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The central pseudoknot in 16S ribosomal RNA is needed for ribosome stability but is not essential for 30S initiation complex formation

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ABSTRACT

To examine the function of the central pseudoknot in 16S rRNA, we have studied *Escherichia coli* 30S subunits with the A_{18} mutation in this structure element. Previously, this mutation, which changes the central base pair of helix 2, C_{18}–G_{917}, to an A_{18}×G_{917} mismatch, was shown to inhibit translation *in vivo* and a defect in initiation was suggested. Here, we find that the mutant 30S particles are impaired in forming 70S tight couples and predominantly accumulate as free 30S subunits. Formation of a 30S initiation complex, as measured by toeprinting, was almost as efficient for mutant 30S subunits, derived from the tight couple fraction, as for the wild-type control. However, the A_{18} mutation has a profound effect on the overall stability of the subunit. The mutant ribosomes were inactivated by affinity chromatography and high salt treatment, due to easy loss of ribosomal proteins. Accordingly, the particles could be reactivated by partial *in vitro* reconstitution with 30S ribosomal proteins. Mutant 30S subunits from the free subunit fraction were already inactive upon isolation, but could also be reactivated by reconstitution. Apparently, the inactivity in initiation of these mutant 30S subunits is, at least in part, also due to the lack of essential ribosomal proteins. We conclude that disruption of helix 2 of the central pseudoknot by itself does not affect the formation of a 30S initiation complex. We suggest that the *in vivo* translational defect of the mutant ribosomes is caused by their inability to form 70S initiation complexes.

INTRODUCTION

The central pseudoknot in 16S ribosomal RNA, first predicted by Pleij *et al.* (1) is a universally conserved structural element in small subunit RNAs (2–5). For *Escherichia coli* this pseudoknot is presented in Figure 1. Models for the three-dimensional structure of 16S rRNA in the *E.coli* 30S subunit predict three major domains. The 5′ domain constitutes the body, the central domain is incorporated in the platform and the 3′ domain is present in the head of the subunit (6,7). The central pseudoknot structure, located in the center of the 16S rRNA molecule, connects these three domains (6,7).

So far, two other pseudoknot structures were predicted in 16S rRNA, based on phylogenetic comparison (8,9). Powers and Noller (10) showed that the pseudoknot predicted in the 530 hairpin region was essential for ribosome activity. Recently, the other proposed pseudoknot, formed by a long-distance interaction between nucleotides $C_{866}A_{865}$ and $G_{570}U_{571}$, was proven to be indispensable for translation (11).

The central pseudoknot structure was studied by Brink *et al.* (12), using the specialized ribosome system. In this system *E.coli* cells harbor a plasmid with an *rrnB* operon under the control of the P_L promoter. This *rrnB* operon encodes, so-called, specialized 16S rRNA with an altered anti Shine–Dalgarno (ASD) sequence. Also plasmid-encoded is a *cat* gene with a corresponding Shine–Dalgarno (SD) sequence. CAT mRNA, transcribed from this gene is only recognized by ribosomes that contain the specialized 16S rRNA. The level of CAT protein production therefore reflects the *in vivo* translational activity of these ribosomes. In this way, mutations introduced in the specialized 16S rRNA can be tested for their impact on ribosome functioning. Since the specialized ribosomes do not translate other messengers than the CAT mRNA, they represent a dispensable pool of ribosomes that do not interfere with the translation of cellular genes. Mutations introduced in these ribosomes therefore do not cause defects in growth. After 2 h induction of the P_L promoter, specialized ribosomes make up about 80% of the ribosome population in the cell.

Brink *et al.* (12) found that replacing the middle base pair in helix 2 of the central pseudoknot by a mismatch abolished ribosomal activity to a level less than 10% of the control. Replacement by another base pair maintained ribosome functioning. This necessity for complementarity in helix 2 also holds for the first and last base pair (our unpublished data). Ribosomes with a disrupted central basepair in helix 2 showed correct processing of the 5′ end of the 16S rRNA but they did not form polysomal complexes on the mRNA. This suggested that the functional defect is related to translation-initiation (12).

Here, we describe the *in vitro* analysis of specialized ribosomes in which the central base pair of helix 2, C_{18}–G_{917}, was replaced by an A_{18}×G_{917} mismatch. We harvested the cells after slowly

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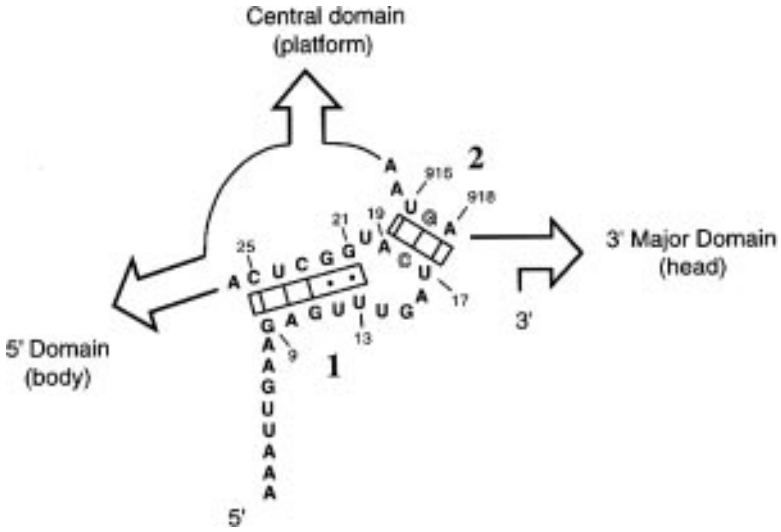

Figure 1. Schematic diagram of the central pseudoknot structure connecting the three major domains in 16S rRNA. The secondary structure is according to Stern*et al.* (7). The central pseudoknot consists of helix 1 (nucleotides 9–13/21–25) and helix 2 (nucleotides 17–19/916–918). The arrows indicate the relative orientation of the three major domains protruding from the pseudoknot structure. The mutated C_{18}-G_{917} basepair is presented with open letters.

cooling the culture, allowing run-off translation. Under these conditions, cell-free extracts do not contain polysomal complexes. Sucrose gradients show that the A_{18} mutation causes a decreased presence of specialized ribosomes in the 70S tight couples fraction. Nevertheless, mutant 30S subunits isolated from this fraction are almost as active in 30S initiation complex formation as the proper control. Therefore, the disruption *per se* of the central base pair of helix 2 does not affect this step in translation-initiation. However, the mutant particles turn out to be unstable and they easily lose some of their ribosomal proteins. Mutant 30S subunits isolated from the free subunit fraction are already inactive upon isolation due to a deficiency in ribosomal proteins.

We suggest that mutant 30S initiation complexes are defective in the association with the 50S subunit. Protection studies and models for the 30S subunit suggest that the central pseudoknot is located at the subunit interface (6,7,13). Disruption of helix 2 could therefore affect the interaction between the 30S and the 50S subunit.

MATERIALS AND METHODS

Strains, media and plasmids

Escherichia coli strain K5637 encodes the thermolabile λ repressor (cI857), was constructed by Dr D. H. Miller and has been described (14). Cells were grown in LC medium (15). When appropriate, ampicillin (Sigma) was added to a final concentration of 100 mg/l. Plasmid $pP_LASDX\text{-}Spc^R\text{-}CATX$, in this paper referred to as pASC, encodes the specialized ribosome system. K5637 cells containing this plasmid were used as a source of specialized 30S subunits. pASC was derived from plasmid pASDX-PSDX-hGH (14) and has been described (12). The *rrnB* operon on pASC encodes 16S rRNA with an altered ASD sequence and is under the transcriptional control of 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 (16). Chromosomally encoded 30S subunits were derived from cells harboring pASC in which the *Kpn*1–*Apa*1 896 bp fragment in the 16S rRNA gene was replaced by a 300 bp murine DNA fragment. These cells do not produce specialized ribosomes.

Isolation of 30S subunits

30S subunits were isolated essentially as described before (15). Strain K5637 harboring pASC was used for isolating specialized 30S subunits with a wild-type central pseudoknot in their 16S rRNA. A mutant derivative of this plasmid was used for the isolation of specialized 30S subunits containing the A_{18} mutation. 70S ribosomes and free 30S subunits were prepared by centrifuging the S30 extract through a 15–30% sucrose gradient in 10 mM Tris–acetate, pH 7.5, 4.2 mM Mg acetate, 60 mM NH_4Cl, 0.1 mM EDTA. Fractions containing 70S tight couples or 30S free subunits were collected and the magnesium acetate concentration was adjusted to 10 mM. Fractions were pelleted by centrifugation for 5 h at 50 000 r.p.m. in a 50.2 Ti rotor. To keep the isolation conditions comparable, we treated the free 30S subunits in an identical way to the 70S tight couples. Pellets were dissolved in 10 mM Tris–acetate, pH 7.5, 4.2 mM Mg acetate, 60 mM NH_4Cl, 0.1 mM EDTA and dialyzed against the same buffer but with 1.2 mM Mg acetate to dissociate the couples (if present). 30S subunits were prepared by 10–30% sucrose gradient centrifugation in the same buffer. The fractions containing 30S subunits were collected, the Mg^{2+} concentration raised to 10 mM, and the 30S subunits pelleted by centrifugation for 5 h at 50 000 rpm in a 50.2 Ti rotor. Pellets were resuspended overnight in 20 mM Tris–acetate, pH 7.5, 20 mM Mg acetate, 100 mM NH_4Cl, 0,1 mM EDTA, 2 mM dithiothreitol (DTT). 30S subunits were reactivated by incubation for 15 min at 40°C and stored at –80°C.

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

The ratio of specialized to chromosomally encoded 30S subunits in the various fractions was determined by primer extension on 16S rRNA (17,18). The procedure exploits the $C_{1192} \rightarrow U_{1192}$ base substitution in specialized 16S rRNA (12).

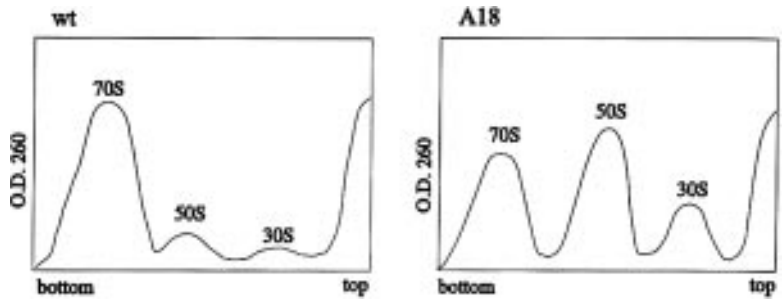

Figure 2. Sucrose gradient profiles from S30 extracts of cells expressing wild-type 30S subunits (wt) or mutant 30S subunits containing the A_{18} mutation. Cells were harvested 2 h after induction of specialized ribosomes synthesis. Profiles were prepared using 15–30% sucrose gradients, as described in Materials and Methods.

Toeprint analysis

CAT mRNA containing the specialized SD sequence 5′ GUGUG was synthesized *in vitro*, using SP6 RNA polymerase (Pharmacia). Plasmid pGEMCAT-SDX (15), containing the specialized *cat* gene under control of the SP6 promoter, was linearized by restriction in the *Bam*HI site, situated about 750 bp downstream of the *cat* gene start. *In vitro* transcription was performed as described by Krieg and Mellon (19) but the DNase treatment was omitted. CAT mRNA was purified by phenol extraction and column chromatography through Sephadex G50, precipitated with ethanol and dissolved in H_2O to a concentration of 0.5 µg/µl. Primer extension inhibition (toeprinting) was performed essentially as described by Hartz *et al*. (20). The primer, 5′ GCAACTGACT-GAAATGCCTC, complementary to residues 64–83 downstream of the *cat* gene startcodon, was end-labelled with [γ-^{32}P]ATP. Toeprinting reactions were performed in standard buffer (10 mM Tris–acetate, pH 7.5, 10 mM Mg acetate, 60 mM NH_4Cl, 0.1 mM EDTA). Reaction mixtures contained 150 nM primer, 20 nM CAT mRNA, 30S subunits as specified in Results, 1 µM $tRNA_f^{Met}$ (Boehringer–Mannheim) and 0.5 U/µl RNase inhibitor (RNA-guard, Pharmacia). After incubation for 7 min at 37°C, to allow the formation of initiation complexes, and addition of 0.05 U/µl AMV reverse transcriptase (Promega), primer extension was performed for 15 min at 37°C. Extension products were separated on an 8% polyacrylamide/8 M urea gel. The relative toeprinting efficiency was determined by measuring the radioactivity of the inhibited and uninhibited extension products in the gels, using a Betascope 603 Blot Analyzer (Betagen), and defined as the ratio between inhibited extension and total primer extension (21).

Partial *in vitro* reconstitution of 30S subunits

30S ribosomal proteins were extracted from chromosomally encoded 30S subunits with LiCl and urea, as described by Leboy *et al.* (22). The extract was dialyzed against buffer A (30 mM Tris–acetate, pH 7.5, 20 mM Mg acetate, 500 mM NH_4Cl, 2 mM DTT). The molar amount of ribosomal proteins in the extract was according to the molar amount of 30S subunits, used for the extraction. Reconstitution was performed by adding 0.1 vol buffer A, containing different concentrations of ribosomal protein extract, to the complete toeprint reaction mixture (see above), except for reverse transcriptase. The final concentrations in the reconstitution-toeprint buffer were 13 mM Tris–acetate, pH 7.5, 12 mM Mg acetate, 110 mM NH_4Cl, 0.1 mM EDTA, 0.2 mM DTT. The mixture was incubated for 10 min at 37°C to allow reconstitution and formation of the 30S initiation complex. Then, reverse transcriptase was added and primer extension was performed as described above.

Hybridization of oligodeoxynucleotides to the 3′ end of 16S rRNA

The oligodeoxynucleotides 5′ TAAGGAGGT and 5′ TAAGTG-TGT, complementary to the 3′ end of chromosomally encoded and specialized 16S rRNA, respectively, were prepared on a Gene Assembler (Pharmacia) using phosphoroamidite chemistry. The oligonucleotides were passed through Sephadex G25 (medium) and 5′-end labelled with [γ-^{32}P]ATP. Excess [γ-^{32}P]ATP was removed by filtration through Sephadex G25 (medium). For hybridization to 16S rRNA, 100 pmol of oligonucleotides were added to 25 pmol of 30S subunits in standard buffer (see above) in a total volume of 50 µl. After incubation for 20 min on ice, 10 min at 37°C and 10 min on ice, samples were filtered through presoaked nitrocellulose (Schleicher & Schuell, BA 85, 0.45µm). Filters were washed twice with 1 ml ice-cold standard buffer, air dried, and counted in scintillation fluid (Carboluma Lumac-LSC) using a Beckmann LS 5000TD scintillation counter.

RESULTS

Disruption of the central base pair in helix 2 of the central pseudoknot impairs formation of 70S tight couples *in vivo*

We used K5637 cells harboring plasmid pASC as a source for specialized ribosomes with a mutant or a wild-type central pseudoknot. For simplicity, specialized ribosomes harboring the wild-type or the mutant pseudoknot will be referred to in this paper 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 of specialized ribosome synthesis and the S30 extracts were prepared and layered on sucrose gradients containing 4.2 mM Mg acetate. At this magnesium concentration, active 30S subunits stay associated with the 50S subunit to form 70S tight couples. The profiles obtained after centrifugation are shown in Figure 2. Cells expressing ribosomes with the A_{18} mutation showed a profile with very prominent free subunits peaks. Control cells, expressing 30S subunits with the wild-type central pseudoknot showed a dominant peak of 70S tight couples, while free 30S and 50S subunits were almost absent.

By primer extension, using the spectinomycin resistance marker present on specialized 16S rRNA, but not on chromosomally encoded rRNA (16), we determined the relative amount of

specialized 30S subunits in the different fractions taken from the gradient (Table 1). In the wild-type 70S tight couple fraction (wt/70S) the specialized ribosomes represented 78% of the population while the 70S fraction from mutant cells (A_{18}/70S) contained only 28% specialized ribosomes. The rest of the mutant ribosomes accumulated in the free 30S subunit fraction, A_{18}/30S, which consisted for 87% of specialized 30S subunits. We calculated that only 20% of the mutant ribosome population was in the tight couples. Apparently, the A_{18} mutation interferes with the association to 50S. The mutation does not influence the total amount of specialized 30S subunits synthesized per cell (data not shown).

Table 1. Proportion of specialized 30S subunits present in 70S and 30S fractions

Sequence/ fraction[a]	% specialized 30S[b]
wt/70S	78 ± 4
A_{18}/70S	28 ± 5
A_{18}/30S	87 ± 3
A_{18}/70S (column)[c]	66 ± 4

[a]70S and 30S sucrose gradient fractions from S30 extracts of cells expressing wild-type 30S subunits (wt) or mutant 30S subunits containing the A_{18} mutation. [b]Relative levels of specialized 30S subunits were determined as described in Materials and Methods. Values are the average of two independent experiments. [c]As A_{18}/70S but after affinity column treatment (see text).

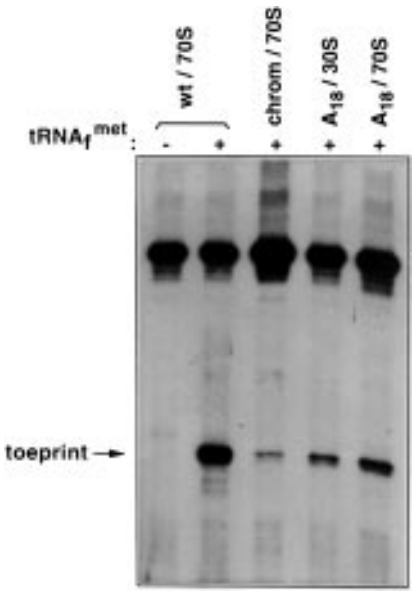

Figure 3. Detection of 30S initiation complexes on specialized CAT mRNA. Toeprinting reaction mixtures contained 72 nM 30S subunits. Fractions are described in Table 1. The chrom/70S fraction contains only chromosomally encoded 30S subunits and is derived from cells not expressing specialized ribosomes. The position of the reverse transcriptase stops (toeprints), 13 nucleotides downstream of the startcodon of the *cat* gene, is indicated by an arrow.

Mutant 30S subunits isolated from 70S tight couples are active in 30S initiation complex formation

We measured the efficiency of 30S initiation complex formation on CAT mRNA, containing a specialized SD sequence, using the toeprinting method (20). Figure 3 shows that formation of a ternary complex on the CAT mRNA is strictly $tRNA_f^{Met}$ dependent (lanes 1 and 2) and specific for specialized ribosomes (lanes 2 and 3). We calculated that specialized 30S subunits bind 20–30 times better to the CAT messenger than chromosomally encoded 30S subunits (data not shown).

The toeprint intensities of the mutant particles (lanes 4 and 5) seemed decreased as compared to the wild-type control (lane 2). However, one should take into account that the 30S subunit fractions contain variable amounts of specialized and chromosomally encoded 30S subunits (Table 1), for which we have to make a correction (see Table 2 legend).

When we then calculated the toeprint efficiency, it turned out that mutant 30S subunits from the 70S tight couple fraction were almost as active (34%) in 30S initiation complex formation as the wild-type (43%, Table 2, compare A_{18}/70S and wt/70S, before partial reconstitution). Apparently, 16S rRNA in which the central basepair of helix 2 of the central pseudoknot is disrupted can be assembled into 30S subunits that are active in initiation complex formation. Unlike the particles derived from tight couples, mutant 30S subunits from the free subunit fraction, A_{18}/30S, were severely impaired in the formation of a ternary complex. This was, at least in part, due to the loss of ribosomal proteins (see below).

Mutant 30S subunits are unstable but can be reactivated by partial reconstitution using a total 30S ribosomal protein extract

In an attempt to isolate pure mutant 30S subunits, we used an affinity chromatography procedure (15). In this system a DNA oligonucleotide, complementary to the 16S rRNA 3′ end of chromosomally encoded 30S subunits, is attached to a column-matrix. The column retains the chromosomally encoded 30S subunits while the specialized 30S run through. For wild-type 30S subunits an almost homogeneous (>97%) and active population could be obtained, starting from a fraction containing 70–80% specialized 30S subunits (15). Purification of 30S subunits having the A_{18} mutation, however, was not straightforward. Starting with the 70S tight couple fraction which contained only 28% specialized 30S, we achieved an enrichment to 66% (Table 1).

Unfortunately, and in contrast to wild-type 30S subunits, the mutant particles showed a three-fold decrease in toeprinting efficiency upon column treatment [Table 2, compare A_{18}/70S and A_{18}/70S (column), before partial reconstitution]. Apparently, the pseudoknot mutation causes instability in the 30S subunits which, in combination with the purification procedure, diminishes their activity.

A possible explanation for the inactivation of the mutant particles was the loss of ribosomal proteins needed for 30S initiation complex formation. To test this idea we performed partial *in vitro* reconstitution by adding total 30S proteins to the ribosome fractions. Table 2 shows that the mutant particles could indeed be reactivated. In particular, the mutant 30S subunits inactivated by the column treatment showed a strong increase in activity upon addition of ribosomal proteins [Fig. 4 and Table 2, A_{18}/70S (column) after partial reconstitution]. Addition of two molar equivalent of TP30 extract to this fraction led to complete restoration of the toeprinting efficiency. This indicates that the inactivation of mutant 30S subunits by affinity chromatography is due to a loss of ribosomal proteins. The mutant 30S subunits in the A_{18}/30S fraction, which already have a low activity upon isolation, also showed an increase in initiation complex formation

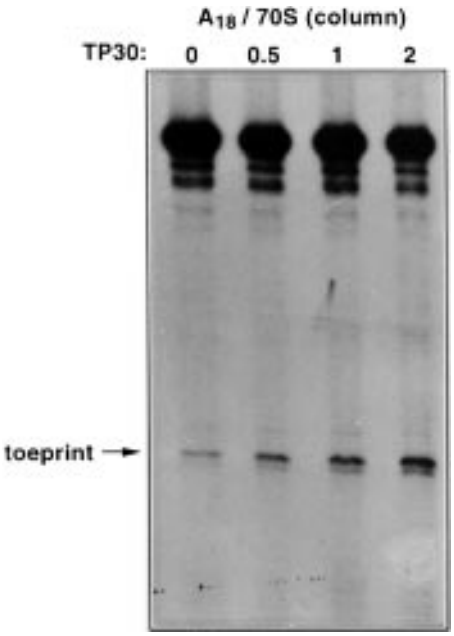

Figure 4. Detection of 30S initiation complexes on specialized CAT mRNA after partial reconstitution. Toeprinting reaction mixtures contained 36 nM 30S subunits from the A_{18}/70S fraction after affinity column treatment (Table 1). Molar equivalents of 30S ribosomal protein extract (TP30) added to the reaction mixtures are indicated. The position of the reverse transcriptase stops (toeprints), 13 nucleotides downstream of the startcodon of the *cat* gene, is indicated by an arrow.

upon reconstitution. This shows that the defect in the mutant free 30S subunits is, at least in part, also due to loss of ribosomal proteins.

Table 2. Relative toeprinting efficiencies of specialized 30S subunits on specialized CAT mRNA (%)[a]

Sequence/ fraction	Before/	After partial reconstitution[b]		
		0 TP30	0.5 TP30	2 TP30
wt/70S	43 ± 4	36 ± 3	36 ± 3	20 ± 2
A_{18}/70S	34 ± 4	9 ± 2	18 ± 3	16 ± 3
A_{18}/30S	7 ± 2	2 ± 0.2	6 ± 1	8 ± 2
A_{18}/70S (column)	11 ± 1	3 ± 0.5	8 ± 0.5	17 ± 2

[a]Toeprinting reaction mixtures contained 72 nM 30S subunits from the wt/70S, A_{18}/70S or A_{18}/30S fraction. In case of the A_{18}/70S (column) fraction, the reaction mixture contained 36 nM 30S subunits. Fractions are described in Table 1. To compare the activity in the different samples, we normalized the toeprinting efficiencies to a constant concentration of 72 nM specialized 30S subunits, usingdata of Table 1. To justify this extrapolation we verified that the toeprint intensity is proportional to the specialized 30S concentration by performing experiments with two- and four-fold decreased concentrations of 30S subunits. The presence of chromosomally encoded 30S subunits in the samples contributes little to the toeprint intensity (Fig. 3). Nevertheless, we compensated for this minor activity. Data before reconstitution represent the average of four independent experiments and data after reconstitution are the average of two independent experiments.
[b]Indicated are the molar equivalents of total 30S ribosomal proteins added to the reaction mixture.

The toeprinting activity of wild-type 30S subunits did not increase after addition of the extract. Rather, higher amounts of total ribosomal protein decreased the activity of these 30S subunits, as shown in Table 2 (compare wt/70S, 0 TP30 and 2 TP30). This effect was probably related to the excess of ribosomal protein S1 over 30S subunits in the sample. Free S1 competes with ribosomes for binding to the ribosome binding site on the messenger (23,24).

The reconstitution buffer by itself had a deleterious effect on the activity of the mutant 30S subunits (Table 2, compare A_{18}/70S before and after partial reconstitution, 0 TP30). Wild-type 30S subunits were nearly unaffected by the change in conditions. The adverse effect of high salt on the mutant ribosomes can also be interpreted in terms of lower stability of the 30S particles with a disrupted central pseudoknot.

Binding of a DNA oligonucleotide to the 3′ end of 16S rRNA of mutant 30S subunits is decreased in a nitrocellulose filter-binding assay

Accessibility of the 3′ end of the 16S rRNA is obligatory for 30S initiation complex formation on the mRNA. 30S subunits inactivated in ternary complex formation by low magnesium treatment (25) or by the lack of ribosomal protein S21 (26) showed no oligonucleotide binding to their 16S rRNA 3′ end (27).

Here, we tested the binding of a nonamer complementary to the 3′ end of specialized 16S rRNA. The labelled oligonucleotide was added in four-fold molar excess over 30S subunits that contain the wild-type or the mutant central pseudoknot. Bound nonamer was measured in a nitrocellulose filter binding assay. As shown in Table 3, mutant 30S subunits isolated from the free subunit or 70S tight couple fraction, A_{18}/30S and A_{18}/70S, respectively, bound less oligonucleotide than wild-type 30S subunits, wt/70S. The oligonucleotide did not bind to chromosomally encoded 30S subunits and the counts therefore did not need to be corrected for the variable amounts of these 30S particles present in the samples.

Table 3. Nitrocellulose filter binding assays

Sequence/ fraction[a]	% 30S active in oligo binding[b]
chrom/70S[c]	0
wt/70S	69 ± 4
A_{18}/70S	16 ± 4
A_{18}/30S	17 ± 2

[a]70S and 30S sucrose gradient fractions from S30 extracts of cells expressing wild-type 30S subunits (wt) or mutant 30S subunits containing the A_{18} mutation.
[b]Nitrocellulose binding values of a nonamer deoxyoligonucleotide complementary to the specialized 16S rRNA 3′-end sequence. Values are the average of four independent experiments and represent the percentage of the specialized 30S subunits that bind the oligonucleotide.
[c]70S sucrose gradient fraction from an S30 extract of cells not expressing specialized ribosomes. This fraction therefore contains only chromosomally encoded 30S subunits. The binding value of this fraction represents the percentage of the chromosomally encoded 30S subunits that bind the oligonucleotide.

As a control that the isolation procedure by itself was not harmful for the quality of the ribosomes, we tested the binding of an oligonucleotide complementary to the 3′ end of chromosomally encoded 16S rRNA. This nonamer bound stoichiometrically to the chromosomally encoded 30S subunits in all of the tested fractions (data not shown), indicating that the poor oligo binding to the mutant 30S subunits was not due to the isolation procedure. We suspect loss of ribosomal proteins from mutant ribosomes on the nitrocellulose filter and the consequent release of the oligonucleotide.

DISCUSSION

We have investigated the activity of ribosomes in which helix 2 of the central pseudoknot in 16S rRNA was disrupted by a mutation changing the central base pair of this helix into a mismatch. This $C_{18}A$ mutation was shown by Brink *et al.* (12) to inhibit translation *in vivo*. By sucrose gradient analysis, these authors showed that the mutant 30S subunits accumulate in the 30S fraction and do not form polysome complexes. Therefore, a defect in translation initiation was suggested. Taking this into account, we concentrated our research on the formation of the 30S initiation complex.

We observed that mutant 30S subunits, when derived from the 70S tight couple fraction, have an efficiency in initiation complex formation that is almost as high as the control. Complementarity of the central basepair in helix 2 is therefore, although obligatory for completing the initiation process *in vivo* (12), not essential for performing the first step in this process *in vitro* i.e. specific and efficient binding to the ribosome binding site on the mRNA and facilitating codon-anticodon interaction in the P site of the 30S subunit (28).

The strong preference of the specialized CAT mRNA for specialized 30S compared to chromosomally encoded 30S in the toeprint assay shows the importance of the SD interaction for the formation of an initiation complex. The accessibility of the ASD sequence in the 30S subunit can be tested by the binding of a complementary oligonucleotide (25–27). We showed that mutant 30S subunits from the tight couple fraction were inefficient in oligo-binding, implying an unavailable ASD sequence. On the other hand we had measured efficient 30S initiation complex formation. This paradox needed an explanation.

A possible clue came with the inactivation of mutant particles by the affinity chromatography treatment. This method can potentially be used to prepare a homogeneous fraction of specialized 30S subunits without loss of activity in initiation complex formation (15). We showed here that the inactivation of the mutant 30S subunits by the column was probably due to the loss of ribosomal proteins since we could reactivate the particles by adding a total 30S ribosomal protein extract. In the oligo-binding assay, we also diluted the 30S fractions during the washing step of the nitrocellulose filtration procedure. We suspect that this dilution step, possibly in combination with the adsorption to the filter surface, is detrimental to the mutant particles. Understandably, in the toeprint assay, where such a step is absent, we found no decrease in activity.

The observation that the loss of ribosomal proteins inactivates a (mutant) ribosome, implies that these proteins are important for initiation complex formation and oligo binding to the 16S rRNA 3′ end. For the mutant 30S from the free subunit fraction, we analyzed the ribosomal protein content and found a decreased presence of S1, S2, S18 and S21 (29). S1 and S21 are essential for initiation complex formation (26,30) and S21 is also important for an accessible 3′ end (27). The reduced affinity in the mutant 30S subunits for S1 and S21 may therefore account for their conditional inactivity.

The mutant 30S active in forming an initiation complex were derived from 70S tight couples and represented 20% of the total mutant population. This number is significantly higher than the almost negligible amount of ribosomes found in the polysomal fractions by Brink *et al.* (12). A similar phenomenon was observed in the study of another pseudoknot in *E.coli* 16S rRNA, formed by the interaction between nucleotides $G_{570}U_{571}$ and $C_{866}A_{865}$ (11). In the translation incompetent mutant A_{571}, formation of polysomes was severely inhibited while tight couple formation was not disturbed. Unlike polysomes, 70S tight couples are not supposed to contain mRNA or tRNA. The similar behavior of these two translationally inactive mutants suggests more stringent demands on 30S subunits for incorporation into a programmed 70S complex than into a tight couple.

In the group of Brakier–Gingras, the implications for translational activity of substitutions $U_{13}A$, $A_{914}U$, and the double mutation were examined *in vivo* and *in vitro* (31–33). These pseudoknot mutations (for their positions, see Fig. 1) impaired growth when cells depended on the mutant ribosomes (33). In toeprint experiments, using a 50/50 mix of mutant and wild-type 30S subunits, a 25% decrease in 30S initiation complex formation was observed. Also, the 915–921 region in the mutant 16S rRNA showed a modest increase in accessibility for oligonucleotide binding and for modification of G_{917} by kethoxal. Since helix 2 is formed by basepairing to a part of this region, the authors suggest that an undisrupted central pseudoknot is necessary for ternary complex formation (33). However, the incorporation of mutant ribosomes into polysomes was still 60% of the incorporation observed in the wild-type control (32). If the major defect *in vivo* in these mutant 30S subunits is in initiation, one would expect their presence to be less prominent in the polysomal complexes. Here, we studied 30S subunits with a more seriously disrupted helix 2. Translational activity is absent and the mutant ribosomes do not form polysomes (12). Nevertheless, the mutant 30S are almost fully capable of forming a 30S initiation complex, indicating that disruption of helix 2 causes a defect other than ternary complex formation.

A_{18} mutant 30S subunits do not form programmed 70S complexes and were therefore suggested to have a defect in translation-initiation (12). Given our finding that 30S initiation complex formation is not affected, the most obvious translational defect would be the association of this complex with the 50S subunit.

Baudin *et al.* (13) showed that in 16S rRNA the 770–930 region together with nucleotides 19 and 20 were protected from chemical modification upon association with the 50S subunit. This is in agreement with structure models for the 30S subunit (6,7) in which the central pseudoknot region is located at the interface between the 30S and 50S subunit. Disruption of the central basepair of helix 2 might therefore interfere with the interaction between the subunits. The instability of the mutant particles, resulting in an easy loss of several ribosomal proteins predicts also an important role of this pseudoknot in the overall architecture of the 30S subunit. The proposed position of the central pseudoknot at the junction of the three major domains in the 16S rRNA (6,7) would agree with such a structural function.

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