containing 78% wild-type and 22% chromosomally encoded 30S subunits. Free subunits were essentially absent in this extract [14]. On the contrary, in extracts with the A_{18} mutant ribosomes, free subunits dominated the profile [14]. The free 30S subunit fraction (A_{18}/30S) consisted almost completely (87%) of mutant 30S particles. The 70S tight-couple peak (A_{18}/70S) contained only 28% mutant 30S subunits. The remainder is chromosomally encoded.

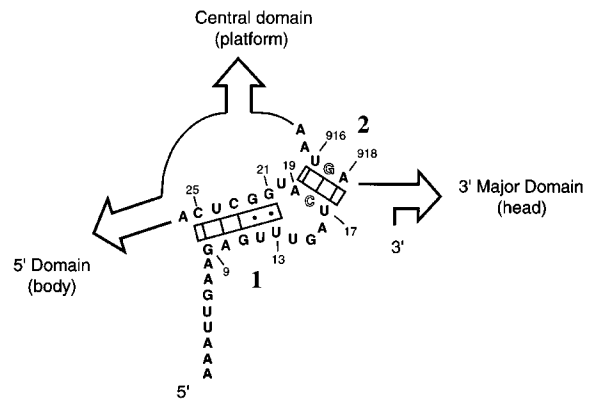

Fig. 1. Schematic diagram of the central pseudoknot structure connecting the three major domains in 16S rRNA. The secondary structure is according to Stern et al. [10]. The central pseudoknot consists of helix 1 (nucleotides 9–13/21–25) and helix 2 (nucleotides 17–19/916–918). The arrows indicate the relative orientation of the three major domains protruding from this structure. The mutated C_{18}–G_{917} base pair is presented with open letters.

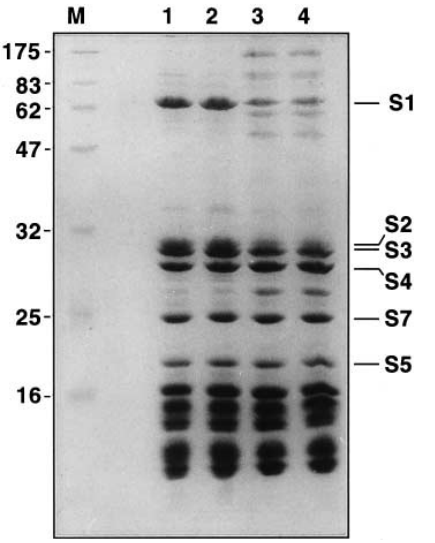

Fig. 2. SDS-polyacrylamide analysis of ribosomal proteins, isolated from 30S subunit fractions (30S fractions are described in the text). Lane 1: wt/70S. Lane 2: A_{18}/70S. Lane 3: A_{18}/30S. Lane 4: C_{1065}-G_{1191}/30S. The molecular mass of the marker proteins is indicated. Distinguishable ribosomal proteins are marked. The extra band between S4 and S7 in lane 3 and 4 represents initiation factor IF3, associated with free 30S subunits.

Apparently, the A_{18} mutation in 16S rRNA, directly or indirectly, inhibits association of the mutant particles with 50S subunits. The mutation does not influence the total amount of specialized 30S subunits synthesized per cell (data not shown).

3.2. Ribosomal protein content of 30S subunits

We compared the ribosomal protein content of mutant 30S subunits derived from either the 70S or 30S fraction to the wild-type pattern. Since our wild-type isolate contained hardly any free subunits [14] we only extracted proteins from tight-couple-derived 30S.

Fig. 2 shows the ribosomal proteins separated on molecular mass by electrophoresis on an SDS-polyacrylamide gel. Mutant 30S subunits, isolated from the free subunit fraction (lane 3), showed a significant reduction in ribosomal proteins S1 and S2, as compared to the wt/70S control (lane 1). The A_{18}/70S fraction (lane 2) showed an identical protein pattern as the control. However, since this fraction contained only 28% mutant 30S subunits, a reduction of one or more ribosomal proteins in the mutant particles will not be visible on the gel.

To test whether the protein deficiency in the mutant ribosomes was specifically related to the rRNA defect, we compared the protein profile of our A_{18} mutant with the pattern of a mutant with the base pair substitution U_{1065}–A_{1191} to C_{1065}–G_{1191}. These substitutions stabilize the upper stem of helix 34 in 16S rRNA, which is the binding site for the antibiotic spectinomycin [22–24]. The double mutation C_{1065}–

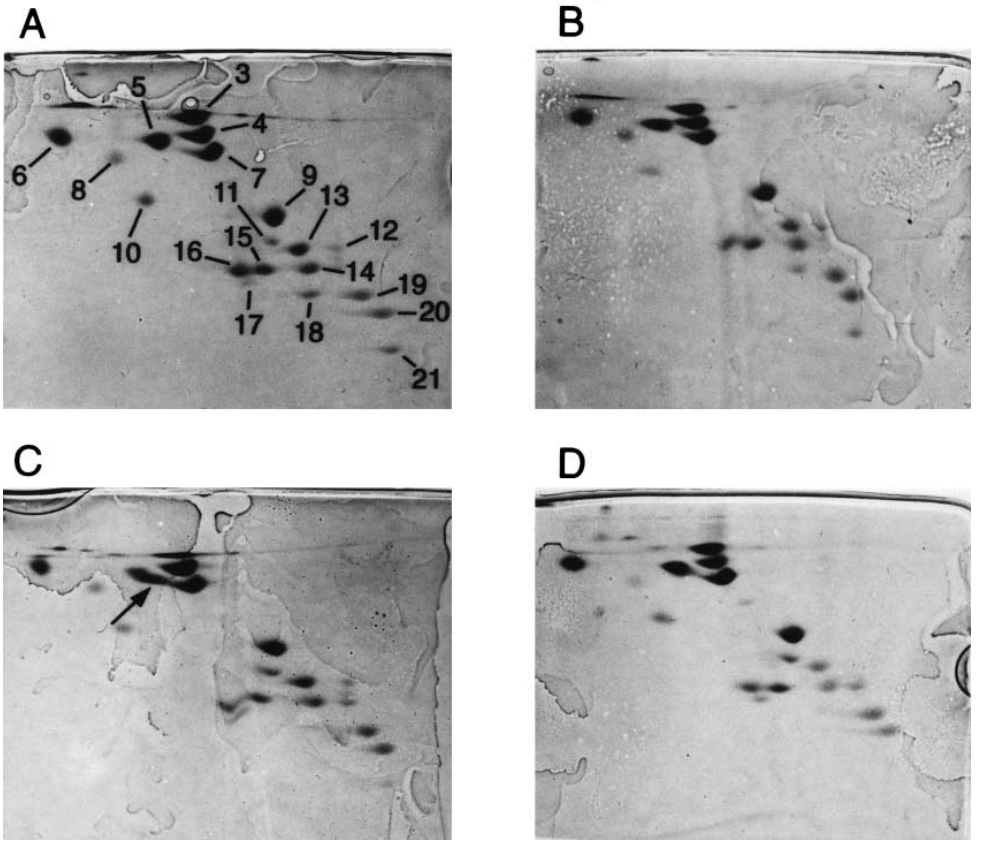

Fig. 3. 2D gel electrophoresis analysis of ribosomal proteins, isolated from 30S fractions. A: wt/70S. B: A_{18}/70S. C: A_{18}/30S. D: C_{1065}–G_{1191}/30S. Ribosomal proteins are marked for the wt/70S fraction [33]. The arrow in (C) indicates the position of the extra protein in the A_{18}/30S fraction. Separation was as described by Geyl et al. [21]. The experiment was repeated 3 times. Only the presence of S18 and S21 was reproducibly decreased.

G_{1191} inhibits translation in vivo and causes accumulation of free mutant 30S subunits (our unpublished results). The free 30S fraction (C_{1065}–G_{1191}/30S) consisted for 85% of mutant particles. Lane 4 shows that these particles had a decrease in the same ribosomal proteins, S1 and S2, as the free A_{18} 30S subunits, indicating that the deprivation of these proteins is not specific for the A_{18} mutation.

Since several ribosomal proteins have a similar molecular mass, their resolution on a 1D SDS gel is poor. Therefore, we also performed 2D gel electrophoresis using urea polyacrylamide gels [21]. In this system the electrophoretic mobility of the proteins, in two dimensions, depends also on their charge. Fig. 3A–D shows the ribosomal proteins in the analyzed fractions, starting from S3, up to S21. Free A_{18} 30S (Fig. 3C), showed a reproducible decrease in amounts of S18 and S21, as compared to the wt/70S control (Fig. 3A). The free C_{1065}–G_{1191} 30S subunits (Fig. 3D) had, again, essentially the same protein profile as the free A_{18} 30S particles. The A_{18}/70S fraction (Fig. 3B) showed, as expected, no difference with wt/70S, as it is mainly composed of chromosomally encoded 30S subunits.

An interesting phenomenon, observed only in free A_{18} 30S subunits, is the splitting of protein S5 into two spots (Fig. 3C, the arrow points at the new spot). In the corresponding 1D SDS gel analysis no S5 satellite band was observed. Therefore, the splitting of the S5 spot in the 2D gel system possibly reflected a difference in charge between S5 and the satellite, rather than a difference in molecular mass. The faster mobility of the putative S5 derivative suggested that it had either lost a negative charge or gained a positive charge, as compared to the authentic S5 protein.

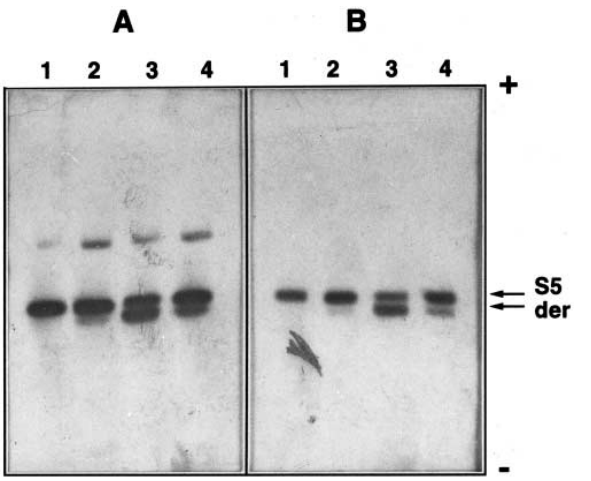

Fig. 4. Western blot analysis of ribosomal proteins with antibodies against ribosomal protein S5. Lane 1: wt/70S. Lane 2: A_{18}/70S. Lane 3: A_{18}/30S. Lane 4: C_{1065}–G_{1191}/30S. Samples in (A) contained 0.5 μg of total 30S ribosomal proteins; samples in (B) contained 0.1 μg of total 30S ribosomal proteins. The positions of ribosomal protein S5 and the S5 derivative (der) are indicated by arrows. Proteins were separated on a urea/8% polyacrylamide gel [21].

3.3. Western blot analysis of S5 and its derivative

To test the relatedness between the two S5 spots, we performed Western blotting using antibodies against S5. The ribosomal proteins of the different fractions were separated on a 1D urea gel using the conditions of the second dimension electrophoresis but with a lower percentage of acrylamide. The results are shown in Fig. 4. In Fig. 4A, 5 times more protein was applied than in Fig. 4B. As can be seen, the faster-running unknown protein reacted specifically with the antibodies against S5. The S5 derivative was present in all the samples, except for the wild-type control (lanes 1 of Fig. 4A,B). The A_{18}/30S sample (Fig. 4A,B, lanes 3) contained, as expected from the Coomassie staining, most of the extra product but also in the C_{1065}–G_{1191}/30S (Fig. 4A,B, lanes 4) and A_{18}/70S sample (Fig. 4A,B, lanes 2) small amounts of the derivative were observed. Comparison of the diluted and undiluted samples shows that mutant 30S present as 70S particles are protected from formation of the S5 derivative, as compared to mutant 30S present as free subunits.

4. Discussion

We have investigated the protein content of mutant 30S subunits in which helix 2 of the central pseudoknot structure in 16S rRNA was disrupted by turning the central base pair into a mismatch. Ribosomes containing this mutation have very little translational activity in vivo [11].

Previously, we showed that the A_{18} mutant 30S subunits, when isolated from the 70S tight-couple fraction, form a 30S initiation complex almost as efficiently as wild-type 30S subunits [14]. These mutant particles are, however, unstable and lose activity when subjected to affinity chromatography and high salt conditions. This was due to loss of ribosomal proteins since partial reconstitution with a total 30S ribosomal protein extract restored activity. Mutant 30S subunits, derived from the free 30S fraction, were already inactive upon isolation. Here, we have analyzed the protein composition of the mutant 30S subunits in an attempt to correlate activity to ribosomal protein content.

The most notable and reproducible change in free 30S subunits with a mutant central pseudoknot is the decrease in ribosomal proteins S1, S2, S18 and S21. Of these proteins, S1 and S21 were shown to be necessary for efficient mRNA binding and 30S initiation complex formation [25,26]. Therefore, the reduced presence of S1 and S21 is in accordance with the low activity of the mutant-free 30S subunits.

The question whether the altered protein composition is a direct or an indirect consequence of the A_{18} mutation is hard to answer. In our type of analysis it is difficult to distinguish between a direct effect of the mutation on assembly or an indirect effect, where the mutation results in a functionally deficient 30S subunit, excluded from the translation cycle and therefore more vulnerable to decay and loss of proteins. The phenotypic defect of mutant C_{1065}–G_{1191} is comparable to that of the A_{18} mutant (our unpublished results). As it appears difficult to imagine how fully different rRNA mutations will lead to loss of the same set of proteins, the suggestion is that the deficiency in translation is the cause rather than the result of the missing proteins. On the other hand, mutation C_{1065}–G_{1191} is in the 3D model for the *E. coli* 30S subunit positioned in the proximity of helix 2 [9,10]. We can therefore not rigorously exclude that mutations in either helix have a direct and identical effect on 30S subunit assembly.

Our 2D urea gels reveal also another aberration in the proteins of free A_{18} 30S subunits. The spot representing ribosomal protein S5 is decreased in intensity and an extra spot, running slightly faster in both dimensions, appears. Western blot analysis using anti-S5 antibodies proved the satellite spot to be a derivative of S5. The derivative is also present on free 30S subunits with the C_{1065}–G_{1191} mutation and on tight-couple-derived A_{18} 30S subunits, though in a much lower proportion. The 1D SDS gel, separating by molecular mass only, shows no splitting of the S5 band, indicating that there is only a marginal difference in molecular mass between mature S5 and its derivative. The increased mobility of the S5 derivative to the minus pole in urea gel electrophoresis should therefore be ascribed to an overall increased positive charge. Wittmann-Liebold and Greuer [27] showed that the N-terminal residue of the *E. coli* S5 protein is acetylated. Absence of the acetyl group would increase the positive charge without appreciably affecting the molecular mass.

Cumberlidge and Isono [28] found that certain mutations in ribosomal protein S4 caused splitting of the spot representing S5. N-terminal amino-acid sequencing indicated that the extra product, which runs slightly faster than the mature S5, was S5 lacking the acetylation of the N-terminal alanine. We observe here an identical splitting of the S5 spot under similar electrophoretic conditions, suggesting that we are dealing with the same phenomenon.

Two papers by Dammel and Noller [29,30] describe a mutant in helix 1 of the central pseudoknot, changing base pair C_{23}–G_{11} into U_{23}–G_{11}. The mutation causes a dominant cold-sensitive phenotype and a defective maturation of the 16S rRNA 5′ end. A ribosomal protein analysis of this mutant, using the same electrophoretic conditions as we do, showed an extra spot at exactly the same position as the presumed unmodified S5. Similar to the A_{18} mutant, the presumed non-acetylated form of S5 was only visible in the free subunits. The cold-sensitive phenotype of the U_{23} mutation could be suppressed by overexpression of a 30S-associated RbfA factor [30]. Overexpression of this factor also suppresses the appearance of, supposedly, unmodified S5. Moreover, in a *RbfA* deletion mutant, which grows poorly at 26°C, the presumed non-acetylated S5 derivative is now also visible in protein gels of wild-type 30S subunits.

Mutations A_{18} and U_{23} are both situated in the central pseudoknot, which is protected by S5 from chemical modification [31]. Recently, Heilek and Noller [32] introduced cysteine residues in positions 21, 99 and 129 of S5, that were used as targets for derivatization with an Fe(II)–EDTA complex. Hydroxyl radicals produced by Fe(II) complexes on position 21 and 129 cleave on many positions in the 9–25 moiety of the central pseudoknot while radicals from position 99 cut in the lower part of helix 1 (see Fig. 1). These data show that the central pseudoknot is in close proximity to various positions in ribosome-bound S5. Mutations in the pseudoknot may therefore change the S5 binding site. Apparently, this does not decrease the stoichiometry of S5 on our mutant 30S subunits but interferes with acetylation. The role of RbfA, which is proposed to interact with the central pseudoknot region in 16S rRNA [30], could be to preserve the proper local 16S rRNA structure.

Undermodification of S5 in 30S subunits with a mutant

pseudoknot was less prominent if the 30S particles were isolated from 70S ribosomes ([30], this paper). This suggests that association with the 50S subunit or the competence of the mutant 30S to associate with 50S can rescue S5 acetylation. The deficiency in association is, however, not the only cause for the modification inhibition since free 30S subunits from the C_{1065}–G_{1191} mutant show a much higher level of acetylation at the same low degree of subunit association. Base pair substitution C_{1065}–G_{1191} is located in the upper stem of helix 34. S5 can not bind independently to this helix [33] but data of Heilek and Noller [32] show that ribosome-bound S5 is in close proximity to helix 34. Consequently, mutations in this stem might also affect the S5 binding site, possibly explaining the incomplete acetylation.

The results mentioned above suggest that certain mutations in 16S rRNA inhibit modification of S5. This implies that S5 acetylation by the product of the *rimJ* gene [28,34], takes place on the 30S subunit or on the 70S ribosome. The mutations in the central pseudoknot and helix 34 are both located in the vicinity of ribosome-bound S5. They could therefore disturb the optimal substrate of the acetylase, i.e. S5 at its non-manipulated binding site, resulting in inhibition of acetylation. Obligatory in situ acetylation would also explain the failure to modify purified S5 in vitro with a ribosome-free extract from *E. coli* cells [28]. Using the same conditions, free L12 could be modified by its acetylase encoded by the *rimL* gene [28,35].

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