Complete Nuclear Magnetic Resonance Signal Assignments and Initial Structural Studies of [^{13}C]Methyl-Enriched Yeast Transfer Ribonucleic Acid†

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ABSTRACT: Carbon-13 methyl enrichment of yeast tRNA in vivo has produced probes that do not perturb native tRNA structure, are located at 19 distinct sequence positions, and are sensitive to structural perturbations of the RNA. Exclusive ^{13}C enrichment of methyl groups was accomplished by design of a medium for optimal growth of cultures and maximal posttranscriptional incorporation of [^{13}C]methyl groups into nucleic acids of a methionine auxotroph of *Saccharomyces cerevisiae*. Carbon-13-enriched tRNA isolated from these cultures and [^{3}H]methyl-labeled tRNA isolated from analogous cultures grown with [^{3}H]methylmethionine were fully methylated as determined by high-performance liquid chromatographic analysis of nucleosides. Ninety-two percent of the radiochemical label was found associated with methylated nucleosides. Carbon-13 NMR spectra of ^{13}C enriched tRNA exhibited prominent high-field, methyl signals between 11 and 60 ppm. Integration of signal area relative to that of the natural abundance (1.1%) ribose carbons indicated a 50–60 atom % [^{13}C]methyl enrichment. Signal assignments have been made for the methyl carbons of ribothymidine, 5-methylcytidine, N^2-methylguanosine, 3-methylcytidine, 1-methylguanosine, 1-methyladenosine, 7-methylguanosine, N^2,N^2-dimethylguanosine, 5-(methoxycarbonylmethyl)uridine, and the 2′-*O*-methyl derivatives of cytidine, guanosine, and uridine. These nucleosides are known to be located at 19 sequence positions in loops and stem regions of the cloverleaf structure and the first position of the anticodon. Addition of Mg^{2+} caused significant line broadening of the yeast tRNA methyl signals, in comparison to other yeast tRNA signals and to all signals of [^{13}C]methyl-enriched *Escherichia coli* tRNA. One possible explanation for the difference between the tRNAs of these organisms is an inherent greater variety of tertiary structures probed by the methyl groups of yeast tRNA vs. that of *E. coli*. Methyl signal chemical shifts moved, on the average, 0.5 ppm upfield with addition of Mg^{2+} and downfield with heat denaturation of the tRNA. The exception to this was the resonance for m^{7}G, which displayed the opposite behavior. Methyl resonances of ribothymidine and 2′-*O*-methyl nucleosides exhibited low-temperature (20–40 °C) downfield shifts in the absence of Mg^{2+} corresponding to temperatures expected to break tertiary interactions between the TψCG loop and the dihydrouridine loop in which these methyls reside. Methyl signals for N^2-methylguanosine and N^2,N^2-dimethylguanosine exhibited significant downfield shifts at temperatures above 40 °C corresponding to temperatures at which secondary structure is expected to dissolve. Relaxation and nuclear Overhauser effect measurements yielded unique solutions for the overall rotational correlation time (10–20 ns) and internal motion correlation times (0.6–2 ps) for the different methyl groups. Thus ^{13}C-enriched methyl groups of yeast tRNA will serve as probes of local dynamics at unambiguously assigned locations throughout the molecule.

The amount of specific nucleic acid available for the study of native secondary and tertiary structure in solution is restricted. This is due to the small amounts in cells and difficulty in purification. Thus, a nondestructive determination of macromolecular conformation is important in order to retain the RNA or DNA for multiple studies. Nuclear magnetic resonance (NMR) spectrometry is a nondestructive means of studying native nucleic acid structure in solution, as well as perturbations of that structure. NMR, in contrast to many other physical chemical techniques, is capable of elucidating both overall dynamics and site-specific internal dynamics of a molecule. However, this technology alone is not enough for understanding the complex structure–function relationships of a macromolecule. In addition, it is important to investigate a biochemically well-defined molecule. Therefore, the study of tRNA structure is a unique opportunity. It is a small nucleic acid (M_r 25 000) and has well-understood biochemistry and genetics. Distinct mutant tRNAs and termination code suppressor tRNAs are potentially available from easily grown strains of both a procaryote, *Escherichia coli*, and a eucaryote, yeast. The only well-characterized X-ray crystallographic structure of tRNA is that of yeast tRNA^{Phe} (Holbrook et al., 1978; Sussman et al., 1978). We have developed methods of studying *E. coli* tRNAs by carbon-13 NMR spectrometry (Tompson et al., 1979). In this paper, we describe the adaptation of these methods to the study of yeast tRNA structure in solution.

Carbon-13 natural abundance is of such low magnitude, 1.1 atom %, that large amounts of material and long NMR accumulation times are required (Bolton & James, 1980; Komoroski & Allerhand, 1972, 1974). Single-carbon resonance resolution by natural abundance ^{13}C NMR is, therefore, essentially unattainable for the available small amounts of a specific nucleic acid. However, we have developed methods that in vivo greatly ^{13}C-enrich specific carbons native to the nucleic acid structure (Tompson & Agris, 1979). Small amounts (5 mg) of the isolated tRNA exhibit ^{13}C-enriched NMR signals in a relatively short accumulation time, and these signals are easily assigned to specific carbons from foreknowledge of the enrichment technique.

The first successful enrichment of carbons native to the tRNA structure was accomplished by incorporation in vivo

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of methyl groups from methionine into the methylated nucleosides of an *E. coli* auxotrophic for methionine (Agris et al., 1975). Subsequently, we and others have been able to effectively enrich the ring carbons of the bases in tRNA at the C_2 of adenine, uracil, and cytosine (Schmidt et al., 1980) and the C_4 of uracil (Hamill et al., 1980). Single-carbon resonance resolution has been demonstrated by us and others for purified, individual species of tRNA enriched in [^{13}C]-methyl groups (Agris & Schmidt, 1980; Yokoyama et al., 1980; Kopper et al., 1983) and C_4 of uracil (Schweizer et al., 1980). Recently, we have accomplished ^{13}C enrichment of the $C_{1'}$-ribose position of *E. coli* tRNA (Schmidt et al., 1983). These studies have shown that a more detailed and accurate understanding of the overall dynamics (Schmidt et al., 1980, 1983; Hamill et al., 1980) and specific-site internal dynamics (Schmidt et al., 1983) of tRNA can be achieved.

Materials and Methods

Preparation of [^{13}C]Methyl-Enriched tRNA. Saccharomyces cerevisiae met$^-$trp$^-$ (RH 762) was grown at 30 °C in a minimal medium composed of 0.145% yeast nitrogen base (without amino acids or ammonium sulfate; DIFCO), 2% glucose, 1% succinic acid, 0.28% KOH, 0.525% ammonium sulfate, 0.30 mM L-tryptophan, and 0.20 mM [^{13}C]- or [^{3}H]methylmethionine (Merck, Canada, and New England Nuclear, Boston, MA). Cells were harvested in stationary phase (1.25×10^8 cells/mL) by centrifugation. Incorporation of methyl groups into tRNA was analyzed, first, with small cultures grown on [^{3}H]methylmethionine. Subsequently, a 30-L culture grown with medium containing [^{13}C]methylmethionine yielded a total of 147 g of cells. Nucleic acids in the amount of 295 mg (A_{260} = 5900 units) were extracted by phenol. Bulk tRNA was purified by DEAE-cellulose[1] column chromatography as previously described (Agris et al., 1975). Unfractionated yeast tRNA, concentrated by ethanol precipitation, was dialyzed extensively against glass-distilled water (pH 6.8). The amount of unfractionated tRNA recovered was 50 mg (A_{260} = 1200 units), and the A_{260}/A_{280} ratio was 1.87. The dialyzed sample was concentrated by evaporation under vacuum to 1.0 mL, exchanged twice with an equal volume of D_2O, and concentrated to a final volume of approximately 0.5 mL. The auxotrophic strain of yeast was a gift from Dr. R. Hütter (The Microbiological Institute in Zurich).

Nucleoside compositions of [^{3}H]methyl-enriched tRNA and [^{13}C]methyl-enriched tRNA preparations were determined. Enzymatic digests of the tRNAs by P1 nuclease and bacterial alkaline phosphatase (Gehrke et al., 1982) were subjected to high-performance liquid chromatographic separation (HPLC), detection by UV absorbance, and quantitation (Davis et al., 1979).

E. coli [^{13}C]methyl-enriched tRNA was prepared from *E. coli* C6 rel$^-$met$^-$cys$^-$ as previously described (Tompson et al., 1979). For analysis of tRNA ^{13}C resonances in the presence of Mg^{2+}, $MgCl_2$ was added to samples at room temperature to obtain a final concentration of 10 mM.

Procedure for Acquisition of NMR Spectra. Nuclear magnetic resonance spectra at 75.5 MHz (^{13}C) were collected with a quadrature-detection Fourier-transform spectrometer of hybrid construction composed of a Cryomagnet Systems 70/50 magnet with 10-mm probe, a Nicolet 1180 computer, a Bruker temperature control unit, and in-house radio-frequency gear. Samples were placed in 0.5-mL microinserts for 10-mm NMR tubes, and the positioning of the insert within the probe was optimized for signal to noise ratio and line shape.

[1] Abbreviation: DEAE, diethylaminoethyl.

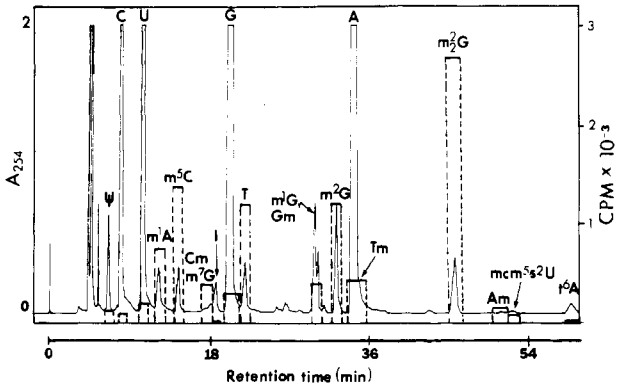

FIGURE 1: [^{3}H]Methyl incorporation into yeast tRNA nucleosides. Yeast strain RH 762 was grown in medium containing [^{3}H]-methylmethionine. Transfer RNA isolated from stationary-phase cells was enzymatically hydrolyzed to nucleosides (Gehrke et al., 1982), and then the nucleosides were subjected to HPLC separation (Davis et al., 1979). The chromatogram depicts the absorbance at 254 nm for this separation (solid line). In addition, fractions containing the major and modified nucleosides were analyzed for radioactivity by liquid scintillation counting. Total radioactivity (cpm) in each nucleoside assayed is depicted in the chromatogram (dashed lines). Ninety-two percent of all radioactivity was found associated with methylated nucleosides.

Yeast tRNA signal assignments were made by comparison of spectra to that of mononucleosides, purchased from Sigma Chemical Co. (St. Louis, MO) and used as standards without further purification. Signal assignments were also corroborated by comparison to assignments for *E. coli* tRNA published earlier (Tompson et al., 1979). Nucleoside-composition analysis of the [^{13}C]methyl-enriched tRNA was an additional aid in making assignments.

Results and Discussion

Incorporation of Methyl Groups into Yeast tRNA. S. cerevisiae strain RH 762 is auxotropic for both methionine and tryptophan. Optimal growth of this yeast in minimal medium was obtained when the supplemental concentrations of methionine and tryptophan were 30 and 60 mg/L, respectively. A 500-mL culture of yeast was grown in the presence of 100 μCi of [^{3}H]methylmethionine (0.08 mg/100 μCi) supplemented with additional nonradioactive methionine (14.92 mg) and tryptophan (30 mg). The culture, harvested by centrifugation, yielded 3.08 g of packed cells. Nucleic acids were extracted with phenol as described under Materials and Methods with a yield of ~130 A_{260} units, and the tRNA (16.4 A_{260} units) was separated by DEAE-cellulose column chromatography.

A portion of the [^{3}H]methyl-enriched tRNA (5 A_{260} units) was enzymatically digested to nucleosides, which were then separated by HPLC. Fractions corresponding to 20 spectrophotometrically defined major and methylated nucleoside peaks were collected, and their radioactivity was determined by scintillation counting. The chromatographic separation and radioactivity for each peak is shown in Figure 1. Ninety-two percent of the radioactivity detected in all the peaks collected was located in methyl-modified nucleosides; the remaining 8% was associated with A, C, G, and U. Seventy-six percent of the total radioactivity was confined to only five nucleosides: m^{1}A, m^{5}C, ribo-T, m^{2}G, and m$_2^2$G. The remaining 16% of tritium incorporation was present in six other methylated nucleosides: Cm, m^{7}G, Gm, Tm, Am, and mcm^{5}s^{2}U.

As the NMR studies were to be conducted on tRNA that was [^{13}C]methylmethionine enriched, it seemed desirable to preclude even the possible 8% incorporation of the methyl carbon into the nucleobase ring structure as might occur

Table I: Nucleoside Composition of [^{13}C]Methyl-Enriched, Unfractionated Yeast tRNA

nucleoside	nmol of nucleoside/ hydrolysate	residue/tRNA
ψ	16.10	3.29
C	98.80	20.20
U	60.27	11.62
m^1A	2.76	0.56
m^5C	5.02	1.03
Cm	0.80	0.16
m^7G	1.21	0.25
I	1.41	0.28
G	96.25	19.68
T	5.57	1.14
m^1I	0.23	0.05
m^1G	2.79	0.57
Gm	1.39	0.28
m^2G	2.91	0.60
A	70.59	14.43
Tm	present	present
m_2^2G	2.63	0.54
Am	0.14	0.03
mcm^5s^2U	present	present
t^6A	2.07	0.42
m^2A	0.20	0.04
i^6A	present	present

through the 1-carbon pool. A hypoxanthine analogue, 6-mercaptopurine (6-MP), has been shown to be an effective inhibitor of de novo purine biosynthesis in *E. coli* (Tompson & Agris, 1979). However, the yeast strain proved to be refractory to 6-MP inhibition in concentrations up to 10^{-4} M. Solubility limitations prohibit use of higher concentrations.

Yeast [^{13}C]methyl-enriched tRNA was prepared as described under Materials and Methods. A total of 60 mg of unfractionated tRNA was recovered from 30 L of culture. Some of this tRNA was used to assess aminoacylation and analyze tRNA nucleoside composition. Assays to determine the ability of [^{13}C]methyl-enriched tRNA to be aminoacylated were conducted as previously described (Tompson & Agris, 1979) with a mixture of radioactively labeled amino acids. The yeast tRNA showed appropriate biological activity with approximately 1400 pmol of amino acids accepted per 50 μg (1 A_{260} unit) of tRNA.

A portion of the [^{13}C]methyl-enriched tRNA (3 A_{260} units) was digested to nucleosides and subjected to HPLC analysis. The results are presented in Table I. These results indicate the incorporation of approximately five methyl groups per average tRNA molecule, 76 nucleosides in length. Thus, the tRNA was fully methylated as determined by comparison to published analyses (Hall, 1971) and sequence data (Sprinzl & Gauss, 1982). Relative amounts of each of the ^{13}C-methylated nucleosides in the tRNA compared favorably with these prior reports, as well as with the [^{3}H]methyl incorporation study shown in Figure 1.

Carbon NMR Signal Assignments of Yeast tRNA. A ^{13}C NMR spectrum of [^{13}C]methyl-enriched yeast tRNA is shown at the top of Figure 2. This spectrum was taken at approximately 70 °C and, therefore, represents the almost completely denatured polynucleotide. Chemical shifts of all the carbon signals compare extremely well with that of the accompanying *E. coli* tRNA spectrum (Figure 2), also taken at high temperature (60 °C), although the signal to noise ratio of the latter is superior. Carbon signals numbered 1–9 represent the ^{13}C-enriched methyl signals of modified nucleosides, whereas signals 10–36 are the ring carbons of the bases and ribose. Signal assignments are listed in Table II.

Yeast tRNA has four types of methylated nucleosides with the methyl group attached to a nucleobase ring carbon: T

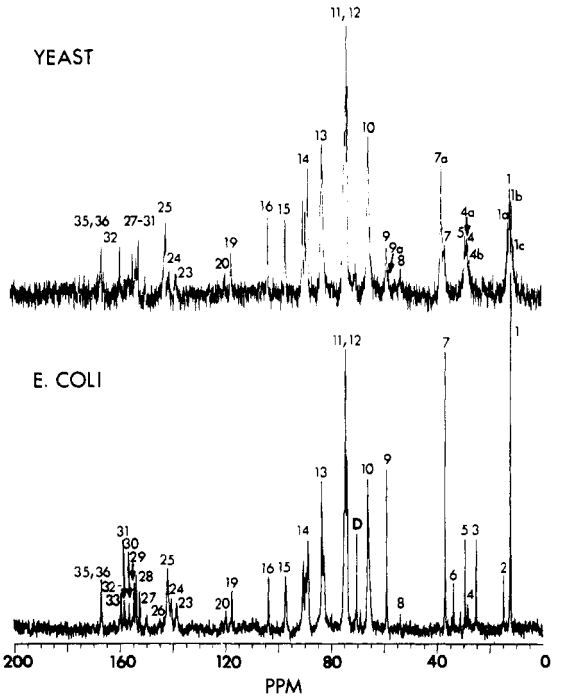

FIGURE 2: ^{13}C NMR spectra of [^{13}C]methyl-enriched yeast and *E. coli* tRNA. Transfer RNA (2 mM) obtained from yeast grown with [^{13}C]methylmethionine was placed in a 0.5-mL microsample insert for a 10-mm NMR tube. The spectrum shown was taken at 72 ± 1 °C and is the result of 10 000 accumulations (±8000-Hz sweep width) acquired with a 60° pulse and 0.95-s delay, including an acquisition time of 0.25 s. The data were processed with 7-Hz line broadening. A spectrum of [^{13}C]methyl-enriched *E. coli* tRNA taken at 60 °C is shown for comparison. This sample was 1.6 mL of a 2 mM tRNA solution in a 10-mm NMR tube. The spectrum is the result of 50 000 accumulations also acquired with a 60° pulse but a shorter delay, 0.75 (3-Hz line broadening). Carbon-13-enriched methyl signals are numbered 1–9. Yeast tRNA signal assignments for all numbered signals are listed in Table II. Signals are numbered according to Tompson et al. (1979) for *E. coli* tRNA.

(m^5U) and Tm (2′-*O*-methyl-T), m^5C, and m^2A. The farthest upfield peaks (1a–c) in the yeast tRNA spectrum are assigned to T, Tm, and m^5C (Table II). T and m^5C represent three of the five methylated nucleosides occurring in the average yeast tRNA, with the former occurring once and the latter twice. T and Tm are found exclusively in position 54 of the TψCG loop. The methylated cytidine occurs predominantly at positions 48 of the extra arm and 49 of the TψCG stem but is also found in the anticodon and anticodon stem. This array of locations, and the incomplete denaturation of the tRNA stem regions, may be contributing to the dispersed chemical shift of peaks 1a–c in comparison to the sharp T signal (peak 1) from the *E. coli* tRNA. *E. coli* tRNA contains only one carbon-bound methyl group, that in T, and it occurs exclusively in loop position 54 of the TψCG sequence. Yeast tRNA does contain m^2A, which would be expected to give a signal at 22 ppm. However, it occurs in such small amounts that a signal is not discernible in the spectrum of unfractionated tRNA.

Three classes of nitrogen-bound methyl groups exist in yeast tRNA: aminomethyls such as m^2G and m_2^2G, ring nitrogen bound methyls of uncharged bases such as m^3C, m^2G, and m^1I, and ring nitrogen bound methyls of bases possibly carrying positive charges such as m^1A and m^7G. With the aid of mononucleoside standards, prior signal assignments for *E. coli* tRNA, and yeast tRNA modified nucleoside composition analysis, signals 4a–b in yeast tRNA have been assigned to m^2G and m^3C. This monomethylated guanosine occurs predominantly in sequence position 10. Signal 5 is assigned to

Table II: ^{13}C Signal Assignments for Yeast tRNA

(A) ^{13}C-Enriched Methyl Signals

signal[a]	chemical shift in tRNA[b]	assignment[c]	location in cloverleaf structure[d]	tRNA species[d]
1 1c	11.4			Ala, Arg, Asp, Cys, Glu
1 1b	11.6	T-54	TψCG loop	Gly, Leu, Ile, Lys, Met, Ser, Thr, Trp, Tyr, Val
1 1	12.2	m^{5}C-49	TψCG stem	Arg, Asp, Glu, Gly, Met-f, Phe, Val
1 1a	13.0	m^{5}C-48	extra arm	Cys, Leu, Ile, Lys, Met-f, Met, Ser, Thr, Tyr
		m^{5}C-40	anticodon stem	Phe
		m^{5}C-34	anticodon-1	Leu
4 4b	27.9			
4 4	28.2	m^{2}G-10	D stem	Arg, Leu, Ile, Lys, Met-f, Met, Phe, Thr, Trp, Tyr, Val
4 4a	28.4	m^{2}G-26	between D stem and anticodon stem	Val
		m^{3}C-32	anticodon loop	Thr
5	29.1	m^{1}G-9	between aminoacyl stem and D stem	Ala, Arg, Glu, Gly, Met-f, Trp, Tyr, Val
		m^{1}G-37	anticodon loop	Asp, Leu
7 7	37.3	m^{1}A-58	TψCG loop	Ala, Arg, Cys, Leu, Ile, Lys, Met-f, Met, Phe, Thr, Trp, Tyr, Val
7		m^{7}G-46	extra arm	Cys, Lys, Met-f, Met, Phe, Trp, Val
7 7a	38.1	m$^2_2$G-26	between D stem and anticodon stem	Arg, Leu, Ile, Lys, Met-f, Met, Phe, Thr, Trp, Tyr, Val
8	53.1	mcm^{5}U-34 (mcm^{5}s^{2}U)	anticodon-1	Arg, Glu, Lys
9 9a	58.4	Cm-32	anticodon loop	Phe, Trp
9		Cm-34	anticodon 1	Phe, Trp
9 9	58.8	Cm-4	aminoacyl stem	Gly
		Gm-18	D loop	Leu, Ser, Trp, Tyr
		Um-44	extra arm	Ser

(B) Signals Common to All tRNAs

signal	chemical shift in tRNA	assignment	
		carbon	moiety
10	65.6	5′	ribose
11, 12	74.0	2′, 3′	ribose
13	83.1	4′	ribose
14	88.2	1′	ribose of uridine and guanosine
	89.2	1′	ribose of adenosine
	90.0	1′	ribose of cytidine
15	96.8	5	cytidine
16	103.2	5	uridine
19	117.1	5	guanosine
20	119.4	5	adenosine
23	138.2	8	guanosine
24	140.2	8	adenosine
25	141.8	6	cytidine
26	149.3	2	pseudouridine
27	152.1	4	adenosine
		4	guanosine
28	153.4	2	adenosine
29	154.2	2	guanosine
30	156.0	6	adenosine
31	157.9	2	cytidine
32	159.9	6	guanosine
35, 36	166.5	4	uridine
			cytidine

[a] Signal numbers refer to signals in Figure 2. For identification of *E. coli* tRNA methyl signals, see Tompson et al. (1979): 2 = ms^{2}i^{6}A; 3 = m^{2}A; 6 = mnm^{5}s^{2}U. [b] Chemical shifts in tRNA are relative to the signal from the 2′ and 3′ carbons of ribose at 74.0 ppm at 65 °C. [c] Assignments were made by comparison of the spectrum of yeast to that of *E. coli* tRNA and its assignments (Figure 1 and Tompson et al., 1979) and by comparison to spectra of mononucleoside standards (Materials and Methods). Number after nucleoside designates position in the standard 76-nucleotide sequence of tRNA. [d] Location within the tRNA cloverleaf structure and within particular tRNA species was obtained from sequence data compilated by Sprinzl & Gauss (1982).

m^{1}G, occurring mostly in position 9. The chemical shift of the methyl group of the mononucleoside m^{1}I is 35.3 ppm under the same solution conditions as those for the tRNA. However, no signal corresponding to that chemical shift is discernible in the tRNA spectrum, most likely due to the low amount of the nucleoside in tRNA. Signals 7 and 7a are assigned to m^{1}A, m^{7}G, and m$^2_2$G, which are found in yeast tRNA on the average as 0.56, 0.25, and 0.57 residue per tRNA, respectively. Each has been located in a specific sequence position: m^{1}A, position 58; m^{7}G, position 46; m$^2_2$G, position 26. The larger of the two signals, 7a, is tentatively assigned to the dimethylated nucleoside m$^2_2$G. Signal 7 is assigned to m^{1}A and m^{7}G because of chemical shift analogy to that of the mononucleosides and that of m^{7}G in *E. coli* tRNA, as well as its particular response to $MgCl_2$ addition and heat denaturation discussed below.

Signal 8 is tentatively assigned to the carboxyl methyl of mcm^{5}U. This nucleoside has been found in the first position of specific yeast tRNA anticodons.

Table III: Methyl Carbon NMR of Unfractionated Yeast tRNA

signal	assignment	integrated areas	T_1[a] (s)	NOE[b]	τ_i[c] (ps)	τ_R[d] (ns)
1, 1a, 1b, 1c	T, m^5C	56	1.1	1.9	2	20
4, 4a, 4b, 5	m^2G, m^1G, m^3C	34	0.7	1.6	2	10
7, 7a	m^7G, m^1A, m_2^2G, m^1I	61	1.3	1.4	0.6	16
8	mcm^5U	20	1.2			
9, 9a	Gm, Cm, Um	40	1.0	1.4	0.8	12
10	5′ ribose	76				
11, 12	2′,3′ ribose	160				
13	4′ ribose					
14	1′ ribose					

[a] Estimated uncertainty of ±10%. [b] Value given is $(1 + \eta)$. Estimated uncertainty ±0.1. [c] Internal rotational correlation time (picoseconds). [d] Overall rotational correlation time (nanoseconds).

The methylation of the 2′-OH of ribose gives rise to signals 9 and 9a. The methyl signal of the mononucleoside Cm is found upfield of that for Gm, Um, and Am. However, an assignment of 9a to Cm can only be tentative at this time for the effect of sequence position on these chemical shifts has yet to be determined. Gm is found exclusively in the dihydrouridine (D) loop, whereas Cm has been found at three different locations.

Methyl carbon-13 enrichment has occurred in some 19 different yeast tRNA sequence locations. These positions exist in the cloverleaf loops, extra arm, anticodon, stems, and positions between stems. In comparison, fewer positions were available in *E. coli* tRNA because positions in stems and between stems do not contain methylated nucleosides (Tompson et al., 1979). For example, yeast phenylalanine tRNA has eight differently methylated nucleosides in nine specific locations (two m^5C's), whereas *E. coli* $tRNA^{Phe}$ has only three methylated nucleosides in three positions. The hypermodified nucleoside wybutosine in yeast $tRNA^{Phe}$ is suspected to have ^{13}C-enriched methyl groups also. This could only be determined from a spectrum of the purified tRNA for the nucleoside occurs only in this one species of yeast tRNA.

Signal assignments for the natural abundance carbon-13 carbon of the base rings and ribose moiety are also shown in Table II. These assignments correspond exactly to those of *E. coli* tRNA as can be seen from Figure 2.

The percentage of ^{13}C enrichment in methyl carbons for the tRNA can be approximated from comparison of methyl signals to those from ^{13}C natural abundance (1.1%) carbons in the absence of nuclear Overhauser enhancement (NOE). Integrated peak areas for a spectrum without NOE are listed in Table III. The total area of signals from ^{13}C-enriched methyl groups equaled 211 relative to 76 for one of the natural abundance ribose carbons occurring 76 times per tRNA molecule. If one approximates the number of methyl groups as five per tRNA, a single methyl group is contributing a peak of area 42.2 relative to a ribose carbon. Thus, the ^{13}C enrichment of the methyl groups was 50–60 atom %.

Effect of Mg^{2+} Addition and Heat Denaturation on Methyl-Signal Chemical Shift. Addition of $MgCl_2$ (10 mM) to 2 mM solutions of yeast tRNA produced significant differences in the methyl carbon NMR spectrum (Figure 3). Of particular interest is the obvious broadening of the methyl signals and concomitant loss in resolution with addition of Mg^{2+}. In contrast, methyl signals from spectra of 2 mM *E. coli* tRNA remained as sharp and resolved after addition of 10 mM Mg^{2+} as they were before (Figure 3). Neither ribose nor base ring carbon signals were significantly affected by the Mg^{2+} for both yeast and *E. coli* tRNAs. Lack of significant changes in line width for these carbon signals, as well as for the methyl signals of *E. coli tRNA*, rules out the possibility

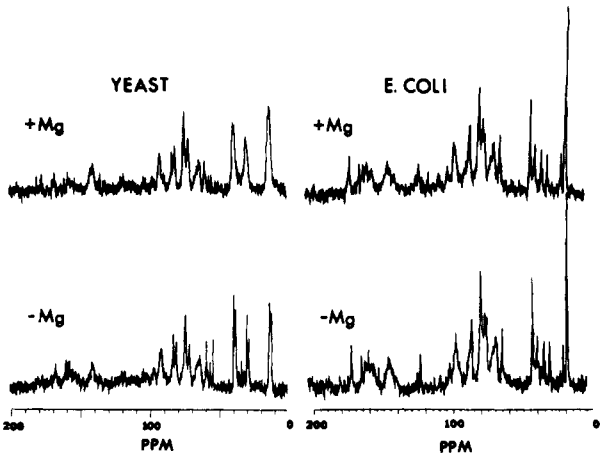

FIGURE 3: Comparison of effects of Mg^{2+} addition on spectra of yeast and *E. coli* tRNAs. All spectra were taken under conditions identical with that for yeast tRNA as explained in the legend to Figure 2, except at 25 ± 1 °C, with or without the addition of 10 mM $MgCl_2$ and with 30 000 acquisitions.

of paramagnetic broadening of the yeast methyl signals. Aggregation of yeast tRNA molecules could be responsible for line broadening, but again, the line broadening would not be expected to be restricted to methyl signals and was not observed for *E. coli* tRNA of equivalent concentration. Therefore, one possible explanation is that the yeast tRNA methyl groups in the presence of Mg^{2+} are probing a greater variety of tertiary structures than are the methyls of *E. coli* tRNA. The same methylated base may have a different chemical shift in one tRNA than in another as seen in our study of purified *E. coli* tRNA species (Kopper et al., 1983).

$MgCl_2$ addition changed the chemical shifts of the yeast tRNA methyl signals (relative to the 2′,3′-ribose carbons at 74.0 ppm). All of the methyl signals moved upfield an average of 0.5 ppm (37.5 Hz) with addition of Mg^{2+} except that of m^7G–m^1A (peak 7, Figure 2), which shifted downfield 0.4 ppm. Similar changes were observed for *E. coli* tRNA methyl signals and were more easily determined for this tRNA due to the lack of line broadening.

Heat denaturation of the yeast tRNA preparation in the absence of Mg^{2+} had the opposite effect on chemical shift: all methyl signals moved downfield with the exception of m^7G–m^1A, which moved upfield. Peak 1, T and m^5C, moved downfield at temperatures between 20 and 40 °C and did not shift thereafter up to 72 °C (Figure 4). This is analogous to the low-temperature downfield shift of T in *E. coli* tRNA (Tompson et al., 1979). Signals assigned to m^1G and Gm, Cm, and Um (5 and 9, respectively) also shifted downfield at low temperatures, whereas m^2G and m_2^2G resonances exhibited a significant shift to low field between 40 and 72 °C. The

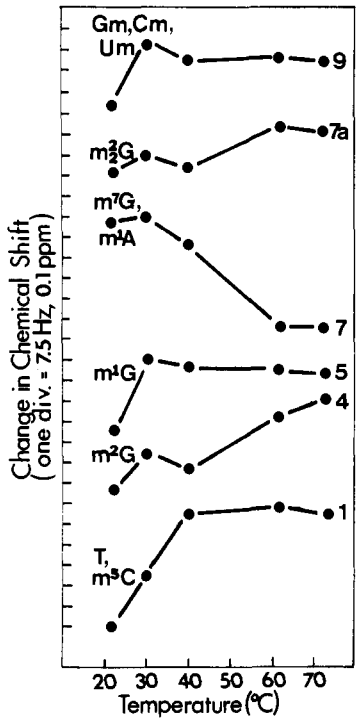

FIGURE 4: Temperature-dependent chemical shifts of yeast tRNA methyl signals. Spectra were taken of 2 mM yeast tRNA as explained in Figure 2 except the temperature was varied between 22 and 72 °C. Changes in chemical shifts of methyl carbons are plotted relative to that of the 2′,3′-ribose signal set at 74 ppm. Thus, results are a qualitative indication of direction and degree of change at the specified temperature. Each unit along the vertical axis represents 7.5 Hz (0.1 ppm). All methyl resonances exhibited downfield shifts except for m^7G–m^1A (peak 7), which moved upfield. Numbers at right side of the graph correspond to the signals in Figure 2.

high-field shifts of m^7G–m^1A occurred after reaching 30 °C. The low temperature-dependent shifts of T and Gm correspond to temperatures expected to break the tertiary interactions between the TψCG loop and D loop in which these nucleosides reside. Other methylated nucleosides directly involved in tertiary interactions include m^5C-48 (to G-15), m^7G-46 (to G-22), m^1A-58 (to T-54), and m^1G-9 (to A-23) according to the yeast tRNA^{Phe} crystallographic structure (Holbrook et al., 1978). The methyl signals from these nucleosides exhibited the expected low-temperature (20–40 °C) change in chemical shifts corresponding to the breakage of tertiary interactions. Chemical shift changes at higher temperatures would be expected for nucleosides associated with stem regions of the molecule. Two methylated nucleoside interactions at the ends of stem regions, m^2G-10 (to C-25) of the D stem and m^2_2G-26 (to A-44) at the very end of the anticodon stem, would be expected to dissolve only at higher temperatures. Indeed, the methyl signals of these nucleosides move significantly to lower field above 40 °C.

Relaxation and NOE Measurements. Yeast tRNA spin–lattice relaxation times (T_1) and values for the nuclear Overhauser enhancement (NOE) were measured at 25 °C in 10 mM $MgCl_2$. T_1 values were measured by progressive saturation (Freeman & Hill, 1971) with seven delays between 0.4 and 4 s. These results are shown in Table III for methyl signals 1–9. T_1 values range from 0.7 to 1.3 s and NOEs from 1.4 to 1.9.

Methyl groups appear most frequently in yeast tRNA directly bonded to bases (e.g., T, m^5C, m^7G, m^1G, m^1A). The next most frequent situation is methyls on amino nitrogens (m^2G and m^2_2G). In both of these situations fast, free diffusional rotation of the methyl group on its axis is expected (London, 1980). With amino methyls, rotation of the amino nitrogen to base bond is too slow to contribute to relaxation of a methyl carbon (Pitner & Glickson, 1975). Thus the motion of a methyl C–H bond contains one fast free internal rotation at approximately the tetrahedral angle, coupled to whatever motion its base undergoes. Because almost all bases are stacked in the yeast tRNA^{Phe} crystal structure—and presumably in solution under "native" conditions—and since the great majority of the methylated bases are base paired as well, the most straightforward expectation is that all bases reorient approximately with the whole tRNA.

Given ^{13}C NMR T_1 and NOE data at 75.5 MHz for yeast tRNA, we can begin to examine motion of methyl groups in light of the simple model of Woessner (1962) for free internal diffusional rotation around a bond linked to a rigid isotropic rotor (Bolton & James, 1980; London, 1980). Overall motion of tRNA is clearly in the nonextreme narrowing region ($\omega_0\tau_R > 1$) at 75.5 MHz (Schmidt et al., 1983). In this case, for each group of peaks, the T_1 and NOE values yield a unique solution for the internal motion correlation time, τ_i, and for the overall motion, τ_R (Table III).

Not surprisingly, given the simplistic nature of the model, τ_R values are not the same for all methyl groups. They range from 10 to 20 ns, the latter being fairly close to other values for tRNA under similar conditions (20–30 ns) (Schmidt & Edelheit, 1981; Patkowski & Chu, 1979; Schmidt et al., 1983; Hamill et al., 1980). Presumably, low values of τ_R reflect some independent motion of bases or sections of the macromolecule. Interestingly, methyl peaks of m^1G and m^2G (which dominate group 2, Table III) show the shortest τ_R. These modified bases are found in yeast tRNA at positions 9 and 10, at the beginning of the D stem. They are hydrogen bonded and sandwiched in the "hinge" region of the yeast tRNA^{Phe} crystal structure and thus might be expected to reflect the motion of the whole molecule. However, there is some question about the stability of the D stem in several tRNA species (Crothers et al., 1974). Our NMR relaxation data may be reflecting transient opening of this region. In contrast, the T and m^5C resonances give approximately the expected overall correlation time. Internal motion is slowest for these peaks ($\tau_i = 2 \times 10^{-12}$ s), possibly because the methyl groups of the modified pyrimidines lie close to neighboring rings. There is evidence for this from ^{1}H NMR work where the T (and to a lesser extent the m^5C) methyl resonances of tRNA^{Phe} from yeast are shifted far upfield due to ring-current effects of nearby nucleotides (Kan et al., 1977; Davanloo et al., 1979).

The key observation is that different methyl groups in tRNA have different relaxation behavior by ^{13}C NMR. This means that these peaks will serve as probes of local dynamics in unambiguously assigned locations all over the molecule. Future studies of individual, purified [^{13}C]methyl-enriched yeast tRNA will include T_2 measurements and NMR frequency dependence of relaxation, yielding detailed pictures of tRNA dynamics.

Registry No. T, 1463-10-1; m^5C, 2140-61-6; m^2G, 2140-77-4; m^3C, 2140-64-9; m^1G, 2140-65-0; m^1A, 15763-06-1; m^7G, 20244-86-4; m^2_2G, 2140-67-2; mcm^5U, 29428-50-0; Cm, 2140-72-9; Gm, 2140-71-8; Um, 2140-76-3; Mg, 7439-95-4.

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Internal Dynamics of Transfer Ribonucleic Acid Determined by Nuclear Magnetic Resonance of Carbon-13-Enriched Ribose Carbon 1†

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ABSTRACT: Carbon-13 enrichment of the C1′ position of the ribose moiety in *Escherichia coli* tRNA has made possible the detailed study of motion in this molecule. Enrichment was accomplished in vivo with a strain, M1R, selected for growth and degree of incorporation of ribose in a stringently defined minimal medium. Purine biosynthesis de novo was blocked with 6-mercaptopurine. Exogenously provided [1-^{13}C]ribose and nucleobases were utilized via the salvage pathway and were required for growth of culture. Carbon-13-enriched transfer RNA in solution at 30 °C exhibited a prominent, broad, asymmetric NMR signal at 91.5 ppm for the C1′ carbon. Upon heat denaturation of the tRNA, three C1′ signals were resolved and could be attributed to the base-specific nucleotides in tRNA: uridine and guanosine at 88.7 ppm; adenosine at 89.5 ppm; and cytidine at 90.6 ppm. Ribose C3′ and C5′ were partially enriched due to scrambling of ribose carbons in vivo. The minimum net isotopic enrichment of C1′ was 33%. Values for the relaxation time T_1 and the nuclear Overhauser enhancement (NOE) at 75.5, 67.8, and 25.2 MHz (^{13}C), the NOE at 50.3 MHz, T_2 at 75.5 MHz, and line widths over the range of 20–75.5 MHz were analyzed in light of several models for internal motion in macromolecules. The data were inconsistent with physically unreasonable constructs involving free internal diffusion of the C1′–H vector about the glycosidic bond. Internal diffusion (wobble) within a cone or jumps between states were models that did fit the data. For diffusion within a cone, the cone half-angle was 15–20°, with a correlation time of about 2×10^{-9} s for internal reorientation. With the two-state jump model, the half-angle for jumps about the glycosidic bond was $14 \pm 2°$ with a lifetime of 2×10^{-9} s.

The ability of macromolecules to interact specifically and successfully in enzymatic reactions is dependent on pliant conformational recognition in three dimensions. Thus, we need to understand not only structure but also structural flexibility and motional capability, particularly internal motion (London, 1980), which is important for mechanisms of catalysis or receptor activation. Nuclear magnetic resonance (NMR) and fluorescence are the techniques most suited for measuring internal motions in macromolecules. Fluorescence is superior from the standpoint of signal sensitivity, but NMR has the advantage of generally offering a nonperturbing probe. Sensitivity limitations are particularly acute in NMR of ^{13}C (1.1% natural abundance), yet it is the nucleus of choice in most dynamic NMR studies of biological molecules, since its re-

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