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# Basepairing potential of the 3' terminus of 16S RNA: dependence on the functional state of the 30S subunit and the presence of protein S21

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## ABSTRACT

The deoxyoctanucleotide 5'd(AAGGAGGT) which is complementary to the 3' terminus of 16S RNA has been used as a probe to measure the potential of this rRNA region to engage in intermolecular basepairing. The site specific binding of the octanucleotide is shown by labeling 16S RNA *in situ* at its 3' end with [$^{32}$P]pCp and T4 RNA ligase (EC 6.5.1.3.). The label can be released as pA[$^{32}$P]pCp by the simultaneous action of RNAse H (EC 3.1.4.34) and 5'd(AAGGAGGT). We show that (1) 30S subunits prepared according to standard procedures, bind less than one copy of 5'd(AAGGAGGT); (2) isolated 16S RNA and 30S subunits inactivated by transient exposure to 0.5 mM $Mg^{2+}$ do not bind the octanucleotide; (3) binding to inactive subunits can be restored by a brief heat treatment; (4) 30S subunits lacking protein S21 do not bind 5'd(AAGGAGGT) even when submitted to heat treatment; (5) addition of protein S21 to subunits lacking S21 restores octamer binding; (6) the apparent exposure of the 16S RNA 3' terminus brought about by protein S21 is accompanied by the potential of the subunits to accept MS2 RNA as messenger; (7) the presence or absence of S1 on 30S subunits has no effect on their octanucleotide binding property.

## INTRODUCTION

The importance of the 3' terminal region of 16S ribosomal RNA for initiation of protein synthesis is documented by a growing number of studies (1-6). The most widely accepted view is that the pyrimidine stretch at the 16S RNA 3' terminus basepairs with a complementary messenger RNA sequence some 10 nucleotides upstream from the initiation codon. For a good understanding of this first step in protein synthesis it is necessary to know if the RNA regions, before they participate in the basepairing reaction, exist as unpaired strands. It is conceivable that they are engaged in intramolecular basepairing and require one or more ribosomal proteins to pass on to the open form.

In the present paper we have examined this question for the 3' terminus of 16S RNA. We have used a deoxyoctanucleotide complementary to the 16S RNA 3' terminus (Figure 1) to study the accessibility of this sequence in isolated 16S RNA and in various 30S subunits. Our results show that the basepairing potential of the 16S RNA region concerned changes drastically with the functional state and protein content of the small ribosomal subunit.

## MATERIALS AND METHODS

*Preparation and reconstitution of ribosomal subunits.* 30S ribosomal subunits were prepared as described (7) except that DNAse was not used here to prepare the S30 extract. Reconstitution was carried out according to Held and Nomura (8). Ribosomal protein S21 was removed from total 30S protein or from the split protein fraction by passing the protein fraction through an affinity column carrying sepharose bound antibodies against S21 (9). Core particles and split proteins were prepared by equilibrium centrifugation of 30S subunits in 44% (w/v) CsCl and 25 mM $MgAc_2$. The split protein fraction contains S1,2,3,5,6,9,10,18 and 21. 30S subunits lacking S1 were prepared according to Tal *et al.* (10).

*Buffers.* Standard buffer contained 10 mM Tris-acetate pH 7.4, 10 mM $MgOAc_2$, 60 mM $NH_4Cl$ and 6 mM redistilled β-mercaptoethanol. In the reconstitution buffer the concentrations are 30 mM Tris-acetate pH 7.5, 20 mM $MgOAc_2$, 320 mM KCl and 6 mM redistilled β-mercaptoethanol.

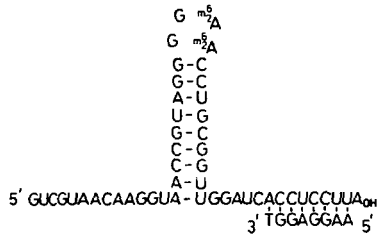


Figure 1. Basepairing scheme of the octanucleotide 5'd(AAGGAGGT) and the 3' terminus of 16S RNA of *E. coli*.

*Oligonucleotides.* The deoxyoligonucleotide (5'-3')d-(AAGGAGGT$_{OH}$) and the ribooligonucleotides (5'-3')ACCUCCUUA$_{OH}$ and (5'-3')ACCUCC$_{OH}$ were synthesized and 5' labeled as previously described (6). One $A_{260}$ unit corresponds to 11 nmoles octanucleotide, 12 nmoles nonanucleotide and 19 nmoles hexanucleotide.

*Preparation of [5'-$^{32}$P] cytosine 3'5' diphosphate.* The procedure of Stanley and Van Kammen (11) was essentially followed with the only exception that [$\gamma$-$^{32}$P]ATP from Amersham was used.

*In situ labeling of the 3' end of 16S RNA.* Preactivated 30S subunits (100 pmoles) were incubated for 1 hour at 15°C with 10 units T4 RNA ligase (PL-Biochemicals) and [5'-$^{32}$P] cytosine 3'5' diphosphate (250 pmoles - 3 µCi/pmole) in 50 µl of 50 mM HEPES pH 7.5, 25 mM KCl, 10 mM $MgCl_2$ and 6 mM 2-mercaptoethanol. The reaction mixture was then layered on a 5-20% sucrose gradient in standard buffer, and centrifuged in a SW56 rotor for 2 hours at 40.000 rpm to separate the labeled 30S subunits from free cytosine 3'5' diphosphate. In general 5 to 10% of the 30S particles were labeled. The 30S peak was precipitated by addition of 2 volumes of ethanol, the pellet dissolved in standard buffer at a concentration of 1 pmole 30S per µl and stored at -80°C after a 30 min heating step at 37°C.

*Binding of oligonucleotide 5'd(AAGGAGGT) to 30S subunits.* All binding experiments are carried out in standard buffer.

*Analysis of sucrose gradients.* Fractionation on sucrose gradients was occasionally performed using a Mico device. In this set up the content of the gradient tube is removed through the pierced bottom by pumping $H_2O$ on top of the gradient. This gives the free oligonucleotide peak on top of the gradient a broad appearance (*e.g.* Figures 10-12).

## RESULTS

### *Binding of 5'd(AAGGAGGT) to 30S Subunits*

The binding of the octanucleotide 5'd(AAGGAGGT) to 30S ribosomal subunits can be shown by filtering mixtures of the two components over nitrocellulose filters at 4°C. An example is given in Figure 2. The stoichiometry of binding in this case is

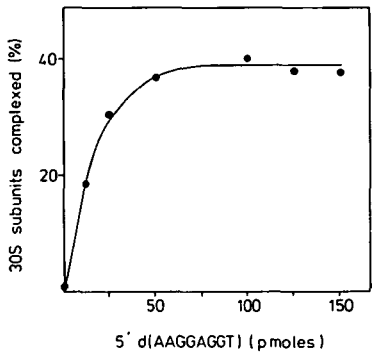


Figure 2. Binding of 5'd(AAGGAGGT) to 30S subunits and retention of the complex on cellulose acetate filters. 25 pmoles of 30S subunits were incubated at 0°C with increasing concentrations of labeled 5'd(AAGGAGGT) during 10 min in 50 µl standard buffer. The reaction mixtures were then filtered over Selectron filters; the filters were washed with 1 ml ice-cold standard buffer. The radioactivity was determined by counting each filter in 5 ml xylene scintillator (1 pmole octanucleotide was 230 cpm).

0.4 moles of octanucleotide per mol of 30S subunit. This number differs for different ribosome preparations and has been found to vary between 0.25 and 0.6. This binding is not changed by preincubating subunits and octanucleotide at 37°C (6). The 30S subunits used, were routinely checked for any activity that would degrade the octanucleotide. Even after prolonged incubation (16 hours at 4°C) of the two components, the labeled octanucleotide was still completely intact as was evident from polyacrylamide gel analysis (not shown). The results indicate some heterogeneity in the 30S subunit population. This aspect is discussed later.

*Binding of 5'd(AAGGAGGT) to Nucleotides 1533-1540 of 16S RNA*

To establish whether the octanucleotide indeed binds to the ribosomal RNA as depicted in Figure 1, we have initially followed the procedure introduced by Steitz and Jakes (3). This involves treatment of the complex between 30S subunits and the oligonucleotide with colicin E3, the disassembly of the ribosome with sodium dodecylsulphate (SDS) and fractionation of the reaction mixture on a non-denaturing polyacrylamide gel. This method reveals the presence of a complex between the colicin fragment and the (label-

ed) octanucleotide (Figure 3, lane 2). Omission of the colicin treatment prevents appearance of the complex (Figure 3, lane 1).

However, a control experiment in which the octanucleotide was added to the reaction mixture after ribosome disassembly in SDS showed the appearance of the same complex (lane 3). Apparently the octamer can associate with the colicin fragment after ribosome dissociation. This results precludes as yet the conclusion that 5'd(AAGGAGGT) binds to the 16S RNA 3' terminus *in situ*. In a further attempt to show that complex formation occurred before SDS treatment we repeated the experiment of lane 2, but added a twelve-fold excess of unlabeled octamer before the SDS treatment. Assuming the non exchangeability of labeled and unlabeled octamer

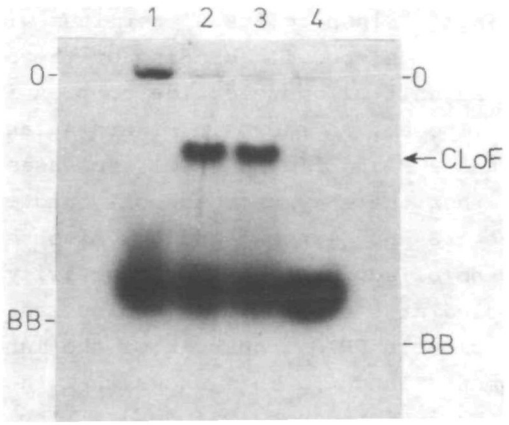


Figure 3. Autoradiogram of the binding of [5'-$^{32}$P] d(AAGGAGGT) to the colicin fragment: analysis on a 15% polyacrylamide gel. A complete reaction mixture contained in 20 µl standard buffer: 100 pmoles 30S subunits, 100 pmoles 50S subunits, 100 pmoles [5'-$^{32}$P] d(AAGGAGGT) and 2 µg of cloacin DF13 (a gift of Dr. F.K. De Graaf). Cloacine DF13 has the same action as colicin E3. Incubation was for 30 min at 37°C. One µl of a 10% solution of sodium dodecylsulphate was then added at 0°C and the reaction mixture was loaded onto the gel (30 x 20 x 0.1 cm) in 5 mM Tris-acetate pH 8, 5 mM magnesium acetate. Electrophoresis was carried out overnight at 4°C and 100 V. Slot 1: reaction omitting cloacin DF13. Slot 2: complete reaction. Slot 3: reaction omitting [5'-$^{32}$P] d(AAGGAGGT) during 37°C incubation; oligonucleotide added after sodium dodecylsulphate treatment. Slot 4: as slot 2 but also added 1200 pmoles unlabeled d(AAGGAGGT) just before the sodium dodecylsulphate treatment. CLoF indicates the position of free colicin fragment. BB is the Bromophenol Blue marker.

in the complex, any octamer binding subsequent to the SDS treatment would thus concern unlabeled octanucleotide. The result is shown in lane 4. As practically all the label appears at the position of the free octanucleotide, this means that either the binding occurred after the disassembly of the ribosome or bound and free octamer are completely exchangeable. Our previous experiments have already shown that octanucleotide bound to 30S subunits exchanges rapidly with the free molecule (6). The experiments of Figure 3 are inconclusive concerning the site specific binding of the octanucleotide. They do, however, illustrate some of the pitfalls encountered in the use of complementary oligonucleotide binding probes.

To solve the problem on the site specific binding of the octamer we have developed a new technique. 16S RNA was labeled *in situ* by coupling [$^{32}$P]pCp to its 3' terminus with the aid of T4 RNA ligase (12). Site specific binding of the octamer will now turn the 30S subunit·oligonucleotide complex into a substrate for RNAse H (13). Indeed, as shown in Figure 4 lane 3 the combined action of RNAse H and 5'd(AAGGAGGT) releases pA[$^{32}$P]pCp from 16S RNA. Neither the enzym nor the oligonucleotide can by itself cleave the 16S RNA (lanes 1 and 2). Also the presence of 50S subunits has no effect on the RNAse H activity (lane 4). If 30S subunits labeled at their 16S RNA terminus as described above are treated with cloacin DF13, the bulk of the label appears in the colicin fragment (lane 6). This shows that the [$^{32}$P]pCp label incorporated in the 30S ribosome is indeed at the 16S RNA terminus. (A minor amount of [$^{32}$P]pCp becomes coupled to an RNA fragment of unknown origin, denoted X in Figure 4). If the experiment shown in lane 6 is followed by RNAse H/octamer treatment, the label in the colicin fragment decreases and new radioactive material appears at the position of pA[$^{32}$P]pCp (and to lesser degrees at the positions of pUpA[$^{32}$P]pCp and pUpUpA[$^{32}$P]pCp) again confirming the site specific binding of 5'd(AAGGAGGT) (lane 5). The incomplete release of the label as evident in Figure 4 is probably a consequence of the non stoichiometric binding of the octamer to 30S ribosomal subunits (vide supra).

It should be noted that very large RNA fragments, that would result from RNAse H cuts at positions sufficiently distant from

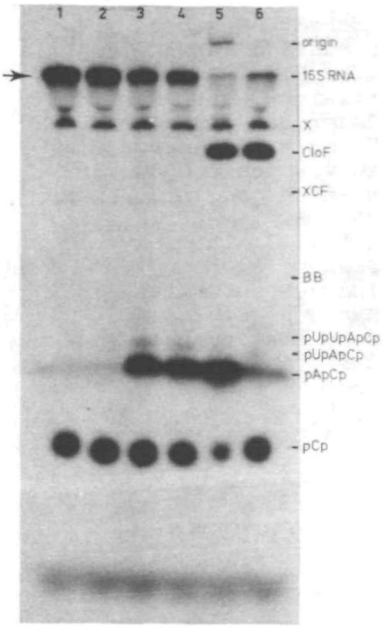


Figure 4. RNA fragments released from [$^{32}$P]pCp labeled 30S subunits by RNAse H and 5'd(AAGGAGGT): autoradiogram of an analysis on a 20% acrylamide gel. The standard reaction included 10 µl standard buffer: 7 pmoles 3' labeled 30S subunits, 1 unit of RNAse H (Enzo) and 60 pmoles 5'd-(AAGGAGGT). Incubation was for 30 min at 37$^{o}$C. The reaction was stopped by phenol extraction and the water phase loaded onto a 20% polyacrylamide gel in 50 mM Tris-borate pH 8.3, 1 mM EDTA and 8 M urea. A 5% polyacrylamide stacking gel was included containing no buffer but 0.1% sodium dodecylsulphate. Electrophoresis was performed at room temperature for 1½ hours and 1000 V. Slot 1: standard reaction omitting RNAse H and 5'd(AAGGAGGT). Slot 2: standard reaction omitting only 5'd(AAGGAGGT). Slot 3: standard reaction. Slot 4: standard reaction including 7.6 pmoles 50S subunits. Slot 5: as slot 4 but including 1 µg cloacine DF13. Slot 6: as slot 5 but omitting RNAse H and 5'd(AAGGAGGT). The sequences of the small oligonucleotides produced were identified by rerunning these fragments next to a partial P1 nuclease digest of isolated 3' labeled colicin fragment (not shown). The arrow indicates the boundary between the 5 and 20% gel.

the 3' end would go unnoticed in the gel system used in Figure 4. We have therefore analyzed the products generated by RNAse H on a 5% acrylamide gel to allow entry of large RNA fragments into the gel. The analysis is shown in Figure 5. In the absence of RNAse H and octanucleotide, we detect the majority of label in the 16S RNA position. Some label appears in a band just below the 16S RNA position and presumably represents a large 16S RNA fragment. Minor amounts of label are found in band X (see Figure 4) and at the position of the bromophenol blue marker. This radioactivity pattern is unchanged when the subunits are incubated with RNAse H alone (lane 2). Incubation of labeled 30S subunits with RNAse H and 5'd(AAGGAGGT) together produces one new band positioned between [$^{32}$P]pCp and bromophenol blue (lane 3). This band corresponds to pA[$^{32}$P]pCp (see legend to Figure 4 for iden-

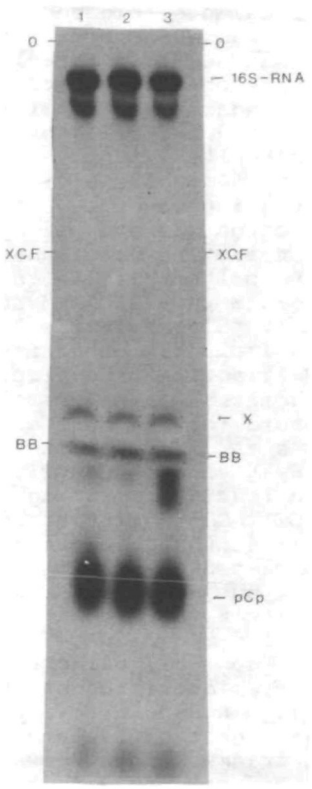


Figure 5. RNA fragments released from [$^{32}$P]pCp labeled 30S subunits by RNAse H and 5'd(AAGGAGGT): analysis on a 5% acrylamide gel. The reaction conditions were identical to those described in Figure 4 with the only difference that the analysis was performed on a 5% polyacrylamide gel in 50 mM Tris-borate pH 8.3, 1 mM EDTA and 8 M urea. Slot 1: 3' labeled 30S subunits incubated alone. Slot 2: 3' labeled 30S subunits incubated with RNAse H. Slot 3: 3' labeled 30S subunits incubated with RNAse H and 5'd(AAGGAGGT).

tification). The important point is that no large 16S RNA fragments are generated by RNAse H/octamer treatment. It is thus unlikely that the octanucleotide binds to internal 16S RNA sequences.

*Binding of 5'd(AAGGAGGT) to Isolated 16S RNA, 30S Core Particles, 30S Subunits and 30S Subunits Lacking S21*

Now that we have demonstrated that the octanucleotide binds only at the target sequence (Figure 1) we can begin testing the basepairing potential of this 16S RNA region. First we have asked whether isolated 16S RNA can form a stable complex with 5'd-(AAGGAGGT). As shown in the sucrose gradient analysis of Figure 6 (panel A) this is not the case. This is not due to any breakdown of the RNA preparation, since octamer binding is restored after incorporation of the RNA into a 30S ribosome by total reconstitu-

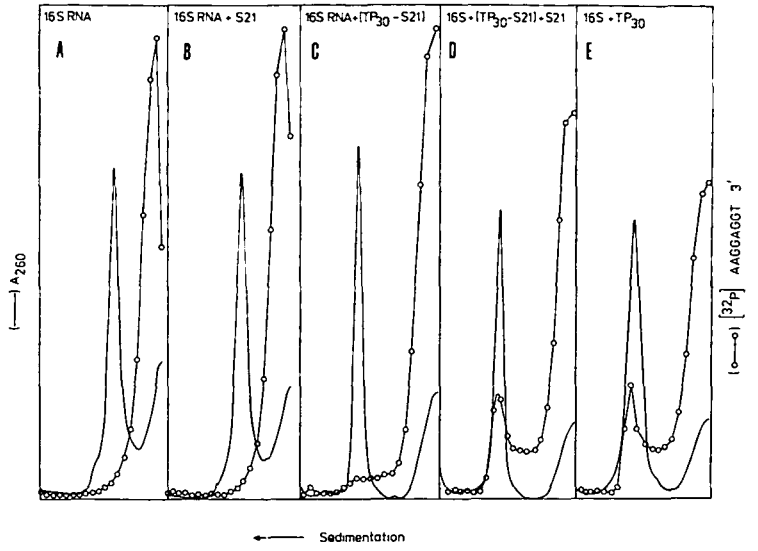


Figure 6. Binding of labeled 5'(AAGGAGGT) to 16S RNA and various 30S particles. Panel A: 16S RNA. Panel B: 16S RNA + 35 pmoles S21. Panel C: 16S RNA + total 30S proteins lacking S21. Panel D: as panel C but in the presence of 35 pmoles purified S21. Panel E: 16S RNA + total 30S protein.
Each sample contained 35 pmoles of 16S RNA and 70 pmoles of labeled octanucleotide. 16S RNA was first heated for 10 min at 41°C in reconstitution buffer. For samples B through E this was followed by another 20 min at 41°C, now including the indicated protein fraction. (The sample of panel A was cooled down after 10 min of incubation). Subsequently all samples were cooled and diluted to standard buffer conditions and then incubated with the octanucleotide for 5 min at 37°C. The final volume of each sample is 170 µl. The analysis was on a 5-20% sucrose gradient in standard buffer. Centrifugation was for 2 hours at 45.000 rpm in a SW-50 rotor.

tion (panel E). Single protein omission experiments reveal that the critical protein required for octamer binding is S21. 30S subunits reconstituted from 16S RNA and total 30S proteins lacking S21 fail to bind the octanucleotide (panel C). Completion of this reconstitution mixture with purified S21 restores octamer binding to control level (panel D). The combination 16S RNA and S21 does not trigger octamer binding (panel B). Control experiments on nitrocellulose filters show that neither S21 nor total 30S proteins retain labeled octanucleotide on the filter. Similarly if the components present in panel E are not subjected to the heat

treatment necessary for reconstitution no binding ensues (not shown). In a series of further controls we have prepared 30S ribosomal core particles and split proteins by CsCl gradient centrifugation (Figure 7). 30S core particles do not bind 5'(AAGGAGGT) (panel A), neither do 30S particles reconstituted from core particles and the split protein fraction from which S21 has been removed (panel C). Supplementation with S21 restores octamer binding to control levels (panel D). S21 by itself cannot induce octamer binding in combination with 30S core particles (panel B). In previous studies we have shown that 30S subunits missing S21 sustain the translation of poly(U) and poly(A,G,U) but not of phage RNA (9).

Further evidence for the indispensability of S21 for the basepairing potential of the 16S RNA 3' terminus is shown in Figure 8. Here the influence of antibodies against ribosomal protein S21 (anti-S21) on the octamer binding properties of 30S sub-

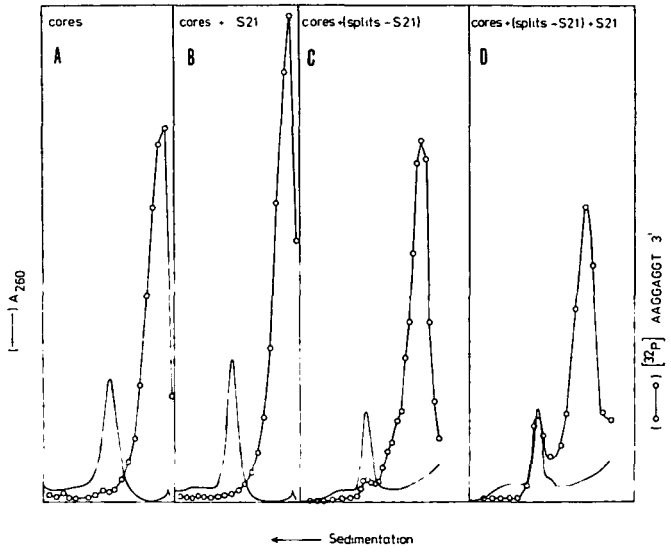


Figure 7. Binding of labeled octanucleotide to core particles and reconstituted 30S particles. Each sample contained 30 pmoles of core particles and 70 pmoles of labeled 5'd(AAGGAGGT). Cores were preincubated with the indicated protein fraction in reconstitution buffer for 10 min at 41°C. The samples were then assayed as in the legend to Figure 6.

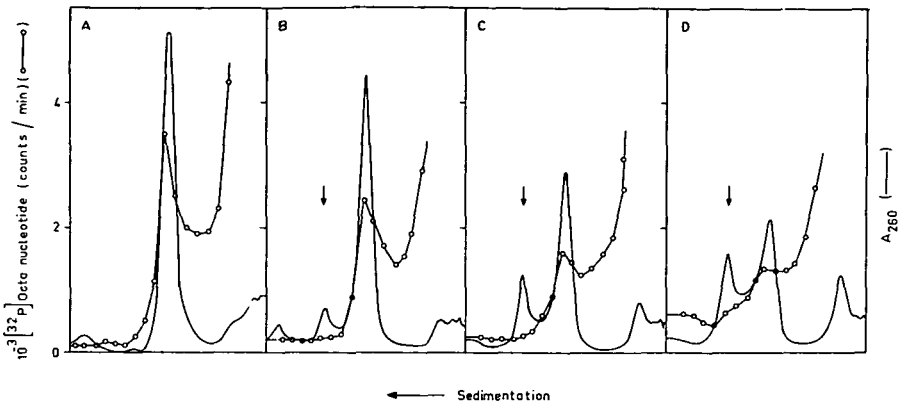


Figure 8. Effect of antibodies against protein S21 on the binding of labeled 5'd(AAGGAGGT) to 30S subunits. Panel A: no anti-S21 present. Panel B: 2 µl anti-S21. Panel C: 4 µl anti-S21. Panel D: 10 µl anti-S21. 30S subunits (35 pmoles) were treated with the indicated amount of anti-S21 in standard buffer for 10 min at 0°C, then 70 pmoles of octanucleotide were added at 0°C and the samples analyzed by sucrose gradient centrifugation as in Figure 6. Arrows point to the position of 30S dimers.

units is measured. Clearly anti-S21 induces the formation of 30S dimers and decreases the octamer binding.

*Correlation Between the Structure of the 3' Terminus and Ribosome Function*

The decreased accessibility of the 16S RNA terminus, induced by anti-S21, is paralled by a decreased activity of these ribosomes in initiation complex formation with MS2 RNA (Figure 9). In contrast the poly(A,G,U) dependent fMet-tRNA binding is not affected by the antibodies (Figure 9). Such a differential inhibition is consistent with studies that imply the 3' terminus of 16S RNA only in phage RNA dependent initiation but not in initiator-tRNA binding induced by synthetic templates (4,5).

*S1 Plays no Detectable Role in the Binding of 5'd(AAGGAGGT)*

The present results implicate S21 as the protein, regulating the basepairing potential of the 16S RNA terminus. Previous reports have presented evidence that this role be reserved for

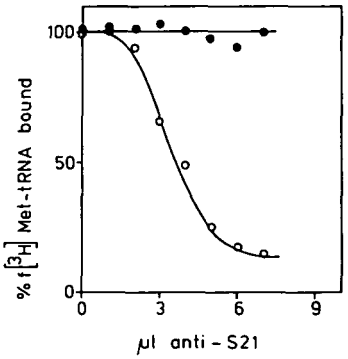


Figure 9. Effect of anti-S21 on f[$^3$H]Met-tRNA binding to ribosomes programmed with phage RNA or poly(A,G,U). Each incubation mixture contained 25 pmoles of ribosomal subunits, optimized amounts of crude initiation factors, 16 µg of MS2 RNA (O——O) or 16 µg of poly(A,G,U) (●——●). The further conditions are as described before (35).

ribosomal protein S1 (14,15). To determine a possible contribution of S1 in this respect we have compared the octanucleotide binding properties of 30S subunits that contain or lack S1. As shown in Figure 10 neither the removal nor the readdition of S1 affects the binding of 5'd(AAGGAGGT). Also antibodies against S1 do not change the accessibility of the 16S RNA terminus. The concentration of anti-S1 used in Figure 10 completely precludes phage RNA translation. The absence of 30S oligomers upon reaction with antibodies against S1 is commonly noticed. S1 is removed from the ribosomes by the IgG preparation upon sedimentation in sucrose gradients (unpublished results).

*Active and Inactive Subunits have Different Octanucleotide Binding Properties*

In 1969 Zamir *et al.* first reported the existence of "active" and "inactive" 30S subunits (16). "Inactive" particles arise by temporary exposure to low $Mg^{2+}$ concentrations. "Active" and "inactive" subunits can be distinguished by several functional criteria (subunit association, non-enzymatic aminoacyl-tRNA binding etc. (17)), and also by a different reactivity of their proteins towards modifying reagents (18). Inactive subunits can be reactivated by a brief heat treatment (17). We have prepared "in-

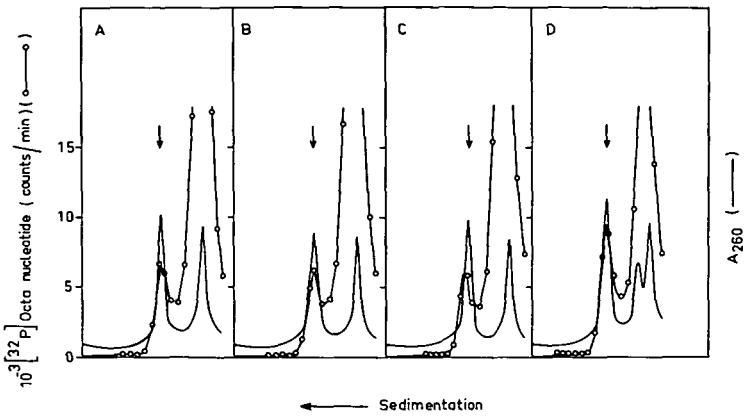


Figure 10. Effect of S1 on the binding of labeled 5'd(AAGGAGGT) to 30S subunits. Panel A: control 30S subunits. Panel B: 30S subunits lacking S1. Panel C: as panel B but the subunits were preincubated with 25 pmoles of purified S1. (A separate experiment revealed that the 30S subunits had bound a substantial part of the added S1 (not shown)). Panel D: as panel A but treated with 10 µl anti-S1. Each sample contained 25 pmoles of 30S subunits and 50 pmoles of octanucleotide. Subunits and octanucleotide were incubated for 5 min at 37°C in standard buffer before analysis. Centrifugation was as in Figure 6. Arrows indicate the 30S position. Bromophenol blue was used to visualize the top of the gradient (see Materials and Methods).

active" 30S subunits by dialysis at 0°C against standard buffer containing 0.5 mM $Mg^{2+}$. After dialysis the $Mg^{2+}$ concentration is raised again to 10 mM but the preparation is kept at 0°C. We find that inactive subunits are unable to bind 5'd(AAGGAGGT). The binding can be restored by heat treatment of the subunits (Figure 11). It should be noted that the octanucleotide is not present during the activation step, but is added after cooling down the subunits again to 0°C. As shown the activation of 30S subunits is accompanied by the recovery of the basepairing potential of the 16S RNA 3' terminus.

*S21 Probably Acts by Exposing the 16S RNA Terminus*

There are at least two ways to interpret the effect of S21 (or the activation step) on the octamer binding properties of 30S ribosomes. In one view the 16S RNA terminus is involved in a long distance intramolecular helix formation. S21 disrupts

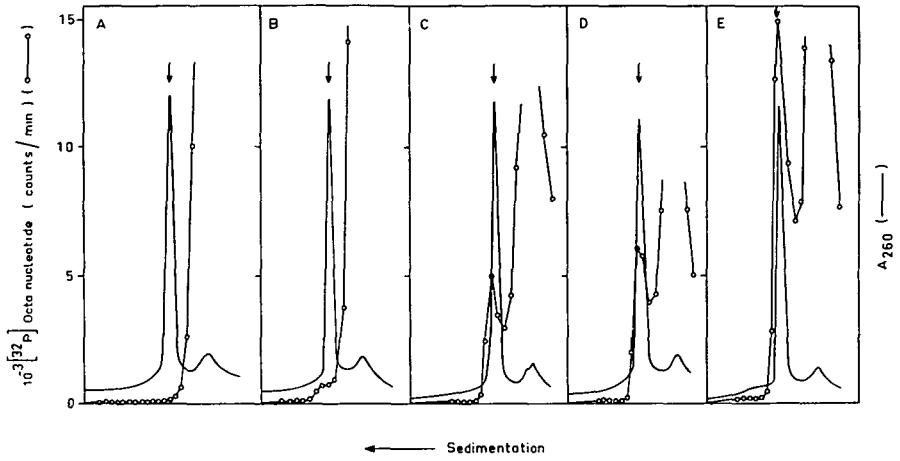


Figure 11. Heat activation of inactive 30S subunits restores octanucleotide binding. 25 pmoles of inactivated 30S subunits (see text) were heated for 15 min at 13, 30, 37, 40 and 45°C, respectively (panels A-E). They were then cooled again, mixed with 50 pmoles of labeled octanucleotide and analyzed on 5-20% sucrose gradients in standard buffer. Centrifugation was for 2 hours at 40.000 rpm in a SW-56 rotor. Arrows indicate the 30S position.

this helix to produce a free single stranded 3' terminus. The other possibility is that the basepairing between the octanucleotide and 16S RNA is directly stabilized by protein S21 for instance by shielding off negative charges. Whether S21 exerts its effect via direct stabilization can be investigated experimentally. If S21 does stabilize the interaction between 30S subunits and the octamer in a direct manner, then the nonanucleotide 5'(ACCUCCUUA) which represents the free 16S RNA 3' terminus, should have a lower affinity for 5'd(AAGGAGGT) than the 30S subunit itself. Consequently increasing amounts of nonanucleotide were mixed with constant amounts of 30S subunits and the labeled octamer. (The order of additions is irrelevant as equilibrium is attained immediately (6)). As shown in the sucrose gradient analysis of Figure 12 the nonanucleotide succesfully competes with 30S subunits for the octanucleotide (panels A-C). This indicates that the association constants of the two complexes are in the same range, which in turn makes it unlikely that the 30S·octamer

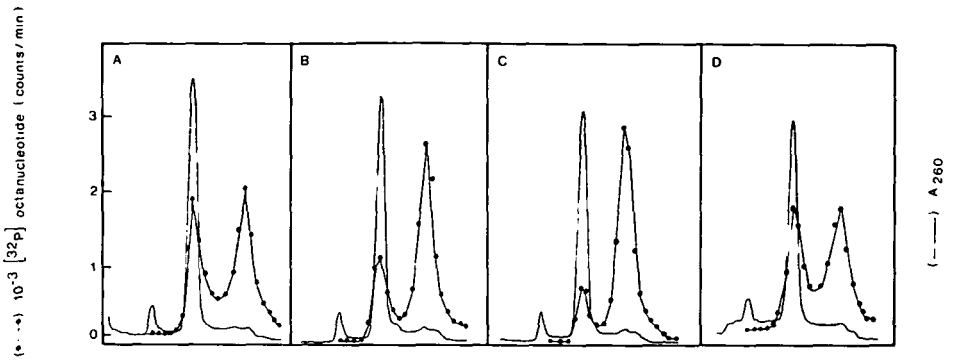


Figure 12. Binding of 5'd(AAGGAGGT) to 30S subunits in the presence of competing oligonucleotides. Panel A: 50 pmoles of 30S subunits and 25 pmoles of labeled octanucleotide. Panel B: as panel A but in the presence of 50 pmoles unlabeled 5'(ACCUCCUUA). Panel C: as panel A but in the presence of 100 pmoles unlabeled 5'(ACCUCCUUA). Panel D: as panel C but 5'(ACCUCCUUA) is replaced by 5'(ACCUCC). All mixtures are incubated for 5 min at 37°C and analyzed as in Figure 11. It may be noted that 5'(ACCUCCUUA) has been shown not to bind to 30S subunits (6).

complex is stabilized by a protein. As an additional control we have examined the competition potential of the hexanucleotide 5'(ACCUCC). This hexanucleotide, which is also homologous to the 16S RNA 3' end, can only form 6 basepairs with 5'd(AAGGAGGT) and its association constant with the octamer can be calculated to be about two orders of magnitude smaller than that of the complex $\overset{5'\ AAGGAGGT}{AUUCCUCCA\ 5'}$ (19). Consistent with this we find that 5'(ACCUCC) cannot compete (at the ratios used) with 30S subunits for the binding of octanucleotide.

## DISCUSSION

In the present paper we show that the octanucleotide 5'd-(AAGGAGGT), complementary to the 3' end of 16S RNA binds exclusively at the predicted sequence in active 30S subunits. The oligonucleotide is therefore a reliable tool to probe the basepairing potential of this functionally important RNA sequence.

Our experiments show that the 3' end of 16S RNA is not "naturally" available for basepairing, since complexes between isolated 16S RNA and 5'd(AAGGAGGT) cannot be detected on gels or

sucrose gradients. This almost certainly means, that the 16S RNA terminus is involved in intramolecular basepairing. Two closely related internal basepairing possibilities have been suggested by Dahlberg and Dahlberg and for convenience reproduced here (Figure 13). Although there is evidence that the intramolecular structure a and/or b (Figure 13) exists in the colicin fragment (15), we think it is unlikely that this can account for the lack of complex formation between 16S RNA and the octamer, since such an intramolecular structure would likewise prevent the association of the colicin fragment with 5'd(AAGGAGGT). However, complexes between the colicin fragment and complementary oligonu-

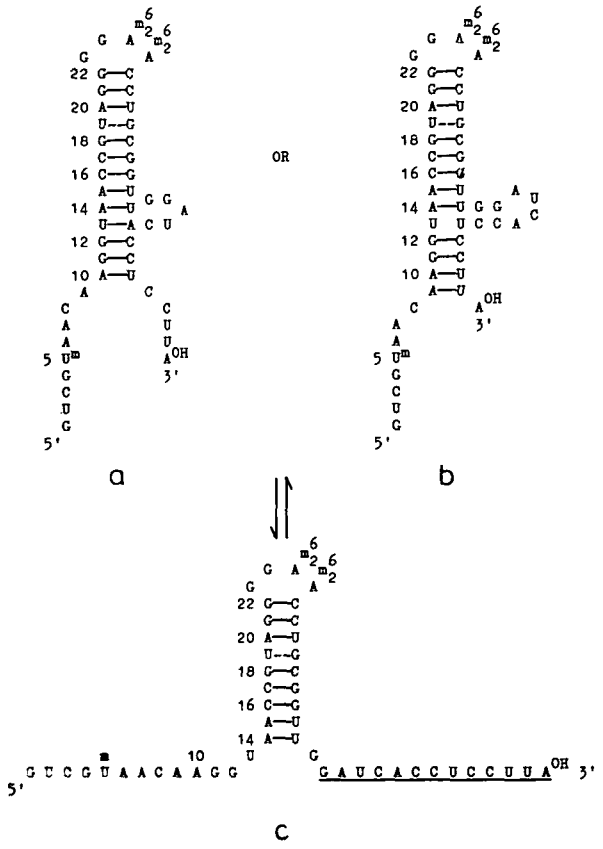


Figure 13. Secondary structure models for the 3' end of 16S RNA as proposed by Dahlberg and Dahlberg (14).

cleotides can readily be detected (e.g. Figure 4, and ref. 3 and 20). Thus we must assume that the 16S RNA 3' end is involved in a long distance interaction to explain its inability to bind 5'd(AAGGAGGT).

Like 16S RNA, 30S subunits that have been transiently exposed to low $Mg^{2+}$ concentrations, so-called "inactive" subunits, fail to bind the octamer. It appears that the 16S RNA conformation in the free molecule and in inactive 30S are the same, at least as far as the 3' extremity is concerned. There is a striking parallel between these data and those obtained by others using electron microscopy to study the structure of 16S RNA in inactive subunits and in the free form (21-23). The authors conclude that the 3' region is associated with a distant part of the same 16S RNA chain. This long distance intramolecular interaction is not visible in intact 30S subunits (22). Indeed we measure the reappearance of the free 3' end upon heat activation of inactive subunits. It was found that antibodies raised against the $m^2_6Am^2_6A$ sequence located at position 24 and 25 from the 3'-end of 16S RNA did only react with "inactive" 30S subunits but not with "active" ones (24). Furthermore studies on 16S RNA modification with kethoxal show that residues 1528 and 1529 are reactive in active but not in inactive 30S subunits (25). (The octamer covers the region 1533-1540). These results point towards a conformational change involving the 3' end region of 16S RNA induced upon activation of 30S subunits. Our data specify that the change in structure involves the exposure of nucleotides 1533-1540 (26, 27).

Our results can also be compared in a meaningful way with the data of Ginzburg and Zamir (18). These authors showed that S21 was one of the ribosomal proteins that reacted with N-ethylmaleimide in inactive but not in active 30S subunits. This result is consistent with the special role played by S21 in modulating the conformation of the 16S RNA 3' end region.

*Effect of S21 on Ribosome Function and 16S RNA Conformation*

The present results show that even heat activated subunits will not bind 5'd(AAGGAGGT) unless S21 is present. The requirement for S21 is reflected in the low stoichiometry of the octanucleotide binding. S21 is present in less than one copy per 30S

subunit and its stoichiometry is variable (28). The effects of S21 on 16S RNA structure are closely paralleled by functional changes in the ribosome. For instance, subunits lacking S21 can only initiate on poly(A,G,U). Initiation on phage RNA is not possible unless S21 is present (9). Other studies have demonstrated that in contrast to poly(A,G,U) initiation on MS2 RNA requires the basepairing potential of the 16S RNA terminus (4, 5, 29). Thus the exposure of the 16S RNA 3' end region by S21 is accompanied by the potential to bind and translate MS2 RNA. In trying to understand how S21 accomplishes its activity it would be of help to know details about its binding site on the 30S subunit. The published data are not unequivocal. The protein can react with the periodate activated 3' ribose (30) and 30S subunits reconstituted from 16S RNA lacking the ultimate 49 nucleotides do not contain S21 (31). There is also evidence that S21 binds to the isolated colicin fragment (32). On the other hand a recent study claims that 30S subunits reconstituted with 16S RNA missing the 3' 160 nucleotides contain all 21 30S proteins (33). Possibly S21 interacts as well with the colicin fragment as with other ribosomal components. In view of our evidence that S21 acts indirectly on the 3' end RNA region, we suggest that the protein helps to melt an internal helix in which the 16S RNA 3' terminus participates. In this connection we notice that S21 like S1 has RNA melting properties (34). Although we refrain from overinterpreting such a result it is at least consistent with the above proposal.

Other workers have for good reasons nominated ribosomal protein S1 for a role in exposing the 16S RNA terminus (14). The present results show that this proposal is probably incorrect. We cannot detect any effect of the absence or presence of S1 on the octamer binding potential of 30S subunits. The binding of protein S1 to the colicin fragment and even the melting of the small extra helix (Figure 13) induced by this protein may indeed be fortuitous (15). Thus our previous proposal that protein S1 functions by opening up regions in messenger RNA that are required for ribosome recognition is strengthened by the present findings (35).

Finally we wish to address the question if and how confor-

mational changes at the 16S RNA 3' end have physiological significance. It is conceivable that once ribosomes have completed the initiation phase it becomes disadvantageous to maintain the full basepairing capacity of the 16S RNA terminus. This may retard or prevent a ribosome from moving away from the initiation site. A mechanism by which ribosomes conceal their 16S RNA 3' terminus may thus be desirable. This can be achieved by basepairing with 5S or 23S RNA. Alternatively, elongating ribosomes may have their 30S moiety in the "inactive" form.

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