The EMBO Journal vol.4 no.4 pp.1019–1024, 1985

Does unpaired adenosine-66 from helix II of *Escherichia coli* 5S RNA bind to protein L18?

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Communicated by R.Garrett

Adenosine-66 is unpaired within helix II of *Escherichia coli* 5S RNA and lies in the binding site of ribosomal protein L18. It has been proposed as a recognition site for protein L18. We have investigated further the structural importance of this nucleotide by deleting it. The 5S RNA gene of the *rrnB* operon of *E. coli* was subjected to primer-directed mutagenesis. To produce the deletion it was necessary to use simultaneously the mutagenic dodecamer dCGGCGCACGGCG and the universal M13 primer dCCCAGTCACGACGTT, and to employ forced annealing conditions. The mutated gene was expressed in an overproducing plasmid derived from pKK3535. Binding studies with protein L18 revealed that the protein bound much more weakly to the mutated 5S RNA. We consider the most likely explanation of this result is that L18 interacts with adenosine-66, and we present a tentative model for an interaction between the unpaired adenosine and the adjacent guanosine-67 of the RNA and glutamine-19 of the protein L18.

Key words: RNA-protein interaction/primer-directed mutagenesis/*Escherichia coli* 5S RNA/ribosomal protein L18

Introduction

Our knowledge of the chemistry of stable protein-RNA interactions is still very limited. Seminal and manifold studies have been performed on the tobacco mosaic virus, the ternary complex of elongation factor Tu, GTP and tRNA, and on various ribosomal protein-RNA complexes. These have yielded little insight into the mechanisms of interaction, apart from the suggested salt bridges between arginines and phosphates, and hydrogen bonds between aspartates and ribose hydroxyls, in the RNA virus (reviewed by Holmes, 1980). Ribosomes, however, provide a rich source of different protein-RNA complexes and a comparison of their RNA moieties has led to the proposal that the protein binding sites fall into two main classes: those constituting regular double helices containing a single unpaired nucleotide and those consisting of irregular helices with non-Watson-Crick base pairings; they are exemplified, respectively, by the binding sites of L18 and L25 on 5S RNA of *E. coli* (reviewed by Noller and Woese, 1981; Garrett *et al.*, 1981, 1984). Several non-ribosomal protein binding sites also fall into the former class (Garrett *et al.*, 1984).

The binding site of protein L18 is centred on helix II of *E. coli* 5S RNA which contains the unpaired adenosine-66 (Peattie *et al.*, 1981; Douthwaite *et al.*, 1982; see Figure 1). A modification-selection experiment was performed to test whether carbethoxylation of this nucleotide inhibited complex formation with L18 (Peattie *et al.*, 1981). There was a strong selection against those molecules modified at A-66 and it was concluded that the latter might participate in L18 recognition.

In the present work, we have investigated further the structural role of A-66 by deleting it. We employed primer-directed mutagenesis to remove the residue from the *E. coli* 5S RNA gene. A system was developed for expressing the mutant 5S RNA and binding studies revealed that the binding affinity of L18 for this RNA was reduced; a structural explanation for the weakened interaction is presented.

Results

Construction of mutagenesis template

A 5S RNA gene was derived from the plasmid pKK3535 (Figure 2) which contains the entire *rrnB* operon from *E. coli* (Brosius *et al.*, 1981) by *Sau*3A digestion. The 1065-bp fragment, containing the end of the 23S RNA gene, the spacer region, the 5S RNA gene and the two operon terminators, was inserted into the vector M13 mp9 (Messing and Vieira, 1982) that had been treated with *Bam*HI and alkaline phosphatase. Single-stranded DNA was isolated from recombinant M13 phages, and the orientation of the insert was established by the capacity of 3′ end-labelled 5S RNA to hybridise to the individual clones, and the ability of clones to generate 'figure eights' on agarose gels (Herrmann *et al.*, 1980). One clone which contained the 'sense' strand of the 5S RNA gene was designated mp911 and used as a template for the *in vitro* mutagenesis.

Deletion of adenosine-66

The mutagenic dodecamer dCGGCGCACGGCG is complementary to all 13 bases of the 5S RNA gene extending from C-60

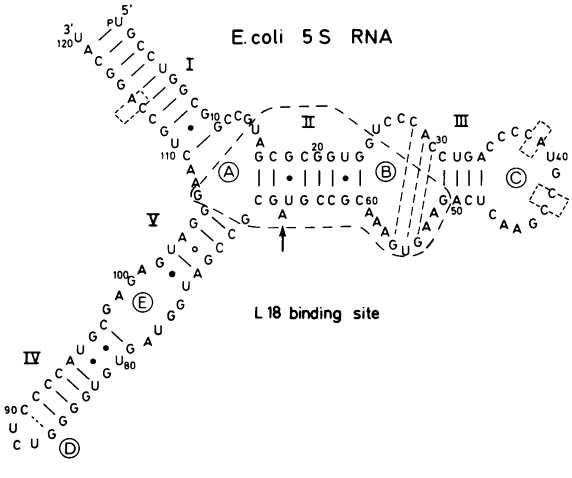

Fig. 1. Secondary structure model of *E. coli* 5S RNA. The boxed areas represent the putative L18 binding region that was probed by chemical reagents and ribonucleases (Peattie *et al.*, 1981; Douthwaite *et al.*, 1982). In this structure adenosine-66 is drawn 'bulged-out' from helix II and indicated by an arrow.

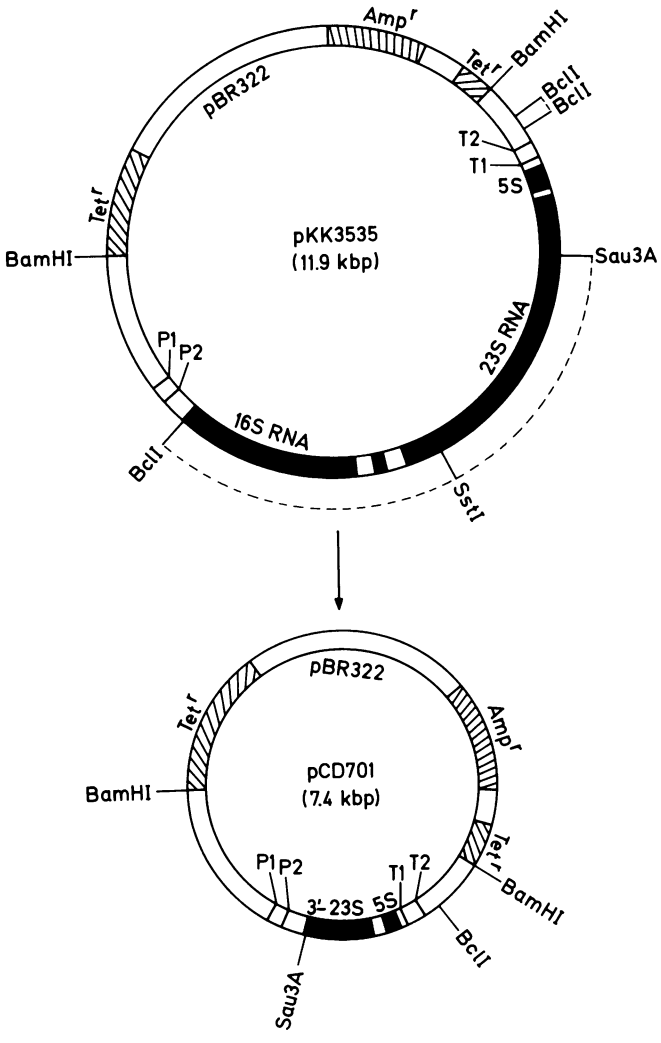

Fig. 2. Construction of the expression plasmid pCD701 from the parent plasmid pKK3535. pKK3535 was isolated from GM48 cells and digested with *Bcl*I, *Sst*I and alkaline phosphatase. The large *Bcl*I fragment (6.3 kbp) was ligated to a 1065-bp *Sau*3A fragment, containing the 5S RNA gene, isolated from pKK3535. The broken lines indicate the regions that were removed from pKK3535 during the construction of pCD701.

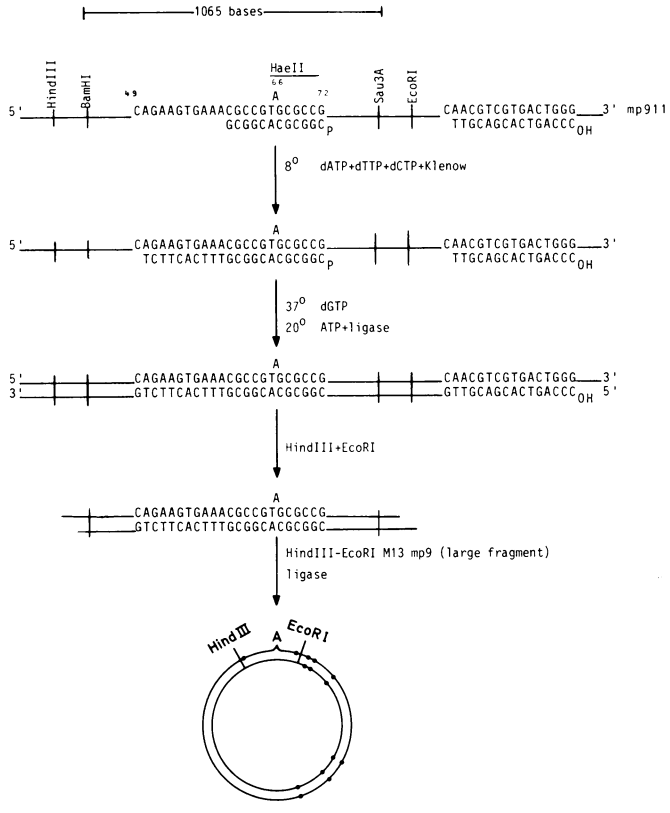

Fig. 3. Outline of the mutagenesis procedure. The gene for 5S RNA, carried on a 1065-bp *Sau*3A fragment, was inserted in the *Bam*HI site of M13 mp9, and a phage (mp911) containing the 'sense strand' was used as the template for primer-directed mutagenesis. The phosphorylated mutagenic primer and the universal M13 primer were annealed and extended as described in Materials and methods. The *in vitro* constructed gapped heteroduplex was digested with *Hind*III+*Eco*RI, and the resulting 1.1-kbp *Hind*III-*Eco*RI fragment was backcloned into the large *Hind*III-*Eco*RI fragment of M13 mp9. Numbers above bases refer to those in mature 5S RNA, and the *Hae*II site in the wild-type gene is indicated. Filled circles in the covalently closed heteroduplex show methylated GATC sites.

to G-72 except for A-66. A computer search of the entire mp911 sequence established the absence of other sites that allowed one mismatch or none. Nevertheless, a primer extension experiment (Zoller and Smith, 1982) did not reveal a secondary priming site at positions 5603–5616 in the M13 genome (van Wezenbeek *et al.*, 1980); annealing to this site involved three mismatches. Therefore, we adopted a procedure employing two primers (Norris *et al.*, 1983). This eliminates the problem of secondary priming within the M13 genome and also reduces the number of non-methylated GATC sites in the *in vitro* synthesised strand, which might diminish *in vivo* mismatch repair (Kramer *et al.*, 1984). Figure 3 outlines the mutagenesis procedure and how the mutagenic primer was forced onto target. After annealing to the target, at low temperature, the mutagenic dodecamer was converted to a docosamer by the Klenow fragment, in the absence of dGTP. The temperature was then raised, dGTP was added and the docosamer was extended in preference to the smaller primers annealed to non-target sites (Gillam and Smith, 1979). The excised 1.1-kbp *Hind*III-*Eco*RI fragment, containing the insert, was then ligated to the large *Hind*III-*Eco*RI fragment from M13 mp9, followed by transformation of competent 71-18 cells. Supernatants originating from 96 recombinants were screened by the dot-blot procedure using increasing temperatures of washing. A 50°C wash revealed two mutants, designated mp912 and mp913, which were subjected to a cycle of segregation, giving 100% and 50% yields, respectively. The non-identity of the mutants was established by a primer extension experiment. This revealed that whereas mp912 contained the desired deletion, mp913 contained mutations 50 nucleotides downstream from the target (Figure 4). Digestion of the replicative forms of mp912 and mp913 with *Hae*II produced different fragmentation patterns on 1% agarose gels (results not shown), providing further evidence that adenosine-66 was deleted in mp912 since a *Hae*II site was destroyed and, also, confirming the different nature of the two mutants.

Construction of an expression plasmid for 5S RNA

The wild-type and mutant 5S RNA genes were excised from pKK3535 and from the replicative form of mp912, respectively, on 1.1-kbp fragments by *Sau*3A digestion and inserted in an expression system as illustrated in Figure 2 for the wild-type gene. The plasmids harbouring the wild-type and mutant 5S RNA genes, in the correct orientation, were designated pCD701 and pCD702, respectively. Both plasmids differ from the parent plasmid pKK3535 in having major parts of the genes for 16S

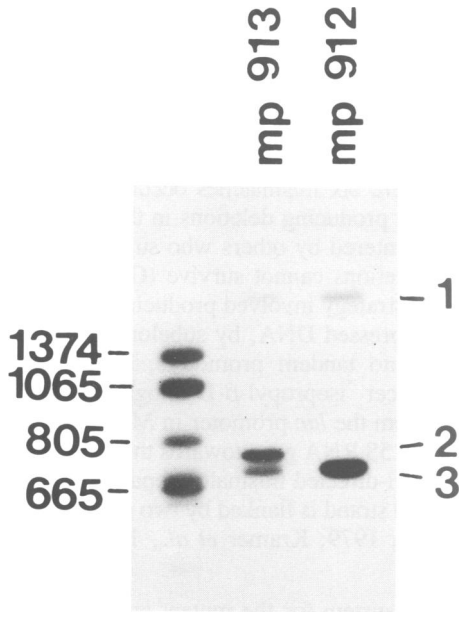

Fig. 4. Primer extension experiment applied to the mutants mp912 and mp913. The 5′ end-labelled mutagenic primer (0.5 pmol) was annealed to ssDNA (0.5 pmol) from the mutants mp912 and mp913 at 25°C, and extension was carried out for 1 h by 0.5 unit Klenow fragment in the presence of 0.5 mM dNTPs. Digestion with *Hind*III was followed by denaturing 5% polyacrylamide gel electrophoresis and autoradiography. **(1)** Secondary priming site in the M13 origin of replication; **(2)** priming site corresponding to bases 110–122 in the 5S RNA gene; **(3)** priming site covering bases 60–72 in the 5S RNA gene. The left-hand track is a size marker derived from a *Sau*3A digestion of pCD701 that was 5′ end-labelled and denatured.

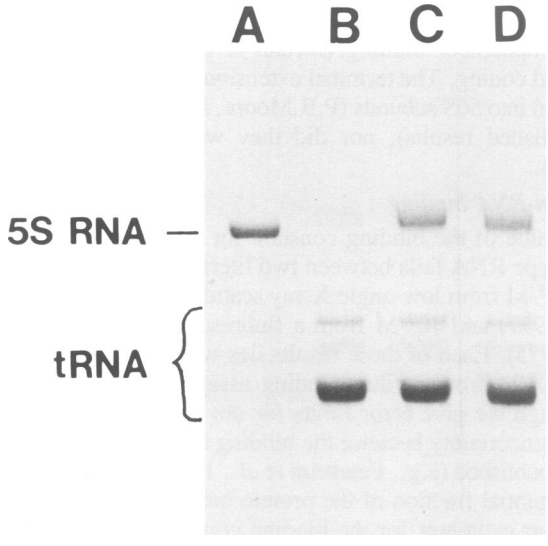

Fig. 5. Analysis of relative amounts of chromosome- and plasmid-encoded 5S RNA. Crude extracts from HB101 cells **(track B)**, HB101 cells containing the wild-type plasmid pCD701 **(track C)**, and HB101 cells containing the mutant plasmid pCD702 **(track D)** were resolved on a 10% denaturing polyacrylamide gel. **Track A** shows a 5S RNA marker isolated from *E. coli* MRE600 ribosomes. The gel was stained for RNA with 0.1% toluidine blue in 7.5% acetic acid.

and 23S RNA removed together with a 146-bp *Bcl*I fragment downstream from the operon terminators. Wild-type and mutant 5S RNA were isolated from chloramphenicol-amplified HB101 cells containing pCD701 and pCD702, respectively, which exhibited identical growth rates (data not shown). 5S RNAs were

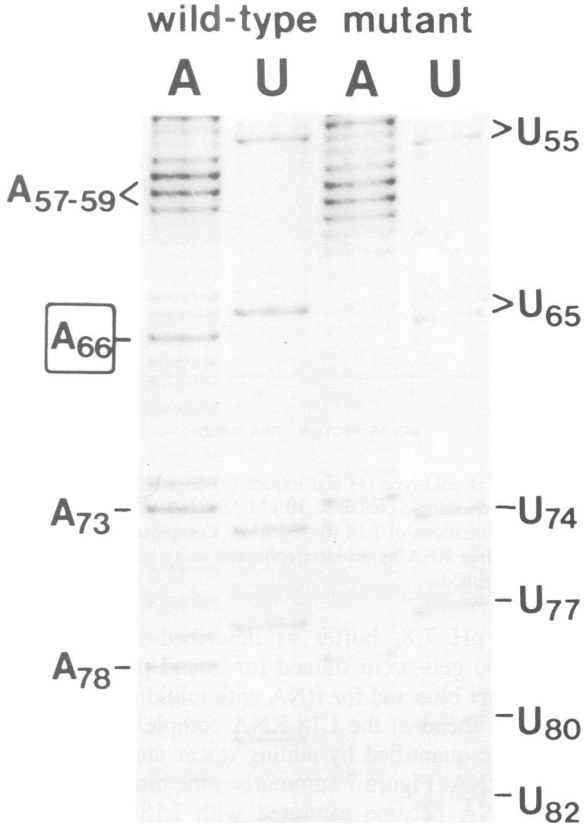

Fig. 6. Chemical sequencing (Peattie, 1979) of wild-type and mutant 5S RNA. The autoradiogram shows part of the adenosine **(A)** and uridine **(U)** tracks from a 12% polyacrylamide sequencing gel. Wild-type and mutant 5S RNA were isolated from HB101 cells containing pCD701 and pCD702, respectively. The numbering of bases in 5S RNA refers to the ordinary 5S RNA of *E. coli*. The deleted adenosine-66 is boxed.

extracted from the phenol-treated cells and the relative amounts of plasmid and chromosome-encoded 5S RNA are depicted in Figure 5. Clearly, the 5S RNA/tRNA ratio is much higher in the cells containing wild-type and mutant plasmids. We estimated by microdensitometry, that the fraction of 5S RNA deriving from the seven *rrn* chromosomal operons was $< 10\%$ of the total in the plasmid-containing cells. Figure 5 also shows that the 5S RNA, isolated from chloramphenicol-treated cells, was slightly longer than the 5S RNA marker prepared from *E. coli* MRE600 cells. In addition to the heterogeneous extension at the 5′ end, that was first observed by Jordan *et al.* (1971), there was also a dinucleotide extension at the 3′ end. The yield of 5S RNA by our purification procedure, which included a preparative gel step, was 2 mg/l culture grown to an A_{650} value of 0.5.

Figure 6 shows part of a chemical sequencing gel (Peattie, 1979) of wild-type and mutant 5S RNA; the figure covers the sequence region from U-55 to U-82. A comparison of the two U-tracks demonstrates that there is a frame-shift between U-65 and U-74 due to a deletion. Similarly, a frame-shift between A-59 and A-73 is evident in the two A-tracks due to the disappearance of A-66. This result was also confirmed by ribonuclease sequencing (data not shown).

Binding studies

The binding of protein L18 to both wild-type and mutant 5S RNA was examined over a range of protein concentrations (0–34 μM), and at a constant RNA concentration (10 μM). The binding experiments were performed by an electrophoretic assay in

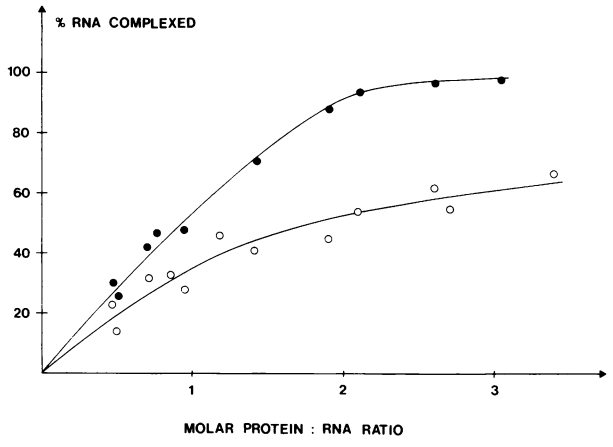

Fig. 7. Binding of wild-type (•) and mutant (○) 5S RNA to ribosomal protein L18. Each sample contained 10 μM 5S RNA (3 x 10^4 d.p.m.), with increasing concentrations of L18 (0 – 34 μM). Complexed RNA was separated from free RNA by an electrophoretic assay as described in Materials and methods.

$T_{30}M_{20}K_{300}$, pH 7.8, buffer as described in Materials and methods. Disc gels were stained for bound protein with Coomassie brilliant blue and for RNA with toluidine blue. The free RNA moved ahead of the L18-RNA complex so that the binding could be quantified by adding tracer amounts of 3′ end-labelled 5S RNA. Figure 7 summarises the binding results. The wild-type RNA became saturated with L18 and its binding behaviour was indistinguishable from that of mature 5S RNA (data not shown). In contrast, no binding above 65% was detected for the mutant RNA.

Disc gels of the L18-mutant RNA complex revealed one band containing the complex and another containing free 5S RNA which was both broader and slower migrating than co-electrophoresed free RNA. Since probing of the mutant RNA complex with cobra venom ribonuclease showed that all of the RNA was complexed, under equilibrium conditions, at the high protein-RNA ratio in Figure 7 (data not shown), we concluded that the mutant RNA migrated anomalously due to complex dissociation during electrophoresis. Assuming, therefore, that a stoichiometric L18-mutant RNA complex existed in the incubation mixture, and that a constant rate of dissociation of the complex occurred during electrophoresis, we could estimate from the equation

$$\frac{d[\text{complex}]}{dt} = -k_{\text{diss.}}\,x[\text{complex}]$$

a dissociation rate constant of 0.02/h. Furthermore, a double reciprocal plot of bound RNA *versus* free protein yielded binding constants for L18 with the wild-type and mutant 5S RNAs of 1.5(±1.0) x 10^6/M and 2.0(±0.7) x 10^5/M, respectively. The former value, with the larger error limits, was checked by measuring complex formation as a function of RNA concentration (0.025 – 7.5 μM) at a constant L18:5S RNA ratio of 4.2:1. A value of 1.4(±0.3) x 10^6/M was obtained by modifying the calculating procedure of Spierer *et al.* (1978).

Discussion

Mutagenesis difficulties

Deleting A-66 from the 5S RNA gene by primer-directed mutagenesis proved very difficult. The secondary priming site, at the M13 origin of replication, contained three mismatched pairs of bases and although we subsequently used the procedure of Norris *et al.* (1983) in which we backcloned the *in vitro* synthesised heteroduplex into M13 instead of pBR322, no mutants were identified (frequency <2%). Only forcing conditions produced the desired deletion with a 1% frequency, but even then the combination of low temperature of annealing with the initial omission of dGTP also generated a different mutant with the same frequency localised in the 3′ terminus of the 5S RNA gene (bases 110 – 122), where six mismatches occur.

Difficulties in producing deletions in the 5S RNA gene have also been encountered by others who suggested that cells containing such deletions cannot survive (Göringer *et al.*, 1984). Our successful strategy involved producing a deletion in a fragment of non-expressed DNA, by subcloning a 1065 bp *Sau*3A fragment with no tandem promoters, and we excluded the gratuitous inducer isopropyl-β-D-thiogalactoside to prevent transcription from the *lac* promoter in M13 mp9. The apparent resistance of the 5S RNA gene towards the mutagenic event may be due to methyl-directed mismatch repair *in vivo*, since the *in vitro* synthesised strand is flanked by two non-methylated GATC sites (Glickman, 1979; Kramer *et al.*, 1984).

Mutant 5S RNA

The expression system for the mutant and wild-type 5S RNAs was constructed with deletions in the large rRNAs such that regulation of plasmid-coded 5S RNA by ribosome feedback was abolished (Jinks-Robertson *et al.*, 1983). This led to production of the 5S RNAs according to the gene dosage. Ribosomal RNA molecules required for ribosome biosynthesis were transcribed from the seven chromosomal *rrn* operons and it is unlikely therefore, that plasmid-coded 5S RNA assembled into ribosomes; this conclusion is supported by the identical growth rates observed for HB101 containing pCD701 or pCD702.

5S RNA isolated after the chloramphenicol amplification procedure exhibited frayed 5′ extensions and an extra two bases at the 3′ terminus. This incomplete processing resulted from chloramphenicol binding (Jordan *et al.*, 1971) and not from plasmid coding. The terminal extensions neither impaired reconstitution into 50S subunits (P.B.Moore, J.Brosius and H.F.Noller, unpublished results), nor did they weaken L18 binding (see below).

Protein-RNA binding

Our value of the binding constant for the complex of L18 and wild-type RNA falls between two literature estimates: values of 8 x 10^5/M from low-angle X-ray scattering (Österberg and Garrett, 1977) and 10^7/M from a fluorescence study (Feunteun *et al.*, 1975). Each of these results lies well below the estimate of 2 x 10^8/M from a filter binding assay (Spierer *et al.*, 1978). Although we give error limits for our results, there is an additional uncertainty because the binding curve in Figure 7, and all those published (e.g., Feunteun *et al.*, 1975), are compatible with a substantial fraction of the protein being inactive. If this is so, then our estimates for the binding constants, and that from the low-angle X-ray scattering, are low, the fluorescence estimate (where it was assumed that L18 bound as a dimer) is a maximum value and the filter binding estimate is high.

Whatever the absolute binding constants, our comparative study clearly demonstrates a 7- to 8-fold decrease in the binding affinity of L18 for the mutant compared with the wild-type RNA. The measurements also revealed that the former complex exhibits a higher dissociation rate constant. These results strongly suggest that L18 forms a bond with A-66. Another explanation for the altered binding characteristics is that the deletion of A-66 leads to either conformational heterogeneity in the 5S RNA or to a change in its tertiary structure. However, probing with single-

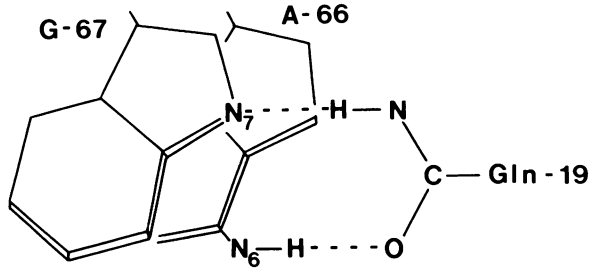

Fig. 8. Tentative interaction between adenosine-66 and guanosine-67 in 5S RNA and glutamine-19 in L18. The carboxamide group and the hydrogen bonds are lying in the major groove of the RNA in a plane that forms an angle of ~45° with the planes of the bases. The hydrogen bonds are indicated by broken lines.

and double-helical specific ribonucleases yielded no evidence for this (unpublished results).

A model

A-66 and G-67 are the most reactive purines towards diethyl pyrocarbonate and dimethylsulphate, respectively, in the whole 5S RNA of *E. coli*, which indicates that their N-7 positions are exceptionally accessible (Peattie *et al.*, 1981). The observation that this guanosine was also strongly protected by the bound L18 (the adenosine could not be tested in the complex), helped to implicate this region in the protein binding site, as did the protection of a ribonuclease A cut preceding the adenosine. This insight was compounded by a modification-selection experiment in which it was shown that RNA molecules exhibiting a carbethoxylated A-66 were strongly selected against during complex formation (Peattie *et al.*, 1981). It has also been established, by trypsin digestion experiments, that whereas the very basic 17 amino acids at the N terminus of L18 are unnecessary for 5S RNA binding, amino acids 18–25 (Leu-Gln-Glu-Leu-Gly-Ala-Thr-Arg) are essential (Newberry *et al.*, 1978); the latter, therefore, either interact directly with the RNA, or they contribute to the correct tertiary structure of L18.

In an attempt to explain these data, we built a space-filling model of helix II of 5S RNA (Figure 1) and tested the interactions with the amino acids of the essential L18 sequence. This exercise demonstrated that a hydrogen-bonded bridge could be formed by the glutamine-19 side chain between N-6 of A-66 and N-7 of G-67 (see Figure 8). In the model, the unpaired adenosine is stacked into helix II in the A-form (Figure 1), causing a small distortion of the phosphate-ribose backbone. The orientation around the glycosyl bond is the favourable *anti* form and the N-7 positions of both A-66 and G-67 are particularly accessible within the major groove of the RNA due to the phosphate-ribose distortion. In the free RNA, the adenosine may also bulge out from the helix thereby rendering the N-7 position even more accessible; our model requires, however, that it is 'locked' into the stacked conformation when L18 is bound.

The model is compatible with all the above-mentioned experimental evidence and provides a working hypothesis for the L18-5S RNA interaction. Location of L18 in the large groove of the RNA, in the vicinity of A-66, also explains both the protection of cobra venom nuclease cuts in helix II by L18 (Douthwaite *et al.*, 1982) and the decreased reactivity of the neighbouring base paired guanosines to dimethylsulphate (Peattie *et al.*, 1981). The model may also be more generally applicable, since other ribosomal and non-ribosomal complexes have also been characterised where a reactive unpaired adenosine neighbours a reactive base paired guanosine, within a helix (Garrett *et al.*, 1984). Moreover, although the A-66 and G-67 are highly conserved in eubacterial 5S RNAs, they change in eukaryotes. The model is still valid, however, as long as nucleotide-66 is a hydrogen bond donor and nucleotide-67 is a purine.

Materials and methods

Plasmids, phages and strains

Plasmid pKK3535 (Brosius *et al.*, 1981) was kindly provided by Harry F. Noller. Construction of plasmid pCD701 involved the removal of two *Bcl*I fragments (5423 and 146 bp) from pKK3535, and insertion of a 1065-bp *Sau*3A fragment into the remaining *Bcl*I fragment as illustrated in Figure 2. The recombinant phage mp911 was produced by inserting the 1065-bp *Sau*3A fragment into the replicative form of M13 mp9 (Messing and Vieira, 1982), which had been treated with *Bam*HI and calf intestinal alkaline phosphatase (Sigma). Plasmids were transformed into *E. coli* strain HB101 or the non-methylating strain GM48 (Marinus, 1973) and *E. coli* strain 71-18 (Messing *et al.*, 1977) was employed as host for M13 phages.

Plasmids and the replicative form of M13 recombinants were isolated by standard procedures, and phages and single-stranded M13 DNA were purified essentially as described by Sanger *et al.* (1980). The integrity of constructed plasmids and phages was established by restriction endonuclease mapping and Southern-blot analysis (Southern, 1979) using *E. coli* 5S RNA-[^{32}P]pCp as a probe.

Primer-directed mutagenesis

The mutagenic dodecamer dCGGCGCACGGCG and the universal M13 primer dCCCAGTCACGACGTT were synthesised by the solid-phase phosphotriester method employing 1-methyl-imidazole as a catalyst (Sproat and Gait, 1984). The oligodeoxynucleotides were purified by DE-52 chromatography and C_{18} reverse phase h.p.l.c.

dCGGCGCACGGCG (200 pmol) was phosphorylated with 100 μM ATP in 30 μl 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT by 3 units polynucleotide kinase (P.L.Biochemicals) at 37°C for 40 min. The reaction was stopped by heating at 70°C for 10 min. The phosphorylated dodecamer (50 pmol) and the universal M13 primer (20 pmol) were annealed to 1 pmol single-stranded mp911 DNA in 40 μl 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl at 56°C for 10 min and at 8°C for 1 h. To this was added 10 μl of a mixture containing 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT and 0.1 mM dATP, dCTP, dTTP and 1 unit Klenow fragment (Amersham). This reaction mixture was maintained at 8°C for 30 min and then at 37°C for 5 min. It was then adjusted to 0.1 mM in dGTP and kept for another 5 min at 37°C and adjusted to 20°C with the addition of 50 nmol ATP, 3 units DNA ligase (Amersham) and a further 1 unit Klenow fragment. Extension and ligation proceeded for 4.5 h at 20°C. The reaction was stopped by extracting with phenol and chloroform, and the DNA was precipitated. The DNA was digested with 6 units *Hind*III+*Eco*RI in 25 μl for 2.5 h at 37°C, and the excised 1.1-kbp fragment was isolated by 1% agarose gel electrophoresis. An aliquot (70 ng) of the excised *Hind*III-*Eco*RI fragment was ligated to 100 ng of the large fragment from M13 mp9 that originated from a similar *Hind*III+*Eco*RI digestion. Transformation of competent 71-18 cells gave 6 x 10^3 recombinants.

Phages from 96 plaques were picked at random and used to infect 71-18 cells in 1.5 ml cultures. Supernatants (40 μl) from individual cultures were spotted on a GeneScreen membrane (10 x 10 cm: NEN), and were denatured in 0.2 M NaOH, 0.6 M NaCl, renatured in 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5, and rinsed in 2 x SSC, before the membrane was baked for 3 h at 80°C. The baked filter was pre-hybridised at 37°C for 1 h in 8 ml 6 x SSC, 10 x Denhardt's solution, 0.2% SDS, and hybridised in 6 ml 6 x SSC, 10 x Denhardt's solution containing [^{32}P]dCGGCGCACGGCG (5 μC_i, 2500 C_i/mmol) for 1 h at room temperature. The labelled probe was purified by 20% polyacrylamide-7 M urea gel electrophoresis. After hybridisation the filter was initially washed with 2 x 70 ml 6 x SSC at room temperature for 10 min and autoradiographed for 2 h. The filter was then washed with 70 ml 6 x SSC at 35°C for 5 min, followed by autoradiography for 2 h. A final wash at 50°C for 5 min preceded autoradiography for 16 h. Phages from isolates that were resistant to washing at 50°C were segregated by plating, and the dot-blot screening repeated. The replicative forms of potential mutants (mp912 and mp913) were isolated, and a digestion with *Hae*II was performed.

A 1064-bp *Sau*3A fragment from the mutant phage mp912 was inserted into the 6.3-kbp *Bcl*I fragment derived from pKK3535 as described for the wild-type construction pCD701. The mutant plasmid was designated pCD702.

Expression and binding

pCD701 and pCD702 in *E. coli* strain HB101 were grown in LB medium containing 50 μg/ml ampicillin to an A_{650} value of 0.5, and then adjusted with chloramphenicol (110 μg/ml), adenosine (300 μg/ml), uridine (300 μg/ml), thiamine (6 μg/ml), and shaken vigorously for 8 h at 37°C. Cells were harvested and washed

with 100 mM sodium acetate, 10 mM magnesium acetate at pH 5.0, centrifuged and resuspended in the washing buffer. The suspension was extracted with phenol for 1 min, and the aqueous layer was re-extracted three times with phenol, washed with chloroform and precipitated with 2.5 volumes ethanol. 5S RNA was purified on a preparative 10% polyacrylamide gel containing 7 M urea.

RNA was renatured and bound to ribosomal protein L18 (Douthwaite *et al.*, 1982) in 30 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 300 mM KCl ($T_{30}M_{20}K_{300}$). Samples were loaded on 8% polyacrylamide disc gels in the same buffer with circulating running buffer, and electrophoresed for ~20 h at 12 mA per tube and 4°C. In some experiments, the KCl concentration in the running buffer was reduced to 60 mM after 2 h to reduce the electrophoresis time; no alterations in the binding characteristics were detected. The disc gels were stained for RNA with 0.1% toluidine blue in 7.5% acetic acid, or for protein with 0.1% Coomassie brilliant blue in 7.5% acetic acid. Stained gels were sliced, and the amounts of complexed and free RNA estimated by the Cerenkov procedure.

Ordinary 5S RNA was extracted from *E. coli* strain MRE600 according to Monier and Feunteun (1971), and *E. coli* protein L18 was prepared by the procedure of Hindennach *et al.* (1971). Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Acknowledgements

We thank Dr. Tom Brown, University of Cambridge, for introducing J.C. to oligodeoxynucleotide synthesis, Jørgen Kjems for his help in setting up this facility here, and Professor Harry Noller, University of California, for kindly providing the plasmid pKK3535. We are grateful to Orla Jensen, Arne Lindahl and Mette Rosing for helping with the manuscript. The research and J.C. were supported by grants from the Danish Natural Science Research Council and the NOVO Fund. S.D. received a long-term EMBO fellowship.

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Received on 11 January 1985