Secondary Structure of 5S RNA: NMR Experiments on RNA Molecules Partially Labeled with Nitrogen-15†

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ABSTRACT: A method has been found for reassembling fragment 1 of *Escherichia coli* 5S RNA from mixtures containing strand III (bases 69–87) and the complex consisting of strand II (bases 89–120) and strand IV (bases 1–11). The reassembled molecule is identical with unreconstituted fragment 1. With this technique, fragment 1 molecules have been constructed ^{15}N-labeled either in strand III or in the strand II–strand IV complex. Spectroscopic data obtained with these partially labeled molecules show that the terminal helix of 5S RNA includes the GU and GC base pairs at positions 9 and 10 which the standard model for 5S secondary structure predicts [see Delihas, N., Anderson, J., & Singhal, R. P. (1984) *Prog. Nucleic Acid Res. Mol. Biol. 31*, 161–190] but that these base pairs are unstable both in the fragment and in native 5S RNA. The data also assign three resonances to the helix V region of the molecule (bases 70–77 and 99–106). None of these resonances has a "normal" chemical shift even though two of them correspond to AU or GU base pairs in the standard model. The implications of these findings for our understanding of the structure of 5S RNA and its complex with ribosomal protein L25 are discussed.

The resonances between 10 and 15 ppm in the proton nuclear magnetic resonance (NMR)[1] spectrum of a nucleic acid taken in H_2O represent base imino protons protected from solvent exchange, usually by hydrogen bonding (Kearns & Shulman, 1974). Assignment of these resonances identifies the bases involved in a nucleic acid's hydrogen-bonded structures and makes possible the exploitation of its imino proton spectrum as a source of information about the molecule's response to environmental perturbations such as its interaction with proteins.

In the late 1970s, Redfield and his colleagues demonstrated that nuclear Overhauser effects (NOEs) can be observed between imino protons in neighboring base pairs in double-helical nucleic acids and that the analysis of these NOEs provides a general means for assigning their resonances (Johnston & Redfield, 1978, 1981; Sanchez et al., 1980; Roy & Redfield, 1981; Hare & Reid, 1982a,b; Roy et al., 1982). The assignment of a large number of downfield resonances in the spectrum of 5S RNA from *Escherichia coli* has been achieved by this means (Kime & Moore, 1983b,c; Kime et al., 1984). Several resonances in the imino proton spectrum of 5S RNA have proven unassignable by NOE techniques, however, because they give few or no NOEs.

As a step toward completing the assignment of the downfield spectra of 5S RNA and its complexes with ribosomal proteins, 5S RNA samples uniformly labeled with ^{15}N were examined by NMR. Like ^1H, ^{15}N is a spin $^1/_2$ nucleus. The resonances of both nuclei are 85–90-Hz doublets in the NMR spectrum of a molecule where the two are covalently bonded. If the resonant frequency of the ^{15}N atom bonded to the proton responsible for a given imino proton resonance can be measured, then the type of base to which the proton belongs can be identified because the frequencies at which ^{15}N UN3 and GN1 nitrogens resonate are well separated (Gonnella et al., 1982). Several spectroscopic techniques exist for making the $^{15}N/^1H$ chemical shift correlations required [see Griffey et al. (1985)]. The base types of almost all the unassigned resonances associated with the fragment 1 moiety of 5S RNA (bases 1–11 and 69–120 of the 5S sequence) were determined in this way (Kime, 1984a,b; Jarema & Moore, 1986).

The spectra obtained with uniformly labeled ^{15}N 5S RNA and fragment also revealed the existence of two UN3 resonances in a densely populated part of the spectrum near 12.7 ppm. These resonances are anomalous in two respects. First, neither gives the aromatic NOE characteristic of an AU base pair or the strong imino NOE associated with GU base pairs. Second, AU imino protons are usually found between 13 and 14 ppm, and GU imino protons appear between 11 and 12 ppm; 12.7 ppm is a typical GC chemical shift. Since a number of NOEs in the 5S spectrum involve resonances having chemical shifts near 12.7 ppm, reexamination of the assignments made on the basis of these NOEs was clearly required.

In this paper, we describe a new method for reconstituting fragment 1 from its constituent oligonucleotides which has enabled us to construct fragment 1 molecules ^{15}N labeled only in bases 69–87 or, alternatively, ^{15}N labeled everywhere except bases 69–87. The ^{15}N-induced splittings observed in the NMR spectra of these samples identify the strand of the nucleic acid to which the protons responsible for each resonance belong.

The spectroscopic analysis of these partially labeled molecules has settled most of the questions raised by the discovery of the two anomalous UN3 resonances. In the process, a significant number of new downfield assignments have been

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[1] Abbreviations: NMR, nuclear magnetic resonance; ADA, alternate delay accumulation; NOE, nuclear Overhauser effect; DSS, 3-(trimethylsilyl)-1-propanesulfonate; FID, free induction decay; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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made, the largest number since the original NOE studies (Kime & Moore, 1983b,c). One of the anomalous UN3 resonances is fully assigned, and the second's assignment is restricted by the data to one of two bases. In addition, the new data sharply restrict the possibilities which can be entertained for the remaining unassigned resonances in fragment 1 and its complex with ribosomal protein L25. The significance of these new data for our understanding of the structure of 5S RNA will be discussed.

Materials and Methods

^{14}N- and ^{15}N-Labeled 5S RNA. The 5S RNA used in these experiments was the product of the plasmid pKK5-1 which carries the 5S RNA gene from the rrnB ribosomal RNA cistron of *E. coli* (Brosius, 1984). The methods used for preparing 5S RNA with normal isotopic content have been described previously (Kime & Moore, 1983b). In order to use this plasmid for the purposes of producing ^{15}N-labeled 5S RNA, it was transduced into *E. coli* strain NG135 (Grindley & Joyce, 1980) which grows on minimal medium. (HB101, the strain normally used as the host for pKK5-1, does not grow on minimal medium.)

NG135/pKK5-1 was grown on a medium containing (per liter) 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 0.5 g of NaCl, 20 mL of 20% glucose, 1 mL of 1 M $MgSO_4$, 10 mL of 0.2% ampicillin, and 10 mL of 0.01 M $CaCl_2$. The nitrogen required was supplied as 0.3 g/L of 99% enriched $^{15}NH_4Cl$ (MSD Isotopes, St. Louis, MO). Cells were grown at 37 °C to a density of about 1×10^8 cells/mL. At this point, 50 mg/L chloramphenicol was added to induce overproduction. Cells were harvested 2 h later. 5S RNA was prepared by phenolization of a postribosomal supernatant made from these cells in the usual manner (Kime & Moore, 1983b).

Fragment 1 was produced by limited digestion of 5S RNA with RNase A as described previously (Kime & Moore, 1983a).

Protein L25. Ribosomal protein L25 was prepared by ion-exchange and Sephadex chromatography in urea starting with either purified 50S subunits or whole 70S ribosomes from *E. coli*, and then renatured following protocols which have been described elsewhere (Kime et al., 1981; Kime & Moore, 1982).

Separation of the Strands of Fragment 1. Fragment 1 preparations were precipitated with ethanol, taken up in 2 mM EDTA, 0.1 M NaCl, and 10 mM cacodylate, pH 6, and then applied to a Sephadex G100 column equilibrated with the same buffer. The column was run at 30–35 °C. Under these conditions, fragment 1 molecules cleaved at the 87, 88, 89 loop dissociate to give a complex of bases 1–11 with bases 89–120, and a single strand comprising bases 69–87. These species resolve on Sephadex and elute appreciably slower than molecules with intact loops, which do not dissociate (Leontis et al., 1986).

Reconstitution of Fragment 1. The purified single strand (bases 69–87) and the two-stranded complex (bases 1–11 and 89–120) were dialyzed separately into 0.1 M KCl, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7. The two components were then mixed to produce a solution 12.0 A_{260nm} units/mL in the complex and 8.0 A_{260nm} units/mL in the single strand, which was heated at 60 °C for 10 min and then allowed to cool slowly to room temperature over 45 min. The product was recovered by ethanol precipitation and purified by chromatography on Sephadex in the manner used for the initial preparation of fragment 1. Quantitative conversion of components into renatured fragment was observed.

Preparation of Samples for Spectroscopy. All samples were prepared for spectroscopy by dialyzing them into the buffers stated at concentrations between 0.1 and 0.3 mM. L25 complexes were formed by mixing renatured L25 with fragment at about 0.1 mM. It was assumed that the optical density of a 1 mM solution of L25 at 276 nm is 0.363 and that the optical density of a 1 mM solution of fragment 1 at 260 nm is 437.0. The ratio of protein to RNA was set at 1.3:1 to ensure saturation of the RNA. Samples were brought to concentrations around 1.0 mM using Centricon-10 ultrafilters.

Spectroscopic Methods. All spectra reported below were obtained in the Fourier transform mode on either the 500- or the 490-MHz NMR spectrometers at the Yale University Instrumentation Center. All ^1H-observe, ^{15}N-decouple experiments were performed on the 490-MHz instrument using a Cryomagnet Systems, Inc., HR-44, wide-band-decouple, proton-observe, 5-mm probe. D_2O at 5% concentration was used for the spectrometer lock, and the chemical shift of the *p*-dioxane added to the samples was assumed to be 3.741 ppm relative to the methyl resonance of DSS at all temperatures, giving a frequency of 489.927141 MHz at 0 ppm on the 490-MHz spectrometer.

Spectra of exchangeable imino protons were obtained by using the twin-pulse method for avoiding excitation of water solvent resonances (Kime & Moore, 1983a) in combination with alternate delay accumulation (ADA) (Roth et al., 1980) to overcome computer word length limitations. Spectra were taken in 8K, 16K, or 32K blocks with a spectral width of 20000 Hz, and the offset was at about 15 ppm except for those spectra involving NOEs from S, where the offset was placed near 11.5 ppm.

NOEs between hydrogen-bonded imino protons were obtained by the one-dimensional difference method. On- and off-resonance spectra were collected in an interleaved manner. Resonances were preirradiated for 0.2–0.3 s at a decoupler power level adjusted to give 50–70% saturation. The NOE difference FIDs were subjected to Lorentzian-to-Gaussian multiplication prior to Fourier transformation in order to improve resolution.

^{15}N–^1H chemical shift correlations were measured by difference decoupling. Samples were continuously irradiated at ^{15}N frequencies at low power (0.1 W) during acquisition of ^1H spectra. On- and off-resonance spectra were accumulated sequentially, with the off-resonance ^{15}N decoupling frequency set 2000 Hz away from the ^{15}N frequencies characteristic of GN1 or UN3 nitrogens. ^{15}N decoupling frequencies were manually set by using a PTS-500 frequency synthesizer mixed with a General Radio 1061 frequency synthesizer operating at 30 MHz. On-resonance spectra were subtracted from off-resonance spectra to reveal the resonances decoupled at each setting of the decoupler. ^{15}N chemical shifts were measured relative to the middle of the four intense ^{15}N resonances of a standard $^{15}NH_4Cl$ sample (Wilmad WGN-01) (at 49.64403 MHz on the 490-MHz spectrometer).

Results

Prior Assignments in Fragment 1. In order that the reader understand the significance of the data presented below, it is necessary to review what was known about downfield assignments in the fragment 1 moiety of the 5S RNA from *E. coli* before these studies began. The top spectrum in Figure 1 is the downfield spectrum of the fragment 1 moiety of 5S RNA (*E. coli*) (at pH 7.2) with the standard nomenclature for its resonances indicated. The lower spectrum in Figure 1 is the downfield spectrum of the fragment 1–L25 complex, also at pH 7.2. A resonance in the spectrum of the complex with the same designation as one in the spectrum of the free RNA gives similar NOEs. Eight resonances can be recognized in the

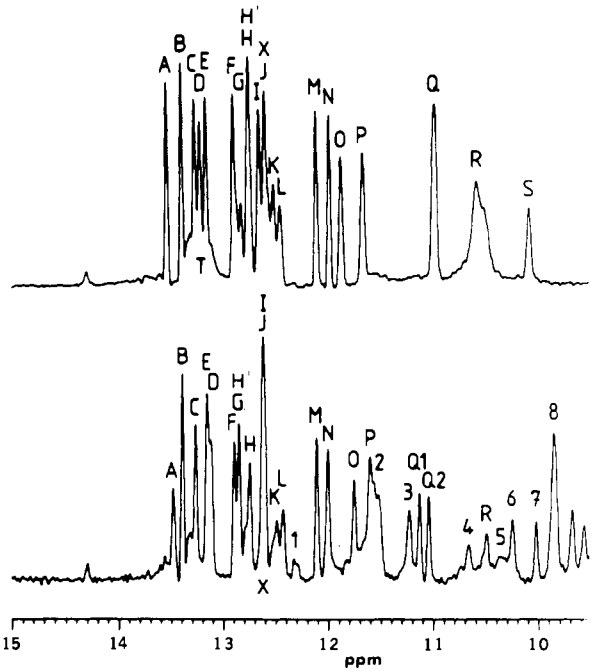

FIGURE 1: Standard downfield spectra for fragment 1 and the complex of fragment 1 and L25. The upper spectrum is that of fragment 1, resolution enhanced, taken in 0.1 M KCl, 4 mM $MgCl_2$, and 5 mM cacodylate, pH 7.2 at 303 K. The lower spectrum was taken under similar conditions and is that of the complex of L25 and fragment 1. The fragment 1 in both samples was derived from ribosomal 5S RNA not the overproduced product. There are small differences in chemical shifts involving resonances J and C between the two species of 5S RNA (Kime & Moore, 1983c) which are of no importance for this study. Resonances are named following Kime and Moore (1983a,b). The spectra themselves are from Kime and Moore (1983b).

spectrum of the complex whose correspondents in the spectrum of free RNA are not obvious (Kime & Moore, 1983c). These complex-related resonances are designated with numerals.

Using NOE techniques, it is possible to demonstrate that resonances J, C, F, B, M, E, and H originate in the terminal helix of the molecule, helix I. They correspond to the imino protons of base pairs G2-C118 through C8-G112, in that order (see Figure 7). Resonances I, N, A, (O, P), (O, Q), and D represent helix IV and assign to base pairs G84-C92 through G79-C97 in the order given (Kime & Moore, 1983b; Kime et al., 1984). Some of the L25 complex-specific resonances do give NOEs, but none can be assigned on that basis (Kime & Moore, 1983c).

The initial series of experiments using ^{15}N, referred to above, concentrated on fragment 1 molecules uniformly labeled with the isotope. Both one-dimensional, difference decoupling experiments and two-dimensional, forbidden echo spectra were taken on samples of fragment 1 and its complex with L25 (Kime, 1984a,b; Jarema & Moore, 1986). The data confirmed that the base types of all the resonances assigned by NOE had been correctly identified. They also demonstrated the existence of two previously undetected UN3 resonances having proton chemical shifts around 12.7 ppm, now designated H′ and X (Kime, 1984a). H′ and X represent bases in the middle of fragment 1, not at its ends (Jarema & Moore, 1986). H′, like most of the resonances in helix IV, changes its chemical shift when L25 binds (Kime, 1984b; Jarema & Moore, 1986); X does not. In addition, the studies on ^{15}N-labeled RNA assigned all the numbered resonances in the L25 complex as to base type, with the exception of resonance 2 for which the data were unclear. Resonance 8 proved to be a superposition of an RNA UN3 resonance and one or more protein resonances (Kime, 1984b).

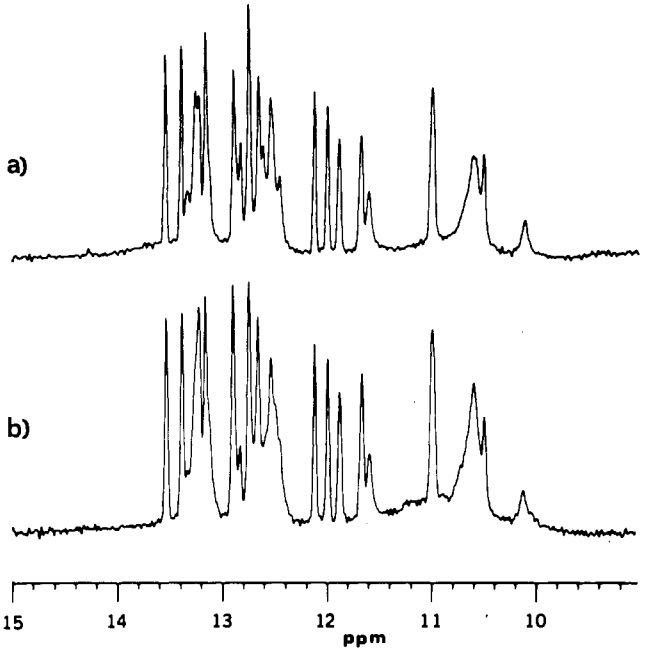

FIGURE 2: Comparison of the spectrum of native and reconstituted fragment 1. Both spectra shown in this figure are of fragment samples at a concentration of about 1 mM in 0.1 M KCl, 4 mM $MgCl_2$, and 5 mM cacodylate, pH 6.0, at 303 K. The top spectrum (a) is that of native, unreconstituted fragment 1. Spectrum b is that of fragment 1 reconstituted from components all of which contained normal ^{14}N.

Preparation of Fragment 1 Molecules Partially Labeled with ^{15}N. The dissociation of fragment 1 preparations in EDTA to yield a species consisting of bases 1–11 ("strand IV") complexed with bases 89–120 ("strand II"), and a single-stranded oligonucleotide, bases 69–87 ("strand III"), has been described elsewhere (Leontis et al., 1986). [Strand I is the oligonucleotide running from G69 to U120 (Kime et al., 1984).] What has recently been discovered is that under the conditions given under Materials and Methods the oligonucleotides produced by dissociation of fragment 1 in EDTA will reassociate.

Fragment 1 reconstituted in this way has electrophoretic properties on native acrylamide gels identical with unreconstituted fragment 1. It binds L25 normally. Figure 2 compares the imino proton spectra of native fragment 1 (a) and a sample of reconstituted material (b) at pH 6.0. They are essentially identical.

[We have described a method for reassociating strand IV (bases 1–11) with strand I (bases 69–120) previously. Although the spectrum of material prepared that way is similar to that of fragment 1, it is not identical (Kime et al., 1984). Molecules reconstituted from strands I and IV would not be suitable for the experiments described below, even if their spectra were normal, however. The labeling patterns possible in preparations of that kind are much less useful.]

Fragment 1 preparations made from both ^{14}N- and ^{15}N-labeled 5S RNA were dissociated in EDTA and their components purified. Reconstitutions were then done to form ^{15}N-labeled strand III + ^{14}N-labeled strands (II + IV) and ^{14}N-labeled strand III + ^{15}N-labeled strands (II + IV). The downfield proton spectra of these two species at pH 7 are shown in Figure 3.

Strand Assignments of Imino Resonances in Fragment 1 in the Presence and Absence of L25. Difference decoupling experiments were run on fragment 1 samples partially labeled with ^{15}N, with and without L25, in order to identify the region of the molecule from which each resonance arises and to

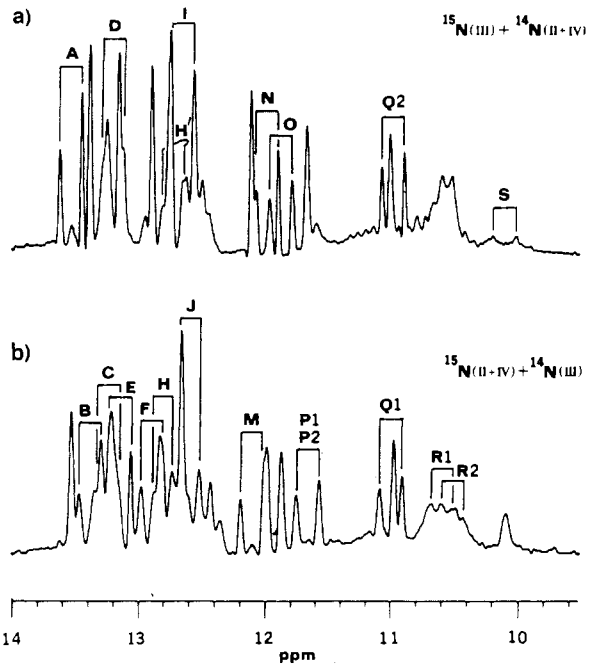

FIGURE 3: Comparison of the spectra of fragment 1 labeled with ^{15}N in strand III only, or labeled with ^{15}N everywhere except strand III. The spectra in this figure were taken in 0.1 M KCl, 4 mM $MgCl_2$, and 5 mM cacodylate, pH 7.2, at 303 K. The fragment concentration was about 1 mM. The top spectrum (a) is that of fragment 1 labeled in strand III (bases 69–87) with ^{15}N. Spectrum b is that of fragment 1 carrying ^{15}N in strands II and IV, bases 89–120 and 1–11, respectively. Many of the resonances which split as a result of the labeling are indicated.

confirm earlier base type identifications. The results are summarized in Table I. Many of the splittings on which Table I is based are obvious in the spectra shown in Figure 3. All resonances assigned previously by NOE methods not only are of the correct base type, as we knew from studies on molecules uniformly labeled with ^{15}N, but also originate in the correct part of the molecule, further confirming their assignments.

Resonances G, 1, and 5 could not be classified because their resonances were too weak to detect in these spectra. Resonances 1 and 5 are weak in all fragment 1–L25 complex spectra observed to date [see Kime et al. (1983c)]. The amplitude of resonance G is known to be low in fragment samples cleaved at bases 87, 88, and 89, as these samples all were (Kime et al., 1984).

Clearly evident in the difference decoupling spectra of strand III labeled molecules is a broad GN1 resonance we designate "G′" having the same chemical shift as G (data not shown). Difference decoupling spectra of uniformly labeled fragment 1 molecules are extremely complex in this region. Our ability to detect G′ in these experiments is due to the simplification achieved by partial labeling.

In the original ^{15}N studies on the fragment 1–L25 complex, the status of resonance 2 was unclear. Resonance 2 is just upfield of P1 and P2, both of which are UN3 resonances, so while it was possible to deduce that 2 could not represent a GN1 imino proton, it was unclear whether 2 was a UN3 imino proton like P1 and P2 or a resonance unaffected by RNA labeling altogether. Inspection of the partially labeled spectra shows that in ^{15}N-labeled strand III molecules complexed with L25, resonance P includes no split components (data not shown). Furthermore, when the labeling is reversed, P becomes a broad doublet whose upfield component is about twice the amplitude of its downfield component. Since 2 is on the upfield side of P, 2 must not be split in these molecules either.

Table I: Strand Assignments for the Downfield Resonances of Fragment 1[a]

reso-nances	strand III	strand II + IV	type	reso-nances	strand III	strand II + IV	type
A	+		UN3	1		nd	(UN3)
B		+	UN3	2		pr	
C		+	GN1	3	+		GN1
D	+		GN1	4		+	GN1
E		+	GN1	5		nd	?
F		+	GN1	6		+	GN1
G	?		(GN1)	7	+		GN1
G′	+		GN1	8	+		UN3
H		+	GN1				
H′	+		UN3				
I	+		GN1				
J		+	GN1				
K		+	GN1				
L		+	GN1				
M		+	GN1				
N	+		GN1				
O	+		UN3				
P1		+	UN3				
P2		+	UN3				
Q1		+	GN1				
Q2	+		GN1				
R1		+	GN1				
R2		+	GN1				
S	+		GN1				
T		+	GN1				
X		+	UN3				

[a] Difference decoupling experiments were performed on samples of fragment 1 which were ^{15}N labeled in strand III or in strands II + IV both in the presence and in the absence of protein L25. On the basis of the results of these experiments, it is possible to classify each resonance on the basis of whether it belongs to strand III or not and to identify it as to base type. The first column lists all the resonances found in the spectrum of free fragment 1. The fifth column lists resonances unique to the complex of fragment 1 with L25. A question mark implies that the data do not settle the point in question. Base type assignments in parentheses were known from earlier work (Kime, 1984a,b; Jarema & Moore, 1986) but not confirmed in this study. nd indicates not detected; pr indicates protein resonance.

Thus, 2 cannot be an imino proton resonance; it must instead be an exchangeable proton belonging to protein L25. It is by far the most downfield-shifted L25 resonance in the complex.

Assignment of the Temperature-Sensitive GU Base Pair in Fragment 1. When fragment 1 was first investigated by NMR, evidence could be found for only two GU base pairs at 303 K, but a third was apparent at 283 K. The stable GUs contribute resonances (P1, Q2) (G81–U95) and (O, Q1) (probably U80-G96) to the spectrum. The temperature-sensitive GU contributes resonances R2 and P2. R2 and P2 both give NOEs to the GC imino proton resonance T.

We earlier advanced the hypothesis that the unstable GU resonances might represent a GU base pair in helix V (Kime et al., 1984). The strongest evidence was that an NOE exists connecting resonance S with a resonance having the chemical shift of H (Kime & Moore, 1986b). Since H represents G112, this NOE appeared to assign S to either the G or the U of G9-U111, preempting the assignment of the R2-P2 GU to those bases, but the evidence was not definitive (Kime et al., 1984). This hypothesis for the assignments of S, R2, P2, and, by implication, T can be tested by using fragment 1 molecules partially labeled with ^{15}N.

Figure 4a shows the spectrum of ^{15}N-labeled strand III fragment 1 at 303 K, and Figure 4b shows the NOEs obtained when resonance P is presaturated at the same temperature in the same sample. While resonance P is not split when strand III is ^{15}N labeled, the two NOEs detected involving P are both to split resonances. The downfield doublet is resonance A, and the upfield one is resonance Q2. This pattern of NOEs and

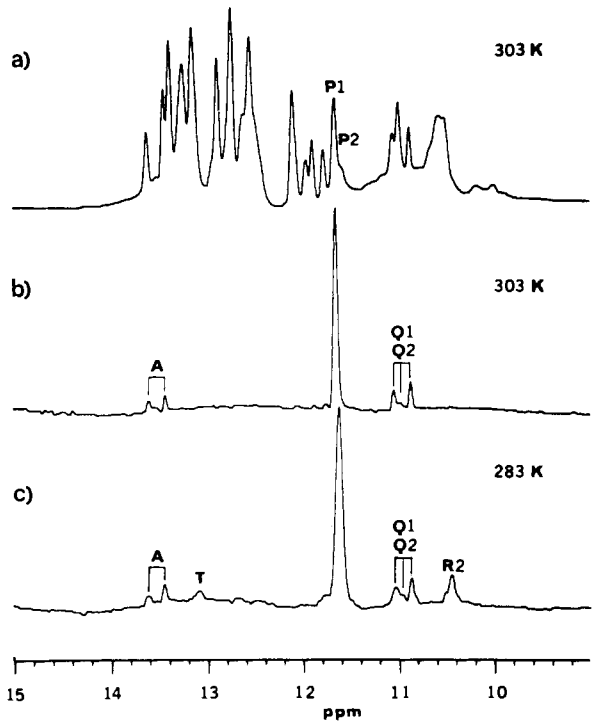

FIGURE 4: NOEs in fragment 1 samples labeled with ^{15}N in strand III. All spectra were taken under the ionic conditions specified in the legend for Figure 3. Spectrum a is that of ^{15}N-labeled strand III fragment 1 at 303 K. Spectrum b is the NOE difference spectrum obtained when this sample was irradiated at the frequency of P at 303 K. Spectrum c shows the NOE difference spectrum observed when resonance P was irradiated at 283 K.

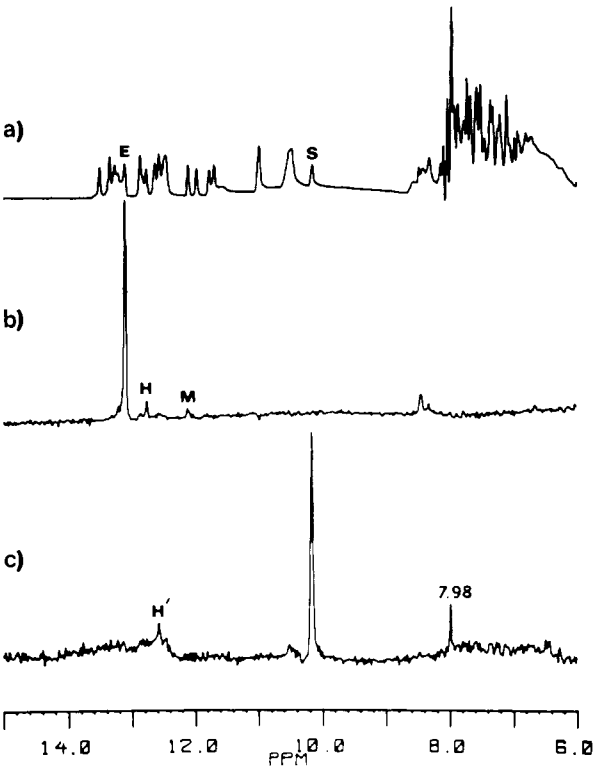

FIGURE 5: NOEs in unlabeled fragment 1 in the presence of Ca^{2+}. A sample of fragment 1 was prepared in 0.1 M KCl, 4 mM $CaCl_2$, and 4 mM cacodylate, pH 7.2. Spectrum a is that of fragment in Ca^{2+} at 303 K. Spectrum b shows the NOEs generated by irradiating resonance E. Spectrum c shows the NOEs obtained when resonance S is irradiated.

splittings corresponds to the expectation based on the assignments of A to U82, Q2 to G81, and P1 to U85 (see Figure 7). At 283 K, the amplitude of resonance P approximately doubles, as expected. The "new" component of P is not split by the labeling of strand III (data not shown). The NOEs given at 283 K (Figure 4c) include both split components seen at 303 K (Figure 4b), and, in addition, NOEs are seen to unsplit resonances at 10.52 ppm (R2) and 13.17 ppm (T), connectivities characteristic of P2 (Kime & Moore, 1983b).

Clearly, neither of the imino protons in the R2-P2 GU originates in strand III nor does the GC imino proton, T, to which this GU relates. Thus, none of these resonances can be assigned to the helix IV–helix V region of fragment 1. Examination of the sequence of fragment 1 shows that the only GU, GC juxtaposition outside of helix IV–helix V is at G9-U111 in helix I. R2, P2, and T must correspond to U111, G9, and G10, respectively. The suggestions made for the assignment of these resonances in 1984 are disproven.

S to H NOE. That S cannot be U111 or G9, as already concluded, is evident in Figure 3a. Resonance S is split when strand III is ^{15}N labeled (see Figure 3a). It follows that the S to H NOE mentioned earlier must not involve H (G112) but must be to H′, the anomalous UN3 resonance which overlaps H.

Direct evidence for an H′ to S NOE has been provided by experiments done on (unlabeled) fragment 1 in buffer containing Ca^{2+} in place of Mg^{2+}. In this buffer, the downfield spectrum differs slightly from the one seen in Mg^{2+}, as can be appreciated by comparing Figures 1a and 5a. Shown also in Figure 5 are two of the NOEs given by fragment 1 in Ca^{2+}, the ones seen when E and S are presaturated. E connects to M, as usual, and to a resonance at 12.75 ppm, by definition H, the resonance of the GN1 of G112. S, however, gives an NOE to a resonance at 12.55 ppm. Thus, the resonance to which S relates by NOE is not the same as the one which follows E in helix I, which is H.

S also gives a conspicuous aromatic NOE in Ca^{2+}-containing buffers. This NOE is hard to observe in Mg^{2+}-containing buffers at pH 7, but at acid pHs, in Mg^{2+}, it is often as strong as the aromatic NOE of an AU base pair. This aromatic resonance is not yet assigned, but it cannot represent a purine C8 proton. This was demonstrated by examining the NOEs given by fragment 1 after labeling it uniformly with ^{2}H at the purine C8 position. These samples were generated by growing cells on minimal medium in the presence of large quantities of adenosine and guanosine labeled with ^{2}H at C8 by exchange. The data showed C8 labeling in 5S RNA at levels greater than 80% but no corresponding decrease in the intensity of the S to aromatic NOE.

Assigning Resonance X. To prove that the resonance to which S connects is H′, difference decoupling experiments were done on fragment molecules labeled with ^{15}N in strand III only, and in strands II and IV only, both in Mg^{2+} and in Ca^{2+}. Figure 6 shows the result. These spectra display only those resonances whose splitting was perturbed by the energy applied to the sample at ^{15}N frequencies during acquisition of ^{1}H FIDs. (A fully decoupled resonance in such a spectrum has a central, negative feature at the chemical shift of the decoupled resonance, flanked by two positive, half-amplitude features having the chemical shifts of the undecoupled doublet.) The spectra in Figure 6 were taken with the decoupler set at frequencies appropriate for decoupling resonances X and H′. Spectrum a was given by ^{15}N-labeled strand III fragment, and spectrum b is of fragment labeled with ^{15}N in strands II + IV, both in the presence of Mg^{2+}. Clearly, resonance H′ at 12.72 ppm, the more downfield of the H′, X pair, belongs to strand III, and X at 12.60 ppm belongs to strand II.

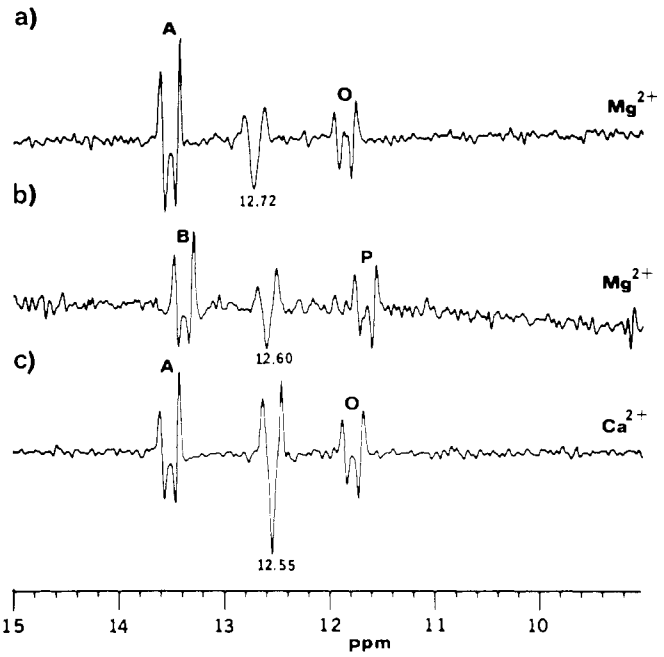

FIGURE 6: Difference decoupling spectra of ^{15}N-labeled samples of fragment 1. Each of the spectra shown was accumulated under the ionic conditions and temperature specified in the legend for Figure 4 except where noted. The spectra were obtained by subtracting a ^1H spectrum obtained while decoupling the sample at ^{15}N frequencies from a similar spectrum accumulated with the decoupler off-resonance. A fully decoupled resonance in such a spectrum has a central negative peak flanked by two positive peaks of intensity $^1/_2$. Resonances are identified in the usual way to assist the reader. Spectrum a shows the difference for a sample of fragment 1 labeled with ^{15}N in strand III when it is decoupled at the resonant frequency of the UN3 ^{15}N's of resonances H′ and X (138.0 ± 0.3 ppm). Spectrum b shows the result of the same experiment done using a fragment 1 sample labeled in strands II and IV only. Spectrum c was accumulated by using the same sample as the one used in spectrum a, under the same conditions, except that all the Mg^{2+} in the specimen had been replaced with Ca^{2+} by dialysis.

Figure 6c shows the difference decoupling spectrum for ^{15}N-labeled strand III fragment 1 in Ca^{2+}. While the chemical shifts of A and O are close to what they would be in a Mg^{2+}-containing buffer, H′ moves upfield to 12.55 ppm, the chemical shift to which S relates by NOE under these conditions (see Figure 5). Thus, the NOE previously interpreted as an H to S NOE is certainly an H′ to S NOE.

The chemical shifts and line widths of both H′ and X do not depend on whether or not fragment 1 is cleaved at residues 87, 88, or 89 (Jarema & Moore, 1986) which makes it improbable that X is U89. Further, it is most unlikely that U120 contributes anything to the downfield spectrum of fragment 1 since there is no base opposite U120 with which it can pair. In addition, the U's in helix I, U5 and U111, are both firmly assigned. Thus, resonance X must represent U103, the only unassigned interior U in the strand II + strand IV part of the molecule.

As for H′, the unassigned U's in strand III at this point are U74, U77, and U87. U87 is ruled out by its terminal position in the molecule; it should contribute nothing to the spectrum. Thus, H′ must be either U74 or U77. Since H′ and S both reside on strand III, and are related by NOE, it is likely, but not proven, that S is either G75 or G76.

RNA–RNA NOEs in Partially Labeled Fragment 1 Molecules Bearing L25. Our initial investigations of the downfield spectrum of the L25–fragment 1 complex led to the discovery of six NOEs involving numbered resonances (Kime & Moore, 1983c). One of these, an NOE identified as "7 to G", is now

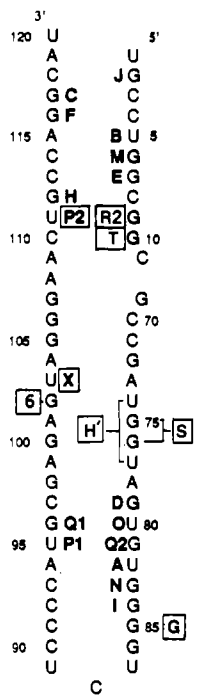

FIGURE 7: Assignments in fragment 1. This figure shows assignments which can be made in the spectrum of fragment 1 on the basis of the data reported in this paper and all prior information. The brackets connected with resonances H′ and S indicate the 2-fold uncertainty which still surrounds their placement. The boxed assignments are those resulting from data presented in this paper. Resonance assignments placed outside the model are still regarded as tentative. Assignments inside the model are regarded as firm. The sequence numbering is that of intact 5S RNA. The helix designations are given in Roman numerals.

explained. Resonance 7 has a chemical shift close to that of resonance S in the uncomplexed fragment. Like S, 7 is a GN1 resonance from strand III. Furthermore, when L25 complexes with fragment, resonance H′ moves downfield and overlaps with resonance G (Kime, 1984b; Jarema & Moore, 1986). The "7 to G" NOE in the complex must be the same as the H′ to S NOE in the free fragment. A second L25–fragment NOE, "2 to 8", involves protein on at least one side and cannot be interpreted in terms of RNA structure alone.

NOE experiments were done on samples of complex made with partially labeled fragment 1. The NOE involving 3 and 1 could not be studied due to the extremely weak intensity of 1 in these samples. The NOE reported between 6 and "I" was observed in samples containing strand III in ^{15}N-labeled form. The "I" resonance was unsplit, proving that it is not I which gives the NOE in question but X which resonates at the same frequency in the complex. In fact, an I to 6 NOE is not permitted by the sequence; 6 is a strand II resonance, and the only G's in the vicinity of the base responsible for I, G84, are on strand III. Resonance 6 is tentatively assigned to G102 by virtue of the assignment of X to U103. (It should be noted that the strong imino proton NOE which might be expected from 6 due to the U which lies opposite it in the sequence has not been detected.)

Discussion

Figure 7 summarizes the fragment 1 assignment information available at this time. Assignments made on the basis of data presented in this paper are boxed to distinguish them from those established earlier. Four new assignments are fully proven by experiments described above, the assignments of P2, R2, T, and X. Another resonance, H′, is assigned to within

a 2-fold ambiguity. Strong hypotheses for the assignments of S, 6, and G can also be advanced.

The assignment of P2, R2, and T extends helix I to include base pairs G9-U111 and G10-C110, consistent with phylogenetic data which show that all eubacterial 5S RNA sequences accommodate base pairs in these positions (Delihas et al., 1984). Resonances in the terminal stem (helix I) of 5S RNA are unaffected by L25 binding while resonances in helices IV and V (see Figure 7) are altered with respect to both chemical shift (Kime & Moore, 1983c) and proton exchange behavior (Leontis & Moore, 1986b). The fact that P2, R2, and T do not respond when L25 binds makes them "helix I like", consistent with the assignments just made.

The instability of the last two base pairs in helix I (R2, P2, T) in fragment 1 can be ascribed to their location at the end of a helix next to the 3′ terminus of one of its strands. It is interesting that the same base pairs are also not observable at 303 K in intact 5S RNA, where there is no covalent discontinuity. The structure of the junction where helices I, II, and V join must be incompatible with stable base pairing at the junction end of helix I.

Resonance H′ has been localized to the center of helix V, but a 2-fold ambiguity remains which cannot be satisfactorily resolved at this point. If H′ is assigned to U74, it is likely that S corresponds to G75, and the aromatic NOE of S can be explained as representing the C2 proton of A101 which lies opposite it. This assignment for H′ suggests there should be a GN1 imino proton resonance for G102, the base opposite U74 in the standard model. There is no evidence for such an NOE. On the other hand, if H′ is assigned to U77, S then is probably G76 and is opposite G100. In that case, the aromatic NOE of S is inexplicable. Moreover, H′ should then be part of an AU base pair, and an aromatic NOE should be seen whose existence is doubtful. Clearly, there are problems in helix V!

H′, S, and X, the three resonances we are certain belong to helix V, are all notable for their unusual chemical shifts and their broad line widths. X, the imino proton of U103, lies opposite A73 in the standard model for 5S RNA secondary structure [see Delihas et al. (1984)], yet it resonates at 12.60 ppm, far from other AUs' imino protons, and does not give the expected AH2 aromatic NOE. H′ is either an AU or a GU. It is "unsatisfactory" in either role for the reasons given in the preceding paragraph. The chemical shift of S, the last of the helix V trio, is so far upfield that it can hardly be considered hydrogen bonded at all. All of these observations point to the fact that helix V is not composed of a number of "ordinary" base pairs interspersed with a few AG and GG mismatches.

The difficulties we face in assembling a coherent picture of the structure of helix V from the available data raise questions as to whether the standard model correctly describes the structure of the helix V portion of free 5S RNA. For this reason, while the assignment of S to G75 or G76 seems likely, it cannot be regarded as certain.

G is the only resonance in the spectrum of fragment 1 which responds to the status of the 87, 88, 89 loop. When the loop is unbroken, G is a full-weight resonance, as conspicuous as F, for example. In cleaved samples, G is much weaker (Kime et al., 1984). The samples of fragment looked at here contain a broad GN1 resonance at the chemical shift of G, G′. Since G is flanked closely by resonances F and H, it could "disappear" from the spectrum by increasing its line width 2- or 3-fold. G′ and G could be the same resonance. It is tempting to suggest that G (G′) is G85's imino proton resonance, which would provide a mechanism to explain its sensitivity to the status of the loop. The G to I NOE this assignment requires would be hard to observe because of their similarities in chemical shift, explaining why it has not been detected.

Within the fragment 1 spectrum, we believe there are six resonances remaining to be assigned, the substoichiometric resonances K and L, and three of the broad upfield resonances which contribute to R. K and L are both GN1 resonances originating in the strand II side of the helix V region. Neither has any known NOEs. Could they represent G105 and G106? The R complex includes three resonances in addition to R2 which is already assigned (see below). All the R resonances represent GN1 protons from outside strand III.

There are only six unassigned G residues outside of strand III, all of them in the helix V region, and there appear to be five resonances to assign to them. In addition, all the internal U's in the helix IV–helix V region on strand II have been assigned. Strand III, on the other hand, has six imino proton-bearing residues in helix V. Two resonances (H′ and S) have been identified as coming from that region, leaving four imino protons (including the terminal G, G69) unaccounted for. Thus, it is clear that despite a sequence inconsistent with a normal, Watson–Crick double helix, and ample spectroscopic evidence that its structure is "unusual", helix V manages to protect its imino protons from exchange with solvent to a remarkable degree. The resistance of this part of the molecule to chemical and enzymatic attack also suggests that whatever its structure the helix V region is far from unstructured single strand [see Kjems et al. (1985)].

The effects of the interaction of L25 with fragment 1 on the latter's downfield spectrum fall into two categories: (1) changes in chemical shift on the part of existing resonances, and (2) the appearance of complex-specific resonances (Kime & Moore, 1983c). The chemical shift changes include all the NOE-assigned resonances in helix IV, except N, and at least resonances H′ and S in helix V.

In the original study on this system, eight resonances in the spectrum of the complex were identified as imino proton resonances unique to it. All of these resonances, except two, were positively identified as to base type by the experiments done on uniformly ^{15}N-labeled fragment 1 (Kime, 1984b). These data and the existing NOE information were assembled into a working hypothesis for the assignment of several of the resonances unique to the complex (Jaerma & Moore, 1986). The suggestion was made that G85 is 6, C86 is 3, and U87 is 1, consistent with the data in hand at the time, but inconsistent with the information presented above. Resonance 6 does not belong to strand III, as it must if it is to correspond to G85.

The true number of complex-specific RNA resonances is considerably smaller than eight. Two new findings are pertinent here. (1) Resonance 2 has now clearly revealed itself as a complex-specific resonance of protein L25, not fragment 1. Its identity is of great interest; its chemical shift suggests that it could represent an exchangeable L25 proton hydrogen bonded to an RNA base. Whatever the case, it no longer needs to be accommodated in schemes for the internal hydrogen bonding of the fragment. (2) It is certain that resonance S and resonance 7 represent the same imino proton as already noted.

A striking difference between the spectra of the free fragment and of complex is that the intensity seen in the R region of the free molecule is dispersed in the spectrum of the complex. R2, the upfield component in the free molecule, remains

at its accustomed position. Around it appear three numbered resonances, 4, 5, and 6. Resonances 4 and 6 are strand II resonances, like the R1 resonances. (Resonance 5 could not be identified.) As already noted, there are six G's in strand II whose imino proton resonances are yet to be assigned and five fragment 1 resonances to assign to them. Resonances 4, 5, and 6 cannot all be new resonances unique to the L25 complex. It is extremely likely that they represent the same imino protons that together account for resonance R1 in the free fragment. [That the number of R components is probably four is indicated by the fact that at pH 5, four components can be resolved in the R region (M. J. Kime and P. B. Moore, unpublished observations). In addition, T_1 relaxation experiments done on fragment 1 show the presence of at least three components differing in relaxation rate in the R region (N. B. Leontis and P. B. Moore, unpublished observations).]

The only truly "new" imino proton resonances created by the interaction with L25 are 3, 8, and 1. Resonance 1 is an extremely weak UN3 resonance which cannot be assigned as to strand at this time. Resonances 8 and 3 are UN3 and GN1 imino protons, respectively belonging to the helix V region of strand III, the only part of helix V where there are any "vacancies". Resonances 8 has to be the U in strand III which H′ is not. If H′ is U74, 8 must be U77; if H′ turns out to be U77, 8 will have to be U74. There are only four G's in the strand III portion of helix V and two resonances to assign to them, S and 3.

What these considerations reveal is that when L25 binds to 5S RNA, the already remarkably well-hydrogen-bonded structure of helix V must "close up" even further, rendering virtually every imino proton in the helix IV–helix V stem sufficiently well protected from solvent exchange to be observed spectroscopically. This qualitative conclusion is consistent with a recent quantitative study showing that L25 binding reduces the imino proton exchange rate of many bases in this same region (Leontis & Moore, 1986b). Footprinting studies have identified this part of the structure as the binding site for L25 (Huber & Wool, 1984).

Among the NOEs observed involving numbered resonances in the L25 complex, there is one reported as "I" to 6 (Kime & Moore, 1983c). The observation that 6 is a strand II resonance and that its NOE is to X, not to I, suggests an assignment for 6. Resonance 6 is likely to be the GN1 proton of G102. It is interesting to note that studies with the (strand I + strand IV) reconstituted molecule mentioned earlier give an apparent R1 to I NOE (Kime et al., 1984) which is not seen in native fragment 1 but which can be satisfactorily explained as a manifestation in free RNA of the X to 6 relationship (D. T. Gewirth, unpublished observation). The qualifications expressed above about the assignment of S, however, hold for the assignment of 6 as well.

In summary, the experiments reported above have provided a positive assignment for resonance 7 in the complex and a probable assignment for resonance 6. They show that 2 is a protein resonance and put useful restrictions on possible assignments for the other resonances unique to the L25 complex. The unassigned, complex-specific NOEs must reflect juxtapositions within helix V. Site-directed mutagenesis experiments are now under way to resolve the remaining assignment problems both in free fragment 1 and in its complex with L25.

The experiments presented above also show that the utility of ^{15}N labeling in studies of the imino proton spectra of nucleic acids can be considerably enhanced by restricting the label to one of the two strands of a duplex. This kind of partial labeling should be possible in many systems.

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