Nuclear Magnetic Resonance Signal Assignments of Purified [^{13}C]Methyl-Enriched Yeast Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: Yeast tRNA^{Phe}, enriched in carbon-13 specifically at the naturally occurring methyl groups, has been produced through biosynthesis, then purified, and analyzed. Transfer RNA^{Phe} was purified from the [^{13}C]methyl-enriched, unfractionated tRNA that had been extracted from a methionine auxotroph of *Saccharomyces cerevisiae* [Agris, P. F., Kovacs, S. A. H., Smith, C., Kopper, R. H., & Schmidt, P. G. (1983) *Biochemistry 22*, 1402-1408]. The yeast had been grown in minimal medium supplemented with [^{13}C]methylmethionine. Transfer RNA^{Phe} purity and the full extent of nucleoside modification were confirmed by high-performance liquid chromatography of constituent nucleosides with simultaneous UV spectral identification and quantitation. Mass spectometry of [^{13}C]methyl-enriched nucleosides and NMR of the tRNA indicated an enrichment of at least 70 atom %. Twelve resolved and prominent carbon-13 NMR signals from the tRNA were seen between 10 and 60 ppm. These have been assigned to 13 of the 14 naturally occurring methyl groups. However, the partially resolved signals assigned to the two 5-methylcytidines could not be assigned to their specific nucleoside positions of either 40 or 49 in the molecule. In addition, the partially resolved signals of the two methyl esters of wybutosine could not be distinguished. The methyl group found not to be enriched with ^{13}C is bound to the ring carbon in the hypermodified nucleoside wybutosine (Y). A 13th enriched signal downfield (120.9 ppm) has been assigned to one of the two carbons added to guanosine to form the third ring in the biosynthesis of Y. The ^{13}C enrichment of this ring carbon demonstrates its origin from the methionine methyl group. Broad ribose carbon signals were the only natural abundance ^{13}C peaks observed under the NMR conditions and accumulation times chosen. Carbon NMR signals were correlated with their corresponding proton resonances by single-frequency selective decoupling of the ^{13}C spectrum. Previous ^{1}H NMR assignments were confirmed except for a peak at 3.03 ppm (^{1}H) that is now assigned to the methyl group of m^{1}A-58. All carbon and proton assignments were accomplished without having to denature the tRNA.

Transfer RNA is a well-understood molecule in terms of biochemistry and genetics. Nuclear magnetic resonance (NMR) spectroscopy is a technology applicable to the study of both native structure and overall and site specific motions of macromolecules in solution. Biochemically well-defined, biologically active molecules, such as nucleic acids, can be studied under physiological conditions by NMR spectroscopy. The imino protons of the secondary and tertiary base pairs, the protons of the modified nucleosides, the protons of aromatic carbons, and the phosphates throughout the tRNA molecule have provided natural NMR probes for ^{1}H and ^{31}P NMR studies, respectively (Kastrup & Schmidt, 1978; Schmidt & Edelheit, 1981; Gorenstein & Goldfield, 1982; Hare & Reid, 1982; Roy et al., 1982). Carbon NMR of specifically ^{13}C enriched carbons has been shown to be feasible (Agris et al., 1975; Hamill et al., 1980; Schmidt et al., 1980; Tompson & Agris, 1979) and informative (Schweizer et al., 1980; Olsen et al., 1982; Schmidt et al., 1983). However, it is ^{13}C enrichment of the site-specific methylated nucleosides in the tRNA structure that has yielded easily assignable spectral resonances (Agris et al., 1975; Tompson et al., 1979), and allowed ^{13}C NMR investigation of internal motion at known structural positions in individual purified *Escherichia coli* tRNA species (Agris & Schmidt, 1983; Kopper et al., 1983).

The methylated nucleosides in yeast tRNA^{Phe} are more extensive than in any *E. coli* tRNA species (Kopper et al., 1983) and widely distributed about the structure. Fourteen methylations occur in 10 nucleosides located in the loops, extra arm, anticodon, base-paired stems, and regions between stems. The specific methylation sites on the tRNA^{Phe} molecule are depicted in Figure 1. Thus, a variety of specific environs of known biological importance (Bjork, 1984) would be probed by such methyl groups in the molecule with the use of ^{13}C NMR. In order to enrich the methyl groups of yeast tRNA for this isotope, a methionine auxotroph has been utilized for in vivo incorporation of [^{13}C]methylmethionine (Agris et al., 1983). Transfer RNA extracted from such a culture demonstrated ^{13}C-enriched NMR signals in relatively short accumulation times.

The three-dimensional structure of yeast tRNA^{Phe} is well characterized from X-ray crystallography (Kim et al., 1972; Holbrook et al., 1978; Sussman et al., 1978). Thus, results from NMR studies of this tRNA may be immediately applied to the structure. This may ultimately give detailed understanding of the presence or absence of local dynamics at biologically important parts of the molecule in solution, for instance, during interaction with aminoacyl-tRNA synthetase and mRNA codons.

Here, we present the first ^{13}C NMR spectra and signal assignments for any purified eucaryotic tRNA, yeast tRNA^{Phe}. In addition, we show that the correlation of the easily assigned

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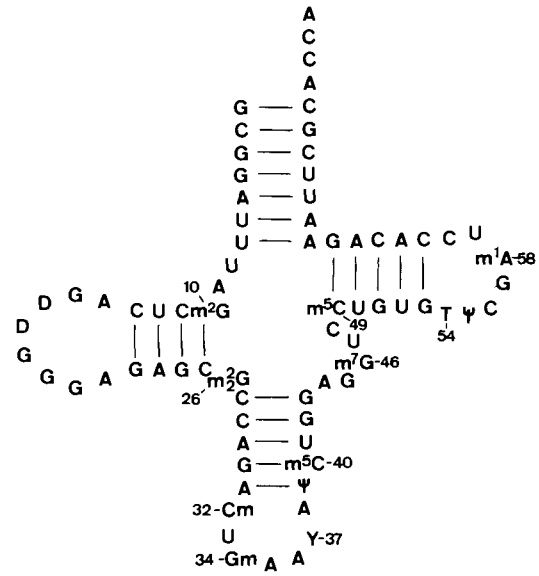

FIGURE 1: Two-dimensional structure of yeast phenylalanine transfer RNA. The specific sites of methylation are indicated by numbers denoting the position in the sequence (Sprinzl & Gauss, 1983).

^{13}C-enriched methyl resonances in the carbon spectrum with the ^{1}H signals is an improved method for making and confirming ^{1}H NMR assignments. Also, [^{13}C]methyl incorporation in vivo has given a new insight into the biosynthesis of the hypermodified nucleoside wybutosine (Y).

Materials and Methods

Preparation of [^{13}C]Methyl-Enriched tRNA. *Saccharomyces cerevisiae* met$^-$trp$^-$ (RH 762) was grown in 15-L batches at 30 °C in minimal media containing [^{13}C]methyl-L-methionine and L-tryptophan (Merck, Quebec, Canada). Cells were harvested in the stationary phase (1.25×10^8 cells/mL) by filtration using a Millipore Pellicon cassette system and a Durapore cassette of 0.5-μm pore size (Bedford, MA). The total yield of cells was 2.5 kg. Crude nucleic acid was obtained by phenol extraction, ethanol precipitation, and extensive dialysis against glass-distilled water. The amount of nucleic acid obtained was 2.5 g. (All nucleic acid concentrations were estimated by UV absorbance at 260 nm and then converted into concentration by using the relationship that 1.0 A_{260} unit is equivalent to 50 μg/mL nucleic acid.) The A_{260}/A_{280} ratio was 1.95. The preparation method extracted only 5S and transfer RNAs, as demonstrated by polyacrylamide gel electrophoresis of the extract. Thus, purification by diethylaminoethyl- (DEAE) cellulose column chromatography was omitted.

Purification of tRNA^{Phe}. The [^{13}C]methyl-enriched yeast tRNA (2.4 g) was fractionated into pure species by benzoylated diethylaminoethylcellulose (BDC) column chromatography. All elution buffers consisted of 0.05 M sodium acetate, pH 5.0, and 0.01 M $MgCl_2$. The gradient elution scheme used for fractionation consisted of the following steps. Step 1 was an 8-L gradient from 0.4 to 0.9 M NaCl. Step 2 was a 6-L gradient from 0.9 to 1.5 M NaCl. Step 3 was an 8-L ethanol gradient consisting of 1.5 M NaCl from 0% to 20% ethanol. The last step consisted of 1.5 M NaCl and 30% ethanol. Column fractions were monitored for UV absorbance at 260 nm and assayed for tRNA^{Phe} by aminoacylation with [^{3}H]-phenylalanine using unfractionated yeast aminoacyl-tRNA synthetase. The fractions showing [^{3}H]phenylalanine incorporation were pooled, and the tRNA was precipitated by ethanol. The amount of tRNA in this pool was 25 mg, and the A_{260}/A_{280} ratio was 2.0. The purity of the tRNA^{Phe} species was determined by aminoacylation of phenylalanine and other amino acids, urea–polyacrylamide gel electrophoresis, and high-performance liquid chromatographic analysis of the constituent nucleosides.

Aminoacylations. Aminoacylation assays were conducted to determine the chromatographic mobility and purity of [^{13}C]methyl-enriched tRNA^{Phe}. These were performed by using [^{3}H]phenylalanine, 60.0 Ci/mmol (New England Nuclear, Boston, MA), and a partially purified homologous yeast extract (Kohli et al., 1979). As a test for purity, the [^{13}C]-methyl-enriched tRNA^{Phe} was assayed by aminoacylation for other tRNA species that may have coeluted from the BDC column with tRNA^{Phe}.

Polyacrylamide Gel Electrophoresis. Urea–polyacrylamide gel electrophoresis (Agris et al., 1974) was used for comparison of unfractionated [^{13}C]methyl-enriched yeast tRNA to *E. coli* and yeast unfractionated tRNA (Sigma Chemical Co., St. Louis, MO). The gels were 12% polyacrylamide and were stained with ethidium bromide. Various amounts of ^{13}C-enriched yeast tRNA (62.0, 50, 37, and 12 μg) were applied along with yeast unfractionated tRNA (54 μg) and *E. coli* unfractionated tRNA (40 μg). Urea gel electrophoresis was also used for determining purity of tRNA by comparing [^{13}C]methyl-enriched tRNA^{Phe} to commercially available tRNA^{Phe} (Boehringer-Mannheim, Indianapolis, IN) and to unfractionated yeast tRNA. The gels were 10% polyacrylamide and were stained with ethidium bromide.

Nucleoside and Isotope Abundance Analysis. Enzymatic hydrolysis of aliquots of tRNA^{Phe} to nucleosides followed by quantitative or preparative HPLC was accomplished by previously published procedures (Gehrke et al., 1982). Preparatively separated nucleosides were collected and concentrated by lyophilization, salt was removed by HPLC, and the nucleosides were concentrated again and then subjected to mass spectrometry (Gerhardt et al., 1983) to determine isotope abundance (Agris et al., 1980). Mass spectra of nucleosides from the ^{13}C-enriched tRNA^{Phe} were compared to spectra of commercially available major and minor nucleosides taken under the exact same conditions.

Sample Preparation for NMR. The remaining portion of the tRNA^{Phe} pool (approximately 22.5 mg) was prepared for NMR by extensive dialysis against glass-distilled water. The sample (5.0 mL) was concentrated 5-fold by vacuum, then exchanged twice with an equal volume of D_2O, and concentrated finally to 0.5 mL. A small amount of concentrated buffer solution was added to achieve the following experimental concentrations: 10 mM $MgCl_2$, 35 mM KH_2PO_4, and 35 mM Na_2HPO_4, pH 7.2. An internal standard, 1,4-dioxane, was added for NMR studies.

NMR Spectra. The tRNA sample was placed in a 0.5-mL microcell fitted in a 10-mm NMR tube. Spectra at 75.5 MHz were obtained with a spectrometer of hybrid construction composed of a Cryomagnet Systems 70/50 magnet and probe, Nicolet 1180 computer, Cryomagnet Systems decoupler, and in-house radio-frequency electronics. Broad-band ^{1}H decoupling at 300 MHz was done with the MLEV-16 technique using a 2-Hz bandwidth and 0.4 W of decoupling power. Selective carbon decoupled proton NMR spectroscopy was accomplished with a 5-mm ^{1}H NMR probe fitted with a broad-banded decoupling coil (Cryomagnet Systems).

Results and Discussion

Purification of [^{14}C]Methyl-Enriched Phenylalanine tRNA. Carbon NMR studies and initial signal assignments have previously been accomplished for unfractionated yeast tRNA

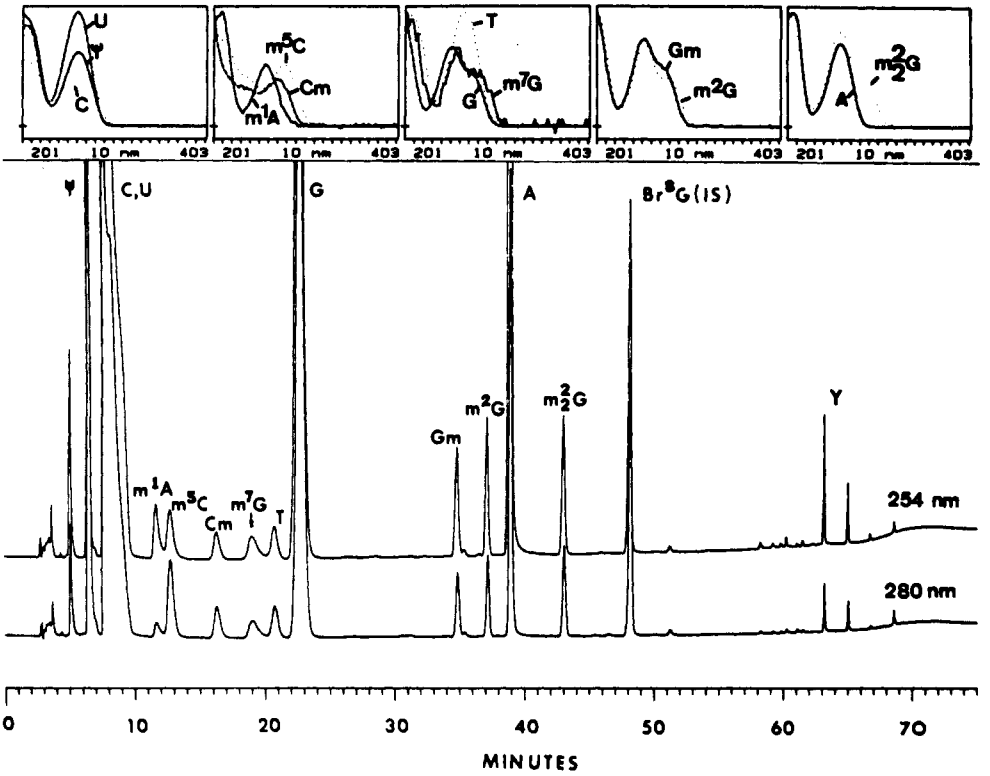

FIGURE 2: Reverse-phase high-performance liquid chromatographic separation of [^{13}C]methyl-enriched tRNA^{Phe} hydrolysate product. Chromatographic parameters and conditions have been previously published (Gehrke et al., 1982). The absorbances at 254 and 280 nm were measured at a sensitivity of 0.02 unit full scale. The total UV spectrum of each peak was measured simultaneously with elutions (top panels). Coincident elution of C and U did not allow application of this particular chromatography to the data of Table I but did give a clear resolution of the minor nucleoside peaks that eluted afterward.

in which the naturally occurring methyl groups had been ^{13}C enriched in vivo (Agris et al., 1983). The good results achieved from the carbon NMR of unfractionated tRNA demonstrated the potential of studying a purified single [^{13}C]methyl-enriched tRNA species. Transfer RNA specific for phenylalanine was chosen because of the ease of purification and expected high yield. Equally important to this choice was the fact that the X-ray crystallographic structure of yeast tRNA^{Phe} is well characterized and that motional data derived from specific sites on the tRNA will eventually lead to a better understanding of how this molecule functions biologically. [^{13}C]Methyl-enriched tRNA was prepared as described under Materials and Methods. A total of 2.5 g of crude nucleic acid was obtained. Due to properties of the yeast cell membrane, the phenol extraction procedure removes only low molecular weight nucleic acids from the yeast cell. This was confirmed by urea–polyacrylamide gel electrophoresis of the crude nucleic acid that displayed bands corresponding to only tRNAs and a faint band corresponding to 5S rRNA.

The unfractionated [^{13}C]methyl-enriched tRNA was then fractionated into single species by benzoylated DEAE-cellulose (BDC) chromatography. Due to the highly hydrophobic nature of the wybutosine nucleoside present in the tRNA^{Phe}, this tRNA remained on the BDC column while most of the remaining tRNA species were fractionated by using salt gradients. Transfer RNA^{Phe} was pooled and concentrated as described under Materials and Methods. Aminoacylation of the tRNA^{Phe} pool yielded 360 pmol of phenylalanine tRNA amino acid acceptance per A_{260} unit. This value is low, but the aminoacylation was performed by using only a yeast extract. No attempt was made at quantitation by optimizing the aminoacylation reaction. However, the pool was assayed for other tRNA species that may have coeluted during the ethanol gradient on the BDC column: tRNA^{Ser}, tRNA^{Leu}, tRNA^{Trp}, and tRNA^{Tyr}. The results of these assays demonstrated the tRNA^{Phe} pool to be of at least 85% purity. This was supported by gel electrophoresis and, particularly, by HPLC nucleoside analysis of the tRNA^{Phe}. Urea–polyacrylamide gel electrophoresis comparing [^{13}C]methyl-enriched tRNA^{Phe} with commercially available tRNA^{Phe} demonstrated very close to single species purity. In addition, the gel electrophoresis analysis under denaturing conditions showed that the tRNA^{Phe} was intact, i.e., did not suffer any nicks in the polynucleotide chain during preparation.

HPLC of Hydrolyzed tRNA^{Phe}. A small portion (15 μg) of the [^{13}C]methyl-enriched tRNA^{Phe} was subjected to complete enzymatic hydrolysis yielding ribonucleosides. Ribonucleosides were then separated and quantitatively analyzed by HPLC chromatography. The resulting HPLC chromatogram is shown in Figure 2. Quantitation of the nucleoside peaks demonstrates very good correlation of residues per molecule with the known sequence of tRNA^{Phe} as shown in Table I. This quantitative analysis offers strong support for the high degree of purity (85%) of the tRNA^{Phe} sample, as well as demonstrating that biosynthesis of modified nucleosides was virtually complete.

Isotopic Abundance Analysis. An additional portion of the tRNA^{Phe} pool (1.0 mg) was hydrolyzed and subjected to HPLC separation of nucleosides. Several peaks corresponding to major and methylated nucleosides were collected. These peaks were desalted by HPLC and subjected to direct probe mass spectroscopy. The mass spectrometry of major and minor nucleosides demonstrated that ^{13}C incorporation was restricted to the methylated nucleosides. Quantitation of the mass spectroscopy data of the thymidine nucleoside showed that isotopic enrichment was approximately 70 atom %.

In corroboration, the ^{13}C isotopic abundance was calculated from a comparison of integrated peak areas for ^{13}C-enriched

Table I: Comparison of Nucleoside Composition of [^{13}C]Methyl-Enriched tRNA^{Phe} to Published Sequence Composition[a]

nucleoside	residues per molecule	
	HPLC analysis	sequence
D	NA	2
Ψ	2.04	2
C	16.00	15
U	12.60	12
m^{1}A	0.84	1
m^{5}C	2.02	2
Cm	0.86	1
m^{7}G	0.72[b]	1
T	1.04	1
G	18.50	18
Gm	0.87	1
m^{2}G	0.86	1
A	16.40	17
m$_2^2$G	0.84	1
Y	NA	1

[a] The number of residues per molecule for each nucleoside in the [^{13}C]tRNA^{Phe} was derived from two independent HPLC analyses (as in Figure 2). The known number of residues was compiled from sequence data (Sprinzl & Gauss, 1983). Nucleosides not analyzed are designated by NA. [b] Low value due to lability of modified nucleoside under conditions of alkaline phosphatase digestion.

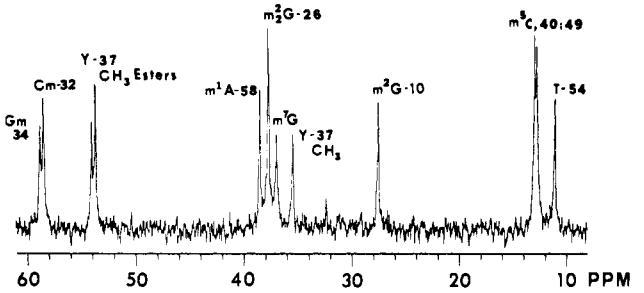

FIGURE 3: Carbon NMR spectrum of [^{13}C]methyl-enriched yeast tRNA^{Phe}. The figure shows only the methyl region of the spectrum, which was taken at 37 °C and is the result of 1900 accumulations acquired with a 90° pulse and a 6-s delay. The data were processed with 3-Hz line broadening.

and natural abundance (1.1%) ^{13}C resonances in the carbon NMR spectrum under conditions of complete T_1 relaxation and in the absence of nuclear Overhauser enhancement (NOE). For instance, the signal area of the individual thymidine methyl peak compared to that of the natural abundance signals from the ribose carbons indicated an isotopic abundance of approximately 85 atom %.

Carbon NMR Signal Assignments of Yeast tRNA^{Phe}. A carbon NMR spectrum of [^{13}C]methyl-enriched tRNA^{Phe} is shown in Figure 3. This figure displays the methyl region from 10 to 60 ppm. The spectrum was taken under broadband ^{1}H decoupling conditions at 37 °C. Experimental conditions of the sample are discussed under Materials and Methods. Signal assignments (Table II) were made initially by comparison with previous spectra from unfractionated yeast and *E. coli* tRNA and from fractionated *E. coli* tRNAs. Also, to aid in the assignment of chemical shifts, peaks were integrated in spectra where the gated decoupling method had been used to suppress nuclear Overhauser enhancements, which would be different for different carbons. The integrated area of the thymidine signal was assigned a value of 1.0, because it is known to occur in yeast tRNA^{Phe} at that frequency, its quantitation by HPLC analysis was 1.04, and the signal was well resolved.

Yeast tRNA^{Phe} contains 14 methyl groups, throughout the molecule, as expected sites of ^{13}C enrichment from methionine. Three of these sites are methyl groups attached to a nucleobase ring carbon. These are T-54, m^{5}C-40, and m^{5}C-49 and are

Table II: Carbon and Proton Chemical Shifts, ^{13}C–^{1}H Coupling Constants, and Signal Assignments for Yeast tRNA^{Phe}[a]

signal	chemical shift (ppm)		coupling constant, $^1J_{CH}$ (Hz)	assignment	location in cloverleaf structure
	^{13}C	^{1}H			
1	11.0	1.00	130	T-54	TΨCG loop
2, 3	12.7	1.56	130	m^{5}C-40	anticodon stem
	12.9	1.60	132	m^{5}C-49	TΨCG stem
b		2.09		Y-37 C–CH$_3$	anticodon loop
4	27.4	2.80	139	m^{2}G-10	D stem
5	35.4	3.91	141	Y-37 N–CH$_3$	anticodon loop
6	36.9	3.79	144	m^{7}G-46	extra arm
7, 8	37.6	2.48	142	m$_2^2$G-26	between D stem and anticodon stem
9	38.4	3.03	147	m^{1}A-58	TΨCG loop
10, 11	53.6	3.69	147	Y-37 methyl esters	anticodon loop
	53.9				
12	58.4	3.65	140	Cm-32	anticodon loop
13	58.7			Gm-34	anticodon loop
5′	63.0			5′ ribose carbon	
2′, 3′	71.0			2′,3′ ribose carbon	
	74.0				
4′	80.5			4′ ribose carbon	
1′	90.0			1′ ribose carbon	
14	120.9			Y-37 ring carbon	anticodon loop

[a] The signal numbers refer to Figure 5. Chemical shifts are relative to dioxane at 67.4 ppm for ^{13}C and 3.74 ppm for ^{1}H. The number after the nucleoside designates the position in the standard 76-nucleotide sequence of tRNA. Location within the tRNA cloverleaf structure was obtained from sequence data. [b] This methyl group was not ^{13}C enriched; thus, no carbon signal was evident.

assigned to the peaks that are farthest upfield in the carbon spectrum. The integral of this area was 2.95. Under these NMR experimental conditions (Figure 3), the T resonance was well resolved. The m^{5}C resonances were slightly resolved from each other by 0.2 ppm (Table II). It is interesting that the environments of the m^{5}C nucleosides could be dissimilar enough to contribute to the resolution of signals. They are also partially resolved in ^{1}H NMR spectra, but by only 0.05 ppm (Davanloo et al., 1979). It is impossible at this time to distinguish between the two m^{5}C signals for purposes of assignment to nucleosides 40 and 49.

The next signal downfield in the carbon spectrum, at 27.4 ppm, is assigned to m^{2}G-10, an aminomethyl (integral = 0.99). This assignment is based on the chemical shift of the mononucleoside standard and previous assignment from unfractionated *E. coli* tRNA (Tompson et al., 1979).

The group of resolved peaks from 35.4 to 38.4 ppm integrated to 5.4 carbons. This set of signals is contributed by the following methyl signals: the ring nitrogen-bound methyls of m^{7}G-46 and m^{1}A-58, the aminomethyl m$_2^2$G-26 (containing two [^{13}C]methyl-enriched carbons), and the ring nitrogen-bound methyl of the Y base 37.

The m^{7}G peak assignment at 36.9 ppm is based on the chemical shift of 36.8–37.1 ppm found in spectra of purified and unfractionated *E. coli* tRNAs (Tompson et al., 1979; Kopper et al., 1983) and on the peak's highly characteristic broadness and chemical shift sensitivity to metal ion concentration and temperature (Agris & Schmidt, 1980). The resonance at 37.6 ppm integrates to two carbons. It was never seen to split into resolved peaks under any conditions. Thus, it most likely is the two methyl carbons of m$_2^2$G-26. The two remaining signals at 35.4 and 38.4 ppm are assigned to the N–CH$_3$ of Y (Figure 4) and the N–CH$_3$ of m^{1}A, respectively. The latter signal responds to raised temperatures and the lack of Mg^{2+} by moving upfield. This is analogous to the methyl resonance of m^{7}G, the only other nucleoside carrying a positive

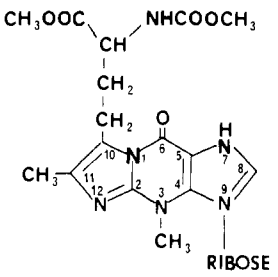

FIGURE 4: Chemical structure of the wybutosine (Y) nucleoside (Blobstein et al., 1975).

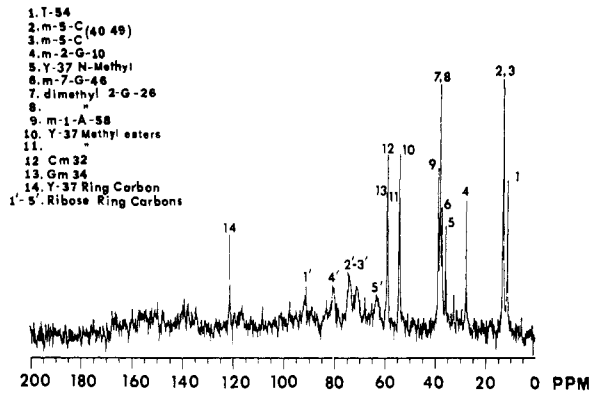

FIGURE 5: Carbon NMR spectrum of [^{13}C]methyl-enriched yeast tRNA^{Phe}. The spectrum was taken under the same conditions as in Figure 3 and shows the one ^{13}C-enriched signal downfield (120.9 ppm) of the methyl region (10–60 ppm).

charge in tRNA under physiological conditions. Spectra of the mononucleosides m^{1}A and m^{7}G show the m^{1}A signal to be downfield of that for m^{7}G, as we have assigned it in the tRNA spectrum. In contrast, the one remaining N–CH$_3$ signal in this area of the tRNA spectrum is upfield of m^{7}G and thus assigned to Y.

The methyl esters attached to Y-37 are assigned to the moderately resolved signals at 53.6 and 53.9 ppm (integral = 1.88). The 2′-O-methylated nucleosides Cm-32 and Gm-34 are assigned to the moderately resolved signals at 58.4 and 58.7 ppm (integral = 2.25), with Gm being the farthest downfield (Kopper et al., 1983).

The remaining methyl that could possibly be a site for ^{13}C enrichment is attached to the nucleobase ring carbon in Y-37. No signal for this methyl was found, suggesting that this site is not ^{13}C enriched and is therefore not derived from the methyl of methionine.

A complete carbon NMR spectrum from 0 to 200 ppm is shown in Figure 5. Table II lists all of the assigned chemical shifts for the enriched methyl carbons and for the naturally occurring ribose carbons. Assignment of the ribose carbon signals is based on previously published carbon spectra (Schmidt et al., 1983). Assignment of the downfield signal at 120.9 ppm is discussed below.

Selective Carbon Decoupling and Observation of the Proton Spectrum. In order to compare all methyl carbon signal assignments with methyl proton assignments, a control proton spectrum (Figure 6A), taken with ^{13}C decoupling about 15 kHz off resonance, was subtracted from each proton spectrum taken with the ^{13}C decoupling frequency corresponding to each [^{13}C]methyl-enriched resonance. Two examples are shown in Figure 6 and two more in Figure 7, where ^{1}H NMR difference spectra reveal fully decoupled peaks by a ^{13}C–^{1}H doublet pointing down and a singlet within the doublet pointing up. Partial decoupling occurs for carbon peaks near the ^{13}C decoupling frequency and appears as a positive doublet of

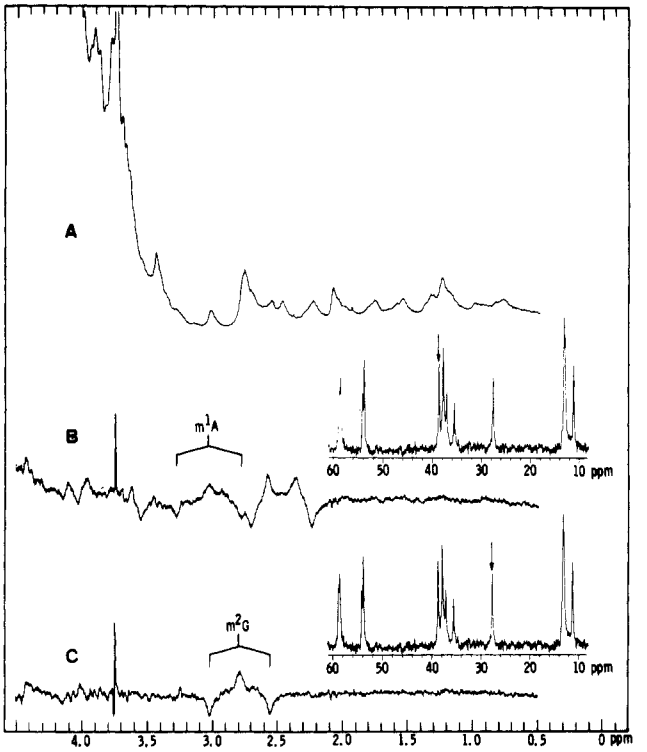

FIGURE 6: Selective carbon decoupling with observation of the proton NMR spectrum. The decoupling radio-frequency field corresponded to a value of B_2 = 125 Hz. (A) ^{1}H spectrum (not a difference spectrum) with ^{13}C irradiation at 118 ppm. The ^{1}H methyl peaks are fully coupled to ^{13}C and consist of a central resonance of ca. 30% of the total area (from residual ^{12}C) flanked by peaks of a doublet generated by ^{13}C enrichment of approximately 70 atom %. (B) As in (A) but with methyl carbon irradiation at m^{1}A, 38.4 ppm. Complete collapse of a proton resonance at 3.03 ppm is seen as well as partial decoupling of peaks at 2.48, 3.79, and 3.91 ppm. (C) Difference spectrum in which the control proton spectrum with carbon irradiation at 118 ppm was subtracted from the ^{1}H spectrum with irradiation at the methyl carbon of m^{2}G, 27.4 ppm. The sharp artifact at 3.74 ppm remains from incomplete subtraction of the prominent *p*-dioxane resonance.

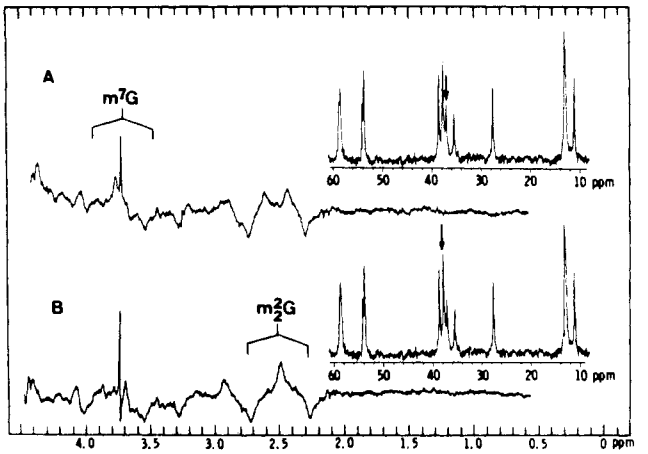

FIGURE 7: Observation of the proton NMR spectra resulting from selective carbon decoupling of two closely occurring methyl carbon signals, m^{7}G and m$_2^2$G. (A) Difference spectrum in which the control proton spectrum of Figure 6A was subtracted from the proton spectrum resulting from irradiation at the methyl carbon of m^{7}G, 36.9 ppm. (B) Difference spectrum as in (A) but with methyl carbon irradiation at m$_2^2$G, 37.6 ppm.

reduced *J* value between the negative doublet of full *J* value. When the ^{13}C carbon signal of m^{1}A at 38.4 ppm was irradiated and the coupled spectrum of Figure 6A subtracted, the difference spectrum of Figure 6B resulted. A completely decoupled peak appears at 3.03 ppm. Three other ^{1}H peaks at

2.48, 3.79, and 3.81 ppm are partially decoupled. They are assigned to m_2^2G, m^7G, and Y–NCH_3, respectively. The peak at 3.03 ppm had been previously assigned to the methylene of dihydrouridine, D-16 (Davanloo et al., 1979). A recent study from a different laboratory left this peak unassigned (Heerschap et al., 1983). The selective decoupling experiment reported here assigns the peak to the methyl of m^1A-58.

Interestingly, the shape and size of the 3.03-ppm resonance match almost exactly the features of the firmly assigned T-54 CH_3 proton peak at 1.0 ppm. In melting experiments the resonance at 3.03 ppm diminishes in intensity and disappears along with the T signals at the same temperature. A-58 is H-bonded to T-54 in the crystal structure and would be expected to follow a similar unfolding pattern. Furthermore, the crystal structure (Holbrook et al., 1978) shows the m^1A-58 methyl group positioned above the plane of the purine ring of G-18. An upfield shift is expected, similar to that experienced by the T-54 methyl due to stacking on G-53. A peak at 3.03 ppm for m^1A would represent a 0.83-ppm upfield shift, compared to an 0.75 ppm for T.

The difference spectrum taken with the ^{13}C decoupling frequency corresponding to the methyl carbon of m^2G (24.4 ppm) is shown in Figure 6C. The positive peak observed corresponds to the previously published chemical shift of m^2G in the proton spectrum (Davanloo et al., 1979). Parts A and B of Figure 7 are also difference spectra but taken with the ^{13}C decoupling frequencies corresponding to the methyl carbon of m^7G (36.9 ppm) and m_2^2G (37.6 ppm), respectively. These two spectra demonstrate that even closely occurring carbon signals may be sufficiently distinguished in the decoupling experiment as to determine the associated protons unambiguously.

Methyl ^{1}H NMR assignments correlated to ^{13}C resonances and H–C scalar coupling constants for all tRNA^{Phe} methyl groups are reported in Table II. Since the entire proton spectrum of yeast tRNA^{Phe} has been previously published, it was possible to compare all proton and carbon-13-enriched signals by the carbon decoupling method. Every assigned ^{13}C-enriched carbon peak was decoupled in separate experiments. Only the irradiation of the enriched carbon-13 signal at 120.9 ppm resulted in no observable change in the proton spectrum.

Assignments of methyl and methylene peaks in tRNA ^{1}H NMR spectra have relied on thermal melting studies where resonances are followed from high temperature (unfolded) to low temperature (folded) (Kan et al., 1977). This method breaks down when peaks undergo slow chemical exchange dynamics, disappearing from one position and reappearing at another, a situation often encountered (Kan et al., 1977; Kastrup & Schmidt, 1978; Davanloo et al., 1979). The high temperature and extended acquisition times of this method can lead to hydrolysis and degradation of the molecule and to the formation of unnatural, but stable, intermediate tertiary structures (Lindahl et al., 1967). We have introduced here an alternative assignment method based on scalar coupling of ^{13}C and ^{1}H. By correlating ^{13}C and ^{1}H spectra via specific ^{13}C decoupling, we have made complete assignments of methyl resonances in the ^{1}H spectrum of tRNA^{Phe} under native structure conditions. Recently another heteronuclear method, which involves ^{17}O labeling, has been shown to be advantageous in assigning ^{31}P NMR signals (Joseph & Bolton, 1984; Petersheim et al., 1984).

Carbon-13 Enrichment of Wybutosine. The structure of the hypermodified wybutosine (Y) nucleoside that occurs 3′ to the anticodon in yeast tRNA^{Phe} is shown in Figure 4. At first glance, there are four expected sites for ^{13}C enrichment by [^{14}C]methylmethionine, the two methyl esters, the methyl attached to the ring nitrogen, and the methyl attached to the ring carbon. Carbon NMR signal assignments have been made for the enriched carbons present in the methyl esters and attached to the ring nitrogen (see Figure 5 and Table II). The tRNA has 14 methyl groups but only 13 carbons appear in the region 0–60 ppm, the methyl resonance frequencies. All 13 have been assigned to methyls other than the group attached to the ring carbon of Y. Thus, we conclude that the Y–CCH_3 is not ^{13}C enriched and not derived from the methyl of methionine.

The only ^{13}C-enriched signal remaining initially unassigned was the downfield peak at 120.9 ppm. Irradiation at this carbon's signal frequency produced no significant change in the proton spectrum. (A long range coupling would not have appeared under the spectral conditions used.) Therefore, this ^{13}C-enriched signal was emanating from a carbon without directly bonded protons yet was derived from the methyl of methionine. The peak area integrates to a single carbon enriched by 70 atom %. Of course, the signal could be the same carbon of 15 different nucleosides throughout the structure, each carbon enriched only 5%. However, the sharpness of this peak, in contrast to the chemical shift nonequivalence of enriched ring carbons of the major nucleobases (Schmidt et al., 1980; Olsen et al., 1982), strongly suggests that this downfield signal must have entered tRNA at the ring carbon of a single modified nucleobase. Thus, it is assigned to one of the two carbons in the third ring of the Y base (Figure 4). These are the only carbons without protons that could be derived from the methyl of methionine.

The posttranscriptional biosynthesis of Y in yeast tRNA^{Phe} is only partially understood. The precursor molecule has been shown to be guanosine with the 3-amino-3-carboxypropyl group added from methionine (Huimin et al., 1973; Munch & Thiebe, 1975). Our work has shown that the methyl esters added onto the side chain and the N–CH_3 all derive from the methyl donor methionine but that the C–CH_3 does not. The mechanism that adds the latter methyl group and the two additional carbons to close the third ring of the nucleobase is not understood. A molecule such as pyruvate or another three-carbon molecule could be condensed with guanosine to form the third ring and C–CH_3. Chemical addition of α-bromo ketones to guanosine has been demonstrated (Kasai et al., 1971). An alternative mechanism is initial methylation at the N^1 position of guanosine resulting in N^1-methylguanosine, a frequently occurring methylated nucleoside in tRNA, followed by the insertion of a two-carbon molecule between the N^1-carbon and the N^2-amino group to close the third ring. In either case, one of the two carbons in the new ring structure has become ^{13}C enriched via [^{13}C]methylmethionine. Whether methionine donates the methyl group at the N^1 position of guanosine or whether methionine is involved in the synthesis of the three-carbon molecule mentioned still remains to be determined. However, we hypothesize that biosynthesis includes a three-carbon substrate addition to guanosine. The methionine-derived ^{13}C-enriched carbon would be bound to the N^1 position and the Y side chain, which is also derived from methionine.

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REFERENCES

Agris, P. F., & Schmidt, P. G. (1980) *Nucleic Acids Res. 8*, 2085–2091.

Agris, P. F., Powers, T., Soll, D., & Ruddle, F. (1974) *Cancer Biochem. Biophys. 1*, 69–77.

Agris, P. F., Fujiwara, F. G., Schmidt, C. F., & Loeppky, R. N. (1975) *Nucleic Acids Res. 2*, 1503–1512.

Agris, P. F., Tompson, J. G., Gehrke, C. W., Kuo, K. C., & Rice, R. H. (1980) *J. Chromatogr. 194*, 205–212.

Agris, P. F., Kovacs, S. A. H., Smith, C., Kopper, R. A., & Schmidt, P. G. (1983) *Biochemistry 22*, 1402–1408.

Bjork, G. R. (1984) in *Processing of RNA* (Apirion, D., Ed.) pp 291–330, CRC Press, Boca Raton, FL.

Blobstein, S. H., Gerbert, R., Grunberger, D., Nakanisi, K., & Weinstein, I. B. (1975) *Arch. Biochem. Biophys. 167*, 668–673.

Davanloo, P., Sprinzl, M., & Cramer, F. (1979) *Biochemistry 18*, 3189–3199.

Gehrke, C. W., Kuo, K. C., McCune, R. A., Gerhardt, K. O., & Agris, P. F. (1982) *J. Chromatogr. Biomed. Appl. 230*, 297–308.

Gerhardt, K. O., Rice, R. H., & Gehrke, C. W. (1983) in *The Modified Nucleosides of Transfer RNA, II* (Agris, P. F., & Kopper, R. A., Eds.) pp 93–128, Alan R. Liss, New York.

Gorenstein, D. G., & Goldfield, E. M. (1982) *Biochemistry 21*, 5839–5849.

Hamill, W. D., Jr., Horton, W. J., & Grant, D. M. (1980) *J. Am. Chem. Soc. 102*, 5454–5458.

Hare, D. R., & Reid, B. R. (1982) *Biochemistry 21*, 5129–5135.

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

Holbrook, S. R., Sussman, J. L., Warrant, R. W., & Kim, S. H. (1978) *J. Mol. Biol. 123*, 631–660.

Huimin, J. L. Nakanishi, K., Grunberger, D., & Weinstein, I. B. (1973) *Biochem. Biophys. Res. Commun. 55*, 818–823.

Joseph, A. P. & Bolton, P. H. (1984) *J. Am. Chem. Soc. 106*, 437–439.

Kan, L. S. Ts'o, P. O. P., Sprinzl, M., Van der Haar, F., & Cramer, F. (1977) *Biochemistry 16*, 3143–3153

Kasai, H., Goto, M., Takemura, S., Goto, T., & Matsura, S. (1971) *Tetrahedron Lett. 29* 2725–2728.

Kastrup, R. V., & Schmidt, P. G. (1978) *Nucleic Acids Res. 5*, 257–269.

Kim, S. H., Quigley, G. J., Suddath, F. L., McPherson, A., Sneden, D., Kim, J. J., Weinzierl, J., & Rich, A. (1972) *Science (Washington, D.C.) 179*, 285–288.

Kohli, J., Kwong, T., Altruda, F., Soll, D., & Wahl, G. (1979) *J. Biol. Chem. 254*, 1546–1551.

Kopper, R. A., Schmidt, P. G., & Agris, P. F. (1983) *Biochemistry 22*, 1396–1401.

Lindahl, T., Adams, A., & Fresco, J. R. (1967) *J. Biol. Chem. 242*, 3129–3134.

Munch, H. J., & Thiebe, R. (1975) *FEBS Lett. 51*, 257–258.

Olsen, J. I., Schweizer, M. P., Walkiw, I. J., Hamill, W. D., Jr., Horton, W. J., & Grant, D. M. (1982) *Nucleic Acids Res. 10*, 4449–4464.

Petersheim, M., Mehdi, S., & Gerlt, J. A. (1984) *J. Am. Chem. Soc. 106*, 439–440.

Roy, S., Papastiavios, M. Z., & Redfield, A. G. (1982) *Biochemistry 21*, 6081–6088.

Schmidt, P. G., & Edelheit, E. B. (1981) *Biochemistry 20*, 79–86.

Schmidt, P. G., Tompson, J. G., & Agris, P. F. (1980) *Nucleic Acids Res. 8*, 643–656.

Schmidt, P. G., Playl, T., & Agris, P. F. (1983) *Biochemistry 22*, 1408–1415.

Schweizer, M. P., Hamill, W. D., Jr., Walkiw, I. J., Horton, W. J., & Grant, D. M. (1980) *Nucleic Acids Res. 8*, 2075–2083.

Sprinzl, M., & Gauss, D. H. (1983) in *Modified Nucleosides of Transfer RNA II* (Agris, P. F., & Kopper, R. A., Eds.) pp 129–226, Alan R. Liss, New York.

Sussman, J. L., Holbrook, S. R., Warrant, R. W., Church, G. M., & Kim, S. H. (1978) *J. Mol. Biol. 123*, 607–630.

Tompson, J. G., & Agris, P. F. (1979) *Nucleic Acids Res. 3*, 765–779.

Tompson, J. G., Hayashi, F., Paukstelis, J. V., Loeppky, R. N., & Agris, P. F. (1979) *Biochemistry 18*, 2079–2085.