Volume 17 Number 21 1989 Nucleic Acids Research

Physical studies of 5S RNA variants at position 66

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Received May 15, 1989; Revised and Accepted September 15, 1989

ABSTRACT
Two variants of the 5S RNA of *E. coli* have been examined by imino proton NMR spectroscopy, one of them a deletion of A66 (Christiansen, J., Douthwaite, S.R., Christensen, A. and Garrett, R.A. (1985) EMBO J. **4**, 1019–1024) and the other a replacement of A66 with a C (Goringer, H.U. and Wagner, R. (1986) Biol. Chem. Hoppe-Seyler **367**, 769–780). Both are of interest because the role the bulged A in helix II of 5S RNA is supposed to play in interactions with ribosomal protein L18. The data show that the structural perturbations that result from these mutations are minimal, and assign the resonances of some of the imino protons around position 66. Some mutations at or near position 66 greatly reduce the L18-dependent increase in the circular dichroism of 5S RNA at 267 nm first observed by Bear and coworkers (Bear, D.G., Schleich, T., Noller, H.F. and Garrett, R.A. (1977) Nucl. Acids Res. **4**, 2511–2526).

INTRODUCTION
Many prokaryotes contain a homologue of *E. coli*'s ribosomal protein Ll8 that binds to 5S RNA in the helix II-helix III region (bases 16–69). In all 5S RNAs Helix II has an unpaired base two base pairs in from its proximal end, and in *E. coli* that base is A66 (Figure 1) (1). A66 is unusually reactive, and when its hydrogen bonding groups are derivatized, the Ll8-binding constant of 5S RNA is reduced (2). Since the binding sites for several other ribosomal proteins include stems with similar bulged A's, it has been suggested that they are important in RNA-protein interactions (3).

Two groups have tested the role of A66 by site-directed mutagenesis. Christiansen and coworkers studied a 5S RNA from which A66 was been deleted, 'des-A66 RNA', and Wagner's group made a 5S RNA in which A66 is changed to a C, 'C66 RNA' (4, 5, 6). Deletion of A66 reduces the Ll8 binding constant about 7-fold, while its replacement with a C has a negligible effect. *E. coli* grows at nearly the same rate when infected with plasmids producing either mutant 5S RNA constitutively as it does when infected with similar plasmids carrying the wild type gene.

The investigation of des-A66 RNA and C66 RNA by imino proton NMR spectroscopy, whose results are reported below, was undertaken for two reasons. First, in order to assess the significance of the binding studies done with these mutants it would be useful to have some information about the effects they have on the structure of 5S RNA. Second, very little spectroscopic information exists about the proximal end of helix II in 5S RNA because it is not part of any of the large nucleolytic fragments that have proven so important in NMR studies of 5S RNA (7, 8). It appeared likely that examination of the spectral changes brought about by mutation at position 66 might yield insights into the structure of that part of the molecule (see 9).

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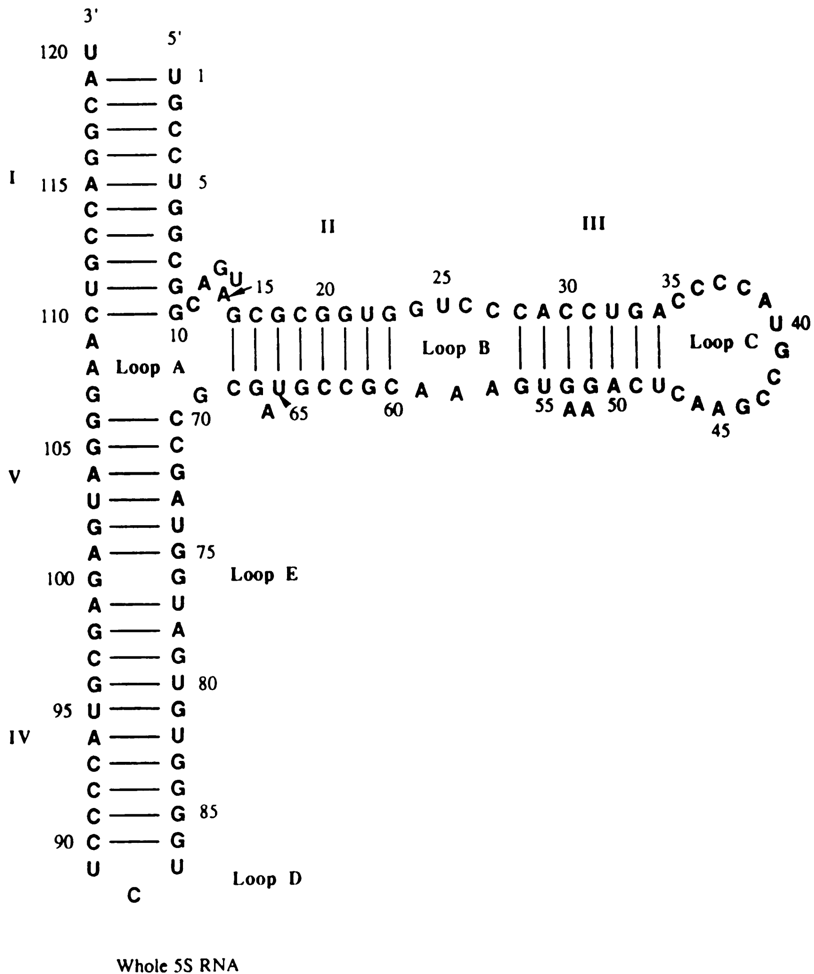


Figure 1. 5S RNA from *E. coli*. The sequence of 5S RNA from *E. coli* is displayed in the generally accepted, 3 stem secondary structure (see 1).

The spectroscopic changes induced by the deletion of A66 are surprisingly modest, suggesting that the structure of 5S RNA is perturbed very little by this deletion. Replacement of A66 with a C, on the other hand, appears to induce a new base pairing pattern around position 66 in which both the position and the type of the bulged base changes. Thus the structural alteration brought about by the mutation that has the lesser effect on L18 binding is larger than the alteration that results from the mutation that has the larger effect on binding. In addition, the data identify some new resonances in helix II, as expected.

In the course of these investigations we attempted to compare the Ll8 binding constants of these 5S mutants with those of wild type 5S RNA using circular dichroism (CD) changes as the assay (10). We verified that 5S RNA and C66 RNA have similar binding constants (6), but to our surprise, found that the L18 binding constant for des-A66 RNA could not

be measured by this technique because little or no CD change could be observed. [Egebjerg and coworkers have recently reported the same finding (11).] The helix junction region of 5S RNA must play a role in the structural alteration that causes the L18-induced CD change.

MATERIALS AND METHODS

Plasmids.

Plasmids pCD701 and pCD702 were given to us by Drs. Jan Christiansen and Roger Garrett. pCD701 contains the 3′ end of the rrnB cistron, including a portion of the 23S RNA gene, the 5S gene, and the associated terminators, positioned downstream of normal ribosomal promoters. pCD702 is identical except for the deletion of A66 in the 5S gene (4). pC66 is a derivative of pKK3535; it carries a complete rrnB cistron whose 5S RNA has been mutated at position 66 (6). Wild type 5S RNA was produced using the overproducing plasmid pKK5-1, which is similar to pCD701 (12).

5S RNAs.

5S RNAs were overproduced in *E. coli* HB101 cells containing plasmids following protocols described elsewhere (13). The yield of des-A66 RNA per weight of HB101/pCD702 was comparable to that of 5S RNA from HB101/pKK5-1, but the yield of C66 RNA from HB101/pC66 was less than half. The isolation and purification of plasmid-produced 5S RNAs was carried out as described earlier (13). C66 RNA was further purified by HPLC on a Nucleogen DEAE 500-10 column using a gradient of 0.40–0.65 NaCl in 20% CH_3CN, 4 mM $MgAc_2$, 50 mM MES, pH 6.0, at room temperature.

NMR Methods.

Samples were prepared for spectroscopy by dialysis into appropriate buffers (see below), and brought to their final concentrations (1 mM or higher) by ultrafiltration using Centricon-10 ultrafilters (Amicon). The final samples contain 5% to 10% D_2O for the spectrometer lock and a small amount of dioxane as a chemical shift standard. It is assumed that the chemical shift of dioxane is 3.741 ppm relative to the methyl resonance of DSS at all temperatures.

Spectra were obtained in the Fourier transform mode on the 490 MHz NMR spectrometer of the Yale University Chemical Instrumentation Center. Imino proton spectra were collected using the twin-pulse method for water suppression (13). Spectra were taken in 8K blocks with a spectral width of 15,000 Hz, and the offset at about 15 ppm. Nuclear Overhauser effects (NOEs) were obtained by the one dimensional difference method. On- and off-resonance spectra were collected in an interleaved manner. Resonances were pre-irradiated for 0.2 at a decoupler power lever adjusted to give 50% to 80% saturation.

L18 Binding Assay.

The L18 used in these studies was purified from *Bacillus stearothermophilus* as described previously (9, 16) and given to us by Dr. D.T. Gewirth. BL18 has a sequence which is about 50% identical to that of EL18 (17), and is functionally compatible with the 5S RNA from *E. coli* (18). It is much more stable than EL18 in solution.

5S RNA was dissolved at a concentration of 1 μM in 0.3 M KCl, 20 mM $MgCl_2$, 30 mM tris, pH 7.4 at 20°C. The circular dichroism of the sample at 267 nm was then followed as aliquots of a 0.1 mM solution of BL18 in the same buffer were added. Measurements were made with an Aviv 60 DS circular dichroism spectropolarimeter.

The binding constant for the L18/5S RNA interaction, K, is given by K = [Cmplx]/PR, where [Cmplx] is the concentration of L18–5S complex, and P and R are the

concentrations of free L18 and free 5S RNA, respectively. Data were analyzed assuming that the increase in ellipticity observed at 267 nm is proportional to [Cmplx]. Taking the total concentration of protein to be P_0 and the total concentration of RNA to be R_0:

$$[Cmplx] = 1/2[(P_0 + R_0 + K^{-1})-((R_0 + P_0 + K^{-1})^2-4P_0R_0)^{1/2}]. \quad (1)$$

Each series of ellipticity increments was fitted to equation (1) using a nonlinear least-squares procedure that yielded values for K and the saturating value for the ellipticity change.

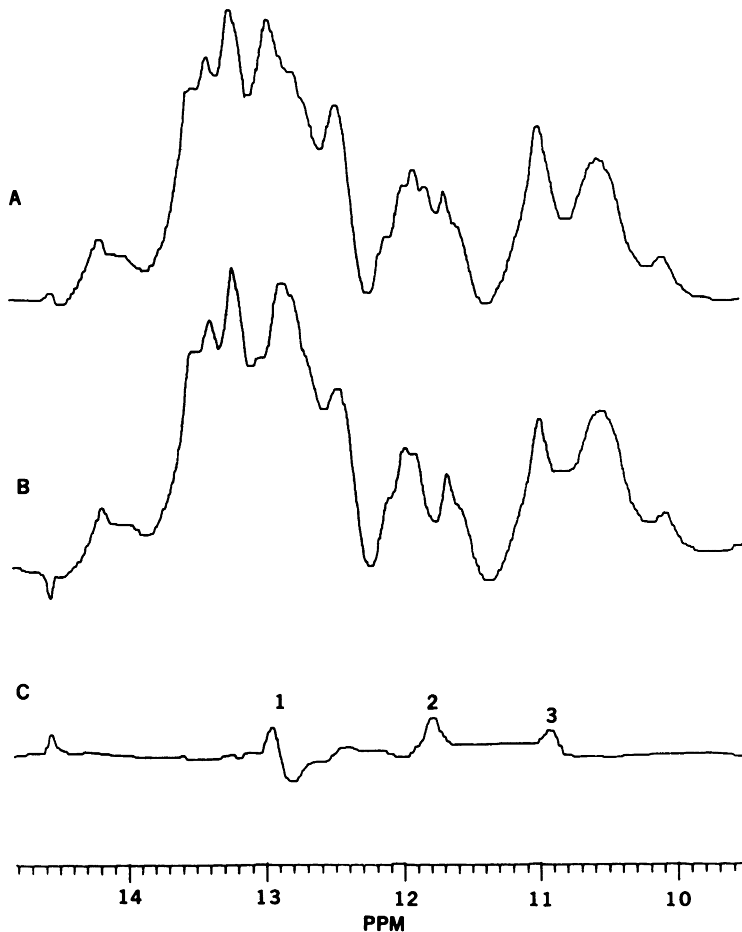


Figure 2. The imino proton spectra of des-A66 RNA and 5S RNA compared. Imino proton spectra were obtained as described in Materials and methods for samples of des-A66 RNA (spectrum A) and 5S RNA (spectrum B) in 0.1 M KCl, 4 mM $MgCl_2$, 5mM cacodylate, pH 7.0 at 303K. Spectrum C is the difference between them (A−B) obtained after appropriate scaling. The features in the difference spectrum are numbered arbitrarily for ease of reference.

RESULTS

The downfield proton spectrum of des-A66 RNA.

The resonances observed between 10 and 15 ppm in the proton NMR spectrum of a nucleic acid in H_2O represent its base imino protons. The resonances of these protons are usually not observed unless they are protected from exchange with solvent, and base-pairing is the most common mechanism of protection (19). Thus the downfield spectrum of an RNA reports primarily on its secondary structure.

Figure 2 compares the downfield spectra of wild type 5S RNA (Figure 2b) and des-A66 RNA (Figure 2a). Both spectra were taken at 303K in 0.1 M KCl, 4 mM $MgCl_2$, 5 mM cacodylate, pH 7.0. The bottom spectrum is the difference between the two spectra, (mutant-wild type). The mutant spectrum has two resonances at 10.95 ppm and 11.82 ppm, designated '3' and '2' respectively, that are either not present or are much weaker in the spectrum of normal 5S RNA. The only other major feature in the difference spectrum is a resonance (resonance 1) which appears to change in chemical shift from 12.82 ppm (wild type) to 13.00 ppm in the des-A66 mutant. Difference spectra identical to this one in all important respects were obtained using 2 other, independent preparations of both des-A66 RNA and 5S RNA.

Assignment of the altered resonances in the spectrum of des-A66 RNA.

Nuclear Overhauser effect (NOE) experiments were done to obtain further information about the resonances detected in the difference experiment. An NOE experiment can identify the resonances of the protons that are within 5 Å of another. The only protons within NOE range of the imino proton(s) of a given base pair are those in the same base pair or in adjacent base pairs on either side (19).

Figure 3 shows the NOEs given when resonances 1, 2 and 3 are irradiated. The 2,3 region of the spectrum contains several other resonances, (resonances O, P1, Q1 and Q2), which represent the imino protons of G96-U80 and G81-U95. The NOEs marked A in Figure 3b and A, O, P1 and D in Figure 3c are connectivities of these GUs (13). When NOE experiments are done on wild type 5S RNA at the frequencies of resonances 1, 2, and 3, the (2 to 1), (3 to 1), and (3 to 2) NOEs shown in Figure 3 are not seen. (Resonance overlap makes it impossible to make the same statement about the (2 to 3) NOE.)

The magnitude of the NOEs between 2 and 3 (Figures 3b and 3c) suggests that the imino protons they represent belong to the same base pair. Since only GU base pairs have two imino protons, it is reasonable to attribute resonances 2 and 3 to a GU base pair, which is also consistent with their chemical shifts. G18-U65 is likely to be the GU in question since it is the only GU near A66 in the standard model.

From its lack of either a strong imino or aromatic NOE, as well as from its chemical shift, 1 is likely to represent a GC base pair. Its weak NOEs to both resonances 2 (Figures 3b and 3d) and 3 (Figure 3c and 3d) prove that the '2,3' GU is adjacent to the GC whose chemical shift changes when A66 is deleted. Because the imino protons of GU base pairs commonly give NOEs only to the imino protons of base pairs on the 3′ side of the G (e.g. 9), it is plausible that the GC whose imino proton chemical shift alters when A66 is deleted is C19-G64.

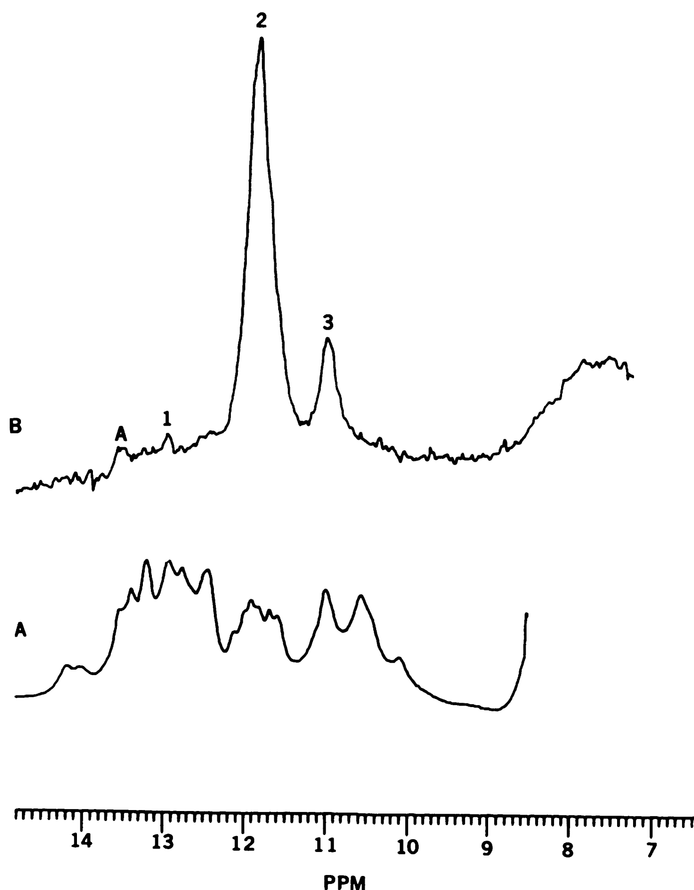
The downfield spectrum of C66 RNA.

Figure 4 compares the spectrum of C66 RNA (Figure 4b) with that of normal 5S RNA (Figure 4a). Both were collected at 303 K in 0.1 M KCl, 4mM $MgCl_2$, 5 mM cacodylate, 0.2 mM EDTA, pH 7.0. The bottom spectrum (Figure 4c) is the difference between the

two, (wild type-mutant). [Note that this difference spectrum is plotted at higher amplification than the one in Figure 2.] The most significant signal in the difference spectrum is the negative feature at 12.75 ppm. Its shape suggests that two or three resonances contribute to it, and its chemical shift suggests that they are likely to be GC resonances.

We were unable to obtain any new NOEs from C66 RNA to help with the interpretation of the (wild type-C66 RNA) difference spectrum. Nevertheless, many of its features can be understood in the framework of the hypothesis of Meier and coworkers (5) that in C66 RNA, C66 should pair with Gl8 to make a CG pair rather than being bulged, and that U65, which ordinarily pairs with Gl8, would become a bulged base (see Figure 5). The effect of this change is to replace a GU with a GC, which should be favored energetically. If Meier and colleagues are correct, a new GC imino proton resonance should be seen in the downfield spectrum of C66 RNA due to C66-Gl8, and quite possibly new intensity representing Cl7-G67, which is likely to be stabilized by the creation of a neighboring GC base pair. The new intensity at 12.75 ppm in the mutant's spectrum is consistent with that interpretation.

The C66 difference spectrum includes three weak, substoichiometric signals that occur



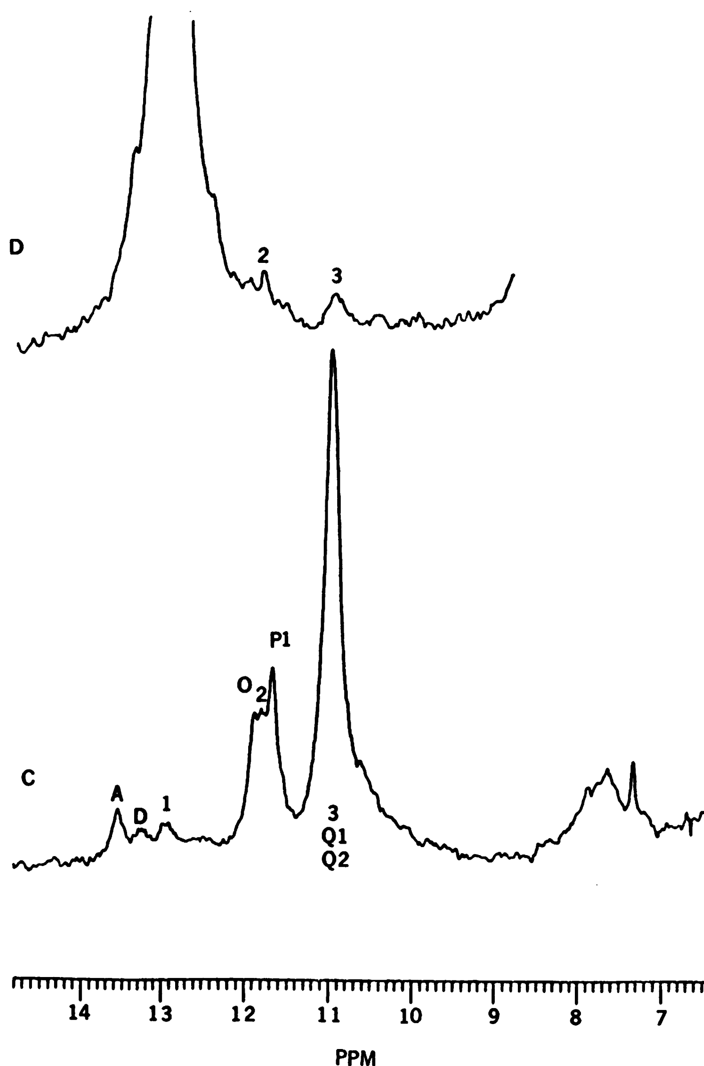


Figure 3. NOE spectra for des-A66 RNA. One dimensional nuclear Overhauser experiments were run on the imino proton resonances of des-A66 RNA samples under the conditions described in the legend for Figure 2. Spectrum A is a normal downfield spectrum of des-A66 RNA shown for reference. Spectrum B is the NOE difference spectrum obtained when resonance 2 (see Figure 2) is presaturated. Spectrum C is the NOE difference spectrum given when resonance 1 is presaturated, and spectrum D is that obtained when resonance 3 is presaturated. NOE difference spectra were obtained as described in Materials and Methods. The significance of resonances designated by letter names is described in the text.

in the spectrum of wild type 5S RNA but not in C66 RNA. Their chemical shifts are 10.83 pm, 12.04 ppm and 12.40 ppm respectively. The resonance at 10.83 ppm is plausibly that of the G in a GU base pair. A likely candidate is G18 in wild type 5S RNA. Similarly the resonance at 12.04 ppm is appropriate in chemical shift to represent U65, the base

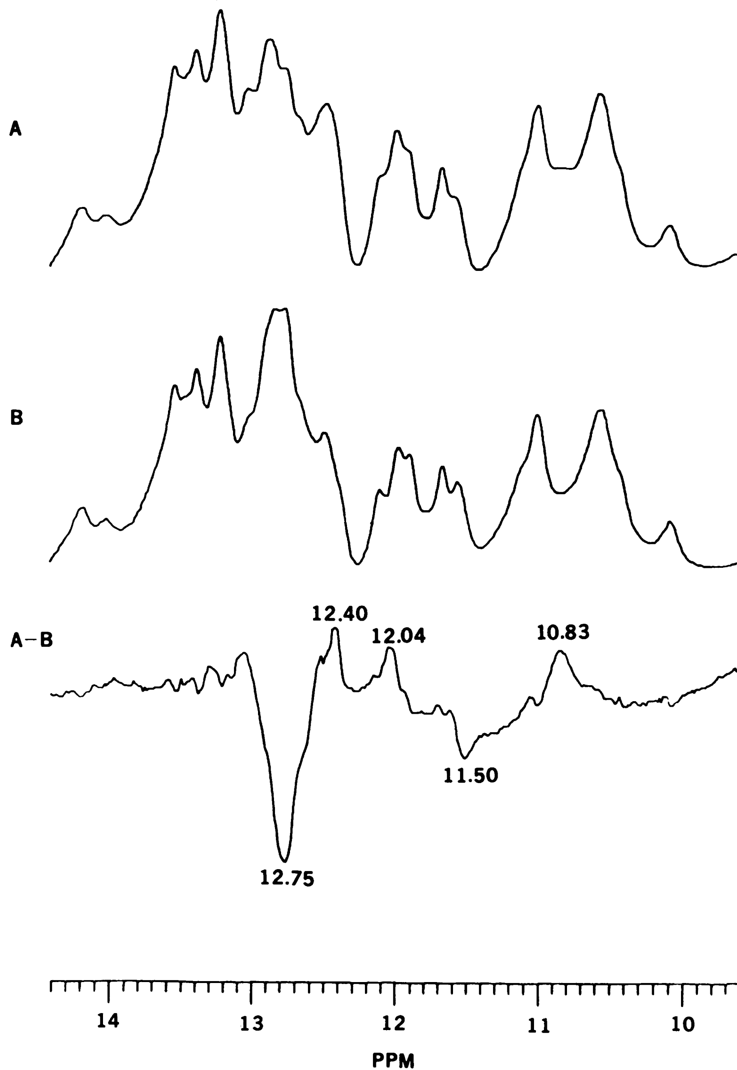


Figure 4. The imino proton spectra of C66 RNA and 5S RNA compared. Imino proton spectra were obtained from samples of 5S RNA (spectrum A) and C66 RNA (spectrum B) as described in the legend for Figure 2. The bottom spectrum ('A−B') is the difference of the two spectra, spectrum A minus spectrum B.

opposite G18. Thus these resonances are likely to be the wild type counterparts of the GU imino proton resonances that are the distinctive features of the spectrum of des-A66 RNA. Their weakness in the wild type spectrum would be explained by destabilization due to proximity to A66.

This hypothesis requires that these same resonances be evident in the (des-A66/wild type) difference spectrum as negative features, i.e., features present in the wild type spectrum but not the mutant. Careful examination of Figure lc confirms the existence of

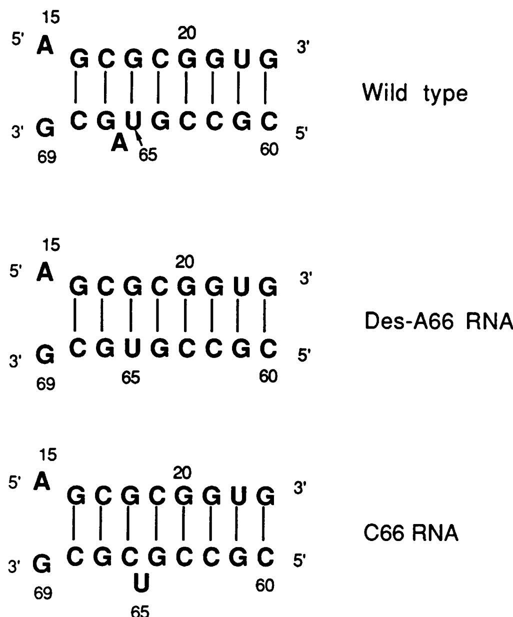


Figure 5. 5S RNA sequences around position 66. The proximal end of helix II of 5S RNA is shown in the form we believe it has in wild type 5S RNA, des-A66 RNA and C66 RNA (see 1). Bases are given the numbers they have in the wild type sequence of 5S RNA.

small, broad, negative features at the chemical shifts in question, adjacent to the 'new' GU resonances, resonances 2 and 3. There can be little doubt that the weak resonances at 10.83 and 12.04 ppm in the (wild type−C66 RNA) difference spectrum represent G18-U65.

What about the resonance at 12.40 ppm? It cannot represent G64, which has already been assigned to a resonance at 12.82 ppm in the wild type spectrum (see above). It is also unlikely that G20's imino proton resonance would be affected either with respect to stability or chemical shift by changing A66 to a C. G20's immediate neighbors are unaltered by that mutation, and nearest neighbors have the strongest influence on base pair stability and imino proton chemical shift. Furthermore, if U65 bulges outward in C66 RNA, as unpaired pyrimidines are believed to do (20), the next nearest neighbor of G20 in the helical stack will be G18-C66 instead of G18-U65, a modest alteration. Thus G20 is unlikely to be responsible for the 12.40 ppm resonance.

We propose that 12.40 ppm may be the chemical shift of the imino proton of G67 in normal 5S RNA. G67-C17 is likely to be destabilized by its proximity to A66 in wild type 5S RNA; its contribution to the wild type spectrum should be less than stoichiometric, as the difference spectrum suggests. Furthermore, bulged A's are thought to insert into helices rather than be turned outwards, away from their axes (19). The ring current effect of A66 should cause G67's imino proton to resonate rather far upfield, and consequently the elimination of that A by mutation should cause its resonance to move downfield. The 0.35 ppm downfield shift required to bring its resonance to 12.75 ppm in response mutation is plausible (9).

Table I. Assignments of Imino Proton Resonances near A66.

Imino Proton	*Chemical Shift (ppm)*
G18	12.04
U65	10.83
G64	12.82
G67	12.40

Legend: The arguments that support these assignments are given in the text. Chemical shifts are determined relative to that of dioxane (3.741 ppm), and should be accurate to within .02 ppm.

The resonance at 11.50 ppm in the C66 difference spectrum is of uncertain origin. It could represent the bulged U65. Its chemical shift value is close to that of an unpaired U residual (22). A modest level of protection might make it detectable at room temperature.

The assignments these observations suggest are summarized in Table 1.

L18 binding.

Bear and coworkers demonstrated several years ago that L18 binding to 5S RNA causes a significant increase in circular dichroism at 267nm, presumably a response of the RNA to the presence of the protein (10). This effect provides a means for measuring L18 binding to 5S RNA at equilibrium. Since the assay techniques used to compare the protein binding properties of 5S RNA and the mutants discussed above in the past were not equilibrium techniques, we thought it would be useful to verify the L18 binding constants of these RNAs by the CD method.

In wild type 5S RNA, the CD increase observed upon saturation with L18 is about 15% (see Table 2). C66 RNA gave an increase in CD of the same magnitude, and the binding constant calculated for its interaction with L18 was 1.3×10^7 M^{-1}, as compared to 1.5 $\times 10^7$ M^{-1} for wild type, consistent with the conclusion of Goringer and Wagner that C66 RNA binds L18 with a strength that is indistinguishable from that of 5S RNA (6). To our surprise, when the same assay was attempted with des-A66 RNA, essentially no CD change was seen. We estimate that the CD increase was no greater than 2%, if different from zero at all, making it impossible to estimate a binding constant by that method. One other molecule was examined the same way, a double mutant which has A's at positions 67 and 72 instead of G's. This double mutant's CD spectrum is also unaffected by the presence of L18.

The RNA samples used for these CD studies were subsequently examined by gel electrophoresis for their protein binding activity (9). All were found to be intact, and, qualitatively, by the gel retardation criterion, all bind L18, as expected (4, 6). The loss

Table II. L18 -induced CD changes and L18 Binding Constants of 5S RNA and Selected 5S Mutants.

RNA species	*Change in CD at 267nm*	*Binding Constant*
5S	16.0 ± 1.6% (3)	1.5 ± 0.6×10^7 M^{-1}
des-A66	2.5 ± 1.4% (2)	–
C66	30.0% (1)	5.3×10^6 M^{-1}
A67,A72	0.7% (1)	–

Legend: 5S RNA and 3 of its mutants were assayed for L18 binding using the circular dichroism method described in Materials and Methods. Reported are the magnitudes of the of the CD increase at 267nm observed at saturation, and the binding constants. Standard errors are given for these measurements. The number of repetitions of each experiment done to obtain these estimates is indicated in parentheses after the estimate of the magnitude of the increase at saturation. No binding constants are given for des A66 and A67,A72 RNAs because the CD changes were too small to permit an accurate analysis.

of the L18 CD signal observed in des-A66 RNA and A67,A72 RNA is not due to the lack of interaction between these RNAs and L18.

DISCUSSION

Of the assignments in Table I, that of G67 is the most problematic. Although the lack of an NOE connecting G18-U65 to C17-G67 is not unexpected in des-A66 RNA, the lack of evidence for that base pair in the des-A66 RNA/5S RNA difference spectrum is surprising, given its apparent presence in the C66 RNA/5S RNA difference. If the removal of A66 from the molecule stabilizes the imino proton resonance of G67, as one might expect, its magnitude should increase and/or its linewidth narrow. The removal of A66 is also likely to alter the magnetic environment of G67, leading to an alteration in the chemical shift of its imino proton resonance (see 9). Either effect should lead to a detectable signal in the difference spectrum, but nothing is seen.

Regardless of the details of the assignments, it is surprising how small the spectroscopic effects are of mutations at position 66 in 5S RNA. Even if an RNA's structure remains constant, replacement of one base pair with another within double helical RNA stems has magnetic effects that can extend as far as 3 base pairs to either side of the altered position (9). Deletion of A66 or its replacement with a C could have affected the imino proton resonances of up to 6 base pairs starting at G16-C86 and ending at G21-C62, but only three base pairs were altered, C17-G67 (probably), G18-U65, and C19-G64.

The NMR data suggest that base pairs C17-G67 and G18-U65, the nearest neighbors of A66, are destabilized in 5S RNA, but nevertheless make a contribution to its imino proton spectrum. But except for that destabilization, the only aspect of the standard secondary structure of 5S RNA in the A66 region that was not detected in these experiments is the G16-C86 base pair. It is possible that G16-C68 is destabilized because of its position at the end of a helical segment. The data also suggest that the ring current shift effects of removing the bulged A either by deletion or replacement with a C are quite small, probably because the local changes in structure brought about by the removal of A66 accidentally compensate each other. The hypothesis of Meier and coworkers that replacement of A66 by a C would cause the bulge to migrate distally by one base provides a satisfactory rationalization of the NMR changes seen (5).

When these observations are considered in light of what is known about the L18 binding of these RNAs, little support can be found for the notion that specific interactions between A66 and L18 play an important role in that interaction. If the local environment around A66 were important it is hard to understand how the structural change brought about by its replacement with a C could be accommodated with so small an effect on L18 binding. The effects of mutation at position 66 on protein binding may have more to do with its influence on helix axis direction and stiffness than on the capacity of A66 for hydrogen bonding with L18.

In the course of this work, quite by accident, we have found that the proximal end of helix II is involved in some way in the L18-related CD effect discovered by Bear and coworkers over a decade ago. Some, but not all mutations at positions 66 and 67 at the base of helix II eliminate the effect altogether, but do so without impairing L18 binding to any major extent. Both des-A66 RNA and A67,A72 RNA, which show little or no CD effect, bind L18 with affinities that are within a factor of 10 of normal. It appears likely that the CD change reflects some L18-induced alteration in the structure of the region of 5S RNA where Helices I, II and V come together, but one that does not make a large

free energy contribution to the L18–5S interaction.

Readers familiar with prior studies of L18 binding will be aware of the large range of the values that have been reported for its binding constant in the past (for discussion, see 5). The scope of the problem is indicated by the fact that we recently obtained a binding constant of 3×10^5 M^{-1} using the gel retardation method (16), a value 50 fold less than the one reported here, even though the ionic conditions, and the materials used in the two measurements were nearly identical. The source of these discrepancies is not understood. We doubt they reflect only the difference to be expected between a true equilibrium binding assay, and one that assays when the system is not at equilibrium. In an effort to further validate the CD method further, we measured L18 binding constants for some of the 5S deletion mutants on which Gewirth reported (15). The binding constants of these mutants relative to wild type measured by the CD assay was the same as determined by gel retardation methods (Popieniek, unpublished data; 15).

ACKNOWLEDGEMENTS

We are indebted to Drs. Christiansen and Wagner for making the plasmids available to us on which this study was based. The derivative of pKK5-1 carrying a 5S gene mutated at positions 67 and 72 was a gift from Kathy Triman and Dr. Harry Noller. We thank Dr. Robert Rycyna for his help in collecting the spectra shown here. This work was supported by grants from the NIH (GM-22778, AI-09167) and NSF (DMB-8698283).

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