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Nucleotides in 16S rRNA that are required in unmodified form for features recognized by ribosomal protein S8

David L.Thurlow*, Chantal Ehresmann and Bernard Ehresmann

Institut de Biologie Moleculaire et Cellulaire du CNRS, 15 rue Rene Descartes, 67084 Strasbourg Cedex, France

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ABSTRACT

Nucleotides in 16S rRNA which are required in unmodified form for specific recognition of ribosomal protein S8 from *Escherichia coli* were identified using a damage-selection experimental approach. Prior to complex formation with S8, 16S rRNA was treated under fully denaturing conditions with either diethyl pyrocarbonate or 25% hydrazine. Following separation of bound from unbound fragments of RNA, those associated with S8 were analyzed for their content of modified bases by treatment with aniline. Nucleotides found to be consistently unmodified in such fragments were located near the base of a stable helix (encompassing bases 581-656) or near the apex of the helix on the 3' proximal side. A minor S8 ribonucleoprotein particle was found to contain fragments which extended in the 3' direction to position 671.

INTRODUCTION

Understanding the detailed mechanism of specific protein-RNA interactions requires precise identification of nucleotides and amino acid residues involved in the process of recognition. To this end, the binding site for ribosomal protein S8 on 16S rRNA has previously been extensively characterized; for the most part by sequencing fragments of RNA which were protected from enzymatic hydrolysis by bound S8 (1-3). Such RNA fragments displayed extensive complementarity and remained associated even in urea containing gels, suggesting a highly stable secondary structure (1-3). Comparisons of several heterologous S8/S15 specific fragments of 16S rRNAs from eubacteria and archaebacteria have revealed extensive conservation of this secondary structure, as well as short stretches of primary sequence and other structural features that may be directly involved in the specific recognition of S8 (4).

In order to examine the S8 binding site in greater detail and to more precisely define which nucleotides are involved in complex formation with S8, we have modified 16S rRNA under fully denaturing conditions prior to reconstitution with S8. Subsequently we analyzed S8 specific RNA fragments,

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including those present in relatively low yield, for their content of modified nucleotides. We reason that RNA fragments which retain bound S8 under such conditions must contain unmodified nucleotides only at those positions where modified bases had interfered with protein binding. Similar damage-selection experiments have been used in the study of protein-tRNA (5) and protein-5S rRNA (6) interactions. We suggest that this approach is a general one that can be used for many protein-RNA interactions and, with appropriate modifications, may also be suitable for identifying amino acid residues in the interacting protein that are essential for specific association.

In this report we identify several adenine, guanine and uracil residues that were unmodified in S8 ribonucleoprotein particles (RNPs) when derived from S8-16S rRNA complexes in which the 16S rRNA had previously been modified with either diethyl pyrocarbonate (DEPC) or hydrazine. The locations of unmodified bases are discussed relative to those that are strongly protected from nuclease digestion by bound S8 and to rebinding of S8 by such protected fragments.

MATERIALS AND METHODS

Isolation of 16S rRNA

Ten g of frozen cells (*Escherichia coli*, strain MRE600) were ground in a chilled mortar with 1-2 g of glass beads (0.1 mm diameter). The slurry was resuspended in 10 ml of 50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 50 mM LiCl; DNase I (RNase-free, Worthington) was added to a final concentration of 25 μg/ml and the solution was incubated at 4°C for 10 min. One tenth volume of 0.1 M Tris-Cl pH 7.6, 1.0 M LiCl, 25 mM EDTA, 5% SDS was added and the resulting mixture was centrifuged for 10 min at 5000 rpm. The supernatant was layered directly onto six 5-20% sucrose gradients in dissociation buffer (10 mM Tris-Cl pH 7.6, 0.1 M LiCl, 2.5 mM EDTA, 0.5% SDS) and sedimented for 20 h at 27000 rpm in a Spinco SW27 rotor. Peaks enriched in 16S and 23S rRNAs were pooled separately and the RNAs were precipitated with two volumes of ethanol at -20°C, collected by centrifugation and resuspended in dissociation buffer. 16S rRNA was repurified by a second cycle of sucrose gradient centrifugation as above and subsequently washed two times with 5 ml of 0.3 M Na-acetate to remove SDS. The final ethanol precipitate was dissolved in doubly distilled water and stored at -20°C at a concentration of 10-20 mg/ml.

Purified ribosomal protein S8 was a generous gift of Dr. Robert A. Zimmermann and was stored at -20°C in 0.05 M Na-acetate pH 5.6, 6 M urea, 5 mM 2-mercaptoethanol at 1.1 mg/ml.

Modification of 16S rRNA

To modify adenine and guanine residues, 200 μg of 16S rRNA were incubated for 1 min at 90°C in 200 μl of 50 mM Na-acetate pH 4.5, 1 mM EDTA containing 1% diethylpyrocarbonate (DEPC). The reaction mixture was then cooled on ice and 50 μl of 1.5 M Na-acetate and 800 μl of ethanol were added. Following precipitation at -80°C for 10 min, the modified RNA was collected by centrifugation, washed once with 200 μl of 0.3 M Na-acetate, reprecipitated and resuspended in doubly distilled water.

For modification of uracil residues, 200 μg of 16S rRNA were incubated in 100 μl of 25% hydrazine for 15 min at 4°C. To stop the reaction, 100 μl of 0.6 M Na-acetate and 800 μl of ethanol were added and the RNA was precipitated, washed and resuspended as described above.

Formation of S8-RNPs

200 μg of 16S rRNA were incubated with 11 μg of protein S8 (molar protein:RNA ratio of approximately 2:1) in 50 μl of 50 mM Tris-Cl pH 7.6, 350 mM KCl, 20 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1.2 M urea (from added protein) at 40°C for 30 min and 4°C for 15 min.

Pancreatic RNase A (Worthington) and calf intestinal phosphatase (Boehringer-Mannheim) were added at enzyme:RNA weight ratios of 1:50 and 1:40 respectively and incubation was carried out at 30°C for 5 min and 4°C for 10 min. The mixture was then loaded directly onto a 10% polyacrylamide slab gel (140 x 160 x 2 mm) in 50 mM Tris-acetate pH 7.6, 1 mM Mg-acetate. Electrophoresis was conducted using constant current (40 mA) at 4°C until the bromophenol blue tracking dye was 1 cm from the bottom of the gel. S8-RNPs were visualized by UV shadowing (7) or by staining with 0.05% Stainsall (Eastman Kodak) in 50% formamide for 30 min in the dark.

Elution of S8-RNPs and labeling 3' termini of subfragments

Bands containing S8-RNPs were sliced from the gel, ground in an Eppendorf 1.5 ml tube and shaken overnight at 37°C in 200 μl of elution buffer (0.5 M NH_4-acetate, 10 mM Mg-acetate, 0.1 mM EDTA, 0.1% SDS). Following centrifugation through a plug of siliconized glass wool, the RNA was precipitated with four volumes of ethanol at -80° overnight. The ethanol precipitate was washed once with 100 μl of 0.3 M Na-acetate and reprecipitated. From 50 to 100 μl of (^{32}P)pCp (37 MBq/ml, Amersham) were added, and dried under vacuum. The samples were resuspended in 20 μl of ligation buffer containing 50 mM Tris-Cl pH 7.6, 15 mM $MgCl_2$, 3.3 mM dithiothreitol, 10 μg/ml bovine serum albumin, 10 μM ATP, 10% dimethyl sulfoxide. Two units of T_4 RNA ligase (P-L Biochemicals) were added and the mixture was incubated at 4°C

overnight. After reducing the volume to about 5 µl by drying under vacuum, 5 µl of 8 M urea in 100 mM Tris-borate pH 8.3, 2.5 mM EDTA (TBE), containing 0.01% bromophenol blue and 0.01% xylene cyanole, were added and the sample was loaded onto a 25% polyacrylamide sequencing gel (300 x 400 x 0.4 mm) in TBE and 7 M urea. Electrophoresis was carried out at 30 mA using TBE as running buffer until the bromophenol blue marker dye had migrated 30 cm. ^{32}P-labeled subfragments were visualized by autoradiography, bands were sliced from the gel and labeled material was eluted by shaking overnight at 37°C in 200 µl of elution buffer containing 10 µg of carrier tRNA. Following precipitation with four volumes of ethanol at -80°C for 4 h, the pellet was washed with 100 µl of 0.3 M Na-acetate and reprecipitated material was resuspended in doubly distilled water.

Sequence analyses and treatment with aniline

Aliquots of labeled S8 specific subfragments from either untreated or modified 16S rRNA were subjected to sequence analyses as described by Peattie (8), except that incubation with aniline was carried out for 5 min. An equal amount of ^{32}P-labeled control and treated subfragments were each treated with 20 µl of 1.0 M aniline-acetate pH 4.5 at 60°C for 5 min, as in the sequencing procedure. Following treatment with aniline and two washes with 20 µl of doubly distilled water, all samples were resuspended in 4 µl of 8 M urea in TBE, containing 0.01% bromophenol blue and 0.01% xylene cyanole, and transferred to fresh Eppendorf tubes. The tubes were counted directly in a scintillation counter and volumes were adjusted so that Cerenkov counts in all tubes for a particular subfragment were within 10% of each other. Samples were then loaded onto 20% gels in TBE, 7 M urea and electrophoresis was carried out at 30 mA using TBE as running buffer until the bromophenol blue marker dye had migrated approximately 20 cm. Radioactive bands were visualized by autoradiography using $(CaWo)_4$ intensifying screens (Ilford) at -80°C.

RESULTS

Isolation of S8-RNPs and 3' labeled subfragments

Following partial digestion of S8-16S rRNA complexes with RNase A, S8-RNPs were isolated on a 10% polyacrylamide gel in 50 mM Tris-acetate pH 7.6, 1 mM Mg-acetate (Fig. 1a). The presence of an S8-RNP is inferred from a band unique to the digestion profile of S8-16S rRNA complexes, as compared to that of 16S rRNA alone (arrow, Fig. 1a). Prior treatment of the 16S rRNA with either DEPC or hydrazine did not prevent isolation of modified S8-RNPs in a similar fashion, although the yield of material was signifi-

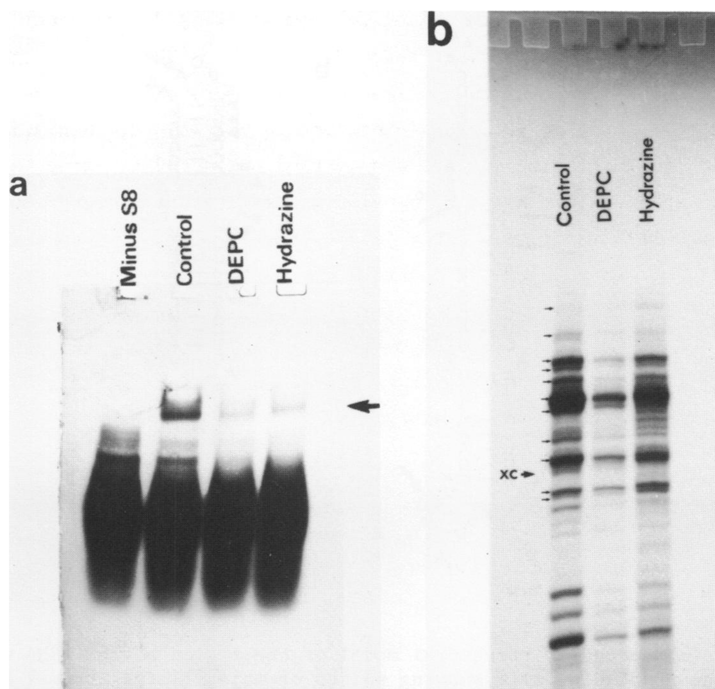

Figure 1. (a) RNPs were isolated on a 10% gel in 50 mM Tris-acetate pH 7.6, 1mM Mg-acetate. Incubation mixtures contained 0.2 mg 16S rRNA which was either untreated (control) or previously modified with 1% DEPC or 25% hydrazine. The arrow indicates the position of the S8-RNP. RNA was visualized by staining for 5 min with 0.05% Stainsall in 50% formamide. (b) [^{32}P]-labeled subfragments from S8-RNPs shown in (a) were resolved on 25% gels in 100 mM TBE, 7 M urea. Electrophoresis was carried out at 30 mA until the bromophenol blue marker dye had migrated 30 cm from the origin. Positions of the xylene cyanole dye (XC) and labeled subfragments eluted for subsequent analyses are indicated by arrows.

cantly reduced in these cases (Fig. 1a).

Subfragments of RNA from RNPs prepared in this manner were eluted from the gel in the presence of high salt and SDS and subjected to labeling at 3' termini with (^{32}P)pCp and T_4 RNA ligase. A highly reproducible pattern of labeled subfragments was observed when products were resolved on a thin gel containing 7 M urea, regardless whether untreated or previously modified 16S rRNA had been used in the formation of the S8-RNP (Fig. 1b). Small arrows (Fig. 1b) indicate the subfragments which were subjected to sequence analysis and cleavage with aniline in this particular experiment. A total of 76 such labeled fragments of S8 specific RNA were analyzed from six separate

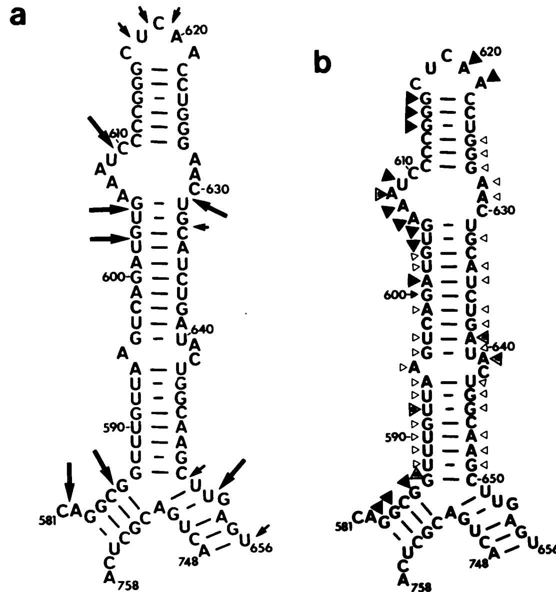

Figure 2. (a) Secondary structural model of the region of 16S rRNA which contains the S8 binding site showing points of major (large arrows) and minor (small arrows) cleavage by RNase A. (b) S8 binding site with nucleotides found to be consistently modified (solid triangles) or consistently unmodified (open triangles) in S8-RNPs made from 16S rRNA previously treated with DEPC or hydrazine. Solid triangles within open ones indicate nucleotides whose sensitivity to aniline varied depending on the length of the particular subfragment. Positions are numbered according to reference 9.

preparations of S8-RNPs. From such analyses it became evident that most subfragments arose from cleavage by RNase A at discrete loci within the hairpin stem, loop and adjacent residues that contain the S8 binding site (Fig. 2a). Cleavage at seven points along the RNA chain (large arrows, Fig. 2a) accounted for more than 90% of the radiolabeled products, although minor bands corresponding to cuts at other residues were always observed (small arrows, Fig. 2a). The exact number of bands, especially in the lower portion of the gel, and the precise distribution of radioactivity among labeled products varied somewhat among separate experiments, but major points of RNase attack were absolutely invariant. Some of the bands corresponding to minor cleavage sites (e.g. bands in lower region of Fig. 1b, without arrows) and a mixture of small fragments which could not be resolved on the urea containing gels may have arisen from contaminating RNase activity in our phosphatase preparation. As evidence we note that one such point of cleavage

occurs between G_{632} and C_{633}: a bond which should not be susceptible to attack by RNase A alone.

Of the bands resulting from major cleavage sites (large arrows, Fig. 2a), ones contained within the 5' proximal half of the stem were far more numerous than those from the 3' proximal half (about 4:1). Furthermore, due to the major point of scission by RNase A between C_{630} and U_{631}, fragments extending to the apex of the helix on the 3' side were recovered in sufficient amounts for analysis in only one experiment.

Susceptibility to cleavage by aniline

The extent of cleavage at a particular nucleotide, following treatment with 1.0 M aniline-acetate pH 4.5 for five min at 60°C, was assessed for labeled S8 specific subfragments from both untreated and previously modified 16S rRNA. In most cases sequencing reactions were performed on subfragments from treated material; in one experiment they were carried out on the unmodified RNA and in another experiment they were done on both sets of subfragments. Using DEPC and hydrazine treated 16S rRNA, we were able to identify in this manner several adenine, guanine and uracil residues between bases 583 and 649 in the 16S rRNA sequence which were either consistently unmodified (i.e., resistant to aniline attack) or consistently modified (i.e. sensitive to cleavage by aniline). Results of these experiments are summarized in Fig. 2b.

Nucleotides 580-616: susceptibility to cleavage by aniline at adenine and guanine residues. Adenine and guanine nucleotide residues indicated by solid triangles (Fig. 2b) always exhibited sensitivity to aniline when present in subfragments of S8-RNPs which had been prepared from 16S rRNA previously modified with DEPC under totally denaturing conditions (1 min, 90°C). As a specific example, one can see in Fig. 3a that there are several points of scission along the RNA chain at adenine and guanine residues between positions 580 and 616 when material previously treated with DEPC was subjected to treatment with aniline (An track, Fig. 3a). The same positions are cleaved by aniline in all four sequencing reactions since subfragments from DEPC treated material were used for the sequence analyses. In this particular case, the absence of bands at corresponding positions in untreated material was verified by performing chemical sequencing reactions on labeled subfragments from control S8 specific RNA (data not shown).

Conspicuously, however, there are several adenine and guanine residues (G_{603}, A_{599}, G_{596}, A_{595}, A_{594}, G_{591} and G_{587}) which were not cleaved by treatment with aniline (An track, Fig. 3a). The resistance to aniline attack

Figure 3. Autoradiograms of sequencing gels for selected [^{32}P]-labeled subfragments isolated as in Figure 1b which were derived from the 5' proximal half of the S8 binding site. Material was subjected to base specific chemical treatments according to Peattie (8) (tracks C, U, G and A) or to the aniline reaction alone (track An). 16S rRNA used for isolation of labeled subfragments was either untreated (control) or modified prior to complex formation with DEPC or hydrazine. Solid triangles indicate those nucleotides which were modified in the S8-RNP, whereas open triangles point to those which were unmodified. Positions of xylene cyanole (XC) and bromophenol blue (BB) tracking dyes are indicated and nucleotides are numbered according to reference 9.

of these same residues can be seen more clearly in a somewhat smaller subfragment from a different experiment (Fig. 3b), in which case susceptibility to aniline can be compared directly to that of untreated material (An track under 'Control', Fig. 3b). In general, this pattern of aniline susceptibility was absolutely reproducible from one subfragment to another and from one preparation of S8-RNP to another. Occasionally, however, certain positions exhibited some variability in their sensitivity to aniline. G_{587}, for example, was most often found to be resistant to aniline (see Fig. 3b), yet sometimes, especially in long subfragments, it was clearly sensitive to aniline induced scission. Similarly, cleavage at A_{608} varied from extensive (Fig. 3a) to slight (Fig. 3b). Consequently, these bases are indicated by a solid triangle within an open one in Fig. 2b.

Nucleotides 580-616: susceptibility to cleavage by aniline at uracil residues. In a similar fashion, using hydrazine treated 16S rRNA to form the S8-RNP, we observed that U_{604} (Fig. 3b) and U_{609} (data not shown) were consistently modified in the S8 specific RNAs, whereas uracil residues 588, 589, 590, 593, 597, and 602 were always unmodified. U_{592} displayed either sensitivity to cleavage by aniline (Fig. 3b) or, occasionally, insensitivity (data not shown) and so it is indicated by a solid triangle within an open one in Fig. 2b.

Nucleotides 620-649: susceptibility to cleavage by aniline at adenine and guanine residues. Nucleotides from subfragments located in the 3' proximal half of the S8 binding site were also examined in this way. Adenine residues 620 and 621 stand out as examples of bases that are highly susceptible to treatment with aniline (Fig. 4a, compare An 'DEPC' and An 'Control' tracks). Notably, positions of all remaining guanine and adenine nucleotides in this fragment (except possibly A_{639} and A_{691}) were not cleaved by aniline; that is, there are no bands in the An 'DEPC' track which were not in the An 'Control' track (Fig. 4a). The series of faint bands between positions 639 and 642 were not observed in other preparations of S8-RNPs (Fig. 4b and data not shown) and so the apparent susceptibility of A_{639} and A_{641} in this one case is probably not significant.

Striking resistance to cleavage by aniline at most adenine and guanine residues from the 3' proximal half of the stem was consistently reproducible (see Fig. 4a,b).

Nucleotides 631-650: susceptibility to cleavage by aniline at uracil residues. Similarly, all uracil nucleotide residues between positions 631 and 650 were observed to be unmodified in the S8-RNPs (Fig. 4b). Fragments

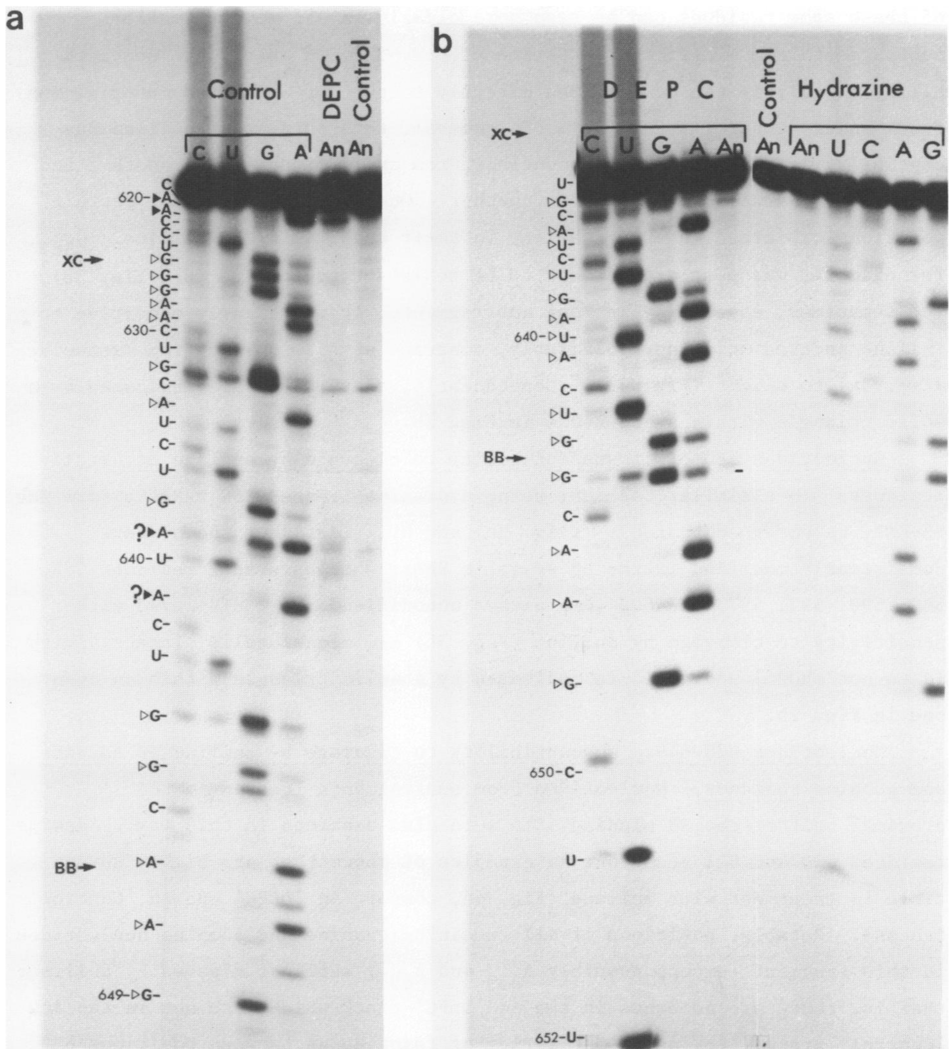

Figure 4. Autoradiograms of sequencing gels for selected [^{32}P]-labeled subfragments isolated as in Figure 1b which were derived from the 3' proximal half of the S8 binding site. Details of the figure are as in Figure 3.

extending in the 5' direction from U_{631} and in the 3' direction from C_{650} were not recovered in the two preparations of S8-RNPs made from hydrazine treated 16S rRNA. We were therefore unable to examine the extent of modification in uracil residues from these portions of the S8 helix.

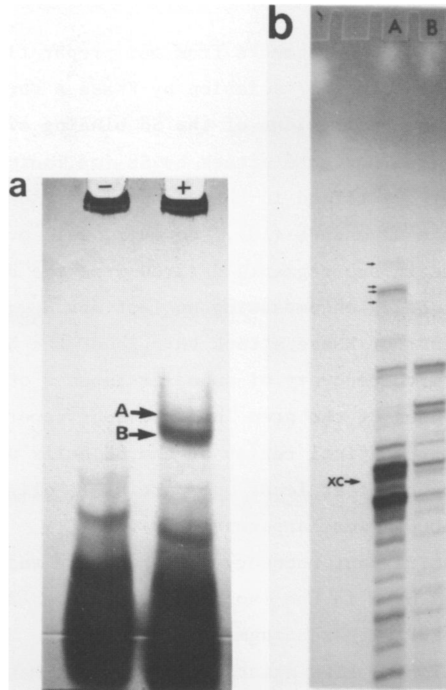

Figure 5. (a) Minor (A) and major (B) S8-RNPs were isolated as described in the legend to Figure 1. (b) [^{32}P]-labeled subfragments from the minor (A) and major (B) S8-RNAs shown in (a) were resolved on a 25% gel as described in the legend to Figure 1. Small arrows indicate the positions of subfragments unique to band A (Fig. 5a) which were eluted and sequenced.

Characterization of two different S8-RNPs

In several preparations of S8 specific RNAs, a region above the main RNP (Fig. 5a, band B) was well resolved as a discrete band (Fig. 5a, band A). The existence of separate S8-RNPs has previously been reported (2), but differences between them have yet to be characterized. To this end we eluted the RNA from bands A and B (Fig. 5a) and performed 3' labeling as usual (Fig. 5b). There were at least four labeled products that were unique to band A (arrows, Fig. 5b). After determining the sequence of these four fragments, it was evident that their 3' ends all originated from cleavage between U_{671} and A_{672}. Their 5' ends were located between C_{636} and A_{628}. The absence of cleavage by RNase A at the usual sites between positions 650 and 656 thus results in a stable, discrete S8-RNP that contains additional residues extending in the 3' direction from the base of the S8 helix to U_{671}.

DISCUSSION

Sequence analyses of subfragments from our preparations of S8 specific RNA show that the major points of scission by RNase A were entirely consistent with previous characterizations of the S8 binding sites (1-3). The extent of protection from nuclease attack by S8 for individual subfragments could not be precisely quantified in these experiments because labeling of 3' termini can be sequence dependent (10). However, well over 90% of the radioactivity was recovered in subfragments derived from the base of a highly stable, but imperfect helix encompassing nucleotides G_{587}-U_{652} (Fig. 2a). The several minor points of RNase attack throughout the helical stem in this study are consistent with recovery of submolar amounts of RNase T_1 oligonucleotides from the apex of the stem in a previous report (1). Even when oligonucleotides from the apical region of the S8 helix were entirely missing, submolar amounts of at least one partial T_1 oligonucleotide from the middle of the stem (AUCUG) were apparent (2,3).

The extent of subfragment recovery can thus vary and probably depends on the amounts of RNase A used in the isolation procedure (2). Our characterization of a minor S8-RNP with subfragments extending to U_{671} confirms that a heterogeneous population of RNPs exists following enzymatic digestion of S8-16S rRNA complexes. The extent of such heterogeneity may also involve other factors such as the concentration of Mg^{+2} ions and the temperature and duration of the incubation. It is thus evident that protection from enzymatic digestion can yield valuable information regarding the general characterization of a particular protein binding site, but is not sufficient in itself to define precisely which nucleotides are directly involved in the process of protein binding.

Other approaches such as comparative sequencing of heterologous S8 sites of attachment have been useful in defining structural features that are essential for interaction with S8. Such studies have demonstrated that several features including short stretches of primary sequence, selected base pairs, internal loops, helical stems of defined length and bulged nucleotides, are highly conserved among S8/S15 binding sites from eubacterial and archaebacterial 16S rRNAs (4).

In the present study we have identified nucleotides in 16S rRNA that are required in unmodified form for binding of protein S8 by using a damage-selection experimental approach. Following partial modification of 16S rRNA with either DEPC or hydrazine under fully denaturing conditions, the damaged RNA was used to reconstitute a S8-16S rRNA complex and, after limited

nuclease digestion, S8 specific fragments of RNA were recovered on a Mg^{+2} containing gel in a discrete S8 RNP (Fig. 1a). The RNA fragments were then subjected to treatment with aniline to determine their content of modified nucleotides (6). This approach yields highly detailed information regarding the bases that are required in intact form for binding of S8, although it does not allow one to determine exactly what role such bases play in the recognition process.

Nucleotides we determined to be unmodified in S8 bound RNA were, for the most part, located within the highly protected base of the S8 helix. In these instances the enzyme protection data agree well with the damage-selection results. It is also interesting to note that bases we have identified as being required in intact form are not always conserved among heterologous S8 binding sites (4). In such cases, interaction with S8 may involve structural aspects of the helix, which is highly conserved. Alternatively, sites along the phosphate backbone may participate, in which case the identity of the individual base may not be decisive as long as the backbone is maintained in a proper conformation.

We were also able to examine in some detail the possible involvement of bases in the apex of the S8 specific helix, because our criteria for involvement in the binding process, susceptibility to cleavage by aniline, could be applied to fragments recovered with very low yields. We thus established that G_{583}, G_{584}, G_{586}, U_{604}, G_{605}, A_{606}, A_{607}, A_{608}, U_{609}, G_{614}, G_{615}, G_{616}, A_{620} and A_{621}, which are all located outside the highly protected base of the stem, are not required in unmodified form for interaction with S8.

Surprisingly, however, the essential nature of nucleotides G_{625}-A_{629}, which are positioned outside the base of the helix, was clearly demonstrated (Fig. 4a). These bases have been reported to be present in S8 RNPs only in submolar amounts (1) or not at all (2,3). Our data suggest that they are required for specific recognition of protein S8. We speculate that the inability of S8/S15 specific 5S RNA to rebind S8 in the case of (11) may have been due to loss of this labile component during deproteinization and reisolation of their ^{14}C-labeled RNA used to test rebinding. Similarly, the absence of these nucleotides from S8/S15 RNA prepared by Muller et al. (12) may explain why their material did not rebind S8. However, the ability of ^{32}P-labeled RNA to rebind S8 in the experiments of Ungewickell et al. (2) is puzzling since bases G_{625}-A_{629} were clearly absent from the fingerprint of their S8 RNA. It should be noted that the rebinding experiments in this case were conducted on material which was not analyzed for oligonucleotide

content. Further evidence which supports our proposed involvement of bases G_{625}-A_{629} in the recognition process is the recently reported crosslink of S8 to nucleotides A_{628}-G_{632} (13). These results confirm that points of close contact with S8 are situated not only near the base of the S8 helix, but also near the apex as well.

Interestingly, mRNA for ribosomal protein L5 contains the sequence CGAAA at positions analogous to the GGAAA sequence in 16S rRNA as deduced by striking homologies in both primary and secondary structure to the S8 binding site (14). Such conservation is not only consistent with the model of autogenous regulation of ribosomal protein synthesis (14), but also constitutes further evidence that nucleotides G_{625}-A_{629} are involved in specific recognition of protein S8.

The utility of damage-selection experiments in providing detailed information on the molecular mechanisms involved in protein recognition is further illustrated by the observation that bases G_{600} and A_{601} were the only consistently modified nucleotide residues that were surrounded by ones which were never found to be modified (Fig. 2b). These results may reflect a loss of direct contact with protein S8 at this point along the RNA chain. The relevance of modified or unmodified bases in protein bound RNA to a particular role in the recognition process, however, depends on the nature of the chemical modification. Carbethoxylation of N-7 atoms in guanine and adenine residues, for example, may interfere with base pairing or stacking interactions, as well as preventing direct contact with points along the protein. Furthermore, treatment of 16S rRNA with hydrazine most certainly interrupts base pairing at modified uracil residues. Residues we have identified as being required in unmodified form may thus be necessary for maintenance of a helical structure rather than for direct contact with the protein. We cannot therefore say definitively which aspects of the protein-RNA interaction were disrupted by modifications made under our conditions, nor can we exclude the possibility that modified bases were nonetheless involved in the association process. Nevertheless, the identification of nucleotides which must be present in unmodified form remains clear.

In such experiments it is essential that all residues must be accessible to modification. Our solution conditions were therefore chosen so as to maximize disruption of secondary and tertiary base pairings. In this particular set of experiments, it was not feasible to isolate modified S8 specific RNA without using S8 in the isolation procedure, and so rigorous control experiments establishing uniform modification were not possible.

However, the possibility that residual secondary structure caused differential susceptibility to modification is unlikely for several reasons. The denaturing conditions under which our modifications were conducted, the location of unmodified residues in single-stranded regions, the presence of modified bases in double-stranded regions and the extent to which our results are in good agreement with earlier characterizations of the S8 binding site all argue against this possibility. Moreover, the several positions which were totally resistant to aniline induced scission as opposed to the many that were highly sensitive are exactly what one would expect if S8 had selected only those 16S rRNA molecules which contained unmodified residues at positions critical to complex formation.

The method of chemically modifying RNA under fully denaturing conditions prior to complex formation with proteins can thus be a powerful tool to characterize the molecular basis for protein-RNA interactions. Its application to a particular situation depends on isolating end-labeled RNA, either before or after association with protein; on reconstitution of a protein-RNA complex with partially modified RNA; on separation of bound from unbound RNA and on the identification of points of scission following treatment with aniline. Precise identification of bases that are required in intact form for complex formation obtained in this manner; taken together with other types of data on a particular protein binding site, including results of enzyme protection experiments, phylogenetically conserved nucleotides, accessibility of nucleotide residues to chemical modification under native conditions, sites of protein-RNA crosslinks and other information regarding secondary or tertiary structural features, should allow a clearer understanding of what role specific aspects of RNA structure play in the recognition process. In principal, a similar approach can be used to identify essential peptides or amino acids in a protein providing that appropriate conditions can be found to partially, but randomly, modify the protein, to reconstitute the complex with modified protein, to separate bound from unbound protein and to quantify the extent of modified residues in bound as compared to unbound protein. As a specific example one possible method of modification is iodination of the protein at tyrosine residues such that an average of one tyrosine per molecule is iodinated. Following reconstitution of the protein-RNA complex with iodinated protein and separation of bound from unbound protein, the complexes protein could be analyzed for content of $[^{125}I]$-tyrosine residues by autoradiography of tryptic peptide maps. Alternatively, reductive methylation using $[^{14}C]$-H_2CO or fluorescent labeling

with dansyl chloride may be used to partially modify the protein so that complex formation is prevented for those molecules containing modifications in sites essential for binding and so that the extent of modification in fragments of RNA bound proteins can be determined. Taken together, these types of information should allow one to establish rigid constraints that must be satisfied by any proposed molecular model describing the interaction.

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*Present address: Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, USA

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