Exploration of the L18 binding site on 5S RNA by deletion mutagenesis

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ABSTRACT

Several deletion variants of *E. coli* 5S RNA have been constructed and produced either *in vivo* or *in vitro* using T7 RNA Polymerase. Their structures and ribosomal protein L18 binding properties have been examined. All of them are similar to wild-type 5S RNA in their helix II-III regions, where L18 binds [Huber, P.W. and Wool, I.G. (1984) *Proc. Natl. Acad. Sci. (USA)* 81, 322-326; Douthwaite, S., Christensen, A., and Garrett, R.A. (1982) *Biochemistry* 21, 2313-2320.], by NMR criteria. However, none of the molecules examined that lack the helix IV-helix V stem bind L18 efficiently, even though that portion of 5S RNA is outside the L18 footprint. The L18 binding site is clearly more than a simple hairpin loop.

INTRODUCTION

Over the past decade much has been learned about DNA and DNA-protein complexes through the study of small DNA oligonucleotides, and progress in understanding RNAs would undoubtedly accelerate if they could also be studied a piece at a time. The RNA "pieces" it is natural to examine are the helical hairpin loops which make up their three-dimensional structures. Studies of this kind have already led to useful results. For example, the interaction of the coat protein of the phage R17, which controls the expression of the phage replicase, has been characterized in detail using the isolated hairpin to which the protein binds (1-4). This hairpin is, of course, much simpler than the intact R17 RNA from which it is derived.

Another hairpin that has proven worthy of study is the fragment 1 domain of the 5S RNA of *E. coli* (see Figure 1) (5). Fragment 1 was first obtained by RNase A digestion of 5S RNA, but a similar molecule, called the pDG07 RNA, was later created by site-directed mutagenesis (6). Both RNAs have structures that closely resemble that of the corresponding sequences in 5S RNA, and both bind ribosomal protein L25 the same way intact 5S RNA does (7; Gewirth, unpublished observations).

Judging from footprinting data, ribosomal protein L18 binds to the helix II-helix III hairpin loop of 5S RNA, the one that is missing from fragment 1 (8,9). Most of that hairpin can be isolated intact from limited RNase A digests of 5S RNA (10). The molecule obtained, which is called fragment 2, contains about 90% of the L18 footprint (see Figure 1). Since fragment 2's conformation resembles that of the corresponding sequences in 5S RNA, it was a surprise to discover a few years ago that fragment 2 does not bind L18 (10).

Several reasons could be advanced to explain fragment 2's failure to bind L18. It lacks the terminal loop of the stem from which it is derived, as well as bases 66-69 of the native sequence, both of which may be important for L18 binding (11,12). It seemed reasonable to suppose, therefore, that an RNA including somewhat more of the 5S

sequence than fragment 2 might bind L18, and thereby constitute a system in which the L18 interaction could be studied.

As a test of this hypothesis, several deletion variants of 5S RNA have been constructed, and their structures and protein binding properties examined. All of them are similar to wild-type 5S RNA in their helix II-III regions by NMR criteria. However, none of the molecules examined that lack the helix IV-helix V stem bind L18 efficiently, even though that portion of 5S RNA is outside the L18 footprint. Our conclusion is that L18 binding must depend on aspects of the structure of 5S RNA that are not represented by footprinting experiments. In contrast to the L25 binding site on 5S RNA, or the coat protein binding site on R17 RNA, L18's binding site is not a simple hairpin loop.

MATERIALS AND METHODS

Strains and Plasmids.

E. coli strains HB101, JM101, and CJ236 were used in these experiments (see 6). Strain BL21 carrying plasmid pAR1219 was the gift of Dr. William Studier of Brookhaven National Laboratory. M13mp18 was purchased from BRL, pUC19 was purchased from IBI, and pKK5-1 was provided by Drs. Harry Noller and Jurgen Brosius (13).

5S RNA and Ribosomal Proteins.

The 5S RNA used in these experiments was the product of the *rrnB* gene on pKK5-1. It was produced and purified as described previously (14). The same method was used to prepare the 5S-like RNAs produced from 5S mutant plasmids *in vivo*. Ribosomal protein bL18 from *Bacillus stearothermophilus* was purified from 70S ribosomes by chromatography on carboxymethyl cellulose and by HPLC on SP5W columns in urea-containing buffers (15). Ribosomal protein L25 from *E. coli* was prepared as described elsewhere (7).

Enzymes and Reagents.

Restriction endonucleases *Hind*III, *Eco*RV, and *Pvu*II were purchased from Promega Biotec, and *Bst*NI was purchased from New England Biolabs, Inc.. T7 RNA Polymerase was purified from *E. coli* strain BL21/pAR1219 according to the protocol provided by Drs. Dunn and Studier (16). Sodium or lithium salts of ribonucleotide triphosphates were purchased from Pharmacia. 100 mM solutions of each triphosphate were prepared by dissolving the dry powder in 50 mM Tris, pH 8 and adjusting the pH of the solution to 8.0 with 1 M Tris base. Concentrations of nucleotides were measured spectrophotometrically using the extinction coefficients supplied by the manufacturer. Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer by Mrs. Grace Sun, for whose help we are grateful. Oligonucleotides were purified by HPLC as described earlier (6).

Construction of Mutant Plasmids.

The methods used to construct and select mutant plasmids have been described previously (6). The 0.5 kb *Hind*III fragment containing the site-directed mutation was excised from M13 and cloned into either the 5.6 kb *Hind*III fragment from pKK5-1 (pKK vector) or the *Hind*III site of pUC19.

Large Scale Preparation of Plasmid DNA.

HB101 strains containing plasmids with 5S RNA genes cloned into the pUC19 vector were grown to saturation in YT broth containing (per liter) 8 g Bacto Tryptone, 5 g NaCl, 5 g Bacto Yeast, and 100 µg/mL

Figure 1: The primary and secondary structure of *E. coli* rrnB 5S RNA (a) and its ribonuclease-resistant fragments (b,c). Helix designations are given as Roman numerals. The extra bases on the ends of the RNA are due to incomplete processing of the plasmid-derived product (18). The binding sites for ribosomal proteins L18 and L25 as deduced from footprinting and chemical modification experiments are shaded. The L18 site is according to Huber and Wool (8), and the L25 site according to Douthwaite et al. (9).

ampicillin. The cells were collected by centrifugation and lysed by a modification of the standard alkaline lysis miniprep procedure (17). Crude plasmid DNA was recovered by ethanol precipitation in the presence of 0.3 M NaAc and purified on a 2.5 x 80 cm column of Sephacryl S-1000 running in 0.1 M NaCl, 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM Na₃N.

Large Scale Transcription *in Vitro*.

Plasmid DNA containing the T7 RNA Polymerase promoter sequence and the RNA sequence of interest was digested with either *Bst*NI or *Eco*RV + *Pvu*II to generate the appropriate 3' end for runoff transcription. The digestions were stopped by extraction with phenol/chloroform, and the DNA fragments recovered by ethanol precipitation.

Large scale transcriptions were carried out in a 10 mL solution that contained 0.1 mg/mL linear template DNA, 5 mM each of GTP, ATP, UTP, and CTP, 45 mM MgCl₂, 5 mM DTT, 0.005% Triton X-100, 40 mM Tris-HCl, pH 8.1, 2 mM spermidine, and 0.1 mg/mL T7 RNA Polymerase. The reaction was normally carried out at 37 °C, although for some transcripts it was found that fewer truncated products were obtained if the reaction was performed at room temperature. After 2-4 h the transcription reaction was stopped by extraction with

phenol/chloroform. The magnesium pyrophosphate produced during the transcription was removed by dialysis against distilled water for 4-6 hours, and the RNA was recovered by ethanol precipitation. The full length transcript was purified by chromatography on Sephadex G75 in a buffer containing 0.1 M NaCl, 10 mM cacodylate, pH 6.0, 5 mM MgCl₂, at room temperature. Some of the transcripts were purified further on a Sephadex G50 column in a buffer containing 8 M urea, 0.1 M NaCl, 10 mM cacodylate pH 6.0 at 60 °C. The RNA was dissolved in the urea buffer and heated to 80 °C for 5 minutes prior to loading on the column to insure complete denaturation. RNAs recovered from the denaturing column were dialyzed against 0.1 M NaCl, 5 mM MgCl₂, 10 mM cacodylate, pH 6.0 at 4 °C to remove the urea, and concentrated by ethanol precipitation.

Gel Electrophoresis and NMR.

RNA samples were assayed for purity and protein binding activity by the gel electrophoretic methods described earlier (14,18). NMR spectra were obtained as described earlier (21, 6).

RESULTS

Production of 5S RNA Deletion Mutants *in Vivo*.

Plasmids pDG06 and pDG09, which code for deletion mutants of 5S RNA, are derivatives of pKK5-1 that can be expressed *in vivo*. The 5S gene on pDG09 lacks sequences coding for bases 35-46 in wild type RNA, and its product could be overproduced in good yield (4mg/gram of cells) (see Materials and Methods). pDG09 RNA runs faster than native 5S RNA on denaturing gels because its molecular weight is lower. Therefore, it was easy to show that more than 90% of the 5S-like RNA obtained from the postribosomal supernatant of HB101/pDG09 cells, under over-producing conditions, was mutant RNA (Figure 2). In cells grown to mid-log phase in the absence of chloramphenicol, chromosomal 5S RNA and plasmid 5S RNAs are produced in roughly equal amounts (6). The ribosomal 5S RNA from cells carrying pDG09 contained no detectable mutant RNA, and the predominant 5S-like RNA in the supernatant was the mutant (Figure 2).

5S RNAs produced from pKK5-1 and its wild-type derivatives are incorporated into ribosomes (6). Therefore, the failure of the pDG09 RNA to appear in ribosomes proves that it is a significantly less efficient participant in the ribosome assembly process than the wild-type 5S RNA. It is interesting that HB101/pKK5-1, which produces excess amounts of wild-type 5S RNA, has a growth rate indistinguishable from that of HB101/pDG09, which produces an apparently useless mutant RNA in equivalent amounts.

Because the L18 footprint does not include any bases in helices IV and V, 5S RNA derivatives lacking these sequences are attractive as candidates for studying the interaction of L18 with 5S RNA. We attempted to produce such a deletion product by the same method that had worked for the pDG09 and pDG07 RNAs (6). Unfortunately, cells carrying plasmid pDG06, a plasmid which codes for a 5S-like RNA lacking bases 70-105, do not accumulate this RNA in their cytoplasm in detectable amounts under overproducing conditions.

Enzymatic Synthesis of RNA *in vitro*.

Because we failed to obtain an RNA lacking helices IV and V *in vivo*, we turned to the T7 RNA polymerase method for making RNAs *in vitro*. T7 RNA polymerase requires a template containing a T7 promoter sequence fused to the start of the gene of interest, followed by a restriction enzyme site which can be cut to define the 3' end of

Figure 2: pDG09 5S RNA ($\Delta(36-47)$) production and ribosome incorporation test. The gel contained 8% acrylamide, 8M urea, and 0.5X TBE. From the left, the first lane is overproduced 5S RNA from HB101/pKK5-1. The second lane is a similar preparation from HB101/pDG09. The third lane is 5S RNA obtained from ribosomes of HB101/pDG05 (another wild-type 5S gene; see 6) that were growing in logarithmic phase. The fourth lane is the 5S RNA obtained from ribosomes of HB101/pDG09 that were growing in log phase. The last lane is the 5S RNA obtained from the post-ribosomal supernatant of HB101/pDG09 cells that were growing in logarithmic phase.

the transcript the template will encode (22). These templates were constructed by site-directed mutagenesis of the 5S RNA gene in pKK5-1.

Two plasmids were produced that give near wild-type 5S products, pDG15, and pDG18. The two differ in the position of their T7 promoters with respect to the 5' end of the 5S gene. In addition, pDG15 has an *EcoRV* restriction site installed near its 3' end whereas pDG18's 3' runoff terminus is determined by a *Bst*VI site. Both were used as parents for a series of deletion mutants. The mutants derived from pDG15 are: pDG19 ($\Delta(70-105)$), pDG17 ($\Delta(78-97)$), and pJK1 ($\Delta(10-69)$, C/U88, U11, U12, C13). [Plasmid pJK1 was constructed by Joseph Kim.] The mutants derived from the pDG18 construct are: pDG21 ($\Delta(70-105)$), pDG21-G109 (= pDG21, A/G109), pDG22 (= pDG21, G/U69, G/A106, G/C107, A/U108, A/G109), and pDG29 (= pDG22, $\Delta(36-45)$). The sequences of these RNA products are presented in Figure 3. Note that the ends of all of these molecules differ from those of wild-type rrmB 5S RNA.

RNA was transcribed from plasmid DNA that had been linearized by restriction endonuclease digestion as described in Materials and Methods. 10 mL transcription reactions produced 7 to 15 mg of homogeneous RNA after purification. This represents a yield of between 500 to 1200 moles of full-length transcript per mole of DNA template, similar what Lowary et al. (22) have reported.

On the quality of T7 RNA polymerase products.

It is important to know whether RNAs made *in vitro* are equivalent to molecules of similar sequence extracted from living cells. For this reason, several comparisons were made between pJK1 RNA, which is a T7 product, and pDG07 RNA, which is an *in vivo* product that has been characterized previously (6). The two have sequences that are the same except at their 5' and 3' termini.

Native gels were used to compare the mobilities of these molecules, and to test their capacities for binding ribosomal protein L25 (Figure 4). pJK1 RNA had nearly the same mobility as pDG07 RNA which was synthesized *in vivo*, and, qualitatively, it binds L25 normally. pJK1 RNA gives a wider band than pDG07 RNA on native gels, but on denaturing gels the *in vivo* and *in vitro* RNAs gave bands of similar sharpness. This shows that length heterogeneity is not the cause of the difference in band width on native gels (see 22). To reduce possible conformational heterogeneities, transcripts were repurified in urea and then dialyzed into native buffers to allow refolding. Alternatively, samples were heated and slow cooled to allow them to reanneal. Neither procedure had any

Figure 3: RNAs transcribed *in vitro* using T7 RNA polymerase. The RNAs which were derived from the genes for the pDG15 or pDG18 5S variants are shown under those structures. [Note that 5S RNA is drawn in a different standard arrangement than in Figure 1 to emphasize a relationship between helices I, II, and III, but the basepairing is identical in both figures.]

effect on band morphology, suggesting that internal sequence heterogeneity might be the cause of the wide bands seen on native gels.

The downfield proton NMR spectra of the two RNAs were also compared. Downfield nucleic acid spectra detect resonances due to base imino protons that are protected from solvent exchange, usually due to hydrogen bonding (23). These spectra are thus sensitive to nucleic acid secondary structure. The pJK1 and pDG07 RNAs have spectra that differ in two respects, one of them trivial, and the other important (Figure 5). The trivial difference has to do with the the chemical shifts of resonances J, C, F, B, M, and E. These resonances originate in the terminal helix of the molecule, helix I (6). The chemical shift differences of these resonances in the spectra of the two molecules reflect their differences in 5' and 3' end sequences.

The non-trivial difference concerns the widths of resonances. Comparison of resonance Z1 in the two spectra, for example, shows that the *in vivo* RNA has a linewidth of 24 Hz, compared to 39 Hz for the *in vitro* RNA, and similar differences occur all the way across the spectrum. Since native gel analysis did not indicate the presence of dimers in the T7 transcript population, this abnormal linewidth must be connected either with the structure of the 3' and 5' ends of the molecule, or to sequence and/or conformational heterogeneities. Nevertheless, the spectrum of pJK1 RNA is interpretable and obviously analogous to that of pDG07 RNA which it closely resembles in sequence. Our conclusion is that the T7 RNA polymerase system, in our hands, can produce RNA hairpins of 70 bases or so that are faithful enough to their DNA template sequences to be useful, but which are not perfect.

Spectroscopy of Deletion Mutants of 5S RNA.

An obvious question to ask about these 5S deletion RNAs is whether their conformations are related to that of

Figure 4: Native gel comparison between pJK1 RNA and pDG07 fragment RNA. The gel contained 10% acrylamide and the running buffer consisted of 0.1 M KCl, 5 mM MgCl₂, 50 mM Tris-borate, pH 7.8. Gels were run at 3.5 V/cm for 16 h. RNA-protein complexes were made 1:2 RNA:protein. The gels were stained for RNA with methylene blue.

the same sequences in native, wild-type 5S RNA. Since these RNAs lack a large, internal, portion of the parent molecule, one would not necessarily expect these molecules to adopt a structure analogous to that of their parent molecule. NMR spectroscopy was used to provide evidence on this point.

The downfield proton NMR spectra of the pDG09 5S RNA $\Delta(36-47)$, wild-type 5S RNA, and their difference are shown in Figure 6. Except for a small number of discrete differences they are quite similar, which strongly supports the view that the structure of pDG09 RNA is very close to that of the parent molecule. The differences involve resonances at 14.15 and 13.45 ppm which are present in the wild-type spectrum but missing from the pDG09 spectrum, and wild-type resonances at 13.15 and 12.40 ppm are reduced in intensity in the pDG09 spectrum as well.

Although none of these resonances have been positively assigned in the wild-type molecule, it is likely that they arise from the lower half of the helix III region (see below). When C36 through C47 are deleted, energy calculations suggest that a new 11 base loop comprised of bases 31-35 and 48-53 is likely to take its place (24). As a result, base pairs U48-A34, C49-G33, A50-U32, and G49-C31 should be lost. The imino protons protected by virtue of their involvement in these base pairs could be responsible for the resonances missing in the pDG09 5S spectrum. Consistent with this hypothesis, we note that 14.15 and 13.45 ppm are typical AU chemical shifts, and 13.15 and 12.40 ppm are typical GC chemical shifts. Furthermore, the spectra of RNAs that contain only the helix II-III stem have resonances near 14.15 and 13.45 ppm that can be shown to represent AU base pairs (see below). The resonances at 13.15 and 12.40 ppm are not AUs.

The 5S RNA deletion mutants which lack helices IV and V were also examined spectroscopically. The downfield proton NMR spectra of the pDG29, pDG22, pDG21-G109, and pDG19 RNAs, synthesized using T7 RNA polymerase, are shown in Figure 7. Also shown for comparison are the spectra of wild-type 5S RNA and fragment 1. [Fragment 1 is shown to identify the resonances in the whole 5S spectrum that belong to helices I, IV, and V.]

As drawn in Figure 1, there could be at most 18 imino proton resonances in 5S RNA originating outside of its fragment 1 domain. Two of these imino protons should come from the G18-U65 basepair adjacent to the bulged

Figure 5: Downfield spectra of pJK1 RNA and the pDG07 fragment. RNA samples were dialyzed into the standard buffer, containing 0.1 M KCl, 4 mM MgCl₂, 5 mM cacodylate, pH 7.2, and the spectra obtained at 303 K. Spectrum a is that of the pJK1 RNA, shown unenhanced. The concentration of RNA in this sample was around 0.5 mM. Spectrum b is that of the pDG07 fragment, also shown unenhanced. The concentration of RNA in this sample was around 1.5 mM. Spectra c and d are the pJK1 and pDG07 fragment, respectively, shown resolution enhanced. The spectra are lettered according to the convention established earlier (6).

A66. They are known to be unobservable by NMR (P. Zhang & P.B. Moore, unpublished observations), leaving 16 resonances expected for the helix II-III region of the molecule. This number agrees well with the 15 imino proton resonances revealed when the spectrum of fragment 1 is subtracted from that of intact 5S RNA (7).

At least nine resonances in the downfield spectra of the 5S deletion mutant RNAs have chemical shifts similar to non-fragment 1 resonances in the spectrum of intact 5S RNA. These resonances are indicated by vertical lines in Figure 7. They correspond to resonances a, b, e, h, j, l, m, and n in the nomenclature of Kime & Moore (7). The remaining 7 non-fragment 1 resonances may also be present, but they cannot be identified with certainty since they are expected to overlap with helix I resonances E, H, P2, R2, and T (which have been identified in these spectra), as well as (possibly) resonances due to the 2 GC and 3 AU resonances expected outside of the helix II-helix III region in pDG29 RNA.

Figure 6: Downfield proton NMR spectra of the pDG09 5S RNA (a), rrnB 5S RNA (b), and their difference [(c) = (a) - (b)]. The RNA in these samples was dialyzed into the standard buffer containing 0.1 M KCl, 4 mM MgCl₂, 5 mM cacodylate, pH 7.2. The concentration of RNA in these samples was around 1 mM. The spectra were obtained at 303 K. Differencing was done at the spectrum level. The chemical shifts of the negative features in the difference spectrum are indicated.

Of the eight resonances in the deletion mutant RNAs which correspond in chemical shift to non-fragment 1 resonances in 5S RNA, three can be shown to arise from helices II or III by independent evidence. The first of these is resonance a, which is sensitive to the deletion of the 36-47 loop in the pDG09 construct. Comparison of the spectra of the pDG22 and pDG29 RNAs shows that resonance a broadens in response to deletions in the loop at the end of helix III. The resonance we call "a" in the spectra of the deletion mutant RNAs is likely to be the same imino proton as resonance a in the spectrum of wild-type 5S RNA. This resonance gives the NOEs expected of an AU base pair (see below); it probably represents A34-U48.

NOE experiments were performed on some of the deletion mutant RNAs in the hope of identifying some long helical progressions characteristic of helices II and III. None were found. The NOE experiments did, however, provide evidence for the existence of the GU basepair in helix II. As seen in Figure 8a, resonance m in the pDG29 RNA gives a NOE to resonance l, the strength of which suggests that these two resonances represent a GU base pair. A weaker NOE also appears at 13.3 ppm, a resonance which represents a GC base pair. The same connectivities

Figure 7: Downfield proton NMR spectra of the pDG29 RNA (a), pDG22 RNA (b), pDG21-G109 RNA (c), pDG19 RNA (d), rrnB 5S RNA (e), and fragment 1 (f), taken in 0.1 M KCl, 4 mM MgCl₂, 5 mM cacodylate, pH 7.2, at 303 K. The concentration of RNA in these samples was between 1 and 2 mM. The spectra are shown resolution enhanced. The vertical lines indicate resonances unique to intact 5S RNA which have similar chemical shifts; see the text for further details.

involving resonances having identical chemical shifts are seen in intact 5S RNA (7), and fragment 2 (10). Since G18-U65 in helix II is NMR-silent due to the bulged A that adjoins it (P. Zhang and P.B. Moore, unpublished results), resonances l and m probably represent U22-G61 in all of these molecules. The structure of the helix II regions of the deletion mutant RNAs thus resemble that of intact 5S RNA.

Most of the AU basepairs were identified in the spectrum of the pDG29 RNA by NOE experiments. The results of a two-dimensional NOESY are shown in Figure 8b. Displayed in this figure are the cross peaks arising

Figure 8: Selected NOEs in the pDG29 and pDG22 RNAs. Panel a shows NOEs arising from resonance m in the pDG29 RNA. The RNA was dissolved in the standard buffer described in the legend to Figure 7. The top spectrum is the reference spectrum, the bottom one is the NOE difference spectrum. Panel b shows the imino to aromatic NOEs in the pDG29 RNA. The mixing time for this spectrum was 200 ms and the sample concentration was 4.5 mM. The sample was dissolved in 0.1 M KCl, 0.3 mM MgCl₂, 5 mM MES, pH 6.0. Otherwise, the conditions under which this spectrum was acquired and processed are as described in (6). The chemical shifts of the cross peaks are indicated. (The crosspeak at 7.02/13.46 ppm is very broad and probably represents an imino to amino NOE.) Panel c shows the NOEs arising from resonance a in the pDG22 RNA. The top spectrum is the reference spectrum, taken in the standard buffer as in (a) above. The bottom spectrum is the NOE difference spectrum obtained when resonance a is presaturated.

from NOEs between imino and aromatic proton resonances. Three strong and sharp cross peaks can be seen connecting resonances at 13.42 ppm with 7.02 ppm, 13.39 ppm with 6.96 ppm, and 13.25 ppm with 6.73 ppm. Two weaker imino-to-aromatic cross peaks are seen between resonances at 13.28 ppm and 6.73 ppm, and 13.22 ppm and 6.69 ppm. Neither one-dimensional nor two-dimensional NOE experiments revealed an aromatic correlate

Figure 9: Native gel analysis of mutant RNAs prepared *in vivo* and *in vitro*. The gels all contained 10% acrylamide and were run as described in the legend to Figure 4. The RNA in each experiment is indicated, as is the addition of ribosomal protein bL18 to the RNA before electrophoresis. A protein to RNA ratio of 2 or 3 to 1 was used in the (+) protein lanes. The gels were stained for RNA with methylene blue.

for resonance a in the pDG29 spectrum, as expected. As Figure 8c shows, however, in molecules like the pDG22 RNA where the loop at the end of helix III is full length, resonance a gives a one-dimensional NOE to an aromatic resonance at 7.68 ppm, indicative of an AU base pair. Thus, six AU resonances have been identified in the pDG29 and pDG22 RNAs, three of which are strong and three of which are weak or variable. Three AUs are expected in the secondary structures of both molecules due to base pairs outside helices II and III, leaving three to be accounted for by sequences within helices II and III. Examination of the proposed secondary structure for this region shows that helix III is expected to contribute three or four AU resonances to the NMR spectrum, depending on whether or not the U25-A57 base pair is stable enough to be NMR-visible. Thus, the correlation between the number of AU resonances expected and the number observed suggests again that a helix III-like secondary structure (Figure 3) does exist in the deletion RNAs.

Comparison of the bL18 binding properties of the 5S RNA deletion mutants.

All of the mutant RNAs constructed in this series of experiments contain virtually the entire L18 binding site, as determined by ribonuclease protection and chemical modification experiments (8,9). The ability of each of them to bind L18 was assayed qualitatively by electrophoresis on non-denaturing polyacrylamide gels with the result shown in Figure 9. [Ribosomal protein bL18 from *B. stearothermophilus* was used in place of the *E. coli* protein because it has better solubility properties. Its sequence is 53% identical to its *E. coli* homologue (25).] The pDG09, pDG15, and pDG18 RNAs bind protein bL18, leaving behind no residual unbound RNA, and form discrete complex bands on gels. These RNAs are thus similar to wild-type 5S RNA. The electrophoretic mobilities of pDG17 and pDG19 RNAs are also affected by bL18. No residual unbound RNA is left behind when these RNAs are challenged with an excess of protein, but discrete complex bands are not observed. The pDG21, pDG21-G109, pDG22, and pDG29 RNAs, on the other hand, are only partially affected by bL18. These RNAs leave behind a significant amount of uncomplexed RNA, and do not form discrete complex bands on the gel. It is interesting to note that this last set of RNAs all give rise to multiple bands on native gels, clearly indicating gross conformational heterogeneity. Repurification and annealing did not alter their apparent complexity on native gels. Note also that

L18 appears to discriminate between these different conformational species. For example, the fastest mobility band in the free pDG21 RNA lane disappears preferentially when L18 is added to the RNA.

Quantitative analysis of binding by gel competition techniques demonstrated that the "full-length" 5S transcripts bind bL18 10-20 fold weaker than wild-type 5S RNA. pDG09 RNA binds about 100 fold weaker, and competition between 5S RNA and any of the helix IV-V deletion RNAs was too weak to measure, which for our system implies an association constant 360-fold less than the wild-type RNA, or weaker (15, data not shown).

DISCUSSION

Many of the mutant RNAs examined in this study were synthesized using T7 RNA Polymerase. Although we believe they were adequate for these studies, the fact remains that our T7 transcripts were not as "good" as their natural counterparts. Both electrophoretically, and spectroscopically they do not appear as homogeneous as they should, an observation which raises questions about the uses to which such molecules may be properly put.

Downfield NMR spectra are sensitive to sequence variation. Single base changes in helical structures that preserve secondary structure can affect the chemical shifts of 3 imino proton resonances on either side of mutation, as well as the chemical shift of the mutated base pair itself (6). Changes that alter secondary or tertiary structure could have even more widespread spectral effects (15). Thus modest levels of sequence variation in an RNA population could be responsible for the spectroscopic and electrophoretic "abnormalities" these molecules display.

If the enzyme's error rate per base is independent of chain length, the spectral quality of transcripts of short chain length should be better than that of long chain length transcripts because the population will contain a greater percentage of perfect molecules. We have examined 5S-like transcripts of nearly normal length, i.e. about 120 bases, (15), as well as some transcripts of about 30 bases in length (P. Zhang, unpublished results). There does seem to be a correlation between chain length and the quality of the downfield spectrum: the longer the transcript, the worse its downfield spectrum is compared to that of a comparable natural RNA.

A second possibility is that the terminal sequences of our T7 transcripts are either interfering with folding, or causing intermolecular aggregation, either of which could have the spectral consequences observed. However, two T7 versions of full length 5S RNAs have been examined which differ only in terminal sequence (15). Their spectra are similar (15).

Unlike the T7-transcribed RNAs, the one mutant RNA produced *in vivo* for this study -- the pDG09 variant of 5S RNA -- behaved as expected. Four basepairs at the end of helix III should have been weak or absent in this RNA, and we found that two resonances had disappeared and two more were weakened in its downfield spectrum, which otherwise is identical to that of wild-type 5S RNA. Earlier Pace et al. (26) showed that 5S RNA molecules lacking bases 42-46 or 42-52 can be reconstituted into ribosomes *in vitro*, and that ribosomes carrying these truncated 5S RNAs are active in protein synthesis. It would be interesting to find out whether pDG09 RNA can function the same way in reconstitution. It is clear that cells having normal 5S cistrons will not perform the experiment for us *in vivo*.

The region of 5S RNA removed or perturbed by the deletion of bases 36-47 is at one end of the region identified as the L18 binding site by Huber and Wool (8), but just outside the main L18-protected region identified by Douthwaite et al. (9). The effect of this deletion on the L18 binding constant is about 15 times greater than is

observed when A66 is deleted (12), and about 100 times greater than found when A66 is replaced with a C (27). The loop beyond helix III clearly makes an important contribution to the L18 interaction.

A tertiary base pairing interaction between bases 41-44 in the loop at the end of helix III and bases 74-77 in helix V has been proposed by Pieler & Erdmann (28), and evidence for another, competing interaction involving bases 35-37 and bases 105-107 at the top of helix V has been claimed by Goring & Wagner (29). Neither can occur in pDG9 RNA because it lacks bases 36-47. Nevertheless, the differences between the downfield spectrum of 5S RNA and pDG9 RNA are small, and all involve resonances assignable to the distal portion of helix III. Furthermore, many of the bases in helix V alleged to be involved in these interactions give resonances that occur at identical chemical shifts in both intact 5S RNA and fragment 1, in which tertiary interactions of the kinds postulated are impossible (7, 21). If the proposed tertiary interactions exist in 5S RNA in solution at all, they must be NMR-silent.

Most of the helix IV-V deletion mutants we made exhibit multiple bands on non-denaturing polyacrylamide gels. These patterns proved resistant to melting and reannealing, suggesting that these RNAs can exist in several conformational states. The order of complexity of the band patterns of these RNAs on native gels is: complex, pDG21 and pDG21-G109; intermediate, pDG22 RNA and pDG29 RNA; single band, pJK1, pDG17, and pDG19 RNAs. Helix stability calculations done on these sequences demonstrated that the RNAs which were the least heterogeneous on native gels had the lowest number of significantly different conformations available to them within 3 kT of the lowest energy conformation (J. Jaeger and D. Turner, personal communication). Those RNAs which were most heterogeneous on native gels had the highest number of significantly different conformations available to them within 3 kT of the lowest energy conformation. In all cases, the energy calculations suggest that the most probable secondary structure for the RNAs from the deletion mutants examined is 5S-like in the helix II-III region. Our NMR evidence supports this conclusion. The chemical shift correspondances for the resonances originating in the helix II-III regions of these RNAs are obvious. Furthermore, some of the resonances clearly assign to analogous base pairs.

The fact that L18 interacts so poorly with RNAs lacking helices IV and V contradicts the hypothesis on which this work was based, namely that molecules only slightly more complex than fragment 2 would bind L18. By footprinting criteria, almost the entire the L18 binding site is contained within the sequences of these constructs, and their structures are largely 5S-like, as hoped. They bind L18 well enough to detect the existence of a weak interaction on gels, which is better than can be said for fragment 2, but still so weakly as to make them useless as a low molecular weight RNA for studying the L18 interaction with 5S RNA. It is difficult to escape the conclusion, then, that L18 is sensitive to aspects of 5S structure that cannot be modeled using a simple hairpin loop. The contrast between L18's binding requirements and L25's in this respect is striking. In light of this conclusion, it is interesting that there is evidence for L18-stimulated conformational alterations in the helix IV-V hairpin, and also for L18 protections in helix I (9,30).

A logical candidate for the structural element L18 requires is the region of the molecule where helices I, II, and V join, which is adjacent to the L18 footprint region on helices II and III. If this region is indeed recognized by L18, then it is not surprising that none of our deletion mutants binds L18 very well. None of them is normal at that junction. In support of this suggestion, we note that the molecules we made which are least constrained in the

junction region, e.g. the pDG19 and pDG17 RNAs, seem to bind L18 better than those molecules which are basepaired throughout, as judged by the native gel data.

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