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Effects of Mutation on the Downfield Proton Nuclear Magnetic Resonance Spectrum of the 5S RNA of *Escherichia coli*†

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ABSTRACT: The imino proton spectra of several mutants of the 5S RNA of *Escherichia coli* are compared with that of the wild type. Three of the variants discussed are point mutations, and the fourth is a deletion mutant lacking bases 11–69 of the parent sequence, all obtained by site-directed mutagenesis techniques. The spectroscopic effects of mutation are limited in all cases, and the differences between normal and mutant spectra can be used to make or confirm the assignments of resonances. Several new assignments in the 5S spectrum are reported. Spectroscopic differences due to sequence differences permit the products of single genes within the 5S gene family to be distinguished and their fates followed by NMR.

Site-directed mutagenesis is proving to be a powerful tool for investigating the relationship of primary structure to function in ribosomal RNAs (Goringer et al., 1984; Gregory et al., 1984; Stark et al., 1984; Jemiolo et al., 1985; Christiansen et al., 1985; Goringer & Wagner, 1986; Meier et al., 1986; Gregory & Zimmermann, 1986). The application of site-directed mutagenesis methods to the solution of a specific problem having to do with the physical properties of the 5S RNA of *Escherichia coli* is discussed below.

The downfield resonances in the proton spectra of nucleic acids dissolved in water, between 10 and 15 ppm, represent

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base imino protons protected from rapid exchange with solvent, usually by hydrogen bonding (Kearns & Shulman, 1974). Thus this region of the spectrum contains information about the secondary structure and any tertiary hydrogen bonding of a nucleic acid molecule. In order to make use of that information, however, the resonances the downfield spectrum contains must be assigned, yet a significant number of resonances in the downfield spectrum of the 5S RNA of *E. coli* cannot be assigned by spectroscopic methods [see Gewirth et al. (1987)]. The experiments reported below demonstrate that assignment problems of this kind can be resolved through the study of appropriate mutant RNAs.

The downfield spectra of three sequence variants of the 5S RNA from *E. coli* are compared with that of the wild type. Two of them have altered sequences at the 3–117 position of the terminal stem of 5S RNA (see Figure 1b). Since these mutants fall in a region of the molecule whose resonances are already assigned, their spectra illustrate the consequences of mutation. The third variant is a deletion of the entire helix II–helix III stem of the molecule, bases 12–69. This molecule closely resembles the principal RNase A resistant fragment of 5S RNA (Douthwaite et al., 1979). Several new downfield assignments are made on the basis of the spectroscopic differences between this molecule and the fragment produced by RNase digestion. The data also show that the spectral alterations generated by mutation can be used as a means for following the fate of the products of a single gene belonging to a multigene family.

Materials and Methods

Strains and Plasmids. *E. coli* strains HB101 (Bolivar & Backman, 1979) and JM101 (Messing, 1983) were provided by Mark Nichols. *E. coli* strains RZ1032 (dut–, ung–, F+, supE44, tetR,) and CJ236 [dut–, ung–, pCJ105 (=F′CmR)] were donated by Dr. Catherine Joyce. M13mp18 was purchased from BRL, and plasmid pKK5-1 was the gift of Drs. Harry Noller and Jurgen Brosius (Brosius, 1984).

Enzymes and Reagents. *E. coli* DNA polymerase I (Pol I), large fragment (cloned), was the generous gift of Dr. Catherine Joyce. T4 DNA ligase was obtained from New England Biolabs, and T4 polynucleotide kinase was purchased from International Biotechnologies, Inc. Oligodeoxynucleotides were synthesized for us on an Applied Biosystems Model 380B DNA synthesizer by Aino Ruusala, for whose help we are grateful. Oligonucleotides were purified by gel electrophoresis.

Construction of Mutant Plasmids. The 0.5-kb *Hin*dIII fragment containing the rrnB 5S RNA gene was excised from pKK5-1 and ligated into the *Hin*dIII site of M13mp18. Recombinant phage were grown on JM101 and the progeny used to infect *E. coli* RZ1032 or CJ236 at a multiplicity of infection of less than 1. The resulting phage were collected and phenol was extracted to yield single-stranded DNA (ssDNA) that contains uracil in place of thymine at a low level (Kunkel, 1985). Mutants were produced from this template essentially by the method of Smith and Gillam (1981). A phosphorylated DNA oligonucleotide containing the desired mutation was annealed to the ssDNA template, extended with Klenow fragment, ligated, transfected into JM101, and plated with a JM101 lawn. A few of the resulting plaques were picked, and the single-stranded DNA extracted from the phage was sequenced to select one that contained the desired mutation.

The replicative form of such an M13 mutant/recombinant was then digested with *Hin*dIII, and the 0.5-kb fragment containing the mutation was ligated into the 5.6-kb *Hin*dIII fragment from pKK5-1 containing the rrnB promotors P1 and P2. The resulting recombinants were then transformed into HB101 and the cells plated in the presence of ampicillin. Resistant colonies were picked and screened for correct structure by restriction endonuclease digestion, and their sequences were verified by double-stranded DNA sequencing.

The following plasmids were constructed in this work: pDG03 (A117-C3), pDG04 (G117-U3), pDG05 (A117-U3), and pDG07. The first three plasmids are identical with pKK5-1 except for the mutations in the 5S sequence indicated in parentheses. (The wild-type sequence is G117-C3.) In pDG07 bases 11–68 of the normal 5S sequence are deleted, and bases 1–10 are joined to bases 69–120 via a three-base bridge whose sequence is 5′-UUC-3′.

Production and Purification of 5S RNA. The method used for production of 5S RNA from cells containing pKK5-1 in the presence of chloramphenicol has been described before (Kime & Moore, 1983b). 5S RNAs containing point mutations were prepared the same way. Similar methods were used to prepare "cytoplasmic" 5S RNA from cells that had not undergone overproduction. By cytoplasmic we mean that 5S RNA which is not incorporated into ribosomes. 5S RNA was prepared from ribosomes as described earlier (Kime & Moore, 1982). The deletion mutant product, "pDG07 fragment", was purified from crude postribosomal RNA by HPLC[1] on a Nucleogen DEAE 500-10 column (Macherey-Nagle) using a gradient of 0.4–0.7 M NaCl in 20 mM cacodylate, pH 6.0, 2 mM magnesium acetate, and 20% CH_3CN at 30 °C.

Fragment 1 was purified from 5S RNA preparations digested with RNase A at 4 °C for 45 min at an enzyme to RNA ratio of 1:100 as described previously (Kime & Moore, 1983a).

NMR Methods. Samples were prepared for spectroscopy by dialysis into 0.1 mM KCl, 4 mM $MgCl_2$, and 5 mM cacodylate, pH 7.2. All samples were about 1 mM in concentration unless otherwise noted. D_2O at 5% concentration was used for the spectrometer lock, and chemical shifts were measured relative to *p*-dioxane, which was included in all samples at about 1 mM. The chemical shift of *p*-dioxane was assumed to be 3.741 ppm relative to the methyl resonance of 3-(trimethylsilyl)-1-propanesulfonic acid.

All spectra were obtained in the Fourier transform mode on the 490-MHz NMR spectrometer at the Yale University Chemical Instrumentation Center. Spectra of exchangeable imino protons were obtained by using the twin-pulse method (Kime & Moore, 1983a), to avoid excitation of the solvent resonance, and alternate delay accumulation (Roth et al., 1980), to overcome computer word-length limitations. One-dimensional spectra were taken in 8K or 32K blocks with a spectral width of 20000 Hz, and the offset was placed at about 15 ppm. All spectra were acquired at 303 K.

Most of the NOEs between imino proton resonances described below 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 s with the decoupler power adjusted to give 50–70% saturation. NOE difference FIDs were subjected to Lorentzian-to-Gaussian multiplication prior to transformation to improve resolution.

Two-dimensional NOE (NOESY) spectra of the downfield region were obtained in both the magnitude and phase-sensitive modes (Macura & Ernst, 1980; Haasnoot et al., 1983; Hare et al., 1985, 1986). The offset was placed in the middle of the imino proton region about 3700 Hz downfield of water and

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; FID, free induction decay; NOESY, nuclear Overhauser enhancement spectroscopy.

the sweep width reduced to fold the upfield buffer resonances into the aromatic region. Each 90° pulse in the NOESY sequence was a 45°-t-45° twin pulse with the delay, t, adjusted to optimize water suppression. A mixing time of 0.2 s was used, the total cycle time was 0.4 s, and spectra were collected in 2K blocks. Phase cycling was performed according to States et al. (1982). For each real and imaginary FID, 288 scans were collected, and 331 t_1 points were collected.

The NOESY data were processed by using the FTNMR program of Dennis Hare. The data were apodized in t_2 by using an unshifted, skewed sine bell 400 points in length and in t_1 by using a skewed sine bell 331 points in length following zero-filling to 1024 points. The two-dimensional matrix was symmetrized by taking the geometric mean of points on either side of the diagonal.

Ring Current Shift Calculations. Ring current shifts were calculated according to the theory of Johnson and Bovey (1958). The ring current intensities and base geometry parameters used were those of Giessner-Prettre et al. (1976). The RNA geometry employed was that of A-form RNA derived by Arnott and Chandrasekaran (personal communication). The shifts calculated in this way are similar to those obtained previously by Arter and Schmidt (1976), Kearns (1976), and Robillard (1977). The intrinsic shift of the UN3 proton of an AU base pair was taken to be 14.35 ppm, and that of the GN1 proton of a GC base pair was assumed to be 13.54 ppm (Robillard, 1977).

RESULTS

Selection of Mutants. Because *E. coli* contains several 5S cistrons of its own, there is no simple way to detect the presence of plasmids carrying mutant 5S genes in transfected cells other than DNA sequencing. Moreover, phenotypically silent variants of the 5S sequence are often as interesting spectroscopically as the ones that might be selectable under more favorable circumstances. For these reasons, the uracil-containing template method of Kunkel (1985) that reduces the probability of recovering unmutated products in M13 cloning experiments was extremely useful. Of the plaques we selected for sequencing, 30–80% were mutant. The ssDNA from only a handful of transfectants needed to be sequenced to find the desired mutation.

Overproduction of Mutant RNA. pKK5-1 and its derivatives express their ribosomal RNA sequences constitutively. The yield of total cytoplasmic 5S product varied from one construct to the next, however. The largest amount of 5S RNA was produced from cells harboring plasmid pKK5-1 (G117-C3) (the wild type), followed in order by those harboring pDG05 (A117-U3), pDG04 (G117-U3), and pDG03 (A117-C3). The production of the A117-C3 product was so poor that spectroscopic characterization was not possible. Typically, we obtained 4.0 mg of 5S RNA/g of HB101/pKK5-1 vs. 2.8 mg/g from HB101/pDG05, 2.5 mg/g from HB101/pDG04, and 0.6 mg/g from HB101/pDG03.

These differences in yield do not seem to be related to variation in the amplification of the different plasmids. Plasmid DNA levels were checked in cells containing the most efficient production plasmid, pKK5-1, and the least efficient, pDG03. Visual inspection of agarose gels stained with ethidium bromide indicated that the quantities of plasmid DNA recoverable from equal amounts of cells were the same in the two strains, both before and after exposure to chloramphenicol. The amplification during growth in chloramphenicol was about 40-fold in both cases.

The T1 terminators of both plasmids were sequenced in the course of characterizing the mutants initially. They were normal. Transfer of the plasmids of hosts other than HB101 had no effect on the differences in production level. The differences in production efficiency, therefore, could reflect either an accidental divergence of promoter sequences or differences in the way the transcripts of these mutant genes are processed. The latter hypothesis is attractive since there seems to be a relationship between the strength of the pairing at the 3–117 position and the amount of product obtained.

Comparison of the NMR Spectra of Wild-Type 5S RNA and the U3-A117 Mutant. Because the mutants of interest all lie in the fragment 1 portion of the molecule (bases 1–11 and 69–120) (see Figure 1b), it was convenient to compare them spectroscopically by using fragment 1 preparations rather than the whole 5S molecule. The downfield spectrum of fragment 1 is simpler than that of intact 5S RNA because its molecular weight is lower, but the two are closely related (Kime & Moore, 1983a,c).

Figure 2 shows the downfield spectrum of fragment 1 derived from HB101/pDG05 (A117-U3) (b), that of wild-type fragment 1 (a), and the difference between them (c). Positive features in the difference spectrum represent mutant resonances that are not found in the wild-type spectrum, and negative features represent the reverse. Difference spectra were also obtained from comparison of the spectra of intact, wild-type and mutant 5S RNA. The pattern of negative and positive features observed was similar to that in Figure 2c. Thus the spectral effects of this mutation are confined to the fragment 1 portion of 5S RNA as expected.

During overproduction the 5S RNA genes of both the host bacterium and the plasmid are active. The result is that none of the resonances shift or disappear completely in the mutant spectrum; each leaves behind a residual resonance at the normal chemical shift. The intensities of the residual resonances suggest that 10–20% of the 5S RNA in the postribosomal supernatant of cells carrying pDG05 is the product of chromosomal 5S genes under overproducing conditions.

Spectroscopic Analysis of the A117-U3 Mutant. Base pair 3–117 lies within the helix I region of 5S RNA. The downfield resonances that originate from this helix have been assigned in the wild-type spectrum by a combination of techniques, the most important of which is the nuclear Overhauser effect (NOE) method (Kime & Moore, 1983b; Kime et al., 1984; Gewirth et al., 1987). The names given the resonances in the wild-type spectrum are shown in Figure 4a, and the assignments established for them are shown in Figure 6.

NOE experiments were done on the mutant spectrum to assign its resonances with the results summarized in Figure 2. Only those resonances are identified whose chemical shifts were affected by the G117-C3 to A117-U3 mutation. The structure of the helix I region is retained in the mutant as evidenced by the fact that the NOE connectivities of its base-pair imino protons are preserved. Resonance C, which corresponds to G117-C3 in the wild-type sequence, disappears in the mutant and is replaced by a new AU resonance at 14.29 ppm, resonance C′ representing A117-U3. The chemical shifts of the imino proton resonances of several of the base pairs next to the 3–117 position are altered: F (G116-C4) by a great deal, B (A115-U5) by a little, M (G6-C114) just detectably, and J (G2-C118) not at all.

Spectrum of the U3-G117 Form of 5S RNA. The downfield spectrum of the mutant 5S RNA fragment containing a U at position 3 in place of a C is shown in Figure 3 and compared with the wild-type spectrum as before. Clearly, the mutant's spectrum has two new resonances in the GU region, one at 12.21 ppm and the other at 11.31 ppm. Again, the new resonances are substoichiometric. The overproduced 5S RNA

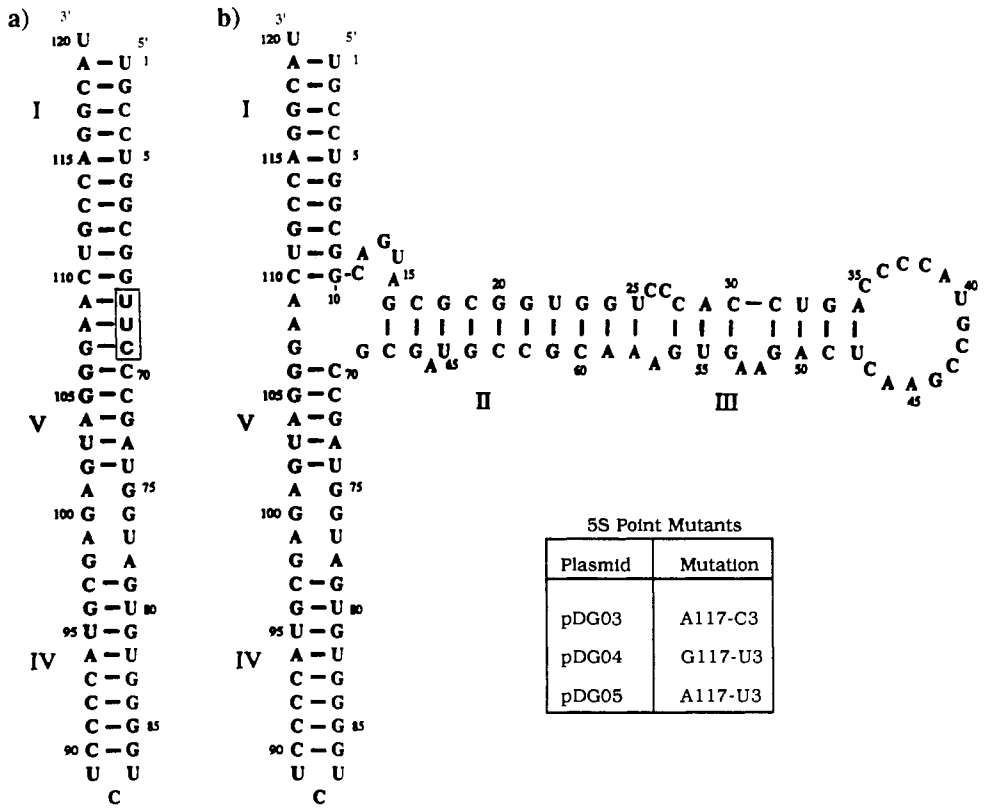

FIGURE 1: Sequences for 5S RNA and its derivatives. The sequence for wild-type 5S RNA (b) is shown in the three-stemmed secondary structure it is believed to assume [see Delihas et al. (1984)]. Sequence a depicts the pDG07 fragment. Nuclease-generated fragment 1 lacks the nucleotides enclosed in the box but includes both C11 and G69. The pDG07 fragment lacks C11 and G69 but is a single covalent polynucleotide.

population contains more host gene product than was the case with the AU mutant because of the lower efficiency of expression of this particular plasmid.

NOE experiments were carried out on the U3 mutant and led to the following findings. Resonances C′ and C″ represent a GU base pair unique to the mutant and must correspond to G117-U3. C′ and C″ connect to F and J resonances at altered chemical shifts, and the connectivity of helix I is again preserved. The perturbations in chemical shift extend past resonance M (G6-C114) to resonance E (G7-C113), which is four base pairs past the site of the mutation.

Production of a Fragment 1 like Molecule in Vivo. A 5S deletion mutant containing helices I, IV, and V was made by the methods used for making point mutations. The sequence of the molecule that is the product of the deleted gene is shown in Figure 1a. It lacks C11 and G69 but includes a UUC sequence to bridge the gap between G10 and C70, the region deleted. This molecule is called the "pDG07 fragment" to distinguish it from fragment 1, the nuclease product that it closely resembles in sequence.

The low molecular weight RNA obtained from the post-ribosomal supernatants of cells carrying pDG07 treated with chloramphenicol is about one-third pDG07 fragment and two-thirds tRNA. The mixture can be separated satisfactorily by HPLC techniques (see Materials and Methods). Fractionation by acrylamide gel electrophoresis showed that the pDG07 fragment prepared in this way is free of contaminating tRNA and that it binds ribosomal protein L25 just as fragment 1 does (data not shown). The yield of pDG07 fragment is about 5 mg/g of cells, an extraordinary overproduction.

Spectroscopy of the pDG07 Fragment. Figure 4 shows the downfield proton NMR spectrum of the pDG07 fragment RNA (b) and of the wild-type fragment 1 RNA (a). While the spectra show obvious similarities, there are also significant differences. The spectrum of the pDG07 fragment spectrum has more resonances in it, but fewer of them are substoichiometric.

The two-dimensional, phase-sensitive NOESY spectrum of Figure 5 displays many of the NOE connectivities within the imino proton region of the spectrum of the pDG07 fragment. One-dimensional NOE experiments were also done. The results are summarized in Table I, and the assignments that result are indicated in Figure 6. Resonances given the same names in parts a and b of Figure 4 represent corresponding protons in fragment 1 and the pDG07 fragment, respectively.

There are three NOEs seen between resonances close together in chemical shift in the pDG07 fragment spectrum that have never been observed in fragment 1 spectra: O–P, I–G, and K–L. All three are marginal because they could represent spillover in the one-dimensional NOE experiments and are only weakly supported by the two-dimensional data when plotted at a lower contour level than in Figure 5 or processed in the absolute magnitude mode (not shown). The putative O–P NOE appears to confirm the longstanding assignment of O to U80. The G–I NOE supports the recent suggestion that G should be assigned to G85 (Gewirth et al., 1987).

Two submolar GC resonances in the fragment 1 spectrum are not observed in the spectrum of pDG07, K and L. The NOE data unambiguously demonstrate that Z3 (G107) is next to a full-strength resonance at 12.34 ppm, which we identify with resonance L in the fragment 1 spectrum. This resonance in turn appears to give an NOE to a strong resonance whose chemical shift is close to that of K in fragment 1 and which we identify with K. Because the "new" base pair responsible for Z3 in the pDG07 fragment flanks L but is a base pair removed from K, it is reasonable that L should be the more strongly affected of the pair. Thus L is tentatively assigned to G106 and K to G105.

That K and L should become strong resonances in the spectrum of pDG07 is consistent with the behavior of R2, P2,

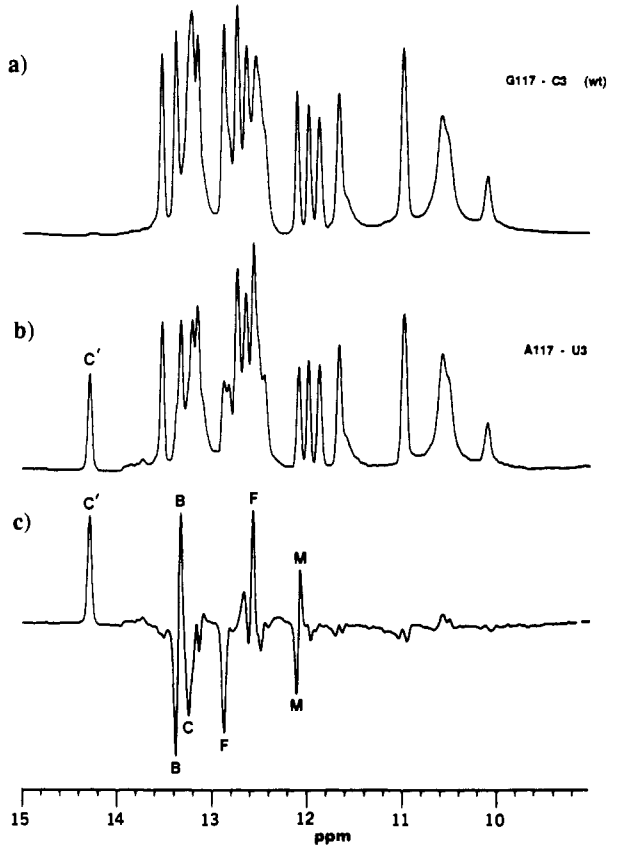

FIGURE 2: Downfield spectra of fragment 1 from pKK5-1 5S RNA and pDG05 5S RNA. Spectrum a is the downfield spectrum of wild-type (pKK5-1) fragment 1. Spectrum b is the downfield spectrum of pDG05 (A117-U3) fragment 1. Spectrum c is their difference [(b) − (a)]. Spectra were obtained as described under Materials and Methods. Only those resonances whose chemical shifts were affected by mutation are labeled. For the complete set of resonance labelings and their assignments, see Figures 4 and 6, respectively.

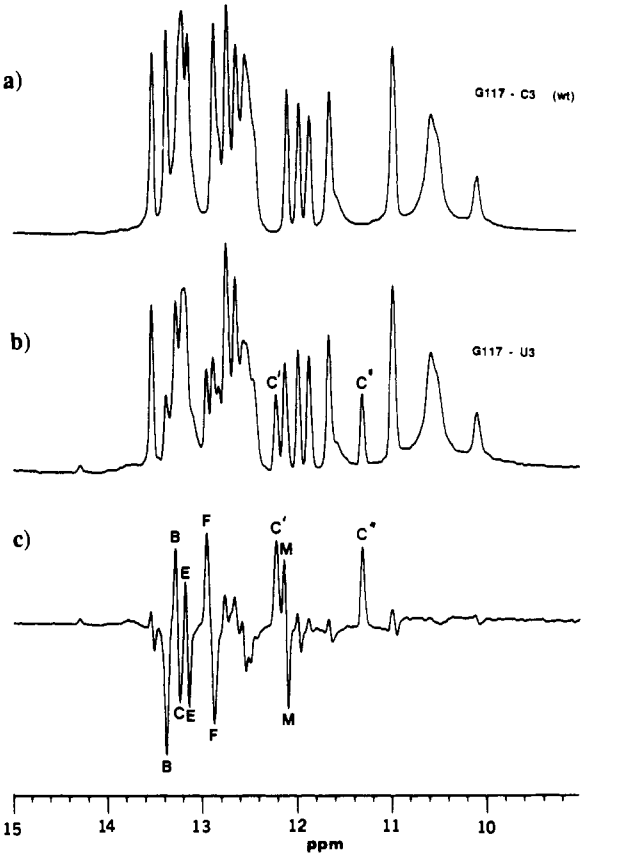

FIGURE 3: Downfield spectra of fragment 1 from pKK5-1 5S RNA and pDG04 5S RNA. Spectrum a is that of wild-type (pKK5-1) fragment 1. Spectrum b is the downfield spectrum of pDG04 (G117-U3) fragment 1. Spectrum c is their difference [(b) − (a)]. See the legend of Figure 2 for further details.

and T on the helix I side of the new base region. In normal fragment, R2 and T are broad resonances, and P2 is not observable at 303 K. Only at 283 K can P2 be seen and the NOEs between these three resonances be demonstrated. In the pDG07 fragment all three are stoichiometric at 303 K and their NOEs conspicuous.

One other observation is of interest. This spectrum shows an NOE from resonance C, D to resonance L. Resonance L is more than molar in strength, indicating that it represents two components. The only neighbor of D (G79) that carries an imino proton and which is unassigned is G98. In the case of C (G117), the neighbor not obviously identified in the pDG07 spectrum is G2, which is responsible for resonance J in the normal spectrum. We favor the view that the C, D to L NOE is evidence that J has the same chemical shift as L in this molecule and have so marked Figure 6.

In support of this view, we note that in the wild-type fragment 1 molecule the C to J NOE is well established. Furthermore, any resonance corresponding to the G98–A78 base pair would be expected to appear in the wild-type fragment 1 spectrum at the same chemical shift as the corresponding resonance in the pDG07 fragment spectrum, since their sequences are identical in that part of the molecule. There is no resonance at the L, J chemical shift in the wild-type spectrum.

The reader will note that the resonances corresponding to J and C have altered chemical shifts in the pDG07 fragment. We believe this reflects an abnormal processing of the ends of this molecule. A similar sensitivity of the chemical shifts of J and C to abnormal maturation has been noted before (Kime & Moore, 1983b).

Use of Mutants To Mark the Products of a Single Cistron in a Multicistron Family. Both the AU and GU substitutions at 3–117 result in molecules whose downfield spectra have resonances in places where the wild-type molecule has none. The presence of these molecules in mixtures that include the normal sequence is easy to recognize.

When cells are growing in logarithmic phase in the presence of 5S RNA producing plasmids, the products of both the plasmid and the chromosomal cistrons for 5S RNA can potentially be used for ribosome synthesis. It is straightforward to compare the abundances of the 3–117 mutant RNAs in the cytoplasmic 5S pool with their abundances in the ribosomal 5S pool spectroscopically.

Figure 7 compares spectra for wild-type 5S, the overproduced mutant 5S, the cytoplasmic 5S RNA pool in log-phase cells containing the A117-U3 plasmid, and ribosomal 5S RNA from the same cells. Clearly there is significant representation of mutant 5S RNA in the log-phase cellular pool.

This material gets incorporated into ribosomes, but the mutant's representation in ribosomes is less than in the cytoplasmic pool. Judging from relative peak heights, about 40% of the RNA in the pool is plasmid product, but only 30% of the ribosomal 5S RNA is mutant.

The same experiment has been done for the U3-G117 mutant (data not shown). The GU variant is incorporated into ribosomes; it is represented in the ribosomal 5S population to about the same degree as it is found in the cytoplasmic pool. Evidence for the incorporation of the pDG07 fragment into ribosomes was sought by gel electrophoresis of low molecular

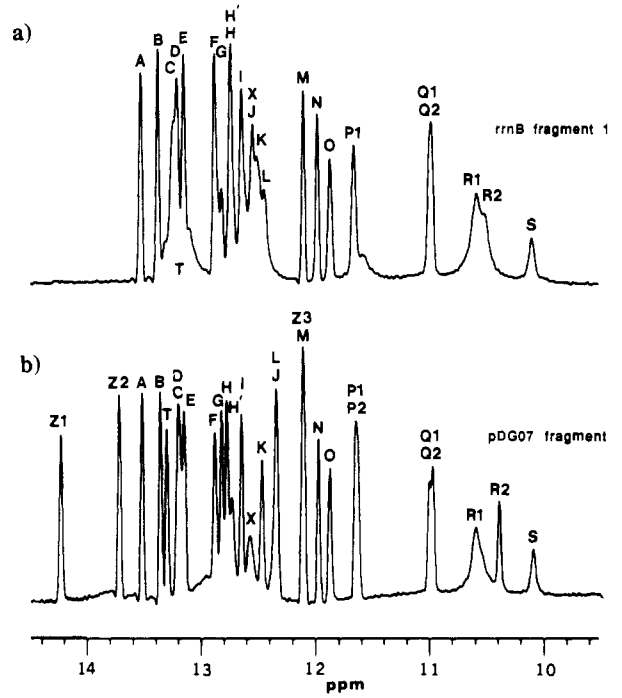

FIGURE 4: Downfield spectra of fragment 1 and of the pDG07 fragment. Spectrum a is the downfield spectrum of wild-type fragment 1. Its resonances are lettered following the convention established earlier (Kime & Moore, 1983b; Gewirth et al., 1987). Spectrum b is that of the pDG07 fragment. Its resonances are named so that resonances having the same names in the pDG07 fragment and fragment 1 correspond to homologous imino protons. Assignments for these resonances are indicated in Figure 6.

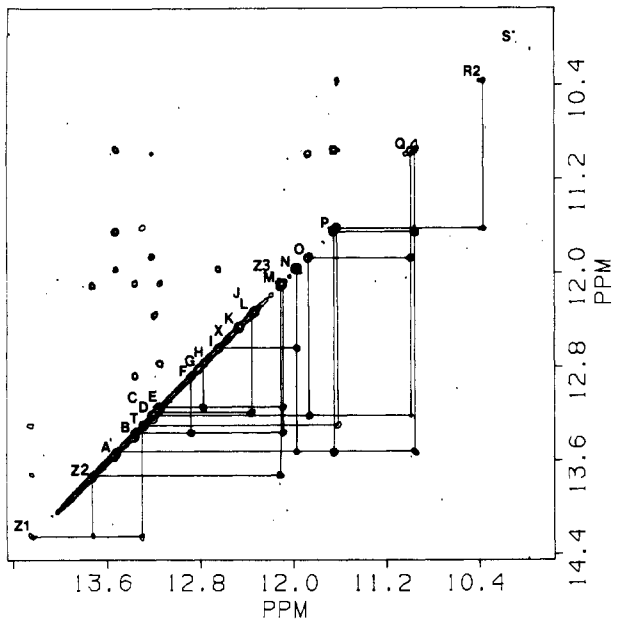

FIGURE 5: Phase-sensitive, two-dimensional NOESY spectrum of the imino proton region of the proton spectrum of the pDG07 fragment. The mixing time used for this spectrum was 200 ms, and the sample concentration was 5 mM. Otherwise, the conditions under which this spectrum was acquired and processed are as described under Materials and Methods.

weight ribosomal RNA; none was detected.

The growth of *E. coli* strains HB101 and DH1 has been examined in both the presence and absence of pKK5-1, pDG04, and pDG05. None of these plasmids affects the growth rates of these strains in broth media. Since the 5S products of these plasmids get incorporated into ribosomes, it is likely that ribosomes containing these 5S variants are

Table I: Chemical Shifts, Assignments, and NOEs in the pDG07 Fragment[a]

resonance	shift (ppm)	diff (ppm)	base	NOEs
J	12.34	−0.21	G2	(C, D), K, (M, Z3)
C	13.20	−0.04	G117	F, H, J, M, O
F	12.88	0	G116	B, (C, D)
B	13.36	−0.02	U5	F, M, 6.94 ppm
M	12.10	0	G6	B, E, (L, J), Z2
E	13.15	0	G7	F, H, J, M, O
H	12.77	0.04	G112	E
P2	11.64	0	U111	A, O, Q, R2, T
R2	10.38	−0.12	G9	T, (P1, P2)
T	13.30	0.10	G10	(P1, P2), Z1
Z1	14.23	–	"U11"	T, Z2, 7.77 ppm
Z2	13.72	–	"U12"	(M, Z3), Z1, 7.10 ppm
Z3	12.10	–	G107	B, E, (J, L), Z2
L	12.34	−0.10	G106	(C, D), K, (M, Z3)
K	12.46	−0.04	G105	(J, L)
X	12.56	0	U103	
H′	12.72	0	U74 or U77	
S	10.08	−0.01	G75 or G76	
D	13.20	−0.01	G79	F, H, J, M, O
O	11.87	0.01	U80	D, (Q1, Q2)
Q1	10.99	0.02	G96	A, D, O, (P1, P2)
P1	11.64	−0.01	U95	A, O, (Q1, Q2), R2, T
Q2	10.96	−0.01	G81	A, D, O, (P1, P2)
A	13.52	−0.01	U82	N, (P1, P2), (Q1, Q2), 7.31 ppm
N	11.97	0	G83	A, I
I	12.64	0	G84	N, G
G	12.82	0	G85	I

[a] Resonances are listed in the order dictated by their assignments to the sequence of the pDG07 fragment starting with the terminal helix and ending with the loop helix (helix IV). Chemical shifts are reported for all resonances to the nearest 0.01 ppm, which is about the accuracy with which they can be measured. The column labeled diff reports the difference in chemical shift between the pDG07 fragment and the normal fragment: diff = pDG07 − normal. The column labeled base identifies the imino proton to which the resonance in question is assigned, and the NOE column lists the NOEs seen when that resonance is saturated. Aromatic NOEs indicative of intra base pair NOEs from UN3 imino protons to AC2 protons are reported by providing their chemical shifts numerically. Bases identified in quotation marks are unique to the pDG07 fragment.

competent in protein synthesis. It is surprising that the presence of the pDG07 product did not disturb their growth rates either. One might have thought it would affect growth by scavenging L25.

Use of Mutants as Aids to Spectral Assignment. Interpreting Chemical Shift Changes due to Mutation. While the spectra of all the 5S variants examined here could be and were analyzed by NOE methods, it is interesting to ask whether studies of mutant molecules can help assign spectra in cases where NOE data are sparse or lacking altogether, a situation that arises in parts of 5S RNA.

In these experiments, base changes were made that resulted in the creation of distinctive new resonances. GUs fall in a sparsely populated part of the spectrum and give strong intra base pair NOEs; AUs usually appear well downfield and also give a distinctive intra base pair NOE. Both should be recognizable even if they give no NOEs to neighboring base pairs, as they were in the cases examined here. In order to make use of the differences between the spectra of mutant and wild-type molecules to assign resonances, however, it is not enough to be able to recognize the resonances of the newly created base pair. It is necessary to be able to identify the resonance in the normal spectrum that represents the base pair altered by mutation. The fact that several resonances corresponding to the immediate neighbors of the mutated base pair

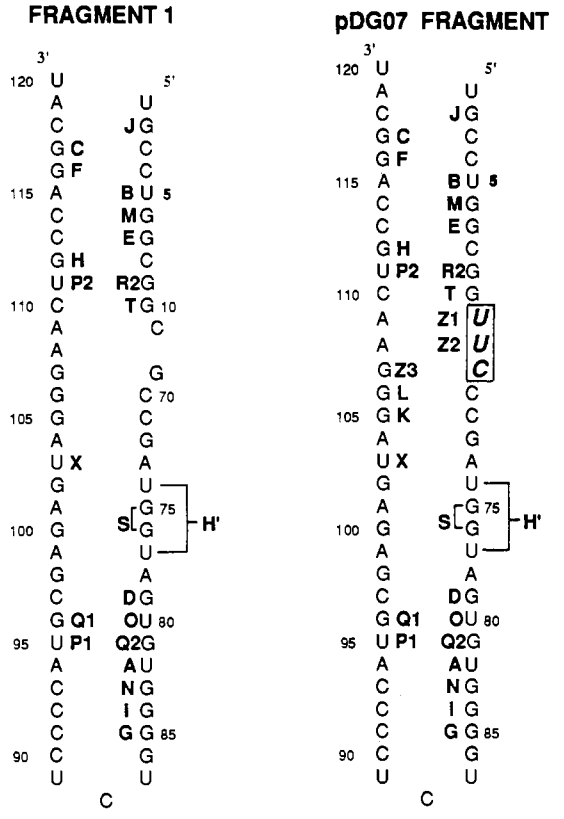

FIGURE 6: Assignments of downfield resonances in fragment 1 and in pDG07 fragment. The assignments given here depend on results reported both in this paper and elsewhere (Kime & Moore, 1983b; Kime et al., 1984; Gewirth et al., 1987).

changed their chemical shifts in the cases examined here makes this identification nontrivial.

Ring Current Shift Effects. It is convenient to think of the chemical shifts of imino protons as consisting of two components. The larger component is the chemical shift the proton has by virtue of the type of base it belongs to and the local molecular arrangement that is protecting it from solvent exchange. In most cases, the arrangement in question is a base pair. The lesser component is the net chemical shift the proton experiences due to its position relative to portions of the molecule that are near it in space but not part of the base pair to which it belongs. Since aromatic ring systems strongly perturb the chemical shifts of neighboring protons ("ring current shift" effects) and since imino protons in nucleic acids generally have other nucleotide bases as their nearest neighbors, these secondary effects reflect primarily the nature and placement of the base pairs that are its neighbors.

When a base pair in a helical region of a nucleic acid is altered by mutation, the imino proton of the newly created base pair will resonate at a chemical shift different from that of the imino proton it replaces either because their intrinsic chemical shifts differ (e.g., a G-C has been replaced by an A-U) or because their orientations in the structure are different (e.g., a G-C has replaced a C-G) or both. It is also to be anticipated that alteration of the sequence will affect the chemical shifts of imino protons in neighboring base pairs because it alters the placement and identities of the aromatic ring systems that influence their chemical shifts, even if the structure is otherwise unperturbed. Both phenomena are clearly evident in the data presented above.

Ring Current Shift Calculations. We began examining the applicability of ring current shift formalism to the problem at hand in response to the observation that resonance M's chemical shift is affected by changes three base pairs away.

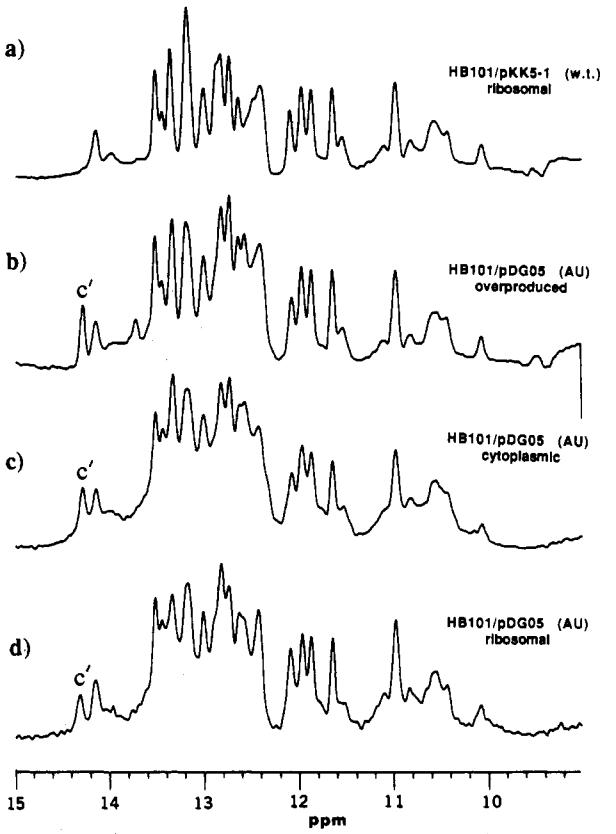

FIGURE 7: Detection of plasmid 5S RNA in ribosomes. Downfield spectra are shown for intact 5S RNA at 303 K and pH 7 as described under Materials and Methods. Spectrum a is that of wild-type (pKK5-1) 5S RNA. Spectrum b is that of the 5S RNA product obtained when pDG05 (A117-U3) is grown in *E. coli* under overproducing conditions. Spectrum c was obtained from the 5S RNA isolated from the cytoplasm of cells carrying pDG05 growing in logarithmic phase under normal (i.e., nonoverproducing) conditions. Spectrum d is that of the 5S RNA purified from the ribosomes of cells carrying pGD05 growing in logarithmic phase under normal conditions. The RNA concentration in the samples used to obtain these spectra was about 0.5 mM.

Ring current shift calculations traditionally treat only nearest-neighbor effects. Arter and Schmidt (1976) had explicitly acknowledged that second neighbors have a measurable influence [see also Giessner-Prettre et al. (1976)]. Was the effect seen in the U3-A117 mutant evidence for a structural change in the terminal stem of 5S RNA or could it be a third-order ring current shift effect?

Since none of the published computations deal with third-order effects, a program was written to make the necessary calculations based on the same approach as its predecessors. It assumes that the geometry of the environments around imino protons in real RNAs are those of the averaged structures found by fiber diffraction. It assumes in addition that intrinsic chemical shifts are invariant, which is equivalent to the statement that hydrogen-bond geometry does not vary from one example of a given base-pair type to another, and that the ring current effect of any base is independent of environment.

Table II lists the ring current shift values computed in this study for the 1983 Arnott–Chandrasekaran structure. Shift effects to third order are given. Third-order effects can be as large as 0.05 ppm or as small as 0.012 ppm. Since chemical shifts can be measured reproducibly to 0.01 ppm from one sample to the next, the change of a base at one position can have a measurable effect on the chemical shift of an imino proton three base pairs away. Therefore, the change in chemical shift of resonance M in response to mutation at the 3–117 position is not proof of a structural alteration.

Table II: Ring Current Shifts[a]

sequence 3′	sequence 5′	shift (ppm) first order	second order	third order
G	C	0.47	0.05	0.02
A	U	0.75	0.08	0.02
C	G	0.20	0.03	0.01
U	A	0.07	0.03	0.02
[A]	[U]			
U	A	0.73	0.17	0.04
C	G	0.31	0.10	0.03
A	U	0.36	0.08	0.03
G	C	0.17	0.07	0.02
5′	3′			
3′	5′			
G	C	0.47	0.07	0.02
A	U	0.96	0.11	0.03
[C]	[G]	0.18	0.04	0.02
U	A	0.09	0.04	0.02
C	G			
U	A	0.95	0.15	0.04
C	G	0.43	0.09	0.02
A	U	0.19	0.06	0.03
G	C	0.14	0.06	0.02
5′	3′			

[a] Ring current shift values for different combinations of base pairs were calculated as described under Materials and Methods. All shifts reported are *upfield* shifts. The predicted chemical shift of a given imino proton is its intrinsic value *minus* the sum of the ring current shift contributions given in this table. Values are computed for AU and GC imino protons flanked by either AUs or GCs as indicated. The base pair whose ring current shifts are sought is the pair in the box. First-order shifts refer to the situation where the base pair in question is the immediate neighbor of the imino proton of interest. Second-order shifts arise when the base pair is the neighbor once removed, etc. For example, an AU base pair with an AU as its first neighbor oriented so that the neighboring AUs U is 5′ to the U of the base pair of interest shifts the imino proton of the AU upfield by 0.71 ppm.

Fourth-order effects were also calculated. They range in magnitude from about 0.015 to 0.007 ppm; one is unlikely to be able to detect an effect of base substitutions four base pairs removed from a given imino proton, provided the overall structure remains constant. The fact that the chemical shift of E changed measurably in response to the G117-U3 mutation suggests that replacement of a GC by a GU at that position has an effect on the structure of the molecule which propagates some distance down the molecule.

Application of Ring Current Shift Predictions to 5S RNA Mutants. Table III compares the observed and calculated chemical shifts of the imino protons adjacent to the 3–117 base pair in the wild-type 5S RNA fragment and in the two mutants. Effects to third order were included. It is clear that predicted shifts are accurate to only 0.25 ppm, similar to previous experience [see Schimmel and Redfield (1980)]. However, the number of resonances affected by mutation is small, and the base-pair type of many of them could be deduced from intra base pair NOEs, even if the connectivities between adjacent imino protons were not. Comparison of the differences observed and the predictions would clearly lead to assignments in these cases.

Application of the same rules to the pDG07 fragment spectrum predicts for the new base region that the resonance of U11 should lie downfield of the resonance for U12 and that the resonance of G107 should be found near 12.20 ppm, all in good agreement with observation. What they cannot do is provide useful predictions for K and L in either fragment 1 or the pGD07 fragment because of the proximity of G105 and G106 to an AG base pair whose ring current shift properties are unknown.

Table III: Chemical Shift Changes due to Base Substitutions at Positions 3 and 117[a]

resonance	chemical shift (ppm) C3-G117 (a)	U3-A117 (b)	U3-G117 (c)	change (ppm) (b) − (a)	(c) − (a)
observed					
J	12.58	12.58	ND	0	–
C	13.25	14.29	(12.21, 11.31)	+1.04	−1.04, −1.94
F	12.89	12.58	12.95	−0.31	+0.06
B	13.39	13.33	13.28	−0.06	−0.11
M	12.12	12.08	12.12	−0.04	0
E	13.15	13.15	13.15	0	0
predicted					
J	12.35	12.30	12.42	−0.05	+0.07
C	13.05	13.77	–	+0.72	–
F	12.83	12.31	12.89	−0.52	+0.06
B	13.38	13.32	13.40	−0.06	+0.02
M	12.04	12.03	12.05	−0.01	+0.01
E	13.00	13.00	13.00	0	0

[a] This table compares the chemical shifts predicted for the imino proton resonances in the vicinity of base pairs altered by mutation with those actually observed. In column a data refer to the pKK5-1 product in which the 117–3 base pair is a GC, the wild-type sequence. Column b lists the results for the pDG05 mutant (A117-U3), and column c provides the data for the pDG04 mutant (G117-U3). Chemical shifts were predicted by using the results reported in Table II. Entries marked (–) represent situations where the value in question could not be observed or could not be calculated, as the case may be. Chemical shifts for sequences involving GUs were calculated by using shift rules computed by means of the algorithm described under Materials and Methods on the basis of the GU geometry found in the amino acid acceptor stem of yeast $tRNA^{Phe}$ (Hingerty et al., 1978).

Discussion

The sequence of the pDG05 product (A117-U3) is identical with that of one of the 5S genes in the rrnD ribosomal RNA cistron of *E. coli* (Duester & Holmes, 1980). An equivalent plasmid could have been constructed by cloning the appropriate segment of DNA directly from the *E. coli* chromosome rather than by site-directed mutagenesis.

The downfield spectrum of fragment 1 preparations made from 5S RNA isolated from the ribosomes of cells lacking 5S-carrying plasmids includes a weak resonance at 14.29 ppm whose source has been obscure (Kime & Moore, 1983b). The results obtained with the pDG05 product make it clear that this resonance represents the rrnD gene product in the 5S RNA population. The intensity of this resonance relative to that of other resonances in the spectrum suggests that 5–7% of the total ribosomal 5S RNA is rrnD product, not the 12% one would anticipate were all 5S cistrons expressed and incorporated equally into mature ribosomes. It is interesting to note that the incorporation into ribosomes of the same sequence produced from pDG05 is less efficient than the incorporation of the standard sequence.

Clearly the spectral changes produced by substituting an AU or a GU for C3-G117 in the terminal stem conform to expectation in many respects. The resonance most altered was that of the imino proton at the mutated position. Its flankers were also affected, as already noted, but to a lesser degree. The general level of agreement found between the chemical shift changes seen in this case and those calculated a priori are consistent with the view that the perturbation to structure of the molecule in the case of the AU substitution was modest but that the replacement of the normal GC with a GU may have had a larger effect.

From a spectroscopic standpoint, the imino proton sequence of helix I in *E. coli* 5S RNA is palindromic. It goes $(GC)_3(AU)(GC)_3$, and the orientation of the GC resonances flanking the central AU relative to the sequence is ambiguous if NOE connectivities alone are the only data available for

making assignments. A study undertaken earlier to resolve this ambiguity (Kime et al., 1984) ultimately led to the conclusion that resonance J should correspond to G2-C118. This assignment of resonances to helix I is definitively proven by the results reported here. Had the earlier work been wrong, the resonances most affected by mutations at 3–117 would have been M, E, and H, not resonances J, C, and F.

In the pDG07 fragment the base of helix V and the top of helix I are stabilized by the hydrogen bonding of the three extra bases inserted to maintain the continuity of the deleted gene. This stabilization is clearly apparent in the increase in the strength of resonances R2, P2, and T and the small alterations in their chemical shifts. These resonances were known from prior studies to represent the bottom of helix I (Gewirth et al., 1987). Their assignments are confirmed. It is clear that resonances K and L also respond to the presence of the new bases, proving that they must belong to the top of helix V. The resonances designated K and L in Figure 4b are correctly assigned in Figure 6. It is still possible that the resonance being called K in the pDG07 fragment corresponds to L in the fragment 1 spectrum and vice versa. Clearly some further work will have to be done.

The magnitude of the variation of ring current shifts to be expected due to local variations in helix geometry was examined by applying the ring current shift prediction algorithm to the structure of yeast $tRNA^{Phe}$ (Hingerty et al., 1978). Although the sample of base-pair juxtapositions available in this molecule is small, two findings emerged: (1) the shifts predicted from the tRNA geometry, on average, agree with those obtained by using Arnott's averaged geometry and (2) local geometric variation contributes significantly to the inaccuracies of ring current prediction techniques when applied to RNA helices of unknown structure.

In conclusion, the data presented in this paper support the view that site-directed mutagnesis is a useful tool in the armamentarium of the nucleic acid spectroscopist. It is also clear that an analysis of the effect of base changes upon the spectrum given by RNAs can provide useful information about their structural effects. They also suggest that NMR could be used as a means for measuring the relative expression of 5S genes.

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