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Fluorescence studies of the accessibility of the 3' ends of the ribosomal RNAs in Escherichia coli ribosomes and subunits

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ABSTRACT

The accessibility of the 3'-ends of E. coli in various states has been probed by reaction, after periodate oxidation, with the fluorescent dye proflavine semicarbazide. Free oxidized 16S and 23S rRNAs each react with 2 equivalents of dye. The 23S rRNA is equally reactive in the 50S subunit and the 70S ribosome. The 16S rRNA 3'-end is accessible in the 30S subunit. In the intact 70S particle, periodate can reach the 3'-end of the 16S rRNA but the dye cannot. The 5S rRNA is relatively inaccessible to periodate oxidation or dye reaction in the 70S particle. Dye-labelled 16S rRNA will reconstitute into 30S particles but they are inactive in polypeptide synthesis. This is apparently due to the inability of the 30S particles to form tight complexes with 50S subunits. Iodide quenching studies indicate that the environment of the 3'-end of 16S rRNA in the 30S particle is different from that of the free rRNA.

INTRODUCTION

Early investigations of structure-function relationships in E. coli ribosomes concentrated heavily on the proteins. Reconstitution, genetics, chemical modification, affinity labeling, cross-linking and immunological methods have all been useful approaches (1). Studies of the role of the RNA and its organization within the ribosome have moved slower for a variety of reasons including the relative paucity of RNA-specific reagents, the comparative difficulty of working with antibodies directed against nucleic acids, and the multiple copies of rRNA genes in E. coli. However, numerous indications now exist that the rRNAs play important functional roles. These include the loss of activity when colicin E3 makes a single cut in the 16S rRNA of 70S ribosomes (2), the association of kasugamycin resistance with a deficiency in methylation near the 3'-end of the 16S rRNA (3), the identification of a region near the

3'-end of the 16S rRNA as important in recognizing valid initiation regions of mRNAs (4), the finding of 23S rRNA located at or near the peptidyl transferase (5), and the evidence that the 5S rRNA may base pair with tRNA bound at the aminoacyl site (6).

Recently several studies have examined the accessibility of regions or individual residues of rRNAs using chemical modification (7), antibodies against unusual bases (8), and nuclease digestion (9). In view of the postulated functional role for the 3'-end of the 23S rRNA and other rRNA regions (10) and previous studies indicating that the 3'-end of the 5S rRNA could tolerate chemical modification (11) we were stimulated to attempt to prepare several 3' fluorescent conjugates of the various rRNAs. In the process of doing this, we were led to a number of observations about the accessibility of the 3' rRNA ends in various structures.

Periodate oxidation is specific for the 3'-ends of RNAs and allows the subsequent formation of fluorescent conjugates by reaction with various hydrazine derivatives (12). The resulting adducts are stable enough at room temperature to permit a variety of studies. Although the stability at elevated temperatures is limited (13) exposure at 37°C can be tolerated without too much loss of the fluorescent dye. In the studies reported here we have used the fluorescent dye proflavine semicarbazide (PSC, 12). Its reactivity with oxidized rRNA free and in ribosomal particles has been measured and the fluorescent properties of some of the conjugates have been characterized.

MATERIALS AND METHODS

Ribosomes, subunits and buffer

Low salt washed 70S ribosomes were prepared from mid-logarithmic *E. coli* MRE 600 as previously described (14). The original MRE 600 strain was generously supplied by Dr. M.S. Friedman of Hunter College. The following buffers were used: A (10mM Tris, pH 7.5 - 30mM NH_4Cl - 10mM $MgCl_2$ - 6mM β mercaptoethanol); B (2.5×10^{-4} $MgCl_2$ - 30mM NH_4Cl - 10mM Tris, pH 7.5, 6mM β mercaptoethanol); C (0.15M NaCl - 0.015M Na citrate, 0.01M EDTA, pH 7.0).

30S and 50S ribosomal subunits were prepared from 70S ribosomes by dialysis against buffer B for 48 hours at 4°C and were separated on 10-30% 38ml sucrose gradients in an SW27 rotor at

25,000rpm for 16 hours. Analytical gradients (5ml) employed an SW50 rotor at 45,000rpm for 1-3 hours.

Ribosomal proteins

Extraction of ribosomal proteins from 30S subunits was performed as described by Traub et al. (14). 30S subunits were stripped of proteins by the LiCl urea method (15). LiCl and urea were added to 30S ribosomes to a final concentration of 2 and 4M respectively. Samples were allowed to stand at 4°C for at least 20 hours. The rRNA was removed at low speed centrifugation.

Ribosomal RNAs

16S, 5S and 23S rRNA were purified from the total 70S particle (14). 70S ribosomes, diluted with an equal volume of buffer C plus 1/5 vol. of 2% bentonite and 1/10 vol. of 10% sodium dodecyl sulfate, were shaken for 5 min. at 4°C. An equal volume of phenol was added, and the mixture was shaken for 5 min. at 4°C. The biphasic solution was separated using a Sorvall SS-34 rotor (5 min. at 10,000rpm). The aqueous layer was withdrawn and reextracted four times by the same procedures. The first two phenol layers were washed with 5mM EDTA (pH 7.0) and the aqueous layer was added to the main solution. Then the rRNA layer was ethanol precipitated twice and dissolved in buffer C (30-40 mg/ml) and 0.2ml of RNA solution was layered onto 36ml of a 5-20% sucrose gradients (in buffer C) and centrifuged for 22 hrs. at 25,000rpm in an SW27 rotor.

The purity of rRNAs was examined by analytical sucrose gradient centrifugation (4 hours at 45,000rpm in an SW50·1 rotor for 16S rRNA). The 16S rRNA sample was capable of reconstitution into active 30S particles. In parallel experiments rRNAs prepared by comparable procedures were heated to 65°C in urea and then examined by 2-15% gradient polyacrylamide gels (13). No significant amount of strand breakage was seen. Note that even if an rRNA sample suffered occasional breaks during isolation or subsequent oxidation, most known mechanisms of breakage would leave a phosphorylated 3'-end incapable of reaction with fluorescent hydrazines.

Fluorescent labeling of isolated rRNAs

Oxidation of the 3' rRNA termini was carried out similarly to the method described by Zamecnik et al. (16). An equal volume of 0.1M NaOAc (pH 5.4) was added to a solution containing 1-5mg of rRNA. A 50-100 fold molar excess of $NaIO_4$ was added and the re-

action mixture incubated for 30 minutes at room temperature in the dark. Then the solution was made 0.2M in KCl, and KIO_4 was allowed to precipitate at 4°C and removed by centrifugation. The rRNA was precipitated twice by 2 volumes of ethanol to remove any traces of KIO_4. The final rRNA solution was redissolved in 1ml of 0.1M NaOAc (pH 5.4).

Proflavine semicarbazide (PSC) was synthesized as described previously (12). Approximately a ten-fold molar excess of PSC was added to the rRNA and the mixture incubated at 37°C for 1.5 hours. The incubation was terminated by ethanol precipitation. Unreacted dye was removed from rRNA by sucrose gradient centrifugation and 10 more ethanol precipitations. The modified rRNA was resuspended in buffer C and dialysed against buffer A for at least 4 hours, then quick frozen and stored at -50°C.

Labeling of rRNA in ribosomes

The following method was used for oxidation and labeling of rRNAs in intact 70S ribosomes or 30S and 50S subunits. An equal volume of 0.1M NaOAc (pH 5.4) was added to 100mg of 70S ribosome pellet and shaken gently for 5 minutes at 4°C. A hundred fold molar excess of $NaIO_4$ was added and the mixture incubated for 30 minutes at room temperature in the dark. The oxidized ribosomes were concentrated by centrifugation in a Spinco SW 50.1 rotor. They were resuspended in 1ml of 0.1M NaOAc (pH 5.4) and a hundred fold molar excess of PSC added. The reaction mixture was incubated for 1.5 hours at 37°C. Then the rRNA was extracted by phenol using the method described above. The rRNA-PSC conjugate was layered on a 12ml gradient containing 5-20% sucrose in buffer C and centrifuged for 5 hours at 40,000rpm in a Spinco SW41 rotor. The rRNA bands were located visually and removed by pipetting. 5S, 16S and 23S rRNA-PSC conjugates were precipitated by 2 volumes of ethanol 10 times. Their purity was examined by analytical sucrose gradient centrifugation.

Reconstitution of 30S subunits with 16S rRNA-PSC conjugate

Reconstitution of 30S particles was performed as described by Traub et al. (14). The purified reconstituted 30S particles were always analyzed by sucrose gradient centrifugation and assayed for activity by monitoring polyphenylalanine synthesis.

Formation of 70S particles from 50S and 30S subunits

Approximately 1mg of reconstituted 30S particles in buffer A was added to 3mg of 50S subunits in the same buffer (1.5 molar excess). The reaction mixture was incubated for 15 minutes at 37°C and then purified by centrifugation through 10% sucrose in buffer A at 45,000rpm for 1.75 hours in a Spinco SW50.1 rotor. The reconstituted 70S particles were analyzed by sucrose gradient centrifugation and assayed for polyphenylalanine synthesis.

Protein synthesis assays

Ribosome activity was measured with a poly(U)-dependent phenylalanine incorporation system (17) using the enzyme fraction of Wood and Berg (18). Each reaction mixture contained 120µg of 70S ribosomes. Each subunit was assayed in the presence of a 1.5 fold excess of the other subunit.

Fluorescence measurements

Static fluorescence measurements were performed on a Perkin-Elmer MPF2A fluorimeter using either 10 x 10mm or 3 x 3mm cuvettes. The excitation wavelength was 440nm. Relative proflavine fluorescence intensities were calculated by comparing peak areas of the emission band. To determine the number of moles of covalently attached dye per mole of RNA the following assumptions were made. The molar extinction coefficient of the bound and free dyes is approximately the same (14,000 at 440nm). The molar extinction coefficients used for rRNAs were 8.2×10^5 for 5S, 1.35×10^7 for 16S, 2.7×10^7 for 23S. The absorbances were determined using a Cary 15 spectrophotometer. Quenching by potassium iodide was performed by adding various concentrations of KI to a sample and recording the change in fluorescence intensity. The results were then analyzed by Stern-Volmer plots.

RESULTS

Reactivity of rRNAs with PSC

The extent of covalent reaction of rRNAs in a 1½ hr. 37°C incubation with the fluorescent dye PSC is summarized in Table 1. If the rRNA has not been subjected to prior periodate oxidation the level of reaction with PSC is quite low. The results shown in Table 1 for free 16S rRNA are typical. In contrast a free rRNA containing an oxidized 3'-terminus reacts with approximately two equivalents of PSC. Based on these results and the known specificity

TABLE 1

Reactivity of rRNAs with Proflavine Semicarbazide

Sample used for PSC reaction	Moles PSC reacted/Mole rRNA		
	16S rRNA	5S rRNA†	23S rRNA
unoxidized free RNA	0.0 - 0.4	nd*	nd
oxidized free RNA	1.95	nd	2.3
oxidized 30S subunit	2.2 - 2.4	--	---
oxidized 50S subunit	-----	0.13	2.3
oxidized 70S ribosome	0.0	0.16	2.1
RNA isolated from oxidized 70S ribosomes	1.85	0.11	nd

* nd is no data

† some contamination with tRNA makes these numbers less precise than those shown for the larger rRNAs

of the periodate reaction we can conclude that the bulk of the covalently attached dye is located at the 3'-end.

The accessibility of the 3'-terminus of rRNAs in ribosomal subunits to the combined effects of periodate and PSC was examined by direct periodate oxidation of intact 30S or 50S subunits. After removal of unreacted periodate, the subunits were treated with a hundred fold excess of PSC as described in Materials and Methods. Considerable uptake of PSC is seen. This presumably represents covalent binding to rRNA, a substantial degree of noncovalent binding, and almost certainly covalent binding to ribosomal proteins damaged at places like N-terminal serines by the periodate treatment (19). To measure the net reaction at the 3'-rRNA termini the rRNAs were removed from ribosomal subunits and the amount of attached PSC measured spectrophotometrically.

The results in Table 1 indicate clearly that the 3'-end of the 16S rRNA is labelled essentially as well in the 30S subunit as it is in free solution. Therefore it is presumably freely accessible to periodate and to PSC. Similarly, the 3'-end of the 23S rRNA is as reactive in the 50S particle as free in solution and therefore also is judged fully accessible. In contrast, the 5S rRNA 3'-terminus appears to be much less reactive in the 50S parti-

cle. This could be due either to inaccessibility to periodate, PSC or both.

The accessibility of rRNAs to PSC was also studied in the intact 70S particle. Here the 16S rRNA which appeared to be fully exposed in the free 30S subunit is now totally unreactive. The 23S rRNA retains its accessibility in the 70S while the 5S RNA remains inaccessible.

The inability of 16S and 5S rRNA to react in the 70S ribosome could be the result of a failure of periodate oxidation or a failure of PSC to reach the oxidized rRNA. These possibilities can be distinguished by purifying rRNA from periodate-treated 70S particles and then subsequently measuring the reactivity of the free rRNA towards PSC. The results in Table 1 show clearly that 16S rRNA becomes oxidized in the 70S particle but 5S rRNA is fairly resistant. Apparently the 3-end of the 5S is firmly bound up in the structure. In contrast the factors responsible for shielding the 16S rRNA 3'-terminus in the 70S particle are apparently more subtle since they can discriminate between periodate and PSC. Presumably the larger size of PSC forms the basis of this discrimination.

Properties of the 16S rRNA-PSC conjugate

Of the 3 rRNAs, only the 16S 3'-end showed an altered environment when ribosomal subunits and the intact particle were compared. Hence this material was selected for further study. Since the 3'-end of 16S rRNA was accessible in the 30S particle it was reasonable to expect that the 3'-PSC-16S rRNA conjugate, prepared by treating free 16S rRNA or isolated from treated 30S particles should be capable of reconstitution. Indeed, 3'-PSC-16S rRNA reconstitutes well with total 30S proteins to give 30S particles which cosediment with 30S prepared with unreacted 16S rRNA. These results are shown in Figure 1.

The protein synthesis activity of the PSC-containing reconstituted 30S particles, however, is low and somewhat variable. The reason for this became apparent when the ability of 30S containing PSC-labelled 16S rRNA to bind to 50S particles was examined. When a sample of 16S RNA with two equivalents of PSC per mole was reconstituted into 30S particles these were totally unable to bind to 50S tightly enough to survive sucrose gradient centrifugation (see Figure 2). If a sample of 30S containing only 1 PSC per 16S

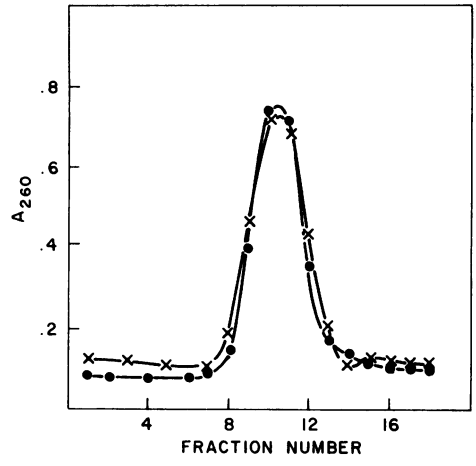

Figure 1
Sucrose gradient sedimentation of reconstituted 30S particles. (•———•) using unoxidized 16S rRNA. (x———x) using periodate oxidized, PSC-conjugated 16S rRNA.

rRNA was used instead, some 70S formation was seen but the resulting particles had very little fluorescence. The results indicate that the presence of two dyes at the 3'-end of the 16S rRNA apparently interfered seriously with the stability of 70S particles. This is reasonable in view of the inaccessibility of the 3'-end of the 16S RNA to PSC reaction in the 70S particle. Since the PSC 3'-link is not completely stable under 30S reconstitution conditions the variable activity of 30S particles containing PSC-treated 16S rRNA presumably reflects the extent of loss of the dye.

The fluorescence of 3'-PSC 16S rRNA and 30S ribosomes containing this material is bright enough for various types of spectroscopic studies. The emission spectrum of free PSC is compared with the rRNA conjugates and reconstituted 30S ribosomes in Figure 3. Note the shift in the emission maximum for 518nm in the free dye to 522nm in the two conjugates. A corresponding shift in absorption is seen from 440nm in free PSC to 444-445nm in the conjugates. The 522nm emission maximum is characteristic of the semicarbazone form of PSC, seen when the dye is reacted with a variety of aldehydes. Thus in the 16S rRNA and the 30S particle the dye is presumably attached to the aldehydes at the oxidized 3'-terminus. Its

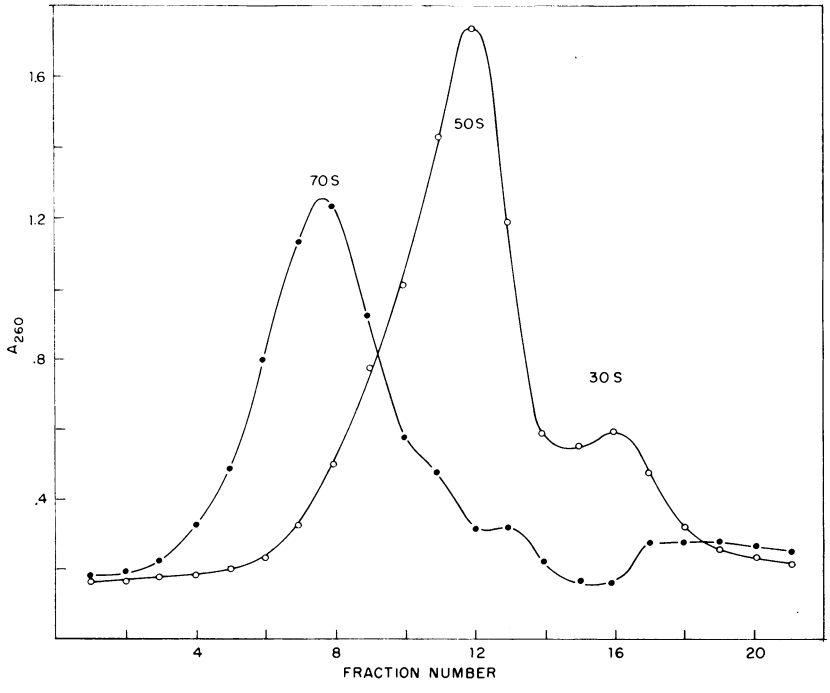

Figure 2
Sucrose gradient sedimentation of a mixture of 30S subunits reconstituted from PSC labelled 16S rRNA and a 3 fold excess 50S subunits (-o-). Note that virtually no 30S is bound tightly enough with 50S to cosediment with the 70S marker (-●-).

environment is apparently not sufficiently different from that in free solution to cause any significant spectral shifts.

Thus far, all of the approaches we have used have failed to detect a significant difference between the environment of PSC on the 16S rRNA and in the 30S ribosome. When a more sensitive technique is used, dynamic quenching with iodide ions, the behavior of these two samples is significantly different. In all the environments we have examined, PSC obeys a linear Stern-Volmer quenching relationship as shown in Figure 4. This is indicative that iodide is quenching by collision although in the absence of lifetime studies other mechanisms cannot be rigorously excluded.

The slope of the quenching plots shown in Figure 4 is proportional to the collisional quenching constant and can serve as a

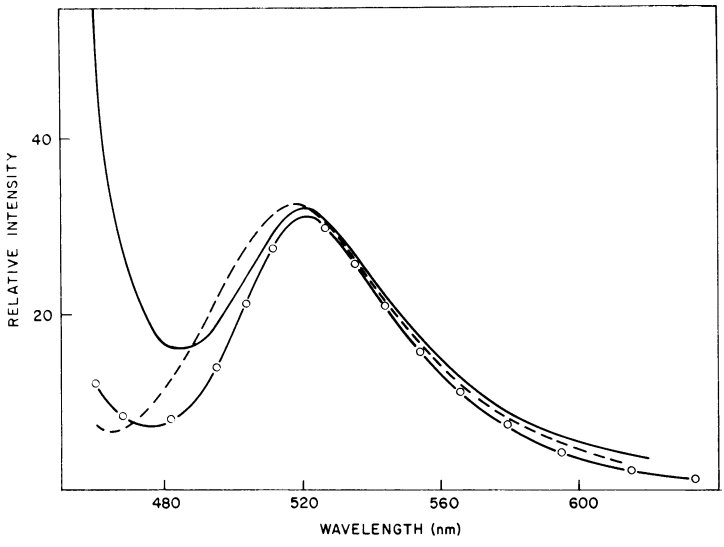

Figure 3
Emission spectra of samples containing proflavine semicarbazide. All were obtained at pH 7.5, 20°C (---), free proflavine semicarbazide in 0.01M phosphate; λ_{max} = 518nm. (o-o), PSC conjugate of 16S rRNA in 30mM Tris HCl, 20mM $MgCl_2$; λ_{max} = 522nm. (——), 30S particles prepared by reconstituting PSC-labelled 16S rRNA in 10mM Tris HCl, 10mM $MgCl_2$, 30mM NH_4Cl; λ_{max} = 522nm.

measure of accessibility. The results indicate that in the 30S particle the 3'-end of the 16S rRNA may be more exposed than in the free 16S rRNA. However, in both samples the dye at the 3'-end is less accessible to quenching than free PSC. Since iodide ion is negatively charged it is not obvious whether to interpret these changes in accessibility as arising from physical occlusion or simple the electrostatic environment. However, the results in Figure 4 show that by quenching, although not by other techniques, the dye at the 3'-end of the 16S rRNA can tell whether or not it is incorporated into a 30S particle.

DISCUSSION

Studies of reactivity of PSC with the 3'-terminus of rRNAs yield a clear pattern of accessibility of the 3'-ends: the 23S is always accessible, the 5S is only slightly accessible, and the 16S is accessible only in the free 30S subunit. Previous studies

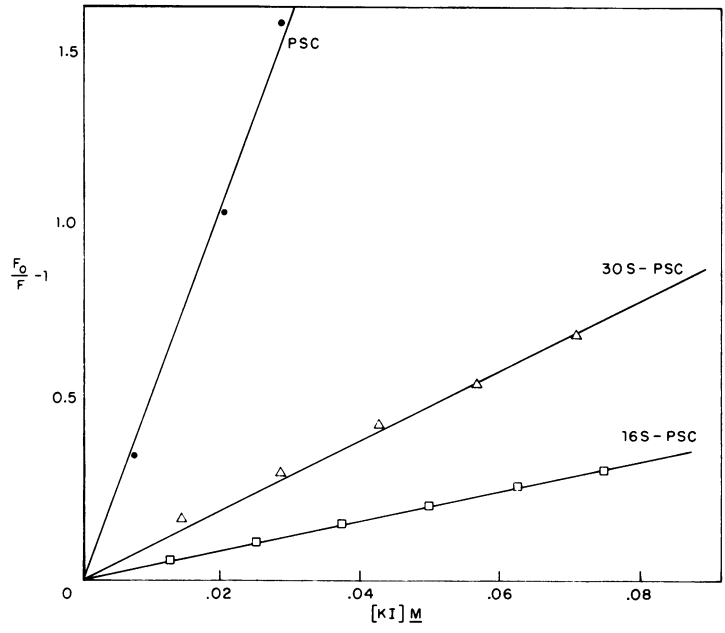

Figure 4
Iodide quenching of free PSC (·), PSC labelled 16S rRNA (□) and reconstituted 30S ribosomes containing this RNA (Δ). Shown are Stern-Volmer plots of $(F_o/F)-1$ versus the molar concentration of iodide. F_o is the relative fluorescence intensity in the absence of quenching; F is the intensity in its presence.

by Fahnestock and Nomura indicated that free 5S rRNA could be periodate-oxidized, reacted with methylamine to form a Schiff's base, reduced and reconstituted into active 50S particles (11). This was used as a decisive argument against a functional role for the 3'-end of the 5S rRNA. Our results support this conclusion.

A number of ribosomal proteins have been shown to react covalently with the 3'-end of the 16S rRNA (20,21) although the functional significance of some of these results remains uncertain (22). Our findings raise the possibility that in the free 30S subunit the 3'-end of the 16S rRNA may retain enough flexibility to reach sites which become inaccessible to it when the structure rigidifies in the 70S complex. The lack of reactivity of periodate-oxidized 16S rRNA with PSC in the 70S ribosome could be due to Schiff's base formation with a nearby ribosomal protein. If so one would have to argue that this protein is not nearby in the

free 30S particle.

The loss in accessibility of the 16S rRNA 3'-terminus upon 70S formation is not at all inconsistent with the role of nearby residues in the recognition of mRNA initiation regions (4). Presumably mRNA interaction with these regions would take place in the free 30S particle and it may even be desirable to break such interaction when the 70S complex is formed. The inability of PSC-modified 30S particles to combine with 50S suggests that the 3'-end of the 16S is located at the subunit interface in the 70S particle. Other studies have reached the same conclusion (10,23). Nuclease digestion studies support this conclusion but indicate that a region near the 3'-terminus of the 16S rRNA may actually be more exposed in the 70S particles than in the 30S (9).

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