Volume 7 Number 3 1979 Nucleic Acids Research

Production of specific site probes of tRNA structure by enrichment with carbon 13 at particular locations

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Received 5 July 1979

ABSTRACT

Escherichia coli C6 *rel met cys* was cultured in a stringently defined minimal medium containing ^{13}C-enriched metabolites in order to (1) achieve maximal ^{13}C isotopic enrichment of tRNA; and (2) produce site specific but natural, non-perturbing NMR probes of tRNA structure and function. Growth conditions were manipulated to achieve optimal culture growth concomitant with maximal *in vivo* incorporation of various ^{13}C-enriched nucleic acid precursors, including L-[*methyl*-^{13}C] methionine, [2-^{13}C] adenine, and [2-^{13}C] uracil. Effective blockage of purine biosynthesis *de novo* was accomplished with the addition of the antimetabolite 6-mercaptopurine to the growth medium. Transfer RNAs specifically ^{13}C-enriched in all methyl groups (57 atom %), C_2 of adenine (60 atom %), and C_2 of uracil (82 atom %) and C_2 of cytosine (73 atom %) have been produced.

INTRODUCTION

Utilization of magnetically active ^{13}C-enriched metabolites and molecular precursors is both a benefit and a necessity to carbon-13 nuclear magnetic resonance (^{13}C-NMR) investigations of macromolecular synthesis, structure, and function (1-6). Our interests lie in transfer RNA and, in particular, the structural dynamics of this molecule in studies examining ribosomal protein synthesis. Many recent investigations of the tertiary structure of tRNA as well as certain dynamic properties have utilized natural abundance ^{13}C-NMR spectroscopy (1,7,8); but, since the natural isotopic abundance of ^{13}C is only 1.1%, it is necessary to enrich specific locations of the molecule with ^{13}C-labeled residues in order to distinguish many of the interesting spectral signals. The ability to produce site specific but natural probes of structure and function in tRNA is therefore essential. Other investigators have incorporated fluorescent probes into tRNA (9,10) and have enriched uridine nucleotides with ^{19}F through chemical modification for ^{19}F-NMR studies (11), but these techniques create structural artifacts (12) and consequently must be viewed with caution. ^{13}C, however, is a natural, non-disruptive probe of

tRNA.

We have accomplished isotopic enrichment through *in vivo* incorporation of various nucleic acid precursors into specific locales of *Escherichia coli* tRNA, including [2-^{13}C] adenine, [2-^{13}C] uracil, and L-[*methyl*-^{13}C] methionine, which is the donor of all methyl groups to methylated nucleosides. Utilization of microbial physiology and selection of the appropriate strains is essential in order to achieve maximal incorporation of the labeled, exogenously provided precursors into tRNA concomitant with optimal growth of the cultures for the eventual isolation of large amounts of tRNA.

In order to obtain optimal cellular growth and incorporation of tRNA precursors (bases and methionine), growth conditions must be stringently controlled. For instance, it is necessary to block purine biosynthesis *de novo*, thus forcing the cells to utilize the salvage pathway and incorporate only preformed bases. This also insures that methyl groups from labeled methionine will not be utilized through the one-carbon pool for biosynthesis *de novo* of major nucleotides.

End product inhibition of biosynthesis *de novo* (13,14) and dependence on the transport mechanism (15) however, is not a practical means of forcing cells to incorporate exogenous labeled tRNA precursors. A continuously high concentration of both AMP and GMP is necessary for complete inhibition, and it is simply not economical to grow cells in a great excess of ^{13}C-bases.

A structural analog of hypoxanthine, 6-mercaptopurine (6-MP), mimics the effects of natural purines (16), is metabolized to 6-ThioIMP, and in this ribonucleotide form, can inhibit the formation of phosphoribosylamine (13,17). It is consequently an effective "pseudofeedback" inhibitor of purine biosynthesis (18). 6-ThioIMP also blocks interconversions of purine nucleotides. It interferes with pathways leading from IMP to both GMP and AMP by inhibiting IMP dehydrogenase, GMP reductase, adenylosuccinate synthetase, and adenylosuccinate lyase (13,17). We report here the use of this drug for ^{13}C-enrichment of tRNA with specific ^{13}C-labeled tRNA precursors (adenine, uracil, methionine) which are incorporated when cells are forced to utilize the salvage pathway.

MATERIALS AND METHODS

Escherichia coli C6 *rel met cys* (19) was grown with aeration in a stringently defined minimal medium at 37°C in a shaking water bath. Strain M1, capable of expressing relatively quickly the genes of the salvage pathway, was screened from cultures of *E. coli* C6. The cells were also tested for methion-

ine and cysteine auxotrophy prior to inoculation of the medium. Culture growth was followed spectrophotometrically by measuring transmission at 555 nm. The culture medium (pH 7) consisted of 22.2 mM KH_2PO_4, 42.4 mM Na_2HPO_4, 18.7 mM NH_4Cl, 1.91 mM $MgSO_4$, 2.2 μM $FeSO_4$, 0.496 mM $CaCl_2$, 27.7 mM glucose, 0.01 mM 6-mercaptopurine, 0.10 mM methionine (or L-[*methyl*-^{13}C] methionine), 0.178 mM uracil (or [2-^{13}C] uracil), 0.180 mM cytosine, 0.110 mM guanine, and 0.054 mM adenine (or [2-^{13}C] adenine). ^{13}C-labeled products were obtained from Merck, Canada. ^{13}C atom % was confirmed by mass spectrometry and ^{13}C-NMR spectroscopy. [2-^{3}H] adenine and [5,6-^{3}H] uracil were obtained from New England Nuclear Corp., Boston, Mass. Each ^{3}H-labeled precursor was substituted for the unlabeled material in early growth studies, and incorporation of the radioactive label into acid precipitable material allowed us to predict an approximate degree of enrichment. Radio-labeling was carried out with 10 ml cultures containing 20 μCi [2-^{3}H] adenine or 5 μCi [5,6-^{3}H] uracil.

Cultures of 30 liters containing the ^{13}C-labeled precursors were harvested by continuous flow centrifugation after eight hours of incubation, at which time the cells were in the late log phase of growth. Nucleic acids were extracted by standard methods (20). Bulk tRNA enriched with either ^{13}C-labeled adenine, uracil, or methyl groups was then isolated by DEAE-cellulose chromatography (21), precipitated with ethanol, dissolved in glass distilled water, and dialyzed extensively against glass distilled water for three hours. The dialysis bath was changed every thirty minutes to remove all traces of ethanol, as determined by gas chromatography of sample aliquots. The tRNA solutions were concentrated by evaporation under vacuum to near dryness and redissolved in H_2O/D_2O (1:1) to give a final volume of 2.5 ml and a concentration of approximately 2.5 mM in preparation for NMR spectroscopy. Dioxane was added to tRNA solutions and to ^{13}C-enriched adenine and uracil solutions as an internal standard for NMR spectroscopy. NMR spectra of 2-^{13}C adenine and 2-^{13}C uracil were taken of aqueous solutions of approximately 5 mM.

Proton-decoupled ^{13}C-NMR spectra were recorded at 25.2 MHz on a Varian XL-100-15 Fourier transform NMR spectrometer with a deuterium lock. The probe used a 12 mm outer diameter sample tube. A Nicolet TT-100 computer recorded and processed the spectra. Dioxane was used as an internal reference (67.4 ppm downfield from external TMS) for chemical shift measurements. Sample temperature was controlled to ± 1.0°C with a Varian temperature control unit. Mass spectra were taken with a CEC 21-110 mass spectrometer (70 ev, 250°C source temperature) coupled to a JEOL-6 computer for recording purposes.

In order to determine the extent of ^{13}C-labeled precursor incorporation

into tRNA, a small sample of tRNA was acid hydrolyzed. Major bases were isolated and separated by high pressure liquid chromatography (HPLC). This procedure for base separation is similar to that established for nucleosides (22). Mass spectrometric measurements were then performed to determine the $^{13}C/^{12}C$ ratio of each precursor in tRNA (Tompson, J.G., Kuo, K.C., Gehrke, C.W., and Agris, P.F., unpublished data). Similarly, ribothymidine residues, which are methylated nucleosides appearing once in each *E. coli* molecule, were isolated to measure the degree of ^{13}C-methyl incorporation in the third sample.

RESULTS

Inhibition of purine biosynthesis de novo and strain selection. 6-Mercaptopurine metabolized to 6-ThioIMP was an effective inhibitor of cell growth even at low concentrations. Figure 1 demonstrates the effect of increasing amounts of 6-mercaptopurine (6-MP) on cell growth in the absence of added purines. A concentration of 10^{-5}M inhibited growth by 83% during the initial seven hours. Cultures were able to overcome the 6-ThioIMP inhibition of

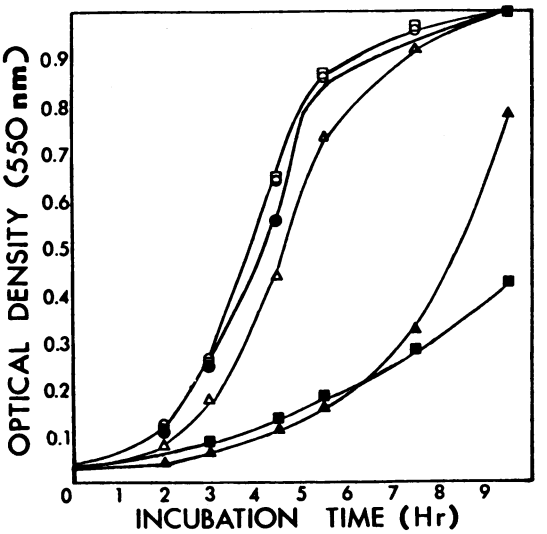

Figure 1. Effect of 6-mercaptopurine (6-MP) on growth of *E. coli* C6 (strain M1) at 37°C. Cells were grown in the absence of exogenous purines with 6-MP concentration varied: 10^{-4} M (■), 10^{-5} M (▲), 10^{-6} M (△), no 6-MP (●). A control culture (○), grown in the absence of 6-MP and with supplemental adenine (0.054 M) and guanine (0.110 M), represented optimal growth. The open square symbols (□) represent growth of a culture in the presence of 10^{-5} M 6-MP when exogenous purines (0.054 M adenine, 0.110 M guanine) are provided.

PRPP-amidotransferase after approximately seven hours and resume purine biosynthesis *de novo*. Consequently, in order to achieve maximal incorporation of a labeled precursor into nucleic acids, it was very important in the experiments to be described that the cultures be harvested before the cells could overcome the 6-ThioIMP block. It was also critical that the culture be harvested before reaching the stationary phase of growth to minimize nucleic acid turnover. The optimal concentration of 6-MP was determined to be 10^{-5}M; this was used in all further experiments.

Cells will grow in the presence of the drug 6-MP by utilizing the salvage pathway when various concentrations of supplemental purines are added to the medium (Figure 2). Addition of increasing amounts of exogenous purines continually improved growth of cultures. Eventually, at concentrations of 0.054 mM adenine and 0.110 mM guanine, the rate and extent of growth approximated that of a culture grown without drug inhibition.

However, we usually found cultures had an extraordinarily long lag period

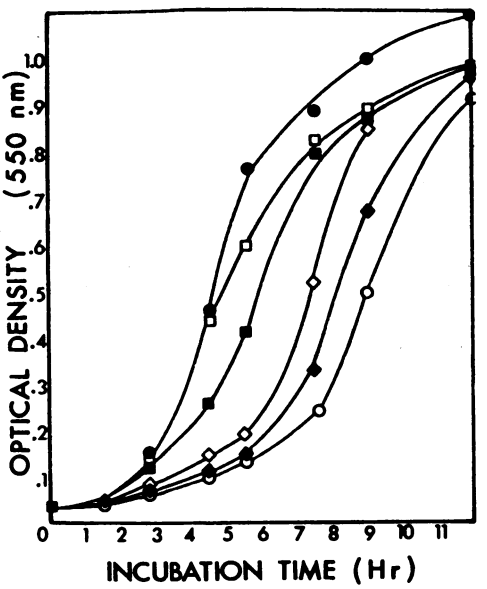

Figure 2. Effect of increasing purine concentration on cell growth in the presence of 6-MP. The closed circle symbols (●) represent growth in the absence of 6-MP and serve as a positive control. The open circle symbols (○) represent growth with 10^{-5} M 6-MP and no supplemental bases and serve as a negative control. Closed diamonds (◆) represent growth with the addition of pyrimidines only (0.178 M uracil, 0.180 M cytosine). Increasing purine concentration (◆, 0.54 mM adenine, 1.10 mM guanine; ■, 5.54 mM adenine, 11.0 mM guanine; □, 0.054 M adenine, 0.110 M guanine) gave increasingly better growth.

--about five hours--before reaching the log phase of growth. In these cases, growth in the presence of 6-MP was not increased by exogenous purines. This indicated that these particular cultures were much slower in their ability to express the enzymes of the salvage pathway for utilizing pre-formed purines. Consequently, we were careful to screen for and successfully isolate a strain (MI) which exhibited short lag periods and which thus had little difficulty in switching to the salvage pathway.

Preparation of [2-^{13}C] adenine enriched tRNA. [2-^{13}C] adenine was specified to have 90+ atom % carbon-13 at the C_2 position only; this was confirmed by mass spectrometry and ^{13}C-NMR spectroscopy (Figure 3). A 10 ml preliminary culture of strain M1 was grown in the presence of [2-^{3}H] adenine; the incorporation of the radioactive label into acid precipitable material followed the culture growth (Figure 4). A sample of tRNA was then isolated from the culture after 5½ hours of incubation. Radioactivity measured in ethanol precipitated

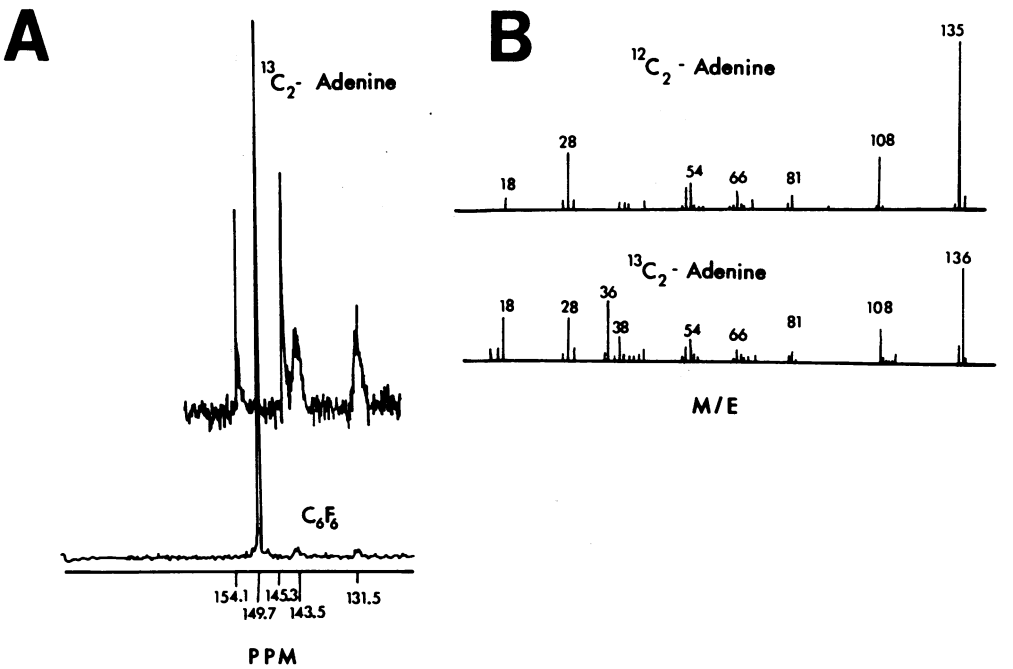

Figure 3. (A) ^{13}C-NMR spectra of [2-^{13}C] adenine. C_6F_6 was the internal standard. The lower spectrum was proton-decoupled while the upper spectrum was not. The nucleic acid precursor is enriched only at the C-2 position (149.7 ppm). (B) Mass spectra of [2-^{12}C] adenine and [2-^{13}C] adenine. The molecular ion of the ^{12}C sample had a mass of 135 units, and that of the ^{13}C-enriched sample a mass of 136 units. The ratio of the two molecular ion signals allowed one to determine the isotopic abundance of the label (90+ atom % ^{13}C).

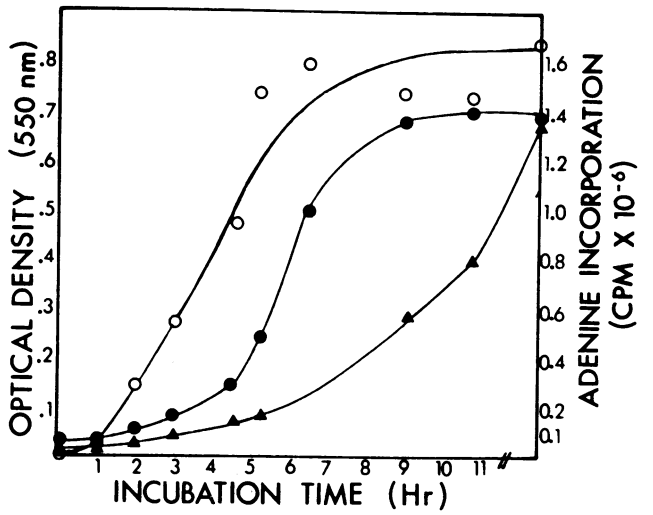

Figure 4. Incorporation of [2-^{3}H] adenine into acid precipitable material from *E. coli* grown in the presence of 10^{-5} M 6-MP. The closed circle symbols (●) represent growth of *E. coli* as measured by changes in the optical density at 550 nm when supplemental purines are provided, and open circles (○) represent ^{3}H-adenine incorporation as measured by cpm of acid precipitable material. The closed diamonds (♦) represent growth of *E. coli* without supplemental purines and serve as a negative control.

material indicated that greater than 70% incorporation of the ^{13}C-labeled precursor into tRNA would be obtainable.

A 30 liter culture of *E. coli* C6, strain M1, was grown in medium containing [2-^{13}C] adenine, the cells collected after 8 hours, and bulk tRNA extracted from the cells. The packed cells (78 g wet weight) yielded approximately 120 mg of bulk tRNA. Mass spectral analysis of isolated base residues from a 100 µg tRNA sample indicated that we had actually achieved 60 atom % ^{13}C enrichment, specifically incorporated at [2-^{13}C] adenine. Isolated guanosine residues carried only the 1.1% natural abundance ^{13}C, which was expected because 6-ThioIMP inhibits the interconversions of purine nucleotides (13, 17). The aminoacylation activity of the sample was measured for seven amino acids and determined to be identical to that of tRNA extracted from *E. coli* grown in medium containing [2-^{12}C] adenine.

E. coli bulk tRNA specifically enriched with [2-^{13}C] adenine dissolved in H_2O/D_2O gave the ^{13}C-NMR spectrum shown in Figure 5. Resonance signal 1 was obtained from the C-2 position of every adenine residue. The "melting"

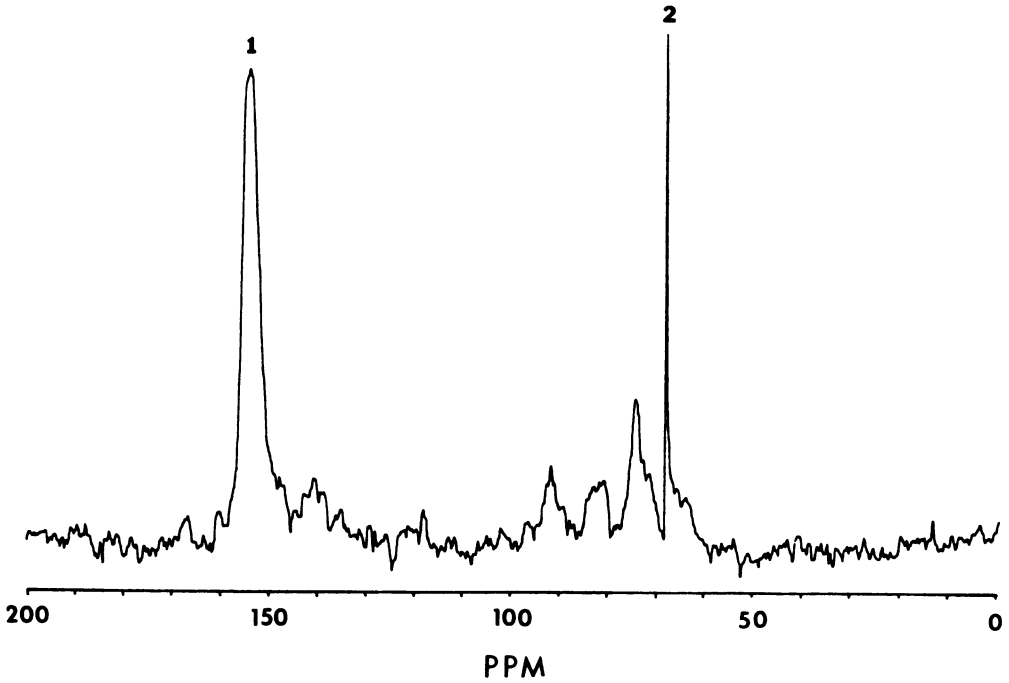

Figure 5. Proton-decoupled, Fourier transformed ^{13}C-NMR spectrum of [2-^{13}C] adenine enriched bulk tRNA. Signal 1 is obtained from the C-2 position of each adenine residue, and signal 2 is the resonance of dioxane, the internal standard. The tRNA was extracted from *E. coli* grown in the presence of 10^{-5} M 6-MP and supplemental purines (0.054 M [2-^{13}C] adenine, 0.110 M guanine) and pyrimidines (0.178 M uracil, 0.180 M cytosine). Spectrum represents signal averaging of 8,192 acquisitions.

of this [2-^{13}C] adenine sample, which gives some information about tRNA structure, has been accomplished and will be reported elsewhere (Tompson, J.G., Hayashi, F., Schmidt, C.F., and Agris, P.F., manuscript in preparation).

At higher temperatures, spectra show improved resolution, and resonance signals due to specific ^{13}C-enriched carbons present in tRNA in lower abundance becoming visible above background. For instance, at low temperatures, the signal arising from the C-2 position of m^2A appears as a "shoulder" on the low field side of the [2-^{13}C] adenine resonance signal. This signal becomes quite prominent as resolution improves at high temperatures, enabling us to identify this carbon located in one particular tRNA nucleoside (P.G. Schmidt, J.G. Tompson, and P.F. Agris, unpublished results). The resonance signal was assigned to m^2A rather than another modified adenosine residue due to the high relative amount of m^2A in 25% of *E. coli* tRNAs (6).

Preparation of [2-^{13}C] *uracil enriched tRNA.* Spectral analysis of [2-^{13}C] uracil, shown in Figure 6, confirmed the 90+ atom % of this labeled precursor. Preliminary studies utilizing the radioactive base, [2-^{3}H] uracil, indicated a 70% incorporation into tRNA.

A 30 liter culture of *E. coli* strain M1 was grown in medium containing [2-^{13}C] uracil, the tRNA extracted from the collected cells and prepared for NMR as done for the [2-^{13}C] adenine labeled culture. Mass spectral analysis of an acid hydrolyzed 4 µg sample showed that we had actually achieved 82 atom % ^{13}C enrichment of C_2-uracil with incorporation of the labeled precursor; 73 atom % enrichment of cytidine residues labeled at the C-2 position was detected as well. This tRNA sample also demonstrated an aminoacylation ability equal to that obtained from *E. coli* grown in medium containing [2-^{12}C] uracil.

The ^{13}C-NMR spectrum of [2-^{13}C] uracil-cytosine enriched bulk tRNA is

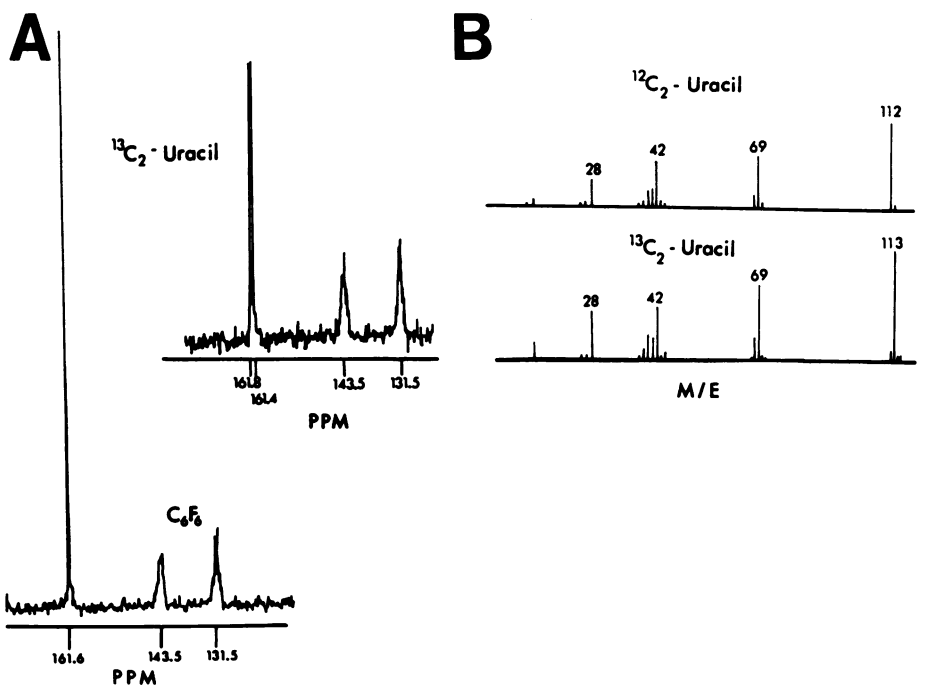

Figure 6. (A) ^{13}C-NMR spectra of [2-^{13}C] uracil with C_6F_6 as the internal standard. The lower spectrum was proton-decoupled while the upper spectrum was not. The precursor is enriched only at the C-2 position (161.6 ppm). (B) Mass spectra of [2-^{12}C] uracil and [2-^{13}C] uracil. The molecular ion of the ^{12}C sample is 112 mass units; that of the ^{13}C-enriched sample is 113 mass units. This sample is 90+ atom % ^{13}C, as determined by the ^{12}C/^{13}C ratio.

shown in Figure 7. Resonance signal 1 was obtained from the C-2 position of every cytosine and signal 2 from uracil C-2. *E. coli* has the ability to convert UTP to CTP with the enzyme CTP synthetase, and this is apparently what has happened, even in the presence of cytosine within the medium. In addition, cytosine is to some extent deaminated to uracil (23). Of all *E. coli* tRNAs sequenced thus far, 28.6% of the nucleosides are cytidines (or modified cytidines), and only 21.2% are uridines (or modified uridines) (24). The resulting biological conversion was an added benefit in that we achieved ^{13}C enrichment of two specific carbon loci in tRNA. Fortunately, the two NMR signals were not overlapping, so that both signals could be studied simultaneously (Figure 7). This doubly-labeled sample has also been "melted", and the resulting spectra, to be reported elsewhere (Tompson, J.G., Hayashi, F., Schmidt, C.F., and Agris, P.F., manuscript in preparation), have given

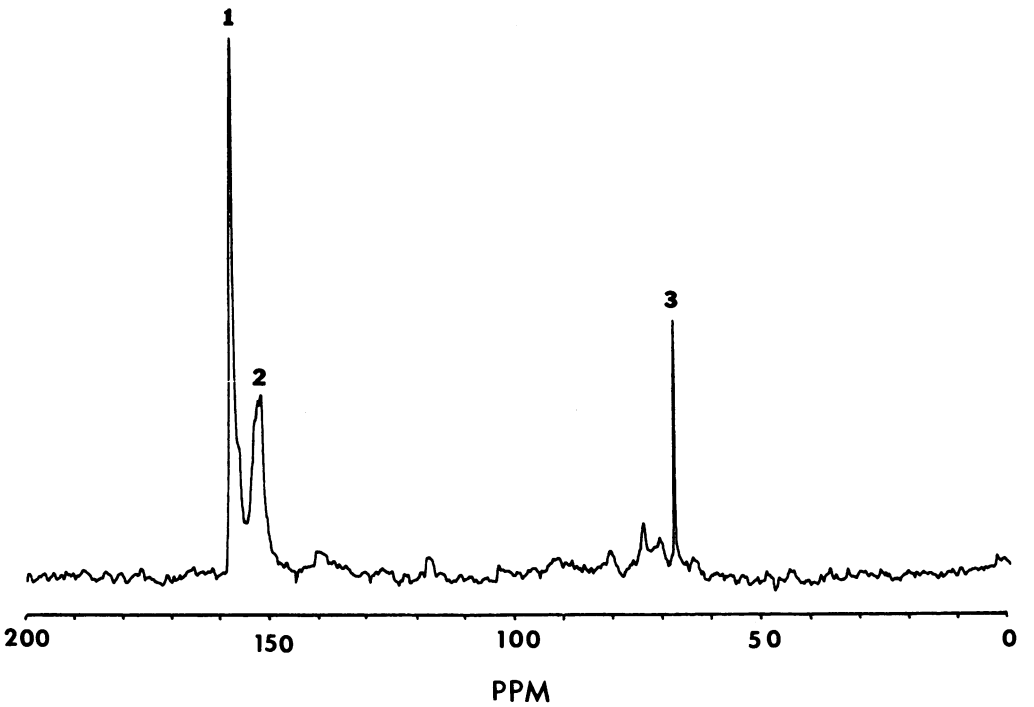

Figure 7. Proton-decoupled, Fourier transformed ^{13}C-NMR spectrum of ^{13}C-uracil/cytosine enriched bulk *E. coli* tRNA. Signal 2 is obtained from the resonance of every C-2 position in each uracil residue, signal 1 from C-2 of every cytosine. Dioxane (signal 3) is the internal standard. The tRNA was extracted from *E. coli* grown in the presence of 10^{-5} M 6-MP with supplemental purines (0.054 M adenine, 0.110 M guanine) and pyrimidines (0.178 M [2-^{13}C] uracil, 0.180 M cytosine) provided exogenously. Spectrum represents signal averaging of 8,192 acquisitions.

structural information concerning the C-2 position of cytosine, which is involved in hydrogen bonds of the tRNA secondary structure, in contrast to the C-2 positions of uracil and adenine that are not involved in normal Watson-Crick hydrogen bonds.

The resonance signal due to the ^{13}C-enriched C-2 position of a common modified nucleoside, dihydrouridine, has been assigned. Natural abundance ^{13}C spectroscopy has shown the chemical shift of dihydroU C-2 to be 154.5 ppm, that of cytosine C-2 to be 157.0 ppm, and that of uracil C-2 to be 152.7 ppm (6). We have identified the dihydroU C-2 signal as a small shoulder on the high field side of the cytosine C-2 signal, between the two dominant signals. This signal becomes quite prominent with increased resolution at higher temperatures, similar to that of m^2A C-2 (P.G. Schmidt, J.G. Tompson, and P.F. Agris, unpublished results).

The resonance signal of the C-2 carbon of another very prevalent modified nucleoside, riboT, was not visible. This signal was probably hidden beneath the uracil C-2 signal, even in high temperature spectra. The modifying methyl group, on C-5, is probably not in close enough proximity to alter significantly the chemical shift of the C-2 carbon.

Preparation of ^{13}C-methyl enriched tRNA. We have previously reported the growth of an *E. coli* methionine auxotroph in the presence of ^{13}C-methyl enriched methionine, extraction of ^{13}C-methyl enriched tRNA, and NMR investigations of this sample (1, 3, 6). The present study, however, included the addition of 6-MP to the growth medium in the hopes of excluding incorporation of any labeled methyl groups into the ring structure of major nucleotides by blocking purine synthesis *de novo*.

Growth studies with varying concentrations of methionine present (Figure 8) indicated the optimal concentration of L-[*methyl*-^{13}C] methionine would be 0.10 mM. A small sample of the ^{13}C-methyl labeled tRNA was acid hydrolyzed and the nucleosides separated by HPLC. One methylated nucleoside, ribothymidine (m^5U), is present once in every tRNA molecule. Therefore, this particular residue was used as a standard. It was isolated, and the $^{13}C/^{12}C$ ratio, as determined by mass spectrometry, indicated a 57 atom % enrichment into methylated nucleosides of tRNA had been obtained.

The ^{13}C-NMR spectrum of this sample at 30^{o} is shown in Figure 9. Methyl carbon signals from seven differently methylated nucleosides were evident: ribothymidine, 2-methylthio-N^6-(Δ-2-isopentenyl) adenosine, 2-methyladenosine, N^6-methyladenosine, 1-methylguanosine, 7-methylguanosine, and uridine-5-oxyacetic acid methyl ester. Signal assignments and this sample's melting pro-

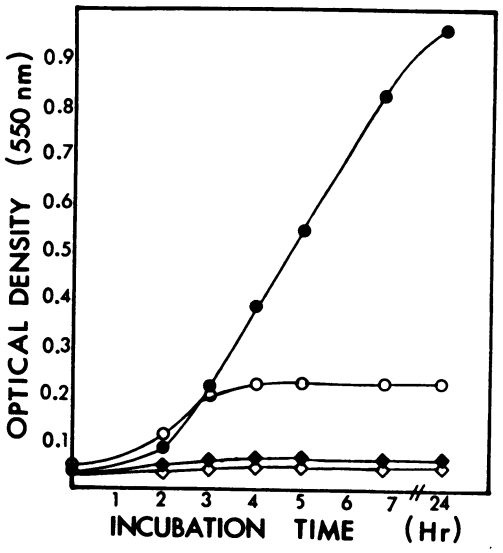

Figure 8. Growth dependence of *E. coli* C6 on methionine. Methionine concentrations were varied from 10^{-6} M (♦) to 10^{-5} M (○) to 10^{-4} M (●). The open diamond symbols (◊) represent the absence of growth when supplemental methionine is not provided.

files, as reported elsewhere (6), have given structural information concerning tertiary interactions of specific locales of the tRNA molecule, such as that of the TΨCG loop with the dihydroU loop.

DISCUSSION

We have demonstrated in these studies the ability to achieve enhanced incorporation of labeled, exogenous bases and methyl groups of modified nucleosides into *E. coli* tRNA when the *de novo* biosynthetic pathway for purine synthesis has been blocked. We accomplished this block with the addition of 6-MP, a hypoxanthine analog, to the culture medium. In the absence of supplemental purines, the cultures were unable to grow with 6-MP because the ribonucleotide form of this drug acted as a pseudofeedback inhibitor of PRPP-amidotransferase. When inhibitors (AMP, GMP, IMP, 6-ThioIMP) bind to allosteric sites of PRPP-amidotransferase, resulting conformational changes are reflected in a decreased affinity for the substrate at the catalytic site (25). This inhibition was found to be only temporary, however. The ability of *E.*

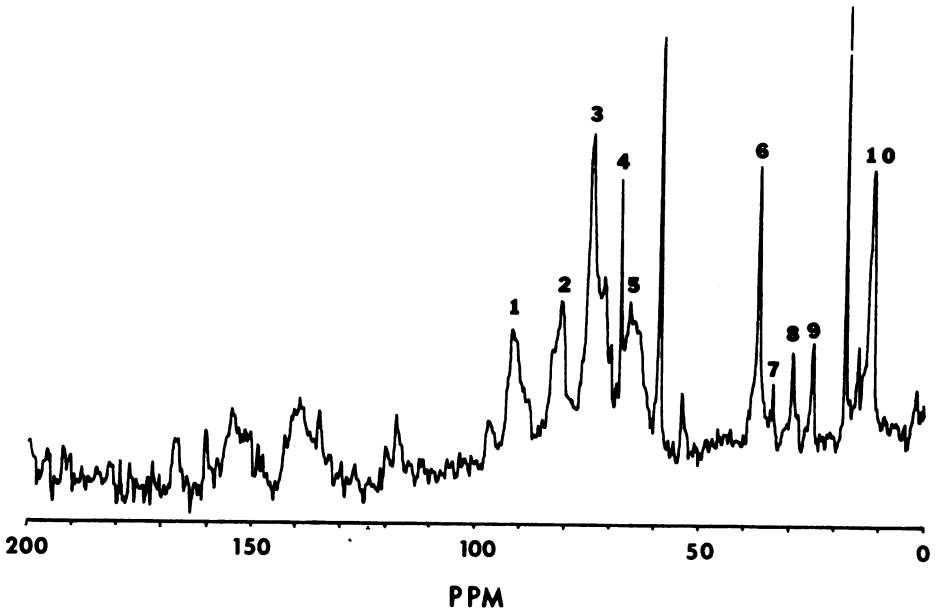

Figure 9. Proton-decoupled, Fourier transformed ^{13}C-NMR spectrum of ^{13}C-methyl enriched bulk *E. coli* tRNA. Methyl resonances, located in the upfield region (0-50 ppm) have all been assigned (6). Two predominant signals, 10 and 6, are obtained from methyl carbon resonances of ribothymidine and 7-methylguanosine, respectively. Natural abundance ribose carbon signals are located in the mid-field region (50-100 ppm) and appear prominant due to the fact that these carbons appear in every nucleotide residue. Resonances of base ring carbons appear in the low-field region as seen in Figures 5 and 7. These do not appear as exceptionally strong signals when they are not specifically enriched. Dioxane (signal 4 at 67.4 ppm) and ethanol (CH_3 signal at 17 ppm, and CH_2OH signal at 59 ppm) were utilized as internal standards in making signal assignments. Spectrum represents signal averaging of 32,768 acquisitions.

coli to overcome inhibition was probably not due to a breakdown of 6-MP which might occur at 37^{o}, because growth of a sample culture inoculated into medium which had been shaking at 37^{o} previously for eight hours was equally inhibited. The ability to overcome the biosynthetic block could have been due to the selection and growth of a mutant, such as one which was unable to make the ribonucleotide 6-ThioIMP from 6-MP or one with an increased capacity for purine biosynthesis *de novo*. We demonstrated the unlikelihood of this possibility by observing growth inhibition of a culture inoculated from a second culture which had already overcome inhibition. PRPP-amidotransferase may be involved in regulating its own biosynthesis. For instance, the conformational

changes experienced by PRPP-amidotransferase with 6-ThioIMP bound to its allosteric sites could prevent the enzyme from repressing its biosynthesis. New PRPP-amidotransferase molecules are then synthesized and may or may not become bound to 6-ThioIMP, depending upon the level of the antimetabolite present. This would explain why longer periods of time were required to overcome growth inhibition with increased concentrations of 6-MP in the absence of supplemental purines.

In order to obtain optimal precursor incorporation into nucleic acids, it was very important that the cultures were harvested in late log phase (7-8 hours) before the cells could overcome the 6-MP block of *de novo* purine synthesis and before extensive nucleic acid turnover could occur. It was equally important, however, that we allowed for maximal cell growth within these bounds for maximal tRNA production and yield during extraction. In taking NMR spectra, a high sample concentration is vital. A decrease in sample concentration by a factor of two would require an increase in NMR signal accumulation time in our measurements by a factor of four. This time element becomes more critical in melting studies where a large number of spectra are required.

Our studies produced ^{13}C-NMR probes in specific locations all around the cloverleaf structure of tRNA (C_2 of adenine, uracil, and cytosine) as well as in very particular locations (methyl groups). It is noteworthy that these carbon probes do not disrupt the native molecular conformation, for they are simply isotopic replacements at natural sites achieved *in vivo*. Specific enrichment and subsequent verification of the enriched macromolecular site provides a valuable means of identifying spectral signals in preparation for examining the macromolecular structure and its changes during interaction with proteins and other nucleic acids.

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ACKNOWLEDGEMENTS

We wish to thank R.K. Dixon for much technical assistance, R.N. Loeppky and J.V. Paukstelis for assistance in NMR spectroscopy and C.W. Gehrke in mass spectrometry, A. Eisenstark and J.T. Parisi for beneficial discussions, and Jackie Curtis and Christy Cox for help in the manuscript preparation.

This work was supported by a grant from NIH (USPH1R01 GM23037) to Paul F. Agris.

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