Nitrogen-15-Labeled Yeast tRNA^{Phe}: Double and Two-Dimensional Heteronuclear NMR of Guanosine and Uracil Ring NH Groups†

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ABSTRACT: ^{15}N1-Labeled hypoxanthine and 1,3-^{15}N-labeled uracil were synthesized chemically and used to prepare labeled yeast tRNA^{Phe} biosynthetically. Maps (500 MHz) of ^{15}N chemical shift vs. proton chemical shift were obtained, for each ring NH group, by means of INDOR (difference heterodecoupling) and also by means of a proton-observe two-dimensional method involving coherences of forbidden resonances of the NH system. Resonances of GC11, T54–m^{1}A58, GU4, and AΨ31 were confirmed, assigned, or reassigned. Ψ39 was found to be in anti conformation, not syn as previously stated. Almost all the uracil NH group resonances could be separated, but most of the GC resonances are too close even in two dimensions to be separately resolved with the observed 20-Hz ^{15}N line width.

Many studies of tRNA's by NMR have used as structural markers the resonances of ring N protons that exchange rapidly with solvent protons (Reid, 1981). These protons are of interest because their resonances are more or less separated from those of the bulk of the tRNA, because their exchange rate is intrinsically interesting as a dynamic probe, and because they are well distributed throughout the molecule. Identification of these proton resonances has proven difficult in the past because one of the methods of choice for assignment, namely, deuterium labeling, is impractical for a labile proton. Fortunately, a large fraction of the ring NH resonances, and many other resonances, can be identified by distinctive nuclear Overhauser effects (NOEs)[1] together with plausible assumptions about secondary and tertiary structure (Johnston & Redfield, 1981; Tropp & Redfield, 1981; Roy & Redfield, 1983; Heershap et al., 1982, 1983a,b).

While direct isotopic labeling is not possible for ring N protons, it is feasible to label chemically stable sites with which these protons interact, either by NOE in the case of nearby carbon protons or by spin–spin coupling in the cast of nitrogen. Thus, an NOE study (Sanchez et al., 1980) of purine C8 deuterated tRNA provided a devastating test of existing resonance assignments and a guide to subsequent work, as did a later NOE study of a purine C2 deuterated sample (Roy et al., 1982). Here the strategy was simply to look for NOEs that disappeared upon deuteration of specific groups to test assignments of resonances to reverse Hoogsteen base pair or Watson–Crick pair protons.

More recently, Griffey et al. (1982) exhibited proton spectra of tRNA labeled with ^{15}N in the uracil N3 and also N1 positions. The isotope ^{15}N has spin $^1/_2$, and a donor nitrogen therefore splits the resonance of its proton into a doublet with a splitting of ~100 Hz, easily seen in favorable cases when it is large compared to the proton line width, which is typically 15 Hz in tRNA. By decoupling the ^{15}N resonances and looking at the proton spectra of the labeled tRNA as a function of the ^{15}N decoupling frequency, they could correlate proton shifts and nitrogen shifts (Griffey et al., 1983a). In subsequent developments, Griffey et al. (1983b) demonstrated further technical advances by using a version of two-dimension (2D) NMR involving stimulation of forbidden or multiple-quantum coherences, to give improved maps of proton vs. ^{15}N shifts. This class of techniques is certain to be useful in a wide range of protein and nucleic acid problems. The studies performed by Griffey et al., besides demonstrating the techniques, established or confirmed several assignments involving GU and GΨ pairs and determined the orientation of Ψ bases in several AΨ pairs as anti.

We also have produced and studied ^{15}N-labeled yeast tRNA^{Phe}, and this paper summarizes our preliminary results. Our study differs from those of Griffey et al. in that we also labeled the purine N1 position of one sample, as well as both the N1 and N3 positions of uracil in a second sample. We used much the same NMR technology as Griffey et al., some of which was kindly described to us prior to publication by Drs. R. H. Griffey, C. D. Poulter, and A. Bax. We were interested in assignments and also in developing the highest resolution possible in the ^{15}N spectral dimension in order to separate resonances in two dimensions that overlap in a one-dimensional proton spectrum. We also hoped that ^{15}N shifts correlated with proton shifts would lead to insight into the relative contributions of ring currents to proton NMR chemical shifts, as opposed to other possible effects such as distortion or strain of hydrogen bonds due to tertiary structure. Finally, we wished to see to what extent these methods would be promising for studies of complexes such as tRNA associated with proteins.

Materials and Methods

Purine N1 labeling was achieved by growing a suitable auxotroph on labeled hypoxanthine (Roy et al., 1982) synthesized in several steps:

(*1*) *Ethyl α-(Hydroxyimino)-α-cyanoacetate.* A 60-mL aliquot of ethyl cyanoacetate was added to a solution of 50 g of $NaNO_2$ in 260 mL of H_2O. A 35-mL aliquot of glacial acetic acid was added gradually, and the resulting yellow

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[1] Abbreviations: NOE, nuclear Overhauser effect; 2D, two dimensional; 2DFE, 2D forbidden echo; SPENDOR, stimulated proton-echo nuclear double resonance; INDOR, internuclear double resonance; Ψ, pseudouridine; EDTA, ethylenediaminetetraacetic acid; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

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solution was stirred until the ester dissolved. A yellow precipitate formed overnight and was separated by decanting, redissolved in 1 L of water, acidified with 50 mL of concentrated HCl, extracted with ether, and evaporated to yield 55 g of product.

(*2*) *Ethyl α-Amino-α-cyanoacetate-p-toluenesulfonate.* Al amalgam was prepared by washing aluminum foil with 0.1 N NaOH, water, and 0.5% $HgCl_2$. A total of 55-g of ethyl α-(hydroxyimino)-α-cyanoacetate was added as an etheral solution to an etheral solution of Al amalgam and fitted with a stirrer and a reflux condenser. A total of 25 mL of water was added over a 30-min period. Reaction of water with Al-Hg kept the solution in reflux. After another 30 min, the solution was filtered through Celite. The residue was ether extracted, combined with the filtrate, and added to 60 g of *p*-toluenesulfonic acid monohydrate in 50 mL of ethanol and 300 mL of ether. After 30 min, the resulting 50 g of crystals was filtered, ether washed, and dried and could be stored indefinitely.

(*3*) *α-Amino-α-cyanoacetamide* (*^{15}N Labeled*) (*Cook et al., 1949b*). The 15 g of the product of the previous step was dissolved in 25 mL of 2 N KOH and extracted twice with chloroform. The chloroform layers were pooled, concentrated to ~25 mL, dried over anhydrous Na_2SO_4, evaporated to a yellow oil, and dissolved in 9 mL of anhydrous ether. This solution of ethyl α-amino-α-cyanoacetate was added to an ice-cooled solution of 6.7 g of $(^{15}NH_4)_2SO$ in 6.7 mL of 1 N KOH and 3 mL of water. After a 1-h stirring, the precipitate was separated and extracted with methanol until all the yellow color was in solution, filtered again, and evaporated to dryness.

(*4*) *5(4)-Aminoimidazole-4(5)-[^{15}N]carboxamide* (*Cook et al., 1949a*; *Shaw et al., 1959*). The residue was redissolved in 40 mL of methanol. A total of 5 g of formamidinyl acetate was added, and the mixture was refluxed for 90 min. The methanol was evaporated, the residue was dissolved in water and applied to an AG1-X4 column (Cl^- form), and fractions having λ_{max} = 269 nm were pooled and evaporated to dryness. Further purification can be done on an AG-1-X8 column.

(*5*) *[1-^{15}N]Hypoxanthine.* The condensation of the previous product with triethyl orthoformate produces hypoxanthine (Richter et al., 1960).

Pyrimidine $^{15}N3$ labeling was achieved by growth of an auxotrophe on [1,3-^{15}N]uracil, synthesized after Lipnick & Fissekis (1979). One gram of [^{15}N]urea and 25 g of anhydrous polyphosphoric acid were stirred at 85 °C, 1.1 mL of propiolic acid was added, and the mixture was stirred 4 h at 85 °C with exclusion of moisture. After 16 h at room temperature, 50 mL of water was added, the mixture was shaken to dissolve the oily residue, and it was kept on ice for 30 min. The precipitate was filtered and washed with water to yield 1.3 g of [^{15}N]uracil.

Yeast was grown in 80-L fermenter lots, with poor yields, typically 100–200 g per growth. Transfer RNA^{Phe} was isolated as described previously (Sanchez et al., 1980), but yields were poor, and we obtained only about 40 units of purine-labeled tRNA and 70 units of pyrimidine-labeled tRNA. Samples were dialyzed extensively against 20 mM EDTA, which was then removed by further dialysis, as described previously. Final buffers were 90% H_2O, 10% D_2O, 0.1 M NaCl, and 10 mM phosphate, pH 7.

Four different methods were attempted to obtain correlation of ^{15}N shifts vs. proton shifts. The most informative methods were difference decoupling or INDOR (for internuclear double resonance), which was previously used and described, for example, by Griffey et al. (1983a), and a 2D method in which coherences are generated transiently between nuclear spin levels for which ordinary magnetic transitions are forbidden. This method was proposed and demonstrated by Bax et al. (1983), and we call it 2DFE for 2D forbidden echo. The two other methods are discussed elsewhere (Redfield, 1983). One of them, SPENDOR [stimulated proton echo nuclear double resonance; see Liao et al. (1974)], with soft ^{15}N 180° pulses whose frequency was varied, yielded fair results at 270 MHz. The other, a 2D version of SPENDOR, was too insensitive for our tRNA samples at 270 MHz, and we did not attempt it at 500 MHz.

INDOR consists of taking the difference between a proton spectrum for which decoupling power is applied, during the free-induction decay, at a frequency well away from any ^{15}N resonance, and a spectrum for which decoupling is at or near the ^{15}N resonance. The frequency of the latter decoupling is stepped through the ^{15}N resonance region in a series of runs to look for the biggest effect. When it is at the exact ^{15}N resonance of some ^{15}NH group in the molecule, the approximately 90-Hz spin–spin splitting of the resonance of the proton of the ^{15}NH group is removed, and the difference spectrum is greatest in amplitude. Since both the range of the relevant features of the proton difference spectrum and the range of action of the nitrogen irradiation cover about 100 Hz, this method is confusing for crowded spectra, but it is still useful for the study of NH groups for which either the ^{15}N resonance or the proton resonance are well separated from other resonances, as is often the case for modified bases in nucleic acids. INDOR is the most sensitive method in general and is most useful for searching for such resonances.

The 2D forbidden echo (2DFE) method is similar to the 2D method already applied to tRNA by Griffey et al. (1983b), except that higher resolution is achieved by the use of a refocusing pulse as demonstrated by Bax et al. (1983) in a small molecule species. The reader is referred to the latter paper for an explanation of the method (sequence 1e in that article). The same sequence was proposed by Bendal et al. (1983). The sequence is as follows: $90_\alpha(H)$, τ_a, $90_x(N)$, $\tau_1/2$, $180_\beta(H)$, $\tau_1/2$, $90_\gamma(N)$, τ_a, followed by proton digitization with time-shared chirped nitrogen decoupling on only during digitization. Here τ_a is 4.5 ms, τ_1 is varied from 0 to 31 ms in 1-ms steps, 90 and 180 indicates 90 or 180° pulses, N or H indicates proton or ^{15}N frequency, and x, α, β, and γ refer to the radio-frequency phases of the pusles, the latter three of which were stepped in intervals of $\pi/2$ relative to their internal clock in a group of 64 pulses at each τ_1. In order to work in H_2O, we replaced the first 90° pulse with a variant of the *J–R* pulse of Plateau & Gueron [1983; see Redfield (1983) for details] and the refocusing pulse by two such *J–R* sequences in succession. Data were treated as described elsewhere (Redfield, 1983) by using the method of States et al. (1982). ^{15}N noise decoupling was applied during signal digitization. A sensitive 500-MHz proton probe with a low-frequency decouple coil was supplied by Cryomagnet Systems (Indianapolis, IN), and they also modified our 270-MHz probe for this purpose. ^{15}N frequency was generated by mixing 2.5 MHz with an offset synthesizer. The synthesizer was chirped digitally for decoupling only, while the 2.5 MHz was digitally generated, phase-shifted, and gated for pulsing or time-shared decoupling. The power amplifier for ^{15}N was preceded and followed by quality filters (Telonic). Further technical details will be described elsewhere by us, but the end result is simply a contour map with one peak for each ^{15}N–H group in the molecule, on a plane for which the coordinates are ^{15}N chemical shift and proton chemical shift. The data reported

Table I: Resonance Shift Assignments of ^{15}NH Groups at 24 °C

(pseudo)uridine N1H or N3H[a]			guanosine N1H		
assignment	^{15}N (ppm)[b]	H (ppm)[c]	assignment	^{15}N (ppm)[b]	H (ppm)[c]
Ψ39 N1	135[d]	10.6	G4(U69), m_2^2G26	144.5[d]	10.4
Ψ55 N1	137[d]	10.96	GC2,27,50	147.7[e]	12.53
T54(m^1A58)	156.5	12.4	GC53, G19(C56)	148.7[e]	12.44
U or D	158[f]	10.6	GC1?	149.5	12.4
U69(G4)	158.5	11.8	GC3,28	147.6[e]	12.23
AU12 or AU52	161	13.6	G15(C48)	147.9	12.2
Ψ55 N3	160.7	11.55		149.1	12.1
AU7	161.5	13.3	GC13	148.5[e]	12.9
AU5	162.3	13.8	m^2G10(C25), GC51	148.3	12.76
AU29,50	162.6	13.2	m^7G46(G22)	151.6[e]	13.2
Ψ39 N3(A31)	163.2	13.23	GC11	147[d]	13.6
	163.2	13.18			
AU6	163.6	14.35			
AU12 or AU52	163.7	13.6			
U8(A14)	164.6	14.2			

[a] All N3H except for Ψ39 and Ψ55. Nonstandard pairing, if any, indicated by parentheses. [b] Error ~0.4 ppm, except as marked, obtained by 2DFE. [c] Error ~0.2 ppm. [d] Error ~1.5 ppm, obtained by INDOR at 270 MHz. [e] Weak and tentative. [f] Observed below 10 °C at 270 MHz. Error ~1.5 ppm.

here were taken in runs of 15 h, but these times, or the tRNA concentration, can almost certainly be decreased when the method is optimized.

The ^{15}N chemical shift scale was established by means of a 2D forbidden echo run on a pyrimidine derivative in $CDCl_3$ described by Bax et al. (1983), which was kindly given to us by Dr. R. H. Griffey. The chemical shift of N3 was assumed to be 160.2 ppm downfield of NH_3, as previously determined by Bax et al. (their compound III), so that our ^{15}N scale is presumably exactly the same as that of Griffey et al. In both cases, a correction was applied to account for the different lock compound used for the chemical shift reference ($CDCl_3$) and tRNA (HDO). Nitrogen chemical shifts are thus downfield from NH_3, while proton shifts are referenced downfield from DSS as usual.

Results and Discussion

The chemical shifts of ^{15}NH groups are summarized in Table I, together with their assignments. Except for a few of the resonances discussed below, these assignments are justified elsewhere (Roy & Redfield, 1983; Heerschap et al., 1982, 1983a,b). For reference, the proton resonance spectrum of an unlabeled sample is shown in Figure 1A.

Purine N1 Labeled Sample. The expected nitrogen NMR region was searched with heteronuclear noise decoupling, first over a wide range and then over a narrow range with a close mesh of frequencies. The INDOR difference spectrum of Figure 1B shows the effect, on a proton resonance at 13.6 ppm, of nitrogen decoupling at 148.5 ppm. This is the point in the nitrogen spectrum where the maximum effect is seen for most of the GC pairs. The effect at 13.6 ppm confirms a previous assignment of this peak to GC11 in the D stem (Heerschap et al., 1982; Roy & Redfield, 1983). The assignment is of interest because this resonance is unexpectedly far downfield for a GC proton so that its correctness could be questioned (except for a similar GC11 pair in *Escherichia coli* $tRNA^{Val}$, there is no other GC resonance known in nucleic acids more downfield than 13 ppm). Furthermore, this proton exchanges with solvent much more slowly than any other imino proton (in about 1 day at room temperature) in this tRNA (Johnston et al., 1979).

The position of the G4 N1 proton resonance is also shown by the spectrum of Figure 1B to be at 10.8 ppm; previously, we knew that either the G4 or the U59 N1 protons of the GU4 pair resonated at 10.8 and 11.8 ppm but did not know which

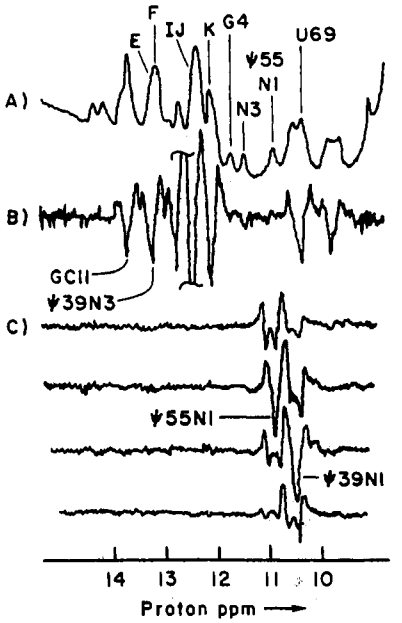

FIGURE 1: (A) The 270-MHz NMR spectrum of yeast $tRNA^{Phe}$. The labels E, F, IJ, and K refer to regions of the spectrum covered in Figures 2 and 3. (B) Difference between decoupling ^{15}N far off-resonance, and at 148.5 ppm, for the purine ^{15}N1 labeled sample. Features mentioned in the text are labeled. (C) Difference decoupling at (from top to bottom) 140, 137.3, 134.7, and 132 ppm for the uracil ^{15}N labeled sample. If Ψ39 N1 were internally hydrogen bonded, a feature should appear in the region between 12 and 13.5 ppm at the point indicated in spectrum B. [The peak marked Ψ39 N1 in (B) does not come from Ψ31 since this sample is not uracil labeled.] The two features at around 10.8 ppm are from the N1 protons of Ψ39 and Ψ55, both of which are clearly externally bonded as judged from their upfield proton shifts.

was which. This method of assignment of GU resonances has been reported previously by Griffey et al. (1982, 1983a).

The other GC peaks are clustered in a rather small region in both H and N directions, and it is difficult to disentangle the several peaks from each other with confidence. Their positions are as expected from an observation by Griffey & Poulter (1983) of a model GC pair G N1 resonance, at 149.4 ppm, and with measurement of the G N1 shift of GC pairs in DNA at around 150 ppm (Diverdi & Opella, 1982).

Figure 2 shows a section of a 2DFE map, for the proton peaks marked IJ and K in Figure 1A. Because of the small amount of this sample, we could not reproducibly resolve single

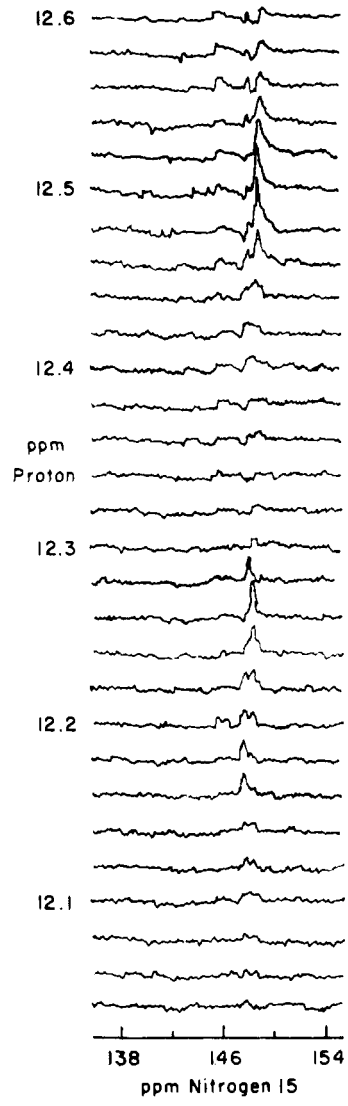

FIGURE 2: Two-dimensional forbidden echo map of the regions marked IJ and K in Figure 1A, for the purine N1 labeled sample. The spacing between lines in the proton dimension is 10 Hz. The total width in the ^{15}N dimension is 1 kHz. This was obtained from 512 by 128 input points, zero filled to double this in both dimensions, over 15 h.

NH groups, and the prominent peaks in Figure 2 are each likely to arise from more than one NH group, fortuitously coinciding. The features at about 146 ppm, and the small feature at 12.6 (protons) and about 148 ppm (nitrogen), are most probably artifacts since they occur in the whole range downfield of 12.6 ppm (proton). Thus the two peaks IJ and K are converted to about four discernible 2DFE peaks, as tabulated in Table I. Peak IJ has been assigned to secondary GC pairs 2, 27, 30, 49, 53, and possibly 1 and peak K to GC pairs 3 and 2, and to the G15–C48 tertiary interaction (Roy & Redfield, 1983).

Two of the methylated bases, m^7G46 and m_2^2G26, have ^{15}N shifts that are slightly unusual on the basis of the tentative data of Table I. These shifts could be due either to methylation per se or to the possibly unusual pairing modes of these bases.

Difference decoupling showed that a proton peak at 9 ppm contains at least one resonance of a GN1 proton whose ^{15}N donor resonates at 130 ppm. This must come from an N1 proton that is not hydrogen bonded to a ring N acceptor. We have tentatively assigned G18 N1H to this resonance (B. Choi, unpublished data).

[1,3-^{15}N]Uracil-Labeled tRNA. We were able to purify about 70 units of this sample, which made a 2D forbidden echo study as well as difference decoupling relatively easy. The most interesting result concerned resonances previously assigned to the Ψ39 N1 and N3 protons (Roy et al., 1982; Heerschap et al., 1983a,b). The corresponding proton resonances of Ψ55 had been assigned convincingly by NOE, and the ^{15}N shifts for these resonances, observed by us on this sample, are 137 ppm for Ψ55 N1 and 160 ppm for ψ55 N3 (Table I). These ^{15}N shifts are in good agreement with those previously found by R. H. Griffey et al. (unpublished results) in both Ψ monomer in tRNA samples labeled at only the U and Ψ N1 position or only the N3 position. In the case of Ψ39, we find that the NH group that resonates at 10.5 ppm for protons resonates at 124 ppm for ^{15}N, strongly inicating that this resonance comes from the N1 proton of Ψ39. From its relatively upfield proton shift, this NH is not internally hydrogen bonded. The other Ψ39 N proton was previously assigned to a group of overlapping resonances at 13.2 ppm. The ^{15}N resonance of this ^{15}NH group is indistinguishable from other overlapping ^{15}NH groups at the same place in the proton spectrum, resonating at about 163 ppm for nitrogen. This resonance must be due to the internal Ψ39 NH, as can be inferred from its large downfield shift relative to both the Ψ55 N1 and Ψ55 N3 proton resonances. It must be an N3 resonance from the ^{15}N shift, and therefore, Ψ31 is in an anti conformation. Figure 1C shows part of a difference-decoupling search around 135 ppm for nitrogen, and there is no sign of any effect at 13.2 ppm for protons. A similar strategy based on specific N1 or N3 labeling has been used previously by R. H. Griffey et al. (unpublished results) to determine the orientation of several Ψ's in Aψ pairs in other tRNAs. The present result may be of interest because the position corresponding to Ψ39 tends to be a Ψ in many tRNAs, whereas the 31 position never is; and this group may be involved in regulation of biosynthesis (Cortese et al., 1974).

Unfortunately, we previously reached exactly the reverse conclusion, namely, that Ψ39 is in the syn conformation, by apparently equally elegant and rigorous means (Roy et al., 1982). We now believe our previous conclusion was incorrect: The most rigorous evidence for the syn conformation was the persistence of an NOE from the 13.2 ppm resonance to an aromatic proton resonance at 6.85 ppm in a purine C2 deuterated sample. This NOE was presumed not to be to the (deuterated) C2 position of A31, which pairs with Ψ39, and was therefore assigned to Ψ39 C6H, since other possibilities such as an NOE to a purine C8H had already been eliminated. If so, the 13.6 ppm Ψ39 NH resonance had to be the adjacent N1H and not the much more distant N3H, and thus, N1 is internal and Ψ39 is syn. Unfortunately, labeling was not complete, and the purine C2 positions were only about 85% deuterated in this sample. Also, the NOE in question was one of three or four NOEs fortuitously overlapping, and the intensity of the observed NOE at 6.85 ppm was difficult to estimate at 270 MHz because of noise and overlap with other broader NOEs. Apparently, it was overestimated by us in our earlier study. Recently, we remeasured this NOE in the same sample (not shown) at 500 MHz and compared it to the residual NOE at 7.73 ppm from the most downfield peak assigned to U6 N1H to the C2 proton of AU7. Again, intensities are hard to estimate, but the latter NOE appears to be one-half to one-third the intensity of the one from 13.2 to 6.85 ppm, which is the same ratio found previously for these NOEs in unlabeled tRNA. This is consistent with the interpretation that both are residual Ψ C2H NOE's in the 85% C2-deuterated sample. Another piece of evidence previously given for the incorrect assignment was that no aromatic NOE was

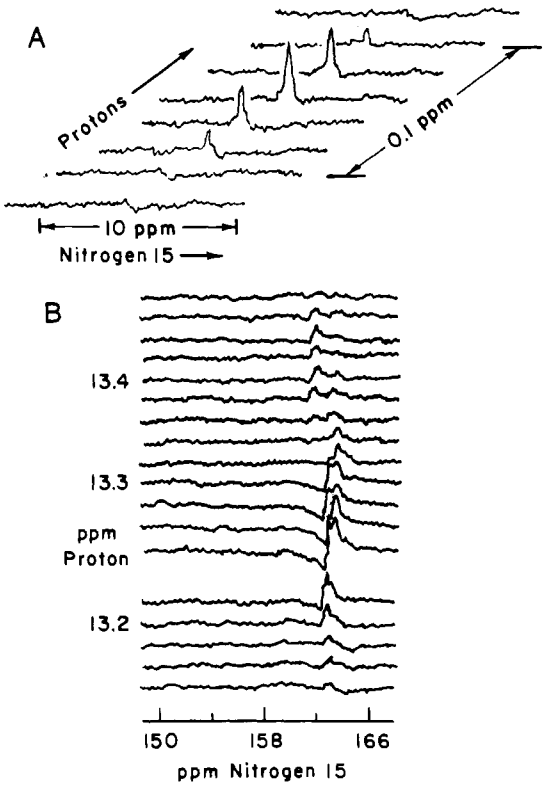

FIGURE 3: Regions of the 2DFE map of the uracil-labeled sample. Conditions and scale are the same as those in Figure 2. (A) single ^{15}NH group of the uracil-labeled sample: T54 ^{15}N1H, 12.4 pm (protons) and 156.5 ppm (^{15}N). (B) Region of the map corresponding to peaks E and F of Figure 1A and containing four U or Ψ ^{15}NH groups.

observed from the upfield 10.6 ppm Ψ39 N proton resonance. Such an NOE would be expected, to Ψ39 C6H, if this resonance is Ψ39 N1H, as we now believe, similar to the previously obseved NOE from the Ψ55 N1H resonance to the Ψ55 C6H resonance. Recently, at 500 MHz, we have found small NOEs (not shown) from this resonance to 7.64 and 6.62 ppm, possibly due to Ψ39 C6 and Ψ39 C1′ protons, respectively. The NOE from 10.6 (Ψ39 N1H) to 7.64 ppm (Ψ39 C6H) may be weak because the C6 proton could be unusually close to sugar protons or because the N1 proton is pulled away from the C6 proton in this tRNA. This NOE is about 15% as strong as the similar NOE from Ψ55 N1H to Ψ55 C6H (from 10.96 to 7.34 ppm, in yeast tRNA^{Phe}).

Another result that we did not fully expect was an ^{15}N-labeled proton resonance at 12.4 ppm, whose ^{15}N shift is 156.5 ppm. This is consistent with, and strong support for, the assignment of the T54 N1 proton that partakes in the T54–m^{1}A58 hydrogen bond interaction to a resonance at this position. This assignment, by Haasnoot et al. (1983), was the most important of the very few differences between our respective completely independent assignment studies of yeast tRNA^{Phe}. We previously tentatively assigned T54–m^{1}A58 to 13.6 ppm, and this assignment is almost certainly incorrect.

A rather good 2DFE map of this sample was obtained at 23 °C and 500 MHz. Figure 3A shows the best peak in this map, which is the presumed T54 N1H resonance. Other peaks were less intense and broader, probably because their proton transverse relaxation rate T_2^{-1} is faster. This rate is relevant during the evolution and refocusing intervals of 4.5 ms, and it also determines the proton line width. The line width in the nitrogen dimension is less than 20 Hz for the peak in Figure 3A. It is easy to show that dipolar interaction between the ^{15}N and its proton should not appreciably relax the forbidden coherences, as it does the allowed ones. This line width is probably due to chemical shift anisotropy of both the ^{15}N and proton, but contributions could also come from dipolar interaction with neighboring protons or motionally narrowed chemical shift variations due to internal motion. We conclude that chemical shift anisotropy broadening, which increases as the square of the magnetic field, is not excessive at 500 MHz, and this conclusion is of considerable interest for future studies in other systems.

Figure 3B shows the region of the 2DFE map for the proton regions marked E and F in Figure 1A. Peak E in Figure 3B is clearly resoved, but peak F at 13.2–13.3 ppm (protons) is somewhat confused; in another 2DFE run, it appeared as a clearer multiplet. It is tentatively resolved into three components (Table I). The skewed form of the peak is presumably an artifact, whose origin we do not understand; this skew is seen less severely in other peaks. All the other U N3H resonances (Table I) were at least as clear as these.

An unexpected peak at 10.6 (protons) and 158 ppm (^{15}N) was found in a preliminary 2DFE run at 270 MHz and 10 °C and was verified in an INDOR run at 5 °C. It is probably due to the N3 proton of a noninternally bonded U or dihydrouracil.

Conclusions

This is the first reported study of any nucleic acid in which the spectrum of ^{15}NH groups has been obtained for a purine ^{15}N1 labeled sample. We have described a method to synthesize [1-^{15}N]hypoxanthine that can be used to produce purine ^{15}N1 labeled nucleic acids. The [^{15}N]purine spectra taken together with the spectra from the [^{15}N]uracil-labeled tRNA and earlier work show that such regiospecific labeling is probably unnecessary in the future for purposes of assigning proton resonances, since the purine and pyrimidine ^{15}N resonances are fairly well separated. Future studies can use tRNA grown in labeled ammonium salt, if this is more economical. However, in a few cases such as GC11 discussed above, specific labeling will be more rigorous. This paper demonstrates, as also previously shown by Griffey et al. (1982, 1983a,b), the usefulness of ^{15}N labeling for assignment purposes and for determining structural details such as pseudouridine conformation. Such labeling provides useful starting points for stepwise NOE assignments and/or provides tests for the correctness of these assignments.

This work also indicates that, in studies of complexes for which both ^{15}N and proton resonances are broader than those in free tRNA, nitrogen labeling of this type is likely to aid in observing those NH groups whose resonances are well separated in either the proton or nitrogen domain, such as thiouridines, pseudouridines, or GU pairs.

In some cases, AU pairs, which are spread over a range of 6 ppm (nitrogen), might also be useful as markers in complex systems. The prospect is dim for using GC base pairs in this way since their ^{15}N shift range appears small, about 3 ppm, and there are more of them, making resolution and identification difficult.

As expected, INDOR is the most sensitive and reliable way to search for a resonance in an otherwise uncluttered region, but 2DFE is the highest resolution method. For this reason, the 2DFE method may still be useful in larger complexes; in any case, its high resolution should make it useful for conformational studies of smaller nucleic acids provided that the phenomenology of the ^{15}N shifts can be worked out. Limited 2DFE studies of proton solvent exchange, paramagnetic ion broadening, diamagnetic ion binding, and, conceivably, nuclear Overhauser effect appear feasible.

No systematic correlations between the directions of proton and of nitrogen shifts are obvious from these data. For example, the AU6 proton resonance is so far downfield that it was suspected of being a reverse Hoogsteen pair before this was disproven (Sanchez et al., 1980), yet its ^{15}N resonance position is similar to that of several other AU pairs. It is certainly true that the ^{15}N shifts are too big to be due to ring current shifts from neighboring bases and must therefore indicate something other than interbase geometry.

The present demonstration is unprecedented in the small amount of material used for any ^{15}N or 2D study. Demonstration of the narrow ^{15}N line width is of special interest for future studies. This paper and a recently publihsed 2D interproton nuclear Overhauser effect study of tRNA (Hilbers et al., 1983) report data on probably the largest molecule for which 2D spectra have been taken, and the this work also shows that semiselective pulses will be usable for such studies, thereby increasing the rate at which data can be gathered. Solid-state studies of ^{15}N-labeled macromolecules and of model compounds are now being reported (Diverdi & Opella, 1982; Harbison et al., 1983; Cross et al., 1983). Thus, these results should be of general usefulness for future studies of other nucleic acids and of macromolecules in general, including ^{13}C-labeled molecules.

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Registry No. ^{15}N, 14390-96-6; ethyl α-(hydroxyimino)-α-cyanoacetate, 3849-21-6; ethyl α-amino-α-cyanoacetate-*p*-toluenesulfonate, 91158-65-5; ethyl cyanoacetate, 105-56-6; *p*-toluenesulfonic acid, 6192-52-5; [1-^{15}N]hypoxanthine, 91158-66-6.

References

Bax, A., Griffey, R. H., & Hawkins, B. L. (1983) *J. Magn. Reson. 55*, 301–315.

Bendall, M. R., Pegg, D. T., & Doddrell, D. M. (1983) *J. Magn. Reson. 52*, 81–117.

Cook, A. H., Davis, A. C., Heilbron, I., & Thomas, G. H. (1949a) *J. Chem. Soc.*, 1071–1074.

Cook, A. H., Heilbron, I., & Smith, E. (1949b) *J. Chem. Soc.*, 1440–1442.

Cortese, R., Landsberg, R., Von der Haar, R. A., Umberger, H. E., & Ames, B. N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1857–1861.

Cross, T. A., Tsang, P., & Opella, S. J. (1983) *Biochemistry 22*, 721–725.

Diverdi, J. A., & Opella, S. J. (1982) *J. Am. Chem. Soc. 104*, 1761–1762.

Griffey, R. H., & Poulter, C. D. (1983) *Tetrahedron Lett. 24*, 4067–4070.

Griffey, R. H., Poulter, C. D., Yamaizumi, Z., Nishimura, S., & Hurd, R. E. (1982) *J. Am. Chem. Soc. 104*, 5811–5813.

Griffey, R. H., Poulter, C. D., Yamaizumi, Z., Nishimura, S., & Hawkins, B. L. (1983a) *J. Am. Chem. Soc. 105*, 143–145.

Griffey, R. H., Poulter, C. D., Bax, A., Hawkins, B. L., Yamaizumi, Z., & Nishimura, S. (1983b) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5895–5897.

Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1983) *Biochemistry 22*, 1–5.

Heerschap, A. Haasnoot, C. A. G., & Hilbers, C. W. (1982) *Nucleic Acids Res. 10*, 6981–7000.

Heerschap, A., Haasnoot, C. A. G., & Hilbers, C. W. (1983a) *Nucleic Acids Res. 11*, 4483–4499.

Heerschap, A., Haasnoot, C. A. G., & Hilbers, C. W. (1983b) *Nucleic Acids Res. 11*, 4501–4520.

Hilbers, C. W., Heerschap, A., Haasnoot, C. A. G., & Walters, J. A. L. I. (1983) *J. Biomol. Struct. Dyn. 1*, 183–207.

Johnston, P. D., & Redfield, A. G. (1981) *Biochemistry 20*, 1147–1156.

Johnston, P. D., Figueroa, N., & Redfield, A. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3130–3134.

Liao, P. F., Hu, P., Leigh, R., & Hartman, S. R. (1974) *Phys. Rev. A 19*, 332–340.

Lipnick, R. L., & Fissekis, J. D. (1979) *J. Labelled Compd. Radiopharm. 17*, 247–254.

Plateau, P., & Gueron, M. (1983) *J. Am. Chem. Soc. 104*, 7310–7311.

Redfield, A. G. (1983) *Chem. Phys. Lett. 96*, 537–540.

Reid, B. R. (1981) *Annu. Rev. Biochem. 50*, 969–996.

Richter, E., Loeffler, J. E., & Taylor, E. C. (1960) *J. Am. Chem. Soc. 82*, 3144–3146.

Roy, S., & Redfield, A. G. (1983) *Biochemistry 22*, 1386–1390.

Roy, S., Papastavros, M. Z., & Redfield, A. G. (1982) *Nucleic Acids Res. 10*, 8341–8349.

Sanchez, V., Redfield, A. G., Johnson, P. C., & Tropp, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 5659–5662.

Shaw, G., Warrener, R. N. Butler, D. N., & Ralph, R. N. P. (1958) *J. Chem. Soc.*, 1648–1655.

States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.

Tropp, J., & Redfield, A. G. (1981) *Biochemistry 20*, 2133–2140.