The EMBO Journal vol.12 no.10 pp.3987-3996, 1993

Formation of the central pseudoknot in 16S rRNA is essential for initiation of translation

Marcel F.Brink, Martin Ph.Verbeet and Herman A.de Boer

Department of Biochemistry, Gorlaeus Laboratories, University of Leiden, Einsteinweg 55, 2333CC Leiden, The Netherlands

Communicated by L.Bosch

The postulated central pseudoknot formed by regions 9-13/21-25 and 17-19/916-918 of 16S rRNA of *Escherichia coli* is phylogenetically conserved in prokaryotic as well eukaryotic species. This pseudoknot is located at the center of the secondary structure of the 16S rRNA and connects the three major domains of this molecule. We have introduced mutations into this pseudoknot by changing the base-paired residues C_{18} and G_{917}, and the effect of such mutations on the ribosomal activity was studied *in vivo*, using a 'specialized' ribosome system. As compared with ribosomes having the wild-type pseudoknot, the translational activity of ribosomes containing an A, G or U residue at position 18 was dramatically reduced, while the activity of mutant ribosomes having complementary bases at positions 18 and 917 was at the wild-type level. The reduced translational activity of those mutants that are incapable of forming a pseudoknot was caused by their inability to form 70S ribosomal complexes. These results demonstrate that the potential formation of a central pseudoknot in 16S rRNA with any base-paired residues at positions 18 and 917 is essential to complete the initiation process.

Key words: central pseudoknot/rRNA/specialized ribosomes/translational initiation

Introduction

Detailed models have been proposed for the arrangement of the 16S rRNA molecule of *Escherichia coli* within the 30S subunit (Expert-Bezançon and Wollenzien, 1985; Brimacombe *et al.*, 1988; Stern *et al.*, 1988; Hubbard and Hearst, 1991). These models suggest that the 16S rRNA molecule forms three major structural domains of which the 5'-domain mainly constitutes the body of the 30S subunit, the central domain is part of the platform and the 3'-major domain is located within the head of this subunit.

At the center of the proposed secondary structure of 16S rRNA, the three domains are connected by an RNA pseudoknot. RNA pseudoknots are formed when several residues in a loop of a hairpin structure form standard Watson-Crick base-pairs with a complementary sequence outside this loop (Pleij *et al.*, 1985). Consequently, a pseudoknot consists of two helices, one forming the stem and the other formed by base-paired residues in the loop of the hairpin. Comparative sequence analyses have shown that there are three potential pseudoknot structures in the 16S rRNA molecule (Pleij *et al.*, 1985; Gutell *et al.*, 1986; Woese and Gutell, 1989). The phylogenetic conservation of these pseudoknots may indicate that the formation of such structures is essential for ribosomal activity. In fact, Powers and Noller (1991) showed that the pseudoknot formed by base-pairing of residues within the loop of the 500-546 hairpin structure is required for ribosome function. In the central pseudoknot, the residues $U_{17}C_{18}A_{19}$ in the loop of the 5'-terminal hairpin structure are base-paired with residues $U_{916}G_{917}A_{918}$ (Pleij *et al.*, 1985). This structure can be considered as a central core element in the 30S ribosomal subunit from which the three major structural domains of 16S rRNA emerge (see Figure 1).

By probing the structure of 16S rRNA within translationally active 30S subunits, Moazed and Noller (1989) provided experimental evidence that 16S rRNA does not adopt a rigid conformation, but rather that alternating structural changes can occur within this molecule. Based on comparative sequence analyses (Neefs *et al.*, 1990), two such conformational switches have been proposed, both of which involve the alternating disruption and formation of the helix formed by residues $U_{17}C_{18}A_{19}$ and $U_{916}G_{917}A_{918}$ (Kössel *et al.*, 1990; Leclerc and Brakier-Gingras, 1991), i.e. disruption and formation of the central pseudoknot.

We have studied the requirement of the potential formation of the central pseudoknot for the function of the 30S subunit by introducing mutations at position 18 and 917, respectively. To determine the effects of such mutations on the ribosomal activity *in vivo*, we used a specialized ribosome system (Hui and de Boer, 1987; Hui *et al.*, 1987). Due to the altered anti-Shine-Dalgarno (ASD) sequence ($^{5'}$CACAC$^{3'}$) at the terminal 3'-end of 16S rRNA, ribosomes containing this rRNA molecule are 'specialized' as they only translate a single mRNA species with a modified Shine-Dalgarno (SD) sequence ($^{5'}$GTGTG$^{3'}$). In the system used in our laboratory, the specialized mRNA species encodes chloramphenicol acetyl transferase (CAT). The suitability of the specialized ribosome system for studying the effect of any mutation in 16S rRNA at the translational level is based on the fact that the specialized ribosomes predominantly translate the CAT mRNA species, while the chromosomally encoded wild-type ribosomes, lacking a complementary ASD sequence, are unable to translate this CAT mRNA. Thus, when determining the accumulation of CAT, the translational activity of specialized ribosomes is assessed without the translational interference of wild-type ribosomes.

Here, we demonstrate that replacing the C residue at position 18 with any other nucleotide, hence affecting the potential interaction between regions 17-19 and 916-918, leads to a reduced translational activity. Introduction of complementary mutations at positions 18 and 917 results in the formation of fully active ribosomes. These results prove that the central pseudoknot indeed exists, and that it is essential for ribosomal function. Mutations in the central

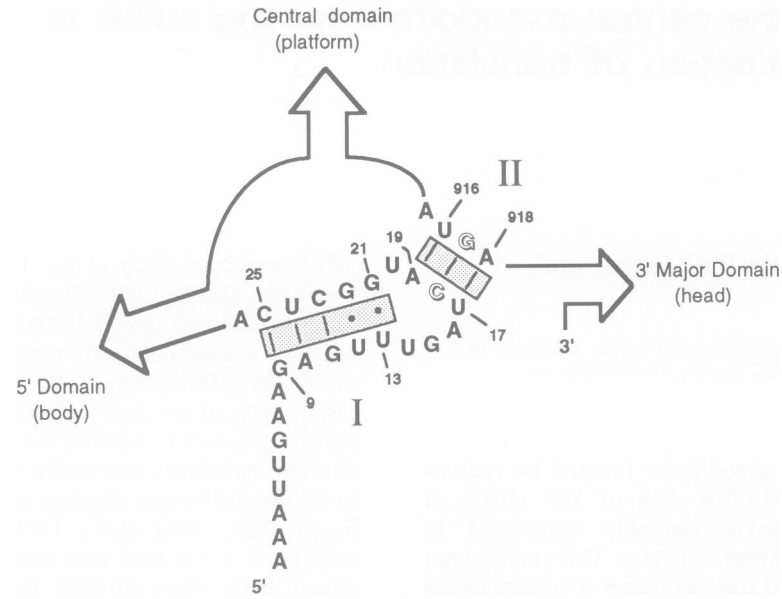

Fig. 1. Schematic diagram of the central pseudoknot connecting the three major domains of 16S rRNA. The sequences and secondary structure are according to Stern *et al.* (1988). The central pseudoknot consists of helix I (nucleotides 9−13/21−25) and helix II (nucleotides 17−19/916−918). These two coaxially stacked helices (indicated by shaded boxes) form a central core element in 16S rRNA. The arrows indicate the relative orientation of the three major domains of 16S rRNA protruding from this structure. The $C_{18}-G_{917}$ base-pair that was subjected to mutational analysis is presented with open letters.

pseudoknot do not affect assembly of mutant 16S rRNA into ribosomal particles, nor do they interfere with the processing of the 5′-end of the molecule. However, 30S subunits with a disrupted pseudoknot are not capable of forming 70S ribosomal complexes, thus implying that these subunits are impaired at the stage of translational initiation.

Results

Introduction of mutations into the central pseudoknot

Figure 1 shows the pseudoknot located at the center of the 16S rRNA molecule together with the relative orientation of the three major structural domains, as seen from the interface with the 50S ribosomal subunit. The central pseudoknot encompasses the base-paired rRNA regions 9−13/21−25 (helix I) and 17−19/916−918 (helix II). In the three-dimensional structure these helices are coaxially stacked and form a central core element in 16S rRNA.

Based on phylogenetic data (Neefs *et al.*, 1990), we have compared the primary and secondary structure of the central pseudoknot, as formed in 232 different 16S-like rRNA molecules. Figure 2 demonstrates that the sequence of helix I varies between the different groups, while there is a consensus sequence for helix II: $U_{17}C_{18}C_{19}/G_{916}G_{917}A_{918}$. Only three of the eukaryotic and four of the eubacterial species analyzed (one of which is *E.coli*) differ from this consensus, having a $U_{19}-A_{916}$ or $A_{19}-U_{916}$ base-pair, respectively. Obviously, despite the sequence variation within the two helices, the secondary structure (i.e. a pseudoknot structure) is universally conserved.

In order to study whether this pseudoknot is required for ribosomal function, substitutions were introduced into helix II. By polymerase chain reaction (PCR) based mutagenesis, the C residue at position 18 was replaced with an A, G or U residue. For the wild-type sequence, the calculated free energy (ΔG_0: Freier *et al.*, 1986) of helix II is −4.1

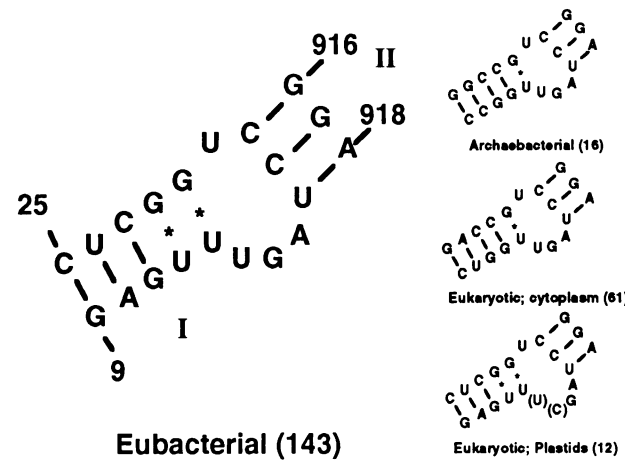

Fig. 2. Phylogenetic conservation of the central pseudoknot. Each pseudoknot represents the consensus primary sequence within the indicated group. Of the total number of different species used for this comparison (indicated within parentheses) a few show minor sequence variations (see text). Helices I and II are indicated (see Figure 1).

kcal/mol. With an A_{18} or G_{18} residue the free energy would be increased up to +0.8 kcal/mol, implying that helix II could not be formed. In contrast, the introduction of U_{18} would result in a moderate increase in free energy (i.e. up to −1.2 kcal/mol) and therefore helix II could be formed containing a $U_{18}-G_{917}$ 'wobble' base-pair. Since helix II can be formed with any sequence, provided that the composing sequences are complementary, it would be possible to restore the central pseudoknot by introducing residues at positions 18 and 917 that complemented one another. Therefore, the mutants $A_{18}U_{917}$, $G_{18}C_{917}$ and $U_{18}A_{917}$ were made.

The effects on ribosomal activity of these disruptive and

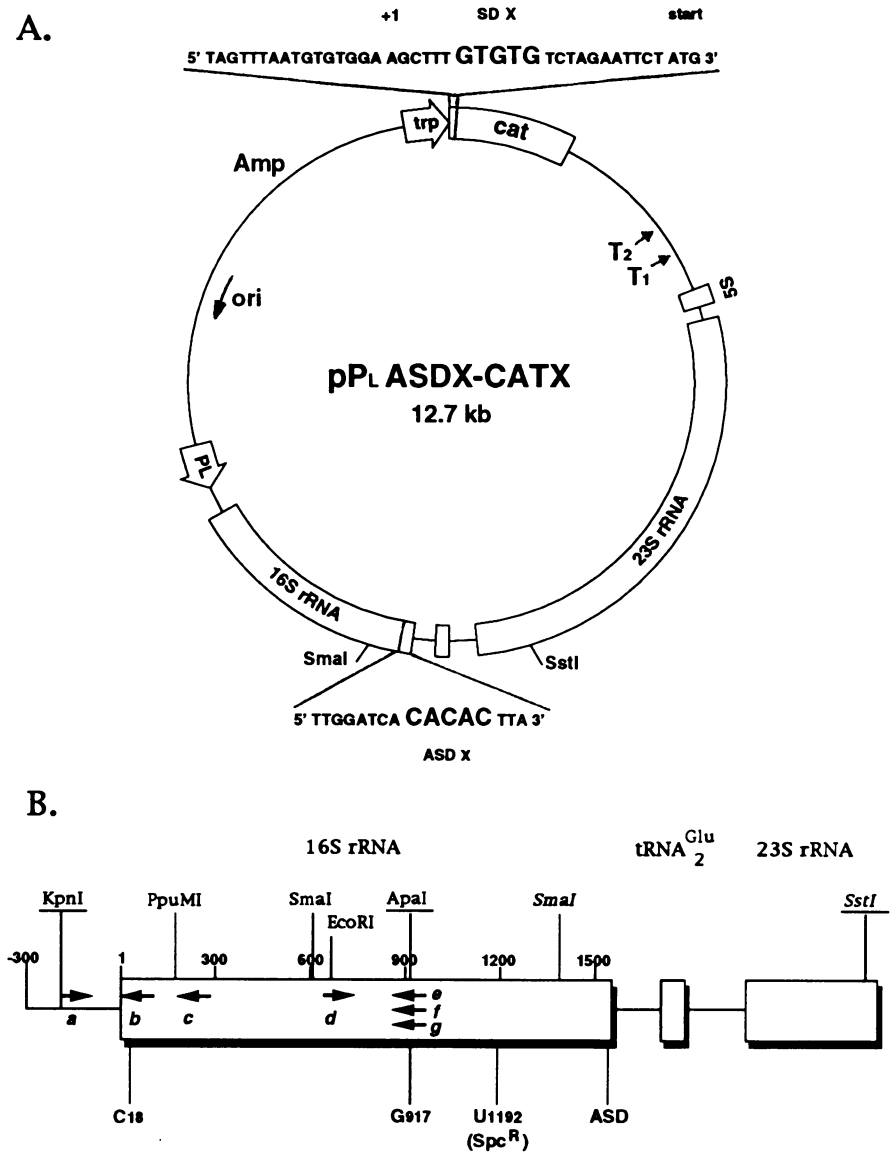

Fig. 3. The specialized ribosome system. (**A**) Structure of the plasmid encoding the specialized ribosome system. The sequence at the 5′-end of the *cat* gene harboring the modified Shine–Dalgarno sequence (SDX: 5′GTGTG3′) and that at the 3′-end of the 16S rRNA gene containing the complementary anti-Shine–Dalgarno sequence (ASDX: 5′CACAC3′) are shown. The *cat* gene is under control of a constitutive *trp* promoter, whereas the rRNA operon *rrnB* is driven by a thermo-inducible lambda P_L promoter. Negative control cells contain a plasmid which is identical to the specialized ribosome system, except for a deletion in the *rrnB* operon between the *SmaI* restriction site at position 1386 of 16S rRNA and the *SstI* restriction site at position 365 of 23S rRNA; pASDΔ(*SmaI*–*SstI*)-CATX. Upon induction of the lambda P_L promoter, cells containing this plasmid do not accumulate specialized ribosomes. (**B**) Location of key restriction sites within the *rrnB* operon and orientation of primers used for PCR. The locations of the mutations at positions 18, 917 and 1192 of 16S rRNA relative to the restriction sites used for construction of the various mutants are indicated. Unique restriction sites are underlined and the *SmaI* and *SstI* restriction sites that flank the deletion in pASDXΔ(*SmaI*–*SstI*)-CATX are shown in italics. The location and orientation of the primers used in PCRs (see Materials and methods) are indicated; (a) positions −180 to −154, (b) positions 4–30, (c) positions 256–276, (d) positions 674–692, (e, f and g) positions 908–933.

compensating mutations in the central pseudoknot were analyzed *in vivo* using a specialized ribosome system (see Figure 3A; Hui and de Boer, 1987; Hui *et al.* 1987). The ribosomal activity was assessed by monitoring the synthesis of CAT either by measuring its enzymatic activity or by metabolic labelling of this protein. The preferential incorporation of L-[^{35}S]methionine into CAT was achieved by labelling in the presence of spectinomycin. Labelling of endogenous proteins by wild-type ribosomes is blocked in the presence of spectinomycin, so only the specialized ribosomes [which were rendered resistant to spectinomycin by a C to U change at position 1192 of the 16S rRNA (Sigmund *et al.*, 1984)], can incorporate label into CAT.

An intact pseudoknot structure is required for ribosomal function

The accumulation of CAT in cells harboring specialized ribosomes with a wild-type or mutated pseudoknot was determined by measuring the enzymatic activity of CAT in whole cell lysates. After induction of the synthesis of specialized ribosomes, samples were taken at 30 min intervals and the CAT activity was determined by measuring the amount of [^{3}H]diacetyl-chloramphenicol formed (c.p.m./OD_{650}). Figure 4A shows that, in the case of specialized ribosomes with the wild-type pseudoknot, the CAT activity increases linearly, while for the A_{18} and G_{18} mutants the CAT activity is barely above the background

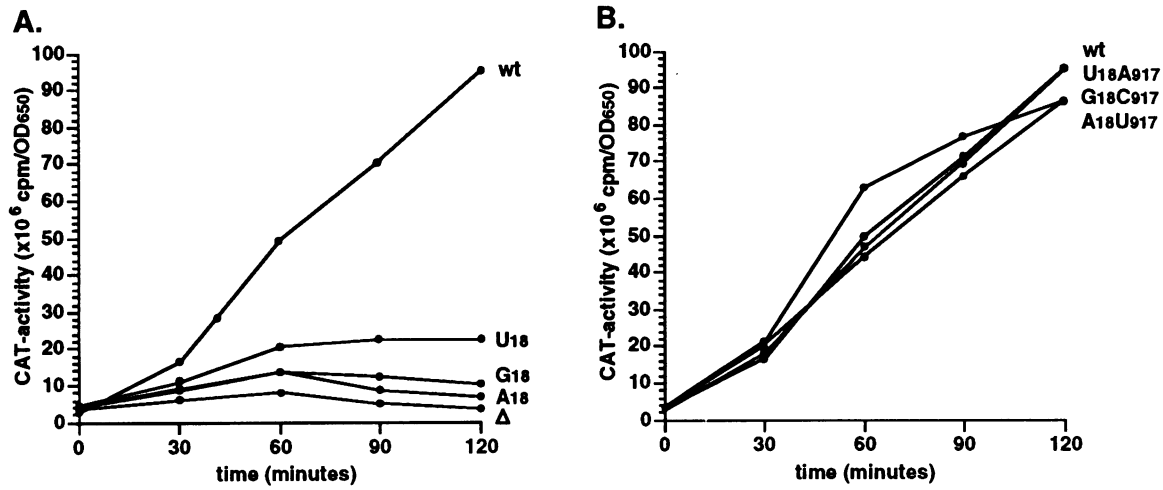

Fig. 4. Ribosomal activity of specialized ribosomes with the wild-type or mutated central pseudoknot. The ribosomal activity of the specialized ribosomes was assessed by measuring the CAT activity in cell lysates. After heat induction of the synthesis of specialized ribosomes (t = 0), samples were taken at 30 min intervals. The CAT activity was determined by measuring the amount of [^{3}H]diacetyl-chloramphenicol formed. (**A**) The CAT activity in cells harboring specialized ribosomes with the wild-type pseudoknot (wt) compared with cells harboring the pseudoknot mutants A_{18}, G_{18} and U_{18} or cells harboring the negative control plasmid (see Figure 3). (**B**) The CAT activity in cells harboring specialized ribosomes with the wild-type pseudoknot (wt) compared with cells harboring the pseudoknot mutants $A_{18}U_{917}$, $G_{18}C_{917}$ or $U_{18}A_{917}$.

level; 2 h after induction, being merely 5% and 10% respectively of the wild-type activity. In contrast, the CAT activity for the U_{18} mutant increases slightly, to 25% of the wild-type level.

To determine whether mutations that would potentially allow the formation of helix II could lead to fully active ribosomes, the CAT activities in cells harboring specialized ribosomes with complementary residues at positions 18 and 917 were determined. Figure 4B shows that the CAT activities in cells harboring such ribosomes increase to the wild-type level. Therefore, these results demonstrate that the central pseudoknot indeed exists and that its restoration, regardless of which Watson–Crick base-pair is introduced, results in full ribosomal activity.

The low CAT activity in cells harboring mutants A_{18}, G_{18} or U_{18} is probably due to a low rate of CAT synthesis. However, it is also conceivable that a reduced translational fidelity, resulting in premature termination or leading to a higher rate of erroneously incorporated amino acids, is the cause of the low enzymatic activity. To determine whether any of the mutant ribosomes synthesize either low amounts of CAT or a truncated and thus enzymatically inactive protein, the size of CAT synthesized by specialized ribosomes with a wild-type or mutated pseudoknot was assessed by SDS–PAGE. Figure 5 shows the protein profile in L-[^{35}S]methionine-labelled cells harboring the indicated mutations. Obviously, the size of CAT synthesized by specialized ribosomes with any of the disruptive mutations (lanes 3, 5 and 7) is identical to the size of CAT synthesized by ribosomes with the wild-type pseudoknot (lane 2) or any of the ribosomes with complementary residues at positions 18 and 917 (lanes 4, 6 and 8). As no truncated forms of CAT are apparent, the translation of the CAT mRNA does not terminate prematurely on ribosomes with any of the disruptive mutations. Moreover, this experiment shows that the amount of L-[^{35}S]methionine incorporated into CAT corresponds to the measured CAT activities (see Figure 4), indicating that the low CAT activity is caused by a low rate of CAT synthesis, rather than by a gross increase in translational errors leading to protein instability or loss of enzymatic activity.

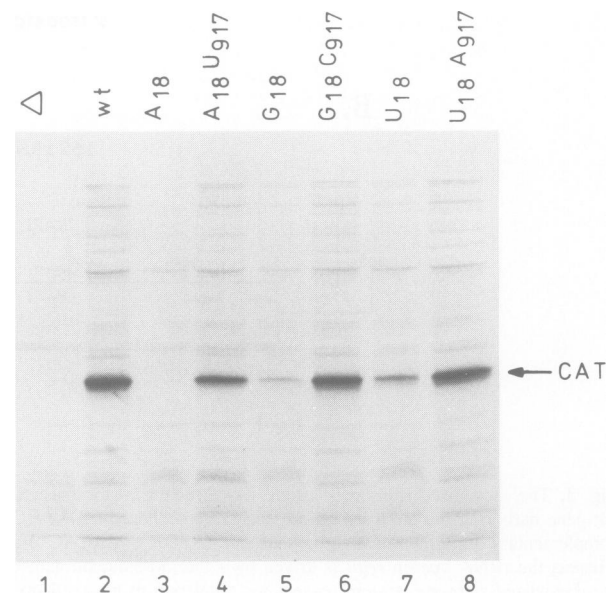

Fig. 5. Protein synthesis by specialized ribosomes with a wild-type or mutated central pseudoknot. Cells were grown for 3 h in M9 medium at 30°C and synthesis of specialized ribosomes was induced by a temperature shift to 42°C for 2 h. Protein synthesis by wild-type ribosomes was blocked by addition of spectinomycin (0.5 mg/ml) and after 15 min *de novo* synthesized proteins were metabolically labelled by addition of L-[^{35}S]methionine (760 μCi/μmol). Proteins were separated by SDS–PAGE (12.5%). Lane 1 shows the proteins synthesized in cells harboring the negative control plasmid (see Figure 3). Lane 2 shows the proteins synthesized by specialized ribosomes with the wild-type pseudoknot and lanes 3–8 show the proteins synthesized by specialized ribosomes with the indicated mutations. The position of CAT is indicated.

Figures 4 and 5 clearly demonstrate that, although the ribosomal activity of the U_{18} mutant is only 25% of the wild-type activity, it is still twice as high as the activity of ribosomes harboring A_{18} or G_{18}. Apparently, ribosomes

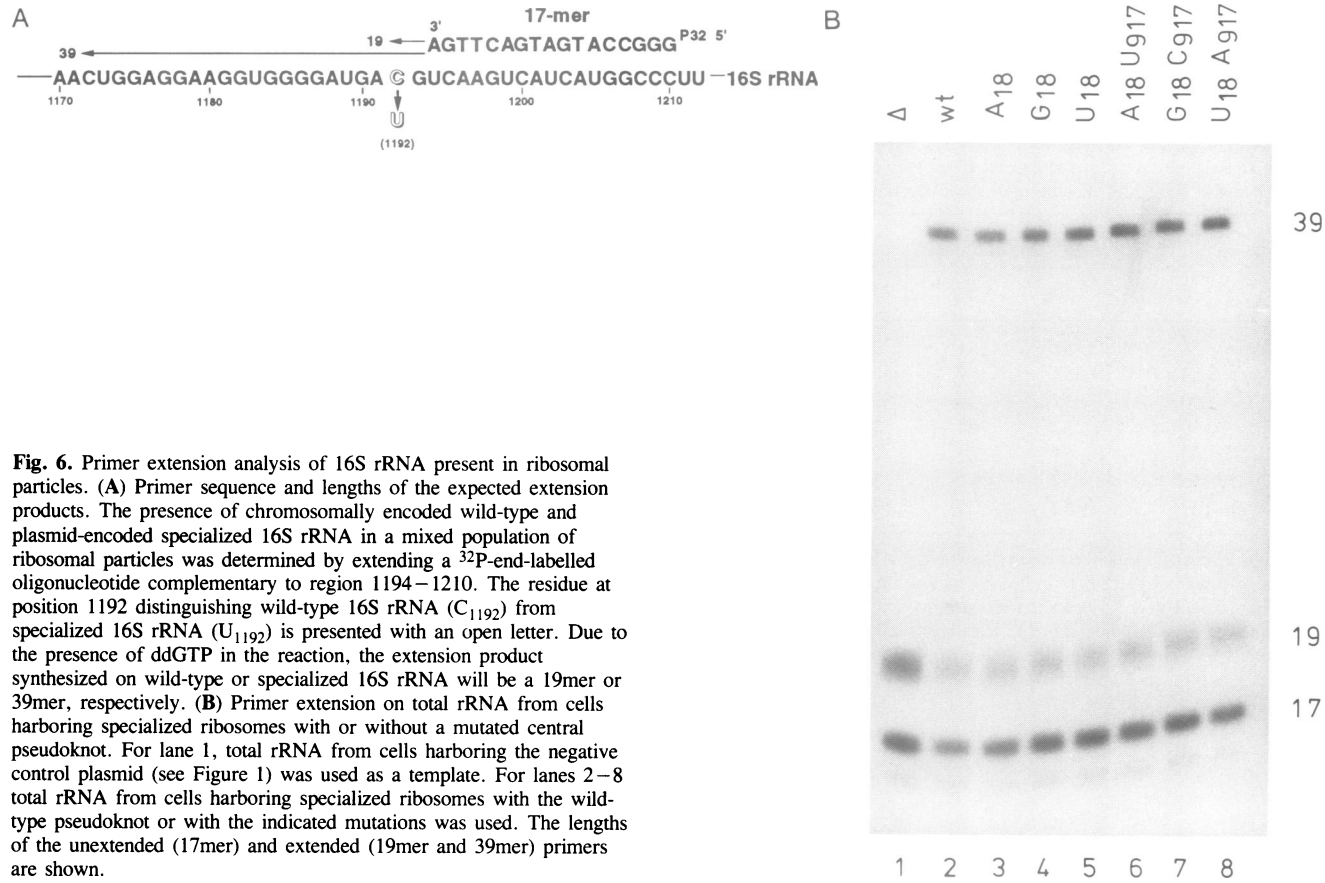

Fig. 6. Primer extension analysis of 16S rRNA present in ribosomal particles. (**A**) Primer sequence and lengths of the expected extension products. The presence of chromosomally encoded wild-type and plasmid-encoded specialized 16S rRNA in a mixed population of ribosomal particles was determined by extending a ^{32}P-end-labelled oligonucleotide complementary to region 1194−1210. The residue at position 1192 distinguishing wild-type 16S rRNA (C_{1192}) from specialized 16S rRNA (U_{1192}) is presented with an open letter. Due to the presence of ddGTP in the reaction, the extension product synthesized on wild-type or specialized 16S rRNA will be a 19mer or 39mer, respectively. (**B**) Primer extension on total rRNA from cells harboring specialized ribosomes with or without a mutated central pseudoknot. For lane 1, total rRNA from cells harboring the negative control plasmid (see Figure 1) was used as a template. For lanes 2−8 total rRNA from cells harboring specialized ribosomes with the wild-type pseudoknot or with the indicated mutations was used. The lengths of the unextended (17mer) and extended (19mer and 39mer) primers are shown.

containing U_{18} are still able to function, albeit slowly due to the formation of a pseudoknot with a weak $U_{18}-G_{917}$ 'wobble' base-pair. As the calculated free energy of a helix is temperature-dependent, it might be expected that the weakened pseudoknot with the central U−G base-pair would be more stable at lower temperatures. The temperature-dependent activity of the U_{18} mutant was studied by analyzing its ribosomal activity at 25, 34 and 42°C. The synthesis of the mutant ribosomes was induced at 42°C and subsequently the temperature was either maintained at 42°C or shifted down to either 25°C or 34°C, before CAT was metabolically labelled. As compared with specialized ribosomes with the wild-type pseudoknot, the relative amount of L-[^{35}S]methionine incorporated into CAT by specialized ribosomes with U_{18} did not appear to increase at any of the lower temperatures (data not shown), implying that the ribosomal activity of the U_{18} mutant is not temperature-dependent.

Mutations in the central pseudoknot do not affect the assembly of 16S rRNA into ribosomal particles

The low rate of CAT synthesis in cells harboring ribosomes with mutations A_{18}, G_{18} or U_{18} is caused either by a low translational activity of such ribosomes or by defective assembly of the mutant 16S rRNA into functional ribosomal particles. To investigate whether the pseudoknot mutations interfere with the assembly of 16S rRNA into specialized ribosomes, the cellular levels of such ribosomes were determined. For that purpose, total rRNA was isolated and the relative levels of plasmid-encoded specialized 16S rRNA and chromosomally encoded wild-type 16S rRNA were determined by the primer extension method using the residue at position 1192 (either U or C) to differentiate between these two rRNA species (Sigmund *et al.*, 1988).

As shown in Figure 6A, a ^{32}P-end-labelled oligonucleotide complementary to the region 1194−1210 of 16S rRNA is extended in the presence of ddGTP using reverse transcriptase. When annealed to chromosomally encoded wild-type 16S rRNA, termination will occur at position C_{1192}, resulting in a 19mer. When annealed to plasmid-encoded specialized 16S rRNA, termination will not occur at position 1192 due to the presence of a U residue; instead, a 39mer will be made. Since the oligonucleotide anneals to the identical sequence in specialized and in wild-type 16S rRNA, the relative intensities of the end-labelled 19mer and 39mer are correlated directly with the relative cellular levels of wild-type and specialized ribosomes, respectively.

Figure 6B shows the results of such a primer extension experiment using total rRNA isolated from a ribosomal pellet which was obtained by high speed centrifugation. In lanes 3−8, which represent the various pseudoknot mutants, the amount of 39mer present is clearly similar to that of the wild-type control (lane 2). This indicates that ribosomal particles with mutations in the central pseudoknot of 16S rRNA accumulate. Indeed, also based on the radioactivity measured in the 19mer and 39mer, the relative levels of specialized ribosomes with any of the indicated mutations are all the same as the wild-type control, i.e. in the range 50−60% (see Table I, left column). Thus, we conclude that the mutations in the central pseudoknot do not interfere with the assembly of the rRNA into ribosomal particles.

It should be borne in mind that total rRNA used in this procedure was isolated from a ribosomal pellet obtained by high speed centrifugation. This pellet not only contains

mature 30S, 50S and 70S ribosomal particles, but could also harbor ribosomal particles that lack a complete set of ribosomal proteins and yet are large enough to be pelleted.

Table I. Relative levels of specialized ribosomes present in whole cell lysates or the 30S, 70S, disome or trisome fractions

Mutant	Whole cell	Fraction			
		30S	70S	disomes	trisomes
Wild-type	54	51	41	34	41
A_{18}	53	53	9	7	9
G_{18}	50	54	6	3	4
U_{18}	59	52	24	22	19
$A_{18}U_{917}$	57	54	33	32	35
$G_{18}C_{917}$	56	59	41	33	36
$U_{18}A_{917}$	59	59	37	37	37

Relative levels of specialized ribosomes having a wild-type or mutated central pseudoknot were determined by measuring the radioactivity (c.p.m.) present in the 19mer and 39mers, as shown in Figure 6B (left column 'whole cell') and Figure 8. Relative levels of specialized ribosomes (%) were calculated as: [(c.p.m. in 39mer)/(c.p.m. in 19mer + c.p.m. in 39mer)] × 100%.

Therefore, it cannot be excluded that the mutations in the pseudoknot impair the binding of some of the ribosomal proteins, or that other stages in ribosome biogenesis such as the processing of the 5′- and 3′-ends of the precursor rRNA are affected.

Mutations in the central pseudoknot do not interfere with processing of the 5′-end of precursor rRNA

Correct processing of precursor rRNA to mature 16S rRNA is essential for the formation of translationally active 30S subunits (Wireman and Sypherd, 1974; Nomura and Held, 1974). Several endonucleases are involved in the maturation process leading to mature 16S rRNA. They do not seem to process the precursor in a fixed sequence of events, and they can act both on naked rRNA and on precursor rRNA which is already assembled into 30S subunits (Srivastava and Schlessinger, 1988).

To assess whether mutations in the central pseudoknot interfere with correct processing, we determined the 5′-end of 16S rRNA that was assembled into ribosomal particles (see previous section) using the primer extension method. Figure 7A shows the ^{32}P-end-labelled oligonucleotide that is complementary to region 19–35 of 16S rRNA and the

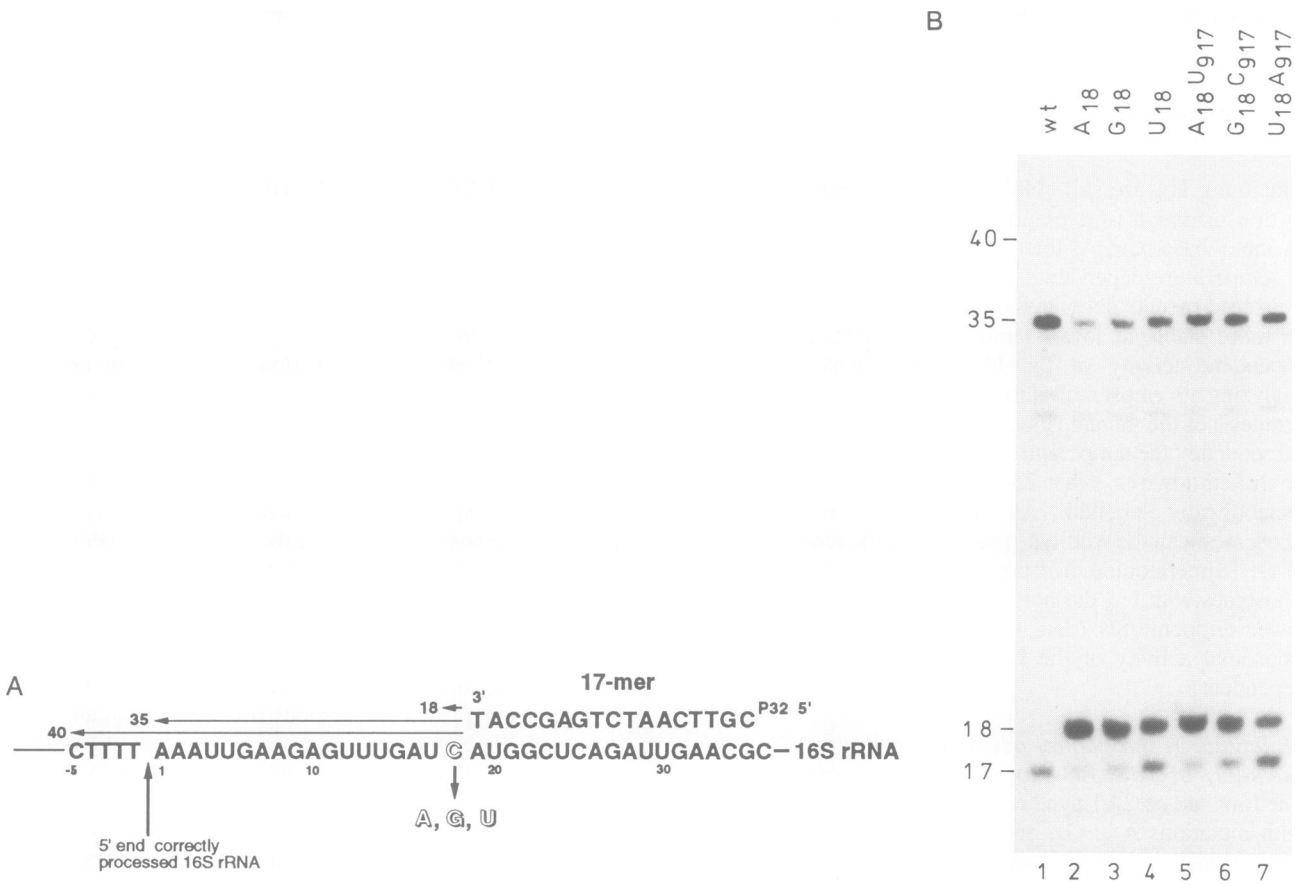

Fig. 7. Primer extension analysis at the 5′-end of 16S rRNA. (**A**) Primer sequence and lengths of expected extension products. The 5′-end of the specialized 16S rRNAs containing a mutation at position 18 was determined by extending a ^{32}P-end-labelled oligonucleotide complementary to region 19–35. The residue at position 18, distinguishing the chromosomally encoded wild-type 16S rRNA (C_{18}) from the specialized 16S rRNA (A_{18}, G_{18} or U_{18}) is presented with an open letter. In the presence of ddGTP in the reaction, the extension product synthesized on the wild-type 16S rRNA will be an 18mer. The size of the extension product synthesized on specialized 16S rRNA will be a 35mer if the 5′-end of the molecule is correctly processed and a 40mer in the case of incorrect processing. (**B**) Primer extension on total rRNA from cells harboring specialized ribosomes with or without a mutated central pseudoknot. For lane 1 primer extension was performed in the absence of ddGTP using total rRNA from cells harboring specialized ribosomes with a wild-type pseudoknot as a template. For lanes 2–7 primer extension was performed in the presence of ddGTP, using total rRNA from cells harboring specialized ribosomes with the indicated mutations as a template. The lengths of the unextended primer (a 17mer) and extended primers (an 18mer and either a 35mer or a 40mer) are shown.

extension products synthesized in the presence of ddGTP. When annealed to chromosomally encoded wild-type 16S rRNA, termination will occur at position C_{18}, resulting in an 18mer. When annealed to plasmid-encoded specialized 16S rRNA with a mutated central pseudoknot, a 35mer run-off transcript will be made, provided the 5′-end of the 16S rRNA is correctly processed. If this is not the case, termination will occur at the next C residue which is five nucleotides upstream of the proper cleavage site (see Figure 7A), resulting in a 40mer.

Figure 7B shows the results of such a primer extension experiment using ribosomes with or without mutations in the pseudoknot. A 35mer is clearly present in all cases, while a 40mer is never observed. This demonstrates that the 5′-end of the mutant 16S rRNAs that are assembled into ribosomal particles, is indeed correctly processed.

Disruption of the central pseudoknot interferes with the formation of 70S ribosomal complexes

Having demonstrated that mutations in the central pseudoknot do not interfere with processing of the 5′-end, we inferred that the disruptive mutations may impair some step in the translation process itself. In order to determine which stage of this process is affected, polysome profiles were prepared and the relative levels of specialized 16S rRNA in the 30S, 70S, disome and trisome fractions were determined using the primer extension method as described in the previous section.

As Figure 8A shows, the polysome profiles of cells containing specialized ribosomes with the wild-type pseudoknot or any of the disruptive mutations are identical. The presence of large amounts of free 30S and 50S subunits in all profiles is inherent in cells containing the specialized ribosome system (M.F.Brink, unpublished data). The proportion of the specialized ribosomes that is engaged in protein synthesis is limited by the fact that their number greatly exceeds the number needed for optimal translation of the CAT mRNA. Therefore, the majority of specialized ribosomes is present as free subunits. As compared with the profile of cells containing specialized ribosomes with the wild-type pseudoknot, no additional peaks or shoulders are observed in the profiles of the disruptive mutants. In particular the absence of peaks that reflect the accumulation of particles smaller than 30S indicates that the disruptive mutations in the pseudoknot do not cause aberrant assembly.

Figure 8B shows that, as illustrated by the presence of a 39mer, in the 30S fraction (right panel) the pseudoknot mutants are abundant (lanes 3–8). Based on the measurement of radioactivity in the 19mer and 39mer, the relative levels of these various mutants were calculated (see Table I). The table shows that, at the time of sampling, more than half of the 30S particles in the cell are plasmid-derived, irrespective of the mutations in the pseudoknot.

In the 70S fraction, however, the 39mers derived from specialized 16S rRNA containing A_{18} or G_{18} (lanes 3 and 4, respectively) are barely detectable. In contrast, for the U_{18} mutant (lane 5), this band is clearly visible even though it appears to be of lower intensity as compared with the 39mer derived from specialized 16S rRNA containing a pseudoknot with the wild-type sequence or compensatory mutations. Calculation of the relative levels demonstrates that, indeed, less than 10% of the 70S fraction is constituted by the A_{18} and G_{18} mutants, whereas the U_{18} mutant is more abundant, constituting 24% of this fraction. In contrast, the 70S fraction contains a significantly higher percentage (33–41%) of specialized ribosomes having a wild-type central pseudoknot or any of the compensatory mutations. Note that this percentage could have been even higher, but, as mentioned above, is limited by the amount of CAT mRNA available. Since the percentage of each mutant is similar in the 70S, disome and trisome fractions (see Table I), we conclude that disruptive mutations clearly affect the formation of 70S particles.

The relative abundance of specialized ribosomes with a mutated pseudoknot in 70S or polysomal fractions appears to be correlated with their measured ribosomal activity. The inactive A_{18} and G_{18} mutants do not form 70S particles at all, whereas the U_{18} mutant is still capable of forming 70S and polysomal complexes, albeit at a reduced rate. The formation of these complexes is not affected when specialized ribosomes contain complementary residues in the pseudoknot. Therefore, these results clearly demonstrate that the ribosomal activity is dependent on the presence of a stable central pseudoknot in 16S rRNA which mediates the formation of 70S ribosomal complexes.

Discussion

The formation of the central pseudoknot structure is required for translational initiation

We have demonstrated that substitutions introduced at position 18 of 16S rRNA dramatically decrease the translational activity of the ribosome. These substitutions neither have a clear effect on the assembly of 16S rRNA into ribosomal particles, nor do they affect the formation of a correctly processed 5′-end. As the levels of specialized ribosomes having disruptive pseudoknot mutations are significantly reduced in 70S as well as polysomal fractions, apparently such mutations interfere with the formation of a 70S ribosomal complex.

The translational activity of the specialized ribosomes with disruptive mutations in helix II was as low as 5% of the wild-type activity for the A_{18} mutant and a mere 10% for the G_{18} mutant. In the case of the U_{18} mutant, in principle, a weakened pseudoknot structure could be maintained due to the potential formation of a U_{18}–G_{917} 'wobble' base-pair. As the activity of this mutant is reduced to 25% of the wild-type level, this may imply that the stability of helix II is correlated with the translational activity of the ribosome. However, we did not observe a correlation between the temperature and the translational activity of the U_{18} mutant. When determined at either 42, 34 or 25°C, the activity of this mutant did not increase relative to the activity of specialized ribosomes having the wild-type sequence. It should be kept in mind that, due to the nature of the thermo-inducible lambda P_L promoter, transcription of the plasmid-borne *rrnB* operon and the assembly of the rRNA occur at 42°C, i.e. before the translational activity of the specialized ribosomes can be determined at the desired temperature. The negative effect sustained when ribosomes containing U_{18} are assembled at 42°C, may not be reversed when the temperature is shifted to either 34°C or 25°C.

Interestingly, Dammel and Noller (1993) found that the inactivation of the ribosome caused by the introduction of a G_{11}–U_{23} 'wobble' base-pair in helix I can be reversed by shifting the temperature. Such ribosomes were functionally impaired at 26°C, whereas ribosomal activity was regained after shifting the temperature up to 42°C. However, in

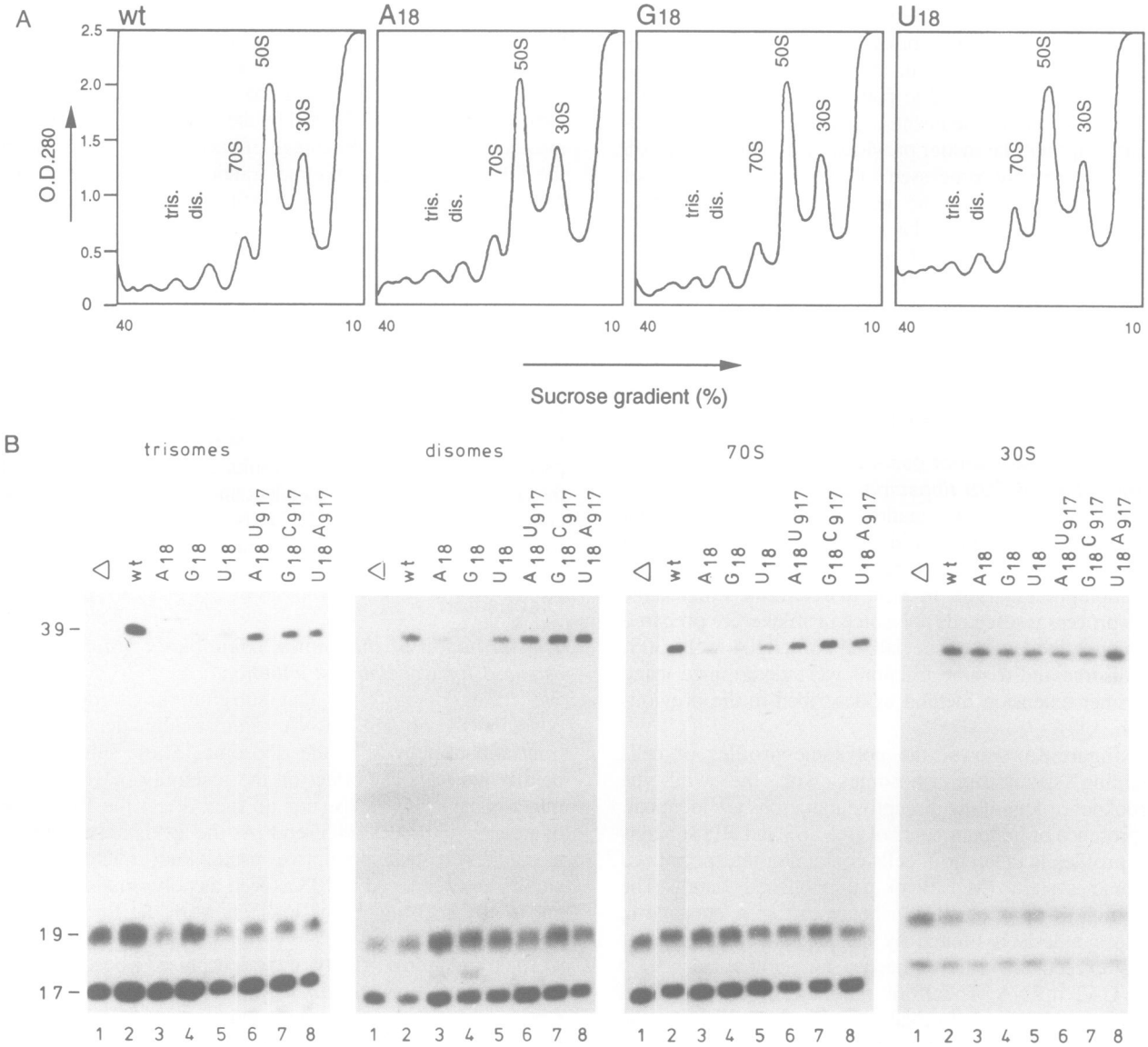

Fig. 8. Primer extension analysis of 16S rRNA present in the 30S, 70S, disome and trisome fractions. (**A**) Polysome profiles of cells containing specialized ribosomes with a wild-type or mutated pseudoknot. Cells were harvested 1 h after induction of the synthesis of specialized ribosomes having the wild-type pseudoknot and the mutations indicated. Polysome profiles were prepared from cell lysates using 10–40% sucrose gradients (see Materials and methods). (**B**) Primer extension on rRNA from polysome fractions of cells harboring specialized ribosomes with or without a mutated central pseudoknot. The relative amounts of wild-type and specialized 16S rRNA in the 30S, 70S, disome and trisome fractions were determined by primer extension analysis (see Figure 6) and quantification of the extension products (see Table I). From left to right the panels represent such an analysis using the trisome, disome, 70S and 30S fractions. For lane 1 in each panel, rRNA from cells harboring the negative control plasmid (see Figure 3) was used. For lanes 2–8, rRNA from cells harboring specialized ribosomes with a wild-type or mutated pseudoknot was used.

contrast to our substitutions in helix II, this perturbation in helix I caused processing anomalies at the 5′-end. Since the incorrect processing was not the cause of the observed cold sensitivity, it was suggested that, at 26°C, the introduced mutation stimulates the folding of the 5′-end into an inactive conformation, whereas after shifting the temperature to 42°C an active conformation is adopted.

While the reduction in translational activity by the introduction of disruptive pseudoknot mutations indicates that formation of the central pseudoknot is important, conclusive evidence for the existence of this structure is provided by mutants having complementary residues at positions 18 and 917. As the translational activity of each of these mutants is the same as that of specialized ribosomes with the wild-type pseudoknot, we conclude that the potential formation of this structure, regardless of its base-pair composition, is essential for full translational activity of 30S subunits. Since 30S subunits containing 16S rRNA with disruptive mutations in the central pseudoknot are not able to form 70S ribosomes, formation of this structure seems to be required to complete translational initiation.

Is the central pseudoknot structure involved in a conformational rRNA switch?

Comparative sequence analyses have shown that pseudoknots may be present in functionally important regions of many classes of RNA molecules (reviewed in Pleij, 1990). Mutagenesis studies have confirmed the existence of such structures and have demonstrated their role in the self-splicing mechanism of group I introns and in the

aminoacylation of the tRNA-like structure of several viral RNA molecules. Furthermore, the formation of pseudoknot structures in certain mRNAs is required for ribosomal frameshifting (reviewed in ten Dam *et al.*, 1992).

In 16S rRNA, in addition to the central pseudoknot, at least two other pseudoknots can be formed (Gutell *et al.*, 1986; Woese and Gutell, 1989). One of these is formed when residues A_{865} and C_{866} in the loop of the 861–866 hairpin structure base-pair with residues U_{571} and G_{570} respectively. Although this structure is supported by phylogenetic data, it has not yet been confirmed by mutational analysis. By introducing disruptive and compensatory mutations into the pseudoknot structure located in the highly conserved G_{530} region, Powers and Noller (1991) showed that this structure is essential for ribosomal function *in vivo*. Moreover, they found that the introduction of moderate pertubations—such as the replacement of the G_{506}–C_{525} base-pair with a G–U wobble base-pair—confers streptomycin resistance.

Our data imply that the formation of the central pseudoknot is required for 30S subunits to complete translational initiation, yet little is known about the function of the central pseudoknot at the molecular level. Pleij *et al.* (1985) and Schimmel (1989) have pointed at the possibility of pseudoknots being involved in conformational RNA switches. Since the folding of RNA into a pseudoknot structure does not result in a substantial increase in thermodynamic stability (Puglisi *et al.*, 1988), there are only small energy barriers between the two alternative RNA structures, namely the pseudoknot conformation and a hairpin conformation with unpaired residues in the loop. In fact, the sensitivity of the G_{917} residue towards kethoxal modification in intact 30S ribosomal subunits appeared to be strongly enhanced upon depletion of magnesium ions and monovalent cations (Hogan and Noller, 1978). Moreover, upon assembly of naked 16S rRNA into 30S subunits, the reactivity of G_{917} to kethoxal is reduced (Moazed *et al.*, 1986). These results indicate that the central pseudoknot can indeed be disrupted, whereas it is stabilized in 30S subunits.

Moazed and Noller (1989) have studied the tRNA-dependent protection of residues within the 16S and 23S rRNA. Ribosome–tRNA complexes therefore were formed *in vitro* mimicking the ribosome in pre- and post-peptidyl transfer stages. Based on their results these authors suggested that conformational changes occur within the ribosomal subunits in order to accommodate the translocation of tRNAs from the aminoacyl site to the peptidyl site and from the peptidyl site to the exit site.

Based solely on comparative sequence analysis, Leclerc and Brakier-Gingras (1991) proposed such a conformational switch involving the alternating formation of the central pseudoknot and a pseudoknot structure formed by the helices $G_9A_{10}G_{11}/C_{23}U_{24}C_{25}$ and $U_{12}U_{13}U_{14}G_{15}A_{16}/U_{911}C_{912}A_{913}$-$A_{914}A_{915}$. Since the residues at positions 18 and 917 do not contribute to the formation of this latter pseudoknot, our results do not provide any evidence to confirm or dismiss this model. Kössel *et al.* (1990) proposed another model also describing the alternating formation of two pseudoknots. In their model, helix II of the central pseudoknot is disrupted in order to allow base-pairing between rRNA regions $U_{14}G_{15}A_{16}U_{17}C_{18}$ and $G_{1530}A_{1531}U_{1532}C_{1533}A_{1534}$. The putative formation of this latter helix implies that the 5′- and 3′-ends of the 16S rRNA molecule must be in close proximity. Although this model is supported by the fact that the terminal 5′- and 3′-ends of the 16S rRNA were cross-linked using psoralen derivatives (Wollenzien and Cantor, 1982), in the current structural models for the folding of the 16S rRNA molecule within the 30S ribosomal subunit (Expert-Bezançon and Wollenzien, 1985; Brimacombe *et al.*, 1988; Stern *et al.*, 1988; Hubbard and Hearst, 1991), these ends are not close enough to allow such an interaction. Since our data demonstrate that 30S subunits containing 16S rRNA with complementary mutations at positions 18 and 917 are completely active, we can conclude that the interaction between residues C_{18} and G_{1530} is not essential for the ribosomal functions tested in this work.

Although current data do not provide conclusive proof for the participation of the central pseudoknot in a conformational switch, if we were to assume that the alternating formation and disruption of this pseudoknot is indeed required for ribosomal function, impairment of its formation (e.g. as described here by preventing the interaction between residues C_{18} and G_{917}) would block such a conformational switch. As a consequence the 30S subunit would be locked into a conformation incapable of, for example, forming a 30S initiation complex, or of associating with the 50S ribosomal subunit and form a 70S ribosomal complex.

Materials and methods

Strains, media and plasmids

The *E.coli* strains K5716 and K5637 used in these studies were constructed by Dr D.H.Miller and have been described previously by Hui and de Boer (1987). In the strain K5716, transcription from the lambda P_L promoter is blocked by the *c*I repressor protein, whereas in strain K5637, harboring the thermolabile repressor protein *c*I857, transcription from the lambda P_L promoter only occurs upon temperature induction. Since prolonged and extensive accumulation of ribosomes with an altered ASD sequence affects the growth rate of cells (Jacob *et al.*, 1987; our own observations), plasmids encoding the specialized ribosome system were amplified in strain K5716. The effect of mutations in 16S rRNA on ribosomal activity was studied after transfer of the plasmids to strain K5637 and subsequent heat induction.

Cells were grown in LB medium containing 10 g/l of tryptone (Difco), 5 g/l of yeast extract (Difco) and 10 g/l of NaCl. Ampicillin (Sigma) was supplied when appropriate, at a final concentration of 100 mg/l.

Plasmid pP_LASDX-CATX was derived from pASDX-PSDRX-hGH (Hui and de Boer, 1987) and contained both the altered ASD sequence and the *cat* gene with a complementary SD sequence. The negative control plasmid pASDΔ(*Sma*I–*Sst*I)-CATX, harbors an *rrnB* operon with a 952 bp deletion between the *Sma*I and *Sst*I restriction sites (see Figure 3B).

Site-directed mutagenesis

Oligonucleotide-directed mutations were introduced using the PCR (Saiki, 1989). Oligonucleotides were synthesized on a 'Cyclone plus' DNA synthesizer (Milligen/Biosearch). PCRs were carried out in 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$ and 0.01% gelatin (Sigma). Fragments of *rrnB* were amplified from 5 ng of pP_LASDX-CATX for 25 cycles using 25 pmol of each primer and 2.5 units of Amplitaq (Cetus). Fragments carrying a mutation at position 18 of 16S rRNA were obtained using the following primers: (a) 5′N18 (5′-ACGGGTACCGGCCG*T*TG-AGAAAAAGCG-3′), (b) N18 (5′-AATCTGAGCCAT*N*ATCAAACTC-TTCAA-3′, where *N* = A, C or T) and (c) 3′N18 (5′-CGGGATCCTA-GGTGAGCCGTTACCCCA-3′). The mutations introduced by these primers in amplified fragments of the *rrnB* operon are presented in italics. The location of the primers in the *rrnB* operon are indicated in Figure 3B. Note that 5′N18 deletes a *Dde*I restriction site near the 5′-end of the amplified fragment, N18 introduces the mutation at position 18 of 16S rRNA, and 3′N18 adds a *Bam*HI restriction site at the 3′-end. The 210 bp fragment amplified by 5′N18 and N18 was digested with *Kpn*I and *Dde*I (BRL). The 462 bp fragment amplified by 5′N18 and 3′N18 was digested with *Dde*I and *Bam*HI (BRL). The respective *Kpn*I–*Dde*I and *Dde*I–*Bam*HI fragments were jointly subcloned into pGEM-7Zf(+) (Promega). Single-stranded DNA was generated using the M13KO7 helper phage and mutations were identified by sequence analysis using the T7 DNA polymerase sequencing kit

(Pharmacia). Plasmids were reconstructed by ligating a *Kpn*I–*Ppu*MI fragment containing the mutation at position 18 combined with the *Ppu*MI–*Sst*I fragment from *rrnB* containing the U_{1192} mutation and the ASDX sequence in 16S rRNA (see Figure 3B).

Fragments carrying mutations at position 917 of 16S rRNA were generated using the following primers: (d) 5′N917 (5′-ATGAATTCCAGGTGTAG-CGGT-3′), (e) A917 (5′-CGGGATCCGGGCCCCCGTCAATT*A*TTT-GAGTT-3′), (f) C917 (5′-CGGGATCCGGGCCCCCGTCAATT*G*ATT-TGAGTT-3′) and (g) T917 (5′-CGGGATCCGGGCCCCCGTCAATT*A*-ATTTGAGTT-3′). The mutations introduced by these primers at position 917 of 16S rRNA are presented in italics. The location of the primers is indicated in Figure 3B. Note that A917, C917 and T917 attach a *Bam*HI restriction site at the 3′-end of the amplified fragment. The 269 bp fragments amplified by 5′N917 in combination with either A917, C917 or T917 were digested with *Eco*RI and *Bam*HI (BRL), subcloned into pGEM-7Zf(+) and mutations were identified by sequence analysis. *Eco*RI–*Apa*I fragments containing the desired mutations at position 917 were combined with *Kpn*I–*Eco*RI fragments containing the complementary mutation at position 18 and reintroduced into the *rrnB* operon containing the U_{1192} mutation and ASDX sequence (see Figure 3B).

Assessment of the ribosomal activity; CAT assays and in vivo labelling of CAT

Strain K5637 harboring a plasmid encoding the specialized ribosome system was grown overnight at 30°C in LB medium containing 100 mg/l ampicillin. The overnight culture was diluted 100-fold and grown for 1 h at 30°C. For the induction of the synthesis of specialized ribosomes, the temperature was shifted to 42°C (t = 0) and cells were grown for another 2 h. Samples of 1 ml were taken at t = 0, 30, 60, 90 and 120 min, the cells were spun down, washed twice with 0.2 ml of 40 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and resuspended in 0.2 ml of 250 mM Tris–HCl pH 7.8. Cells were lysed by freezing and thawing three times using ethanol/dry ice. Cell debris was spun down for 5 min at 15 000 r.p.m. (Eppendorf) and the supernatant was stored at −20°C.

[^{3}H]Acetyl-coenzyme A was freshly prepared according to the procedure of Nordeen *et al.* (1987). For each sample to be assayed, the following solutions were premixed: 0.1 ml H_2O, 62.5 μl CAT assay buffer (400 mM Tris–HCl pH 7.8, 24 mM $MgCl_2$, 300 mM KCl), 20 μl 5 mM coenzyme A (Sigma), 9.75 μl 10 mM sodium acetate, 7.5 μl 100 mM ATP (Boehringer), 0.02 U of *S*-acetyl-coenzyme A synthetase (Sigma), 0.3 μl of sodium [^{3}H]acetate (8.4 Ci/mM; DuPont) and 0.5 μl of 0.5 M chloramphenicol (Sigma). Samples of 0.2 ml of this mixture were aliquoted and incubated for 30 min at 37°C, in order to allow acetylation of coenzyme A. One microliter of the cell extract was added and incubated for another 30 min to allow acetylation of chloramphenicol by CAT. Acetylated chloramphenicol was extracted with 1 ml benzene (Merck). 0.85 ml of the benzene was transferred to a scintillation vial and allowed to evaporate overnight. 5 ml of Econofluor (DuPont) was added and the amount of [^{3}H]acetyl-chloramphenicol was measured in a 1214 Rackbeta scintillation counter (LKB).

Metabolic labelling of CAT with L-[^{35}S]methionine (Amersham) was done as described previously (Hui and de Boer, 1987), except that M9 medium was supplied with all L-amino acids (Sigma) excluding L-methionine.

rRNA analysis: primer extension method

The relative cellular level of specialized ribosomes was assessed using the primer extension method (Sigmund *et al.*, 1988). The synthesis of specialized ribosomes was induced as described above. Exponentially growing cells were harvested 2 h after induction when the A_{600} was between 0.6 and 0.7. Isolation of rRNA and the primer extension method were performed as described by Triman *et al.* (1989). AMV reverse transcriptase was obtained from Promega and ddGTP from Boehringer-Mannheim. Extension products were analyzed on 12.5% (w/v) polyacrylamide–urea gels. The radioactivity present in the 39mer or 19mer extension products was measured using a Betascope 603 Blot Analyzer (Betagen).

Preparation of the polysome profile

Synthesis of specialized ribosomes was induced as described above, except that cells were harvested 1 h after induction. Cells were chilled within 20 s from 42°C to less than 4°C by pouring 50 ml of culture into a 1 liter flask which was submerged in an ethanol/dry ice bath. Polysome profiles were prepared as described by Powers and Noller (1990) with the addition of 5 μg/ml of DNase I (Boehringer) to the cell suspension before cells were lysed by freezing and thawing three times using dry ice/ethanol. The A_{260} of the cell lysate suspensions were measured and 10–15 A_{260} units were loaded on to 10 ml 10–40% sucrose gradients. Gradients were spun for 2 h at 35 000 r.p.m. at 4°C using a TST41.14 swing-out rotor (DuPont) in a Centrikon T11-70 ultracentrifuge (Kontron). Peak fractions containing trisomes, disomes, 70S, 50S or 30S ribosomal particles were collected and ribosomes complexes were precipitated from these fractions with 2.5 vol of ice-cold ethanol. The pellets were resuspended in 0.3 M sodium acetate and rRNA was isolated and subjected to the primer extension method using the oligonucleotide spanning region 1210–1194 of the 16S rRNA (see above).

Acknowledgements

The authors wish to dedicate this paper to the late Professor Piet van Knippenberg who initiated our interest in the central pseudoknot structure. We would also like to thank Professor Leendert Bosch and Dr Jan van Duin for encouragement and invaluable discussions.

References

Brimacombe,R., Atmadja,J., Stiege,W. and Schüler,D. (1988) *J. Mol. Biol.*, **199**, 115–136.
Dammel,C.S. and Noller,H.F. (1993) *Genes Dev.*, **7**, 660–670.
Expert-Bezançon,A. and Wollenzien,P. (1985) *J. Mol. Biol.*, **184**, 53–66.
Freier,S.M., Kierzek,R., Jaeger,J.A., Sugimoto,N., Caruthers,M.H., Neilson,T. and Turner,H. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 9373–9377.
Gutell,R., Noller,H.F. and Woese,C.R. (1986) *EMBO J.*, **5**, 1111–1113.
Hogan,J.J. and Noller,H.F. (1978) *Biochemistry*, **17**, 587–593.
Hubbard,J.M. and Hearst,J.E. (1991) *J. Mol. Biol.*, **221**, 889–907.
Hui,A.S. and de Boer,H.A. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 4762–4766.
Hui,A.S., Jhurani,P. and de Boer,H.A. (1987) *Methods Enzymol.*, **153**, 432–452.
Jacob,W.F., Santer,M. and Dahlberg,A.E. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 4757–4761.
Kössel,H., Hoch,B. and Zeltz,P. (1990) *Nucleic Acids Res.*, **18**, 4083–4088.
LeClerc,D. and Brakier-Gringas,L. (1991) *FEBS Lett.*, **279**, 171–174.
Moazed,D. and Noller,H.F. (1989) *Nature*, **342**, 142–148.
Moazed,D., Stern,S. and Noller,H.F. (1986) *J. Mol. Biol.*, **187**, 399–416.
Neefs,J.M., Van de Peer,Y., Hendriks,L. and de Wachter,R. (1990) *Nucleic Acids Res.*, **18**, supplement 2237–2317.
Nomura,M. and Held,W.A. (1974) In Nomura,M., Tissières,A. and Lengyel,P. (eds), *Ribosomes*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 193–223.
Nordeen,S.K., Green,P.P.,III and Fowlkes,D.M. (1987) *DNA*, **6**, 173–178.
Pleij,C.W.A. (1990) *Trends Biochem. Sci.*, **14**, 143–147.
Pleij,C.W.A., Rietveld,K. and Bosch,L. (1985) *Nucleic Acids Res.*, **13**, 1717–1731.
Powers,T. and Noller,H.F. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 1042–1046.
Powers,T. and Noller,H.F. (1991) *EMBO J.*, **10**, 2203–2214.
Puglisi,J.D., Wyatt,J.R. and Tinoco,I.,Jr (1988) *Nature*, **331**, 283–286.
Saiki,R.S. (1989) In Erlich,H.A. (ed.), *PCR Technology*. Stockton Press, New York, pp. 7–16.
Schimmel,P. (1989) *Cell*, **58**, 9–12.
Sigmund,C.D., Ettayebi,M. and Morgan,E.A. (1984) *Nucleic Acids Res.*, **12**, 4653–4663.
Sigmund,C.D., Ettayebi,M., Borden,A. and Morgan,E.A. (1988) *Methods Enzymol.*, **164**, 673–690.
Srivastava,A.K. and Schlessinger,D. (1988) *Proc. Natl Acad. Sci. USA*, **74**, 7144–7148.
Stern,S., Weiser,B. and Noller,H.F. (1988) *J. Mol. Biol.*, **204**, 447–481.
ten Dam,E., Pleij,C.W.A. and Draper,D. (1992) *Biochemistry*, **31**, 11665–11676.
Triman,K., Becker,E., Dammel,C., Katz,J., Mori,H., Douthwaite,S., Yapijakis,C., Yoast,S. and Noller,H.F. (1989) *J. Mol. Biol.*, **209**, 645–653.
Wireman,J.W. and Sypherd,P.S. (1974) *Nature*, **247**, 552–554.
Woese,C.R. and Gutell,R.R. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 3119–3122.
Wollenzien,P.L. and Cantor,C.R. (1982) *J. Mol. Biol.*, **159**, 151–166.

Received on May 10, 1993; revised on June 22, 1993