Unique Phenylalanine Transfer Ribonucleic Acids in Relaxed Control *Escherichia coli:* Genetic Origin and Some Functional Properties†

Geoffrey R. Kitchingman, Elizabeth Webb, and Maurille J. Fournier*

ABSTRACT: Inhibition of protein synthesis in relaxed control *E. coli* results in the formation of chromatographically unique isoacceptor species of phenylalanine tRNA. The genetic origin and some functional properties of the major unique species of $tRNA^{Phe}$ produced during leucine starvation were investigated. RNA:DNA hybridization analyses revealed that the normally occurring and major unique species of $tRNA^{Phe}$ are generated from DNA sequences which are identical or closely related and that there may be only one such sequence in the *E. coli* chromosome. Results from ^{32}P pulse-chase experiments revealed that the unique $tRNA^{Phe}$ can be converted to a chromatographically normal form upon resumption of cell growth in fully supplemented medium. These findings, taken with earlier results which indicate that the unique species is not derived from preexisting, normally occurring species, indicate that the unique $tRNA^{Phe}$ is a modification-deficient form of the normal species. Comparative studies of the unique and normal phenylalanine tRNAs revealed that the unique species is aminoacylated at a much lower rate than the normal species and is only about 60% as efficient in a tRNA-dependent, poly(U)-directed protein synthesizing system.

Culturing of *Escherichia coli* under conditions which lead to unbalanced growth can result in the formation of chromatographically unique species of tRNA (Wettstein and Stent, 1968; Capra and Peterkofsky, 1968; Waters et al., 1969, 1973; Fournier et al., 1970; Fournier and Peterkofsky, 1975; Huang et al., 1971; Kitchingman and Fournier, 1973–1975; Mann and Huang, 1973; and Chase et al., 1974). In some of the cases cited, the unique species observed were detected in relaxed control (*rel*$^-$) strains following methionine starvation, a condition which results in the formation of tRNA deficient in methylated nucleosides (Mandel and Borek, 1963). tRNA deficient in *N*-(purin-6-ylcarbamoyl)threonine (Powers and Peterkofsky, 1972) and sulfur-containing nucleosides (Harris et al., 1969) can be similarly obtained by threonine or cysteine starvation of *rel*$^-$ *E. coli*. Aside from these special situations, however, unique isoacceptor tRNAs have also been detected in both relaxed and stringent control cells in which protein synthesis was blocked by starvation for leucine or arginine (*rel*$^-$) or by treatment with chloramphenicol (*rel*$^\pm$), puromycin (*rel*$^-$), or amino acid analogs (*rel*$^+$). Because the new species produced during leucine starvation or chloramphenicol treatment cannot be detected in cells unable to synthesize RNA, it seems unlikely that they are derived from preexisting tRNAs. This view is further supported by studies of the compositional changes which occur in the isoacceptor tRNA patterns for leucine and phenylalanine tRNA from *rel*$^-$ *E. coli* during leucine starvation and following restoration of protein synthesis. These results (Kitchingman and Fournier, 1975) show that the major unique species of $tRNA^{Leu}$ and $tRNA^{Phe}$ accumulate as a function of starvation time, are heterogeneous, and appear to be formed at the expense of the normally occurring major isoacceptor species. Upon restoration of leucine there is a decrease in the relative amount of unique $tRNA^{Phe}$, suggesting the possibility that those species can be converted to chromatographically normal forms. However, because the unique species "disappeared" only slowly, it was not clear whether the changes observed were due to conversion or, rather, dilution by newly synthesized normal $tRNA^{Phe}$. The situation with the chloramphenicol-induced tRNAs appears to be analogous. The changes which occur in the $tRNA^{Phe}$ profiles during recovery from chloramphenicol treatment suggest the possibility that these unique species can be converted to normal $tRNA^{Phe}$ (Mann and Huang, 1973).

Taken together, the results strongly suggest that the unique species formed during amino acid starvation or in the presence of chloramphenicol or puromycin are undermodified precursors to the normal species. An alternative hypothesis can be advanced, however, that is also tenable. This second view holds that the unique tRNAs could be derived from tRNA genes that are normally not expressed. During conditions of physiological stress these "silent" cistrons could be derepressed and the unique species produced. Both hypotheses are consistent with the observation that unfractionated tRNA from amino acid starved or chloramphenicol-treated *E. coli* has a lower content of dihydrouridine and, perhaps, 4-thiouridine than tRNA from control cultures (Jacobson and Hedgcoth, 1970; Waters et al., 1973) and the finding that the unique species of $tRNA^{Phe}$ induced by chloramphenicol appear to differ from the normal $tRNA^{Phe}$ only in their content of certain minor bases (Huang and Mann, 1974).

The work described here was undertaken in part to reveal the genetic origin of the major unique phenylalanine tRNA produced during amino acid starvation. A comparative study of some of the biological properties of the unique phenylalanine tRNA was also performed in an attempt to determine what effect their occurrence may have on cellular metabolism. A preliminary report of part of this work was presented at the 1974 Annual Meeting of the Federation of American Societies for Experimental Biology, Minneapolis, Minn. (Kitchingman and Fournier, 1974a), and the 1974 Brookhaven Symposium in Biology on the "Processing of RNA" (Kitchingman and Fournier, 1974b).

† From the Department of Biochemistry, University of Massachusetts Amherst, Massachusetts 01002. *Received August 18, 1975.* Supported by U.S. Public Health Service Grant GM-19351. Taken in part from the Ph.D. dissertation of G.R.K. (in preparation)

Experimental Section

Materials. Radioactive phenylalanine was obtained from either Schwarz/Mann or New England Nuclear. L-[^{14}C]Phenylalanine was uniformly labeled and used at the stated specific activity of 455 mCi/mmol. L-[^{3}H]Phenylalanine was generally labeled and was adjusted to the final specific activities indicated under Methods. [^{32}P]Orthophosphate (carrier-free) was obtained from ICN. Brain-heart infusion media was purchased from Difco Laboratories. Alumina was obtained from the Aluminum Company of America and washed with 1.0 M KCl and water before use. DEAE-cellulose[1] (Whatman DE52) was obtained from Reeve-Angel and prewashed with 0.5 N HCl and 0.5 N NaOH. BD-cellulose (50–100 mesh) and phenoxyacetyl-*N*-hydroxysuccinimide were obtained from Schwarz/Mann. The BD-cellulose was prewashed in buffer that was 0.01 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$, 2 M NaCl, 20% ethanol. Adogen 464 [methyltrialkyl(C_8–C_{10})ammonium chloride] was obtained from Ashland Chemical Co., and Plaskon CTFE-2300 (poly(chlorotrifluoroethylene) powder) from Allied Chemical Co. The RPC-5 sorbent was prepared according to method C described by Pearson et al. (1971). Nitrocellulose filters for nucleic acid hybridization experiments were obtained from Schleicher and Schuell (Selectron filters, 0.45 μ). Acrylamide, bis(acrylamide), and RP-54 x-ray film were purchased from Eastman Kodak. Poly(uridylic acid) was obtained from Miles Laboratories, calf-thymus DNA from the Worthington Biochemical Corp., phosphoenolpyruvate and pyruvate kinase from the Sigma Chemical Co., and T_1 RNase from Calbiochem. A generous gift of pure *E. coli* $tRNA^{Phe}$ was provided by Drs. Bernard Dudock and Bruce Roe. All other chemicals were of reagent grade and obtained from commercial sources.

Methods

Bacteria and Culturing Conditions. E. coli strain CP79 (*arg*⁻, *his*⁻, *leu*⁻, *thr*⁻, *thi*⁻, *rel*⁻) was grown in brain-heart infusion medium (37 g/l.) as a source of DNA and aminoacyl-tRNA synthetase; in the citrate-glucose-salts medium of Vogel and Bonner (1956) for amino acid starvation studies or in preparing [^{32}P]tRNA, in low-phosphate (LP) medium. Fully supplemented Vogel-Bonner medium contained amino acids at concentrations of 20 μg/ml and thiamin at 2 μg/ml. The LP medium contained (per liter): tris(hydroxymethyl)aminomethane base, 12 g; potassium chloride, 2 g; ammonium chloride, 2 g; concentrated HCl, 7.5 ml; 0.44 ml of 10% sodium sulfite; 1 ml of 0.25 M magnesium chloride; 20 ml of 25% glucose; required amino acids at 20 μg/ml; and inorganic phosphate to a final concentration of 0.25 mM. All cultures were grown at 37 °C in a rotary-action shaking incubator. Full growth in supplemented Vogel-Bonner or LP medium corresponded to an optical density of about 1.2 at 450 nm. Leucine starvation was effected by growth in medium supplemented with a limiting amount of leucine. At a concentration of 5 μg/ml growth terminates at about the midoint of the logarithmic phase. Nonstarved control cultures were harvested at the midpoint of the logarithmic phase. When labeling with ^{32}P, 5 ml of a culture grown overnight in LP medium was inoculated into 300 ml of LP medium containing 0.125 mM phosphate and limiting in leucine. Twenty millicuries of $[^{32}P]PO_4^{3-}$ was added 20–30 min after the onset of starvation. All amino acid starved cultures were harvested 6 h after growth ceased.

Preparation of DNA. DNA was prepared by the method of Marmur (1963).

Preparation of Unfractionated tRNA. Bulk tRNA was prepared through the DEAE-cellulose column step as described previously (Fournier and Peterkofsky, 1975): 1 A_{260} unit: the amount of RNA which, when dissolved in 1 ml of 0.01 M Tris-HCl pH 8.9 has an absorbance of 1.0 at 260 nm. 1 A_{260} is equivalent to 50 μg of RNA.

Preparation of Phenylalanyl-tRNA Synthetase. Phenylalanyl-tRNA synthetase was partially purified by a procedure patterned largely after those described by Muench and Berg (1966) and Stulberg (1967) for bulk aminoacyl-tRNA synthetase and phenylalanyl-tRNA synthetase, respectively. All operations were performed at 4 °C. In our procedure the cells were disrupted by grinding with alumina in a mortar with pestle (Nirenberg, 1963) and the resulting paste extracted with buffer that contained: 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM KCl, and 5 mM β-mercaptoethanol. The extract was centrifuged twice, first at 30 000*g* (Sorvall, SS-34 rotor) for 20 min and the resulting supernatant at 200 000*g* for 90 min (Beckman Spinco, No. 65 rotor). The high-speed supernatant derived from 40 g of cells was applied to a column of DEAE-cellulose 2.5 × 37 cm, preequilibrated in buffer that contained 20 mM potassium phosphate pH 7.5, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10% glycerol. After washing the column with two column volumes (350 ml) of the same buffer, bulk aminoacyl-tRNA synthetase was eluted with buffer that was 250 mM potassium phosphate, pH 6.5, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10% glycerol.

The fractions containing the major part (ca. 80%) of the synthetase activity were pooled and the protein was precipitated by the slow addition of solid $(NH_4)_2SO_4$ to a final concentration of 75% (48 g per 100 ml at 4 °C). The protein suspension was stirred for 30 min after the ammonium sulfate was dissolved and the precipitate recovered by centrifugation at 15 000*g* for 20 min (Sorvall SS-34 rotor). The precipitate was solubilized in 5 ml of the 20 mM phosphate buffer described above and dialyzed against fifty volumes of the same buffer with one change. The sample was then applied to a column of DEAE-cellulose, 2.5 × 37 cm, equilibrated in 20 mM potassium phosphate, pH 7.5, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 10% glycerol (same as above), and eluted with a 1-l. linear gradient of phosphate buffer of the same composition and pH, except that the terminal buffer was 250 mM in phosphate. The flow rate was controlled at 0.8 ml/min and 10-ml fractions were collected. The phenylalanyl-tRNA synthetase activity was located by the filter disk microaminoacylation assay described below. Twenty microliters from every third fraction were assayed and the fractions which contained most of the activity were pooled (fractions 41–47 = ca. 60% of the activity). Glycerol was added to a final concentration of 50% (v/v) and the enzyme stored at −20 °C. The specific activity was 5.3 units/mg where one unit is equal to the amount of enzyme that catalyzed the formation of 1 nmol of phenylalanyl-tRNA per min at 25 °C under the assay conditions described below. Phenylalanyl-tRNA synthetase prepared and stored in this way is stable for at least 1 year.

Aminoacylation Procedure. Two procedures were used for the aminoacylation of $tRNA^{Phe}$. Except where indicated, tRNA was aminoacylated at 37 °C in a reaction mixture that contained per ml: 50 mM Tris-HCl, pH 7.5, 18 mM $MgCl_2$, 4 mM ATP, 10 mM β-mercaptoethanol, 5 mM NH_4Cl, 20–200 pmol of $tRNA^{Phe}$, 10 μM [^{14}C]- or [^{3}H]phenylalanine, a mixture of 19 unlabeled amino acids (20 common minus phenylalanine) at 50 μM each, and 0.2 unit of phenylalanyl-

[1] Abbreviations used are: DEAE, diethylaminoethyl; BD, benzoylated DEAE; LP, low-phosphate.

tRNA synthetase. The kinetics of acylation was monitored by sampling the reaction mixture at intervals. Aliquots were precipitated with cold 5% trichloroacetic acid and the precipitate was collected on glass fiber or cellulose nitrate filters (Millipore Corp). After drying at 100 °C, the extent of charging was determined by liquid scintillation counting in toluene–Liquifluor (24:1, New England Nuclear Corp). Samples to be chromatographed or used in hybridization or protein synthesis assays were extracted with an equal volume of 90% phenol after addition of one-tenth volume of 20% potassium acetate, pH 4.5. The aminoacyl-tRNA was then precipitated with alcohol and solubilized in the buffer of choice or, in the case of chromatographic analyses, dialyzed against the initial column buffer. Samples to be cochromatographed were combined just prior to dialysis. For the hybridization analyses the unique and normal phenylalanine tRNAs were aminoacylated with [^{3}H]phenylalanine at a specific activity of 59 Ci/mmol at a final concentration of 2×10^{-6} M.

A rapid microaminoacylation assay was used in the purification of phenylalanyl-tRNA synthetase and tRNA^{Phe}. The method was taken essentially from the procedure described by Cherayil et al. (1968). In this procedure the incubations were conducted on 25-mm filter paper disks (Schleicher and Schuell No. 593-A). When assaying for synthetase, 100 μl of the reaction mixture described above, complete except for the enzyme, was applied to each of a series of numbered paper disks supported on pins on an aluminum foil covered block of styrofoam. Immediately following, an aliquot of the protein fraction to be tested was applied and the impregnated disks allowed to incubate for 15 min at room temperature. The reaction was terminated by dropping the disks into cold (4 °C) 10% trichloroacetic acid. After 1 h the disks were washed for 15 min each in: 5% Cl_3CCOOH (4 °C); 1:1 ethanol–diethyl ether; 1:3 ethanol–ether; and, finally, ether. The last three washes were done at room temperature. The disks were air-dried and counted in toluene–Liquifluor scintillation fluid. The tRNA^{Phe} assays were performed in the same way except that 50–100 μl of the RNA fraction to be analyzed was applied to the disk and the disk was washed with cold 95% ethanol and dried before the enzyme-containing reaction mixture was applied.

Acrylamide Gel Electrophoresis. [^{32}P]tRNA was purified by electrophoresis through 10% polyacrylamide gel slabs (10 × 20 × 0.3 cm) according to the method of DeWachter and Fiers (1971).

Chromatography of tRNA on Benzoylated DEAE-Cellulose (BD-Cellulose). tRNA^{Phe} was enriched by BD-cellulose chromatography by two different procedures. Nonaminoacylated tRNA was fractionated essentially by the method described by Gillam et al. (1967). Where indicated, phenylalanyl-tRNA derivatized with phenoxyacetyl-*N*-hydroxysuccinimide (PAHS) was purified on BD-cellulose according to the procedure developed by Gillam et al. (1968). Both column procedures were performed at room temperature (24 °C).

The unique and normal species of tRNA^{Phe} used in the hybridization and protein synthesis experiments were first separated and enriched by chromatography on BD-cellulose as uncharged tRNA. 450 A_{260} units of bulk tRNA from leucine-starved cells were applied to a column of BD-cellulose (1.5 cm by 25 cm) preequilibrated with buffer that was 0.01 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$, 0.4 M NaCl. The tRNA was eluted with a linear NaCl gradient in the same buffer with limits of 0.4 and 1.1 M. Total volume of the gradient was 300 ml. At the conclusion of the salt gradient (fraction 130) the column was washed with 45 ml of limit buffer and then (at fraction 150) with buffer that was 1.5 M NaCl, 15% ethanol. The flow rate was controlled at 1 ml/min and 50-drop fractions were collected. The tRNA^{Phe} was located by testing every fifth fraction for phenylalanine acceptor activity using the microaminoacylation assay method described above. The fractions containing the major unique and normal phenylalanine tRNAs (fractions 65–85 and 105–140, respectively) were pooled and the tRNA was recovered by ethanol precipitation. The pooled fractions corresponded to approximately 130 A_{260} and 10 A_{260}, respectively, and were determined to have amino acid acceptor activities of 27 and 148 pmol per A_{260}. The individual species were then purified further by RPC-5 chromatography.

In the pulse-chase experiment, [^{32}P]tRNA aminoacylated with unlabeled phenylalanine was mixed with 7 A_{260} tRNA from leucine-starved cells precharged with [^{3}H]phenylalanine (sp act. 24 Ci/mmol) and derivatized with PAHS as described earlier (Kitchingman and Fournier, 1975). The tRNA was then applied to a column containing 3 ml of BD-cellulose equilibrated in buffer that was 0.01 M sodium acetate, pH 4.5, 0.01 M $MgCl_2$, 1 M NaCl. The column was then washed with 10 ml of the same buffer and the derivatized tRNA^{Phe} eluted with a 50-ml linear salt–ethanol gradient with limits of 1 M NaCl and 2 M NaCl–20% ethanol. At the end of the gradient the column was washed with 40 ml of the limit buffer. Two-milliliter fractions were collected. The fractions containing the tRNA^{Phe} were pooled, 5 A_{260} of carrier tRNA (from leucine-starved cells) were added, and the RNA was recovered by ethanol precipitation. The derivatized phenylalanine was released from the tRNA by incubation in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (pH 8.5) for 3 h at room temperature. The tRNA was then dialyzed against 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.0), reacylated with [^{3}H]phenylalanine, and subjected to RPC-5 chromatography. When used on a preparative scale where the addition of carrier tRNA was omitted, the acceptor activities of the tRNA^{Phe} species obtained by this method were 1400–1600 pmol/A_{260}, corresponding to a purity of 80–90%. By analogy, the [^{32}P]tRNA^{Phe} prepared in this way should be of similar purity.

Reversed-Phase Chromatography. Unique and normal phenylalanine tRNAs were separated by high-pressure reversed-phase column chromatography on RPC-5 sorbent (Pearson et al., 1971). Fractionation was performed on either a 0.6 × 69 cm (large) or 0.5 × 30 cm (small) column. The procedures for sample preparation, operation, and assay of both RPC-5 columns have been described in detail elsewhere (Fournier and Peterkofsky, 1975; Kitchingman and Fournier, 1975).

DNA:tRNA^{Phe} Hybridization. RNA:DNA hybridization analyses were carried out essentially as described by Scherberg and Weiss (1970). Alkaline denatured DNA was bound to 47-mm cellulose nitrate filters according to the method of Gillespie and Gillespie (1971) from which 22-mm filters containing 140–220 μg of DNA were cut. Chemical analyses (Meijs and Schilperoort, 1971) showed the variance in DNA content for filters cut from the same large filter to be less than 2%. The filters were incubated in 24-mm scintillation vials in 500 μl of reaction mixture that was 0.03 M sodium citrate, 0.3 M NaCl (2 × SSC), pH 5, 50% (v/v) formamide, and [^{3}H]phenylalanyl-tRNA as indicated. The vials were incubated for 5 h at 28 °C with gentle agitation. The filters were then washed in 2 × SSC, pH 5.0 for 30 min at room temperature, incubated with 36 μg (14 units) of T_1 RNase in 1 ml 2 × SSC, pH 5.0 for 20 min at room temperature, washed twice

with 2 × SSC, air-dried, and counted in toluene–Liquifluor scintillation fluid. Filters containing calf thymus DNA were included as controls for nonspecific binding. Because the degree of nonspecific binding to control filters proved to be influenced significantly by the amount of DNA on the filter, an effort was made to pair only test and control filters which contained similar amounts of DNA. In most cases the amount of DNA on the test and control filters differed by only about 10%. The extent of deacylation of [^{3}H]phenylalanyl-tRNA during the course of the experiment was determined by precipitating aliquots with 5% trichloroacetic acid, collecting the precipitate on Millipore filters, and measuring the acid-insoluble radioactivity by liquid scintillation spectrometry. The saturation hybridization values have been corrected for deacylation. At saturation, from 200 to 350 cpm were bound to each filter after subtracting the activity resulting from nonspecific absorption.

Cell-Free Synthesis of Polyphenylalanine. A cell-free system for studying the poly(uridylic acid)-directed synthesis of polyphenylalanine was established essentially according to the procedure of Nirenberg (1963) except that the S30 was prepared by the method of Zubay et al. (1970). The incorporation reaction contained per 100 μl: 40 mM Tris-acetate, pH 7.8, 20 mM KCl, 5.33 mM $MgCl_2$, 1.73 mM ATP, 0.11 mM GTP, 2 mM β-mercaptoethanol, 5 mM phosphoenolpyruvate, 4 μg of pyruvate kinase, 5 mM each of 19 unlabeled amino acids, 5.27 μg of poly(U), 45 μg of S30 protein, and [^{14}C]phenylalanyl-tRNA as indicated. The reaction mixtures were incubated for 30 min at 30 °C and incorporation was terminated by the addition of 2 ml of 10% CCl_3COOH. The samples were then heated for 20 min at 90 °C and chilled, and the acid-insoluble material was collected on Millipore filters. The incorporation of phenylalanine into protein was determined by measuring the radioactivity by liquid scintillation counting.

Results

Formation of the Unique tRNA^{Phe}. When protein synthesis is interrupted in *E. coli* under conditions which permit the synthesis of RNA to continue, two chromatographically unique isoacceptor species of tRNA^{Phe} are produced which are distinct from the normally occurring species. Figure 1 shows the effect of leucine starvation on the phenylalanine tRNA from a relaxed control (*rel*⁻) strain of *E. coli*. The nonstarved *rel*⁻ cells contain a single species of tRNA^{Phe} while the leucine-starved cells can be seen to possess the normal tRNA^{Phe} and two additional isoacceptor species which are well resolved from each other and from the normal species. While the unique and normal species appear to be homogeneous on RPC-5 or BD-cellulose chromatography, it was discovered (Kitchingman and Fournier, 1975) that each consists of subspecies with different reactivities toward phenoxyacetyl-*N*-hydroxysuccinimide (PAHS), a reagent used in the derivatization and purification of aminoacyl-tRNA (Gillam et al., 1968) and now known to react with the base 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U; Friedman et al., 1974) which occurs in several tRNAs including tRNA^{Phe} from *E. coli* (Barrell and Sanger, 1969).

Unique species of tRNA^{Phe} also accumulate in *rel*⁻ cells during starvation for arginine and following treatment with chloramphenicol or puromycin. The unique species produced under these conditions have similar elution properties, suggesting that they may be identical and, possibly, derived by a common mechanism (Kitchingman and Fournier, 1975, and in preparation). Because the minor unique species comprised only about 2% of the total tRNA^{Phe}, it was considered technically unfeasible to include that species in the characterization studies described below; thus, only the major unique and normal isoacceptor species have been considered.

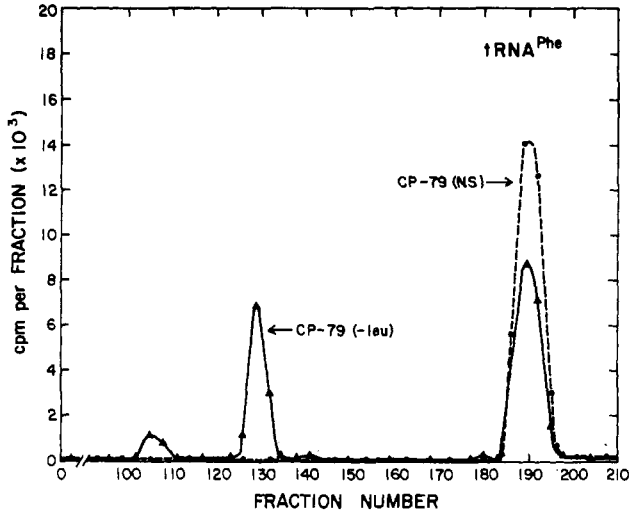

FIGURE 1: Effect of leucine starvation on the phenylalanine tRNA from relaxed control *E. coli*. Samples of tRNA from leucine-starved and nonstarved (mid-log) cultures of *E. coli* CP79 were aminoacylated with [^{3}H]- and [^{14}C]phenylalanine, respectively, mixed, and analyzed by RPC-5 cochromatography as described in Methods, and earlier (Fournier and Peterkofsky, 1975). The sample (^{3}H to ^{14}C ratio about 3:1) was applied to a 0.9 × 69 cm column equilibrated in buffer that was 0.01 M sodium acetate, pH 4.5, 1 mM EDTA, 0.01 M $MgCl_2$, and 0.4 M NaCl, and eluted with a linear NaCl gradient with limits of 0.4 and 1.2 M. Total volume of the gradient was 600 ml; 2-ml fractions were collected. Fractions were precipitated with trichloroacetic acid and the acid-insoluble material collected on Millipore cellulose nitrate filters. The ^{3}H and ^{14}C activity profiles were determined by liquid scintillation spectrometry and the resulting profiles were normalized. The minor unique, major unique, and normal phenylalanine tRNAs are in fractions 100–110, 125–135, and 185–195, respectively. (▲—▲) tRNA^{Phe} from leucine-starved cells (−Leu) [^{3}H]phenylalanine; (●—●) tRNA^{Phe} from nonstarved cells (NS), [^{14}C]phenylalanine.

DNA:tRNA^{Phe} Hybridization. To rule on the question of whether the unique and normal tRNA^{Phe} isoacceptor species are derived from the same or different cistrons, a series of tRNA:DNA hybridization experiments were performed. If the two species of tRNA^{Phe} are transcribed from the same or closely related cistrons the saturation hybridization values for each species should be identical; further, if the two tRNAs are combined, the saturation value should not be affected. If, however, the unique and normal species of tRNA^{Phe} are generated from different genes which have little or no homology, the saturation hybridization values may be the same or different but, when combined, should give a saturation value equal to the sum of the individual values.

Figure 2 shows the results of the saturation hybridization analyses performed. It can be seen that the saturation values for the major unique and normal species are essentially the same and that combining the two isoacceptors does not give an additive result. This result indicates that the nucleotide sequences of the isoacceptor species are closely related. That the two species are derived from the same gene(s) cannot be concluded from this observation, however. Isoacceptors generated from genes which differ by only a few nucleotides could well cross-hybridize under the assay conditions used here. Thus, it can only be concluded that these isoacceptors share a high degree of sequence homology and may well be transcribed from the same DNA sequence.

When the gene dosage is estimated by extrapolation from double-reciprocal plots of the hybridization data (see inset in

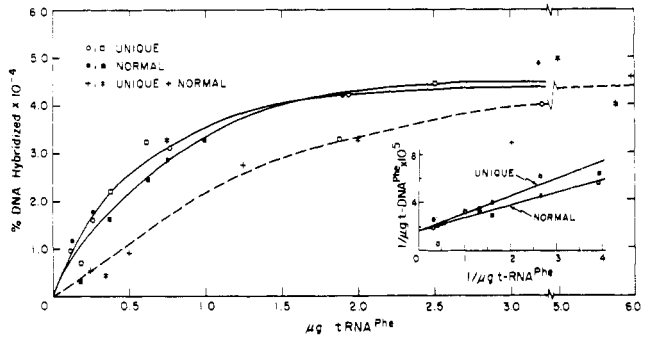

FIGURE 2: Hybridization of unique and normal $tRNA^{Phe}$ to *E. coli* DNA. The major unique and normal species of $tRNA^{Phe}$ were enriched and separated by BD-cellulose and RPC-5 chromatography; the acceptor activities were determined to be 200 and 750 pmol/A_{260} respectively. [^{3}H]Phenylalanyl-tRNA in the amounts indicated was hybridized with filter-bound *E. coli* CP79 DNA as described under Methods. The amount of $tRNA^{Phe}$ hybridized was determined from measurements of the amount of radioactivity bound to the DNA in a complex stable to T_1-RNase. The hybridization values were corrected for the loss of radioactivity resulting from deacylation. Saturation hybridization values were determined for the unique and normal isoacceptor species individually and together: (○,□) unique $tRNA^{Phe}$; (●,■) normal $tRNA^{Phe}$; (+, ±) unique plus normal $tRNA^{Phe}$. Two independent analyses were performed with each isoacceptor species. The inset figure indicates the gene dosage for $tRNA^{Phe}$ in *E. coli* derived from double reciprocal plots of the saturation hybridization results. The pertinent calculations are described in the text.

Figure 2), the results suggest that the *E. coli* chromosome contains only one gene for $tRNA^{Phe}$. At saturation, 4.5×10^{-6} μg of $tRNA^{Phe}$ are hybridized per μg of DNA. This corresponds to 4.5×10^{-4} % of the DNA or, assuming a molecular weight of 2.8×10^9 daltons for the DNA (Cairns, 1963), 1.26×10^4 daltons of homologous DNA. Using 2.8×10^4 daltons as the molecular weight of $tRNA^{Phe}$ (calculated from the known sequence—Barrell and Sanger, 1969) the amount of DNA hybridized corresponds to about 0.5 copy of $tDNA^{Phe}$ per chromosome.

While it seems reasonable to conclude that the results reflect the occurrence of a single gene for $tRNA^{Phe}$ in the *E. coli* chromosome, because of the importance of the measurement to this study, the apparently low hybridization values bear some scrutiny. Of special interest, of course, is the possibility that the method cannot distinguish between one and two copies of a gene and that a second, closely related, $tRNA^{Phe}$ gene exists. Factors which could contribute to a low estimate of the gene dosage include: (1) poor precision owing to error in correcting for deacylation, nonspecific interactions, and loss of DNA from filters during incubation, and (2) the possibility that the bulk of the DNA sequences occur in slightly more than one copy per cell owing to multifork replication.

With regard to precision, this method of analysis yields measurements which may be imprecise to the extent of 30–40% (unpublished results). Thus, the saturation values derived could be close to but still less than one copy per chromosome. To test the possibility that the estimate of the $tRNA^{Phe}$ gene dosage is low because of competition by other tRNAs closely related in sequence to $tRNA^{Phe}$, the saturation value for a preparation of highly purified $tRNA^{Phe}$ was also determined. This material, estimated to be at least 90% pure based on oligonucleotide analyses (Roe and Dudock, personal communication), gave a saturation value of 7.9 μg of $tRNA^{Phe}$/μg of DNA $\times 10^{-6}$ or 0.9 copy of $tDNA^{Phe}$. Although this value is higher than those obtained with the less pure preparations of unique and normal $tRNA^{Phe}$, because of the precision of the method the values can be considered to be in agreement. Thus, while it is possible that competition for $tDNA^{Phe}$ binding sites by non-cognate tRNAs does occur, that contribution does not appear to be of real significance here.

Evaluating the potential effects of multifork replication on the apparent gene dosage is not possible here as the chromosomal location of the phenylalanine tDNA is not known. Under the culturing conditions used to prepare cells for DNA, it seems likely that multifork replication does occur. If a single $tRNA^{Phe}$ gene occurs far from the point where DNA replication originates, the bulk of the DNA derived from a culture of asynchronously dividing cells could appear to possess, on a percentage basis, slightly less than one copy of the gene. Similarly, sequences located near the point of origin of replication would appear to be present in greater amounts. Thus, while the hybridization results presented indicate the occurrence of only one gene for *E. coli* $tRNA^{Phe}$, the possibility of a second, closely related gene cannot be ruled out until the map location of the $tDNA^{Phe}$ is known.

Effect of Restoring Growth on the Unique $tRNA^{Phe}$. While the hybridization results provide additional support to the view that the unique and normal $tRNA^{Phe}$ isoacceptors are related as precursor and product, more compelling evidence would come from a demonstration that the unique species can be converted to a normal form upon recovery from starvation. To this end, a ^{32}P pulse-chase experiment was performed to determine if the major unique $tRNA^{Phe}$ can be converted to a chromatographically normal form following recovery from starvation. [^{32}P]Orthophosphate was added to a culture of cells at the onset of starvation so that only the tRNAs synthesized during the starvation period were labeled. After 6 h of starvation the cells were: (1) recovered by centrifugation, (2) washed and resuspended in fresh fully supplemented medium, and (3) incubated under normal culturing conditions. At various times during the recovery period, the culture was sampled, viable cell counts were performed, and the labeling pattern of the $tRNA^{Phe}$ was analyzed.

One of the purification steps used in the preparation of the $tRNA^{Phe}$ was chromatography of phenoxyacetylated phenylalanyl-tRNA on benzoylated DEAE-cellulose. This method was used for two reasons. First, this step alone yields $tRNA^{Phe}$ that is 60–80% pure. Secondly, we have discovered that the major unique and normal isoacceptors consist of subspecies which can be resolved by RPC-5 chromatography following phenoxyacetylation (Kitchingman and Fournier, 1975; Webb et al., 1975). The basis for the heterogeneity is believed to lie with the base 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U), formerly designated "X", which occurs in the extra arm of several tRNAs. This nucleotide reacts with PAHS and causes the derivatized species to elute later on BD-cellulose or RPC-5 chromatography (Friedman et al., 1974). Because a portion of both the major unique and normal species of $tRNA^{Phe}$ was found to be unreactive with PAHS when derivatized prior to aminoacylation and fractionation, it is assumed that each contains subspecies devoid of 4abu^{3}U. By analyzing phenoxyacetylated tRNA in the pulse-chase experiment it seemed likely that information could be obtained about the sequence of events involved in the secondary modification of $tRNA^{Phe}$

Following chromatography of the PAHS-derivatized $tRNA^{Phe}$ on BD-cellulose the tRNA was recovered, stripped of phenoxyacetylphenylalanine, and reacylated with [^{3}H]phenylalanine, and the subspecies of $tRNA^{Phe}$ was separated by RPC-5 chromatography. Both PAHS-derivatized and nonderivatized $tRNA^{Phe}$ from leucine-starved cells were added to the [^{32}P]$tRNA^{Phe}$ as internal markers before RPC-5 chromatography. The results of this experiment are shown in Figure 3.

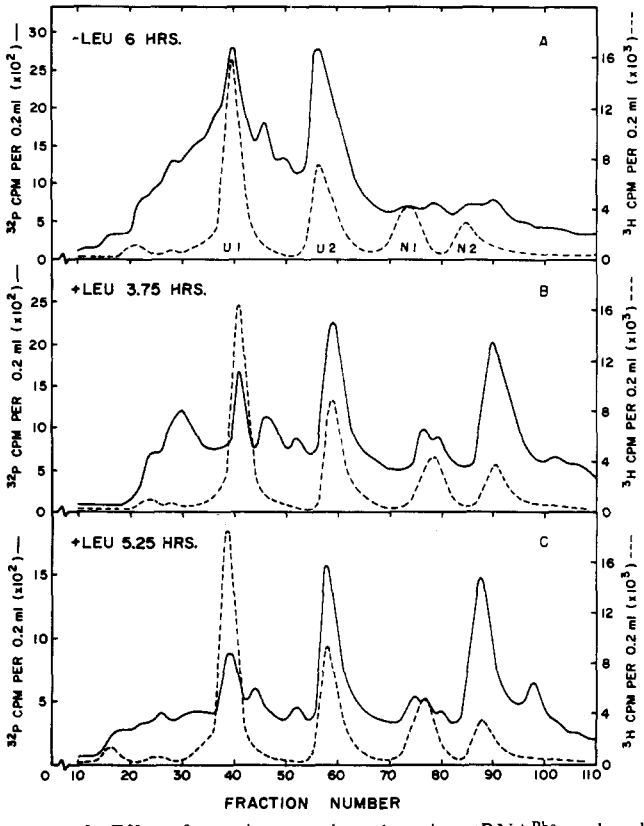

FIGURE 3: Effect of restoring growth on the unique tRNA^{Phe} produced in *rel*⁻ *E. coli* during leucine starvation. Twenty millicuries of [^{32}P]PO_4^{3-} was added to a 300-ml culture of *E. coli* CP79 20-30 min after the onset of leucine starvation. After 6 h of starvation the cells were harvested by centrifugation, washed in LP medium, resuspended in 600 ml of fresh, fully supplemented medium minus ^{32}P, and incubated under normal culturing conditions. At 0, 3.75, and 5.25 h after resuspension in the fresh medium, one-third of the culture was removed for analysis of the tRNA^{Phe}. Bulk [^{32}P]tRNA was prepared by phenol extraction and chromatography on DEAE-cellulose and partially fractionated by electrophoresis through a slab of 10% polyacrylamide gel as described under Methods. The main-band 4S RNA was recovered and the [^{32}P]tRNA^{Phe} prepared by chromatography on BD-cellulose as phenoxyacetylphenylalanyl-tRNA. The tRNA^{Phe} was deacylated, recharged with [^{3}H]phenylalanine, and fractionated by RPC-5 chromatography. The resulting RPC-5 elution profiles are shown. Details of the procedure used to prepare the [^{32}P]tRNA^{Phe} are given under Methods. (—) [^{32}P]tRNA; (- - -) [^{3}H]phenylalanyl-tRNA. The unique tRNA^{Phe} subspecies are designated U1 and U2; the species identified as N1 and N2 are subspecies of the normal tRNA^{Phe}. Species U2 and N2 have been derivatized with phenoxyacetic acid *N*-hydroxysuccinimide (PAHS); U1 and N1 do not react with PAHS.

Panels A, B, and C show the distribution of ^{32}P in the subspecies of tRNA^{Phe} after 6 h of starvation and at 3.75 and 5.25 h after resuspension of the starved cells in fresh, fully supplemented medium devoid of ^{32}P. The unique and normal isoacceptors which do not react with PAHS coelute with the untreated unique and normal isoacceptors and are identified here as U1 and N1, respectively. The unique and normal subspecies which react with PAHS elute later than the corresponding nonderivatizable subspecies and are designated as U2 (unique species 2) and N2 (normal species 2), respectively.

The elution patterns in panel A indicate that only the unique species of tRNA^{Phe} were labeled during the 6-h starvation period—that is, no normal tRNA^{Phe} appears to have been formed during starvation. Although the tRNA^{Phe} was not purified to homogeneity, the ^{32}P profile suggests that U1 and U2 were present in approximately equal amounts. In other similar recovery experiments described elsewhere (Kitchingman and Fournier, 1975) it was observed that cell growth resumed about 1 h after resuspension of the starved cells in fresh medium. Viable cell counts performed at the onset and end of starvation indicated that greater than 90% of the cells were still viable following 6 h of starvation. Analysis of the [^{32}P]tRNA^{Phe} from cells incubated for 3.75 h in fresh medium revealed the occurrence of ^{32}P in both subspecies of the normal isoacceptor. This result indicates that the unique species can be converted to a chromatographically normal form upon lifting the nutritional block.

More ^{32}P appears to be associated with species N2 than N1. Similarly, there was relatively more labeled U2 than U1. After 5.25 h in fresh medium, all four subspecies were still labeled, and, as was the situation at 3.75 h, there was more U2 than U1 and N2 than N1.

Taken together, the results demonstrate that: (1) at least one of the unique species can be converted to normal species, and (2) the conversion of one, perhaps both, of the unique subspecies proceeds only slowly as ^{32}P-labeled unique species persisted for more than 4 h after growth resumed. While the first result indicates that the unique species are undermodified forms of the normal, fully modified tRNA, the apparent slow rate of conversion suggests that the unique species may not be normal intermediates in the biosynthetic pathway for tRNA^{Phe}. The implications of this apparently slow rate of maturation will be discussed below.

Biological Activity of Unique tRNA^{Phe}. Inasmuch as unique species of tRNA^{Phe} accumulate under a variety of physiological conditions, it is reasonable to ask what effect their occurrence may have on cellular metabolism. For this reason and in the hope of gaining more information about the effects of post-transcriptional modification on tRNA function, a comparison was made of some of the biological properties of the unique and normally occurring tRNA^{Phe} species. The unique tRNA^{Phe} examined was the major unique species produced during leucine starvation—the same species used in the hybridization analyses. The functions examined were aminoacylation and the ability to participate in protein synthesis.

When the kinetics of aminoacylation were compared it was revealed that the unique tRNA^{Phe} was charged at a slower rate than the normal tRNA^{Phe}. This difference is perhaps shown most dramatically by comparing the RPC-5 column profiles of tRNA^{Phe} samples that are partially and completely charged. The patterns shown in Figure 4 are for samples of the same tRNA preparation aminoacylated to the extent of 10 and 100%. The resulting profiles have been normalized to equivalent amounts of normal tRNA^{Phe}. Inspection of the profiles reveals that the ratio of normal to unique phenylalanyl-tRNA differs considerably for the two samples. This ratio is 30:70 for the fully charged portion and approximately 70:30 for the sample that was only 10% acylated. Direct comparison of the relative amounts of aminoacylated unique tRNA^{Phe} in the normalized patterns suggests that the unique species is charged at approximately one-fifth the rate of the normal species.

The efficiency with which the major unique tRNA^{Phe} participates in protein biosynthesis was determined with a poly(U)-directed cell-free system. With this procedure the activities of the unique and normal species of tRNA^{Phe} were compared directly. The incorporating system was supplied with precharged tRNA^{Phe} to minimize the effect of endogenous tRNA^{Phe} and to enable us to examine only those steps which occur after aminoacylation.

Figure 5 shows the results of a kinetic analysis in which the rates and extents of polyphenylalanine synthesis with the unique and normal species were compared. It was observed that

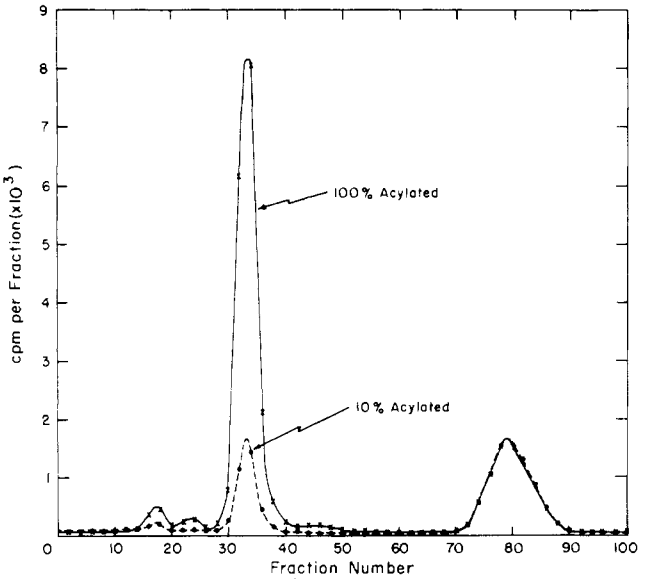

FIGURE 4: Rate of aminoacylation of unique and normal species of $tRNA^{Phe}$. Duplicate samples of unfractionated tRNA from leucine-starved *E. coli* CP79 were aminoacylated in vitro to the extent of 10 and 100% with [^{3}H]- and [^{14}C]phenylalanine, respectively, and analyzed by cochromatography on an RPC-5 column as described in Figure 1 and Methods. The resulting ^{14}C and ^{3}H profiles have been normalized to show equal amounts of the normal $tRNA^{Phe}$ activity. (●—●) 10% acylated, ^{3}H; (x—x) 100% acylated, ^{14}C.

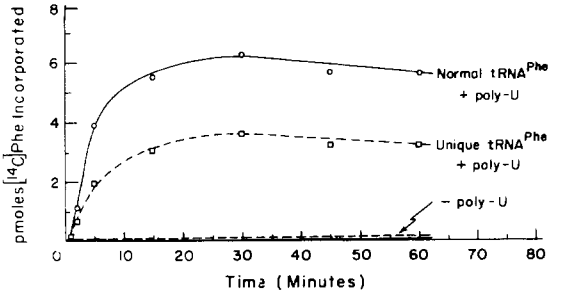

FIGURE 5: Kinetics of polyphenylalanine biosynthesis with unique and normal species of phenylalanyl-tRNA. 8.5 pmol of unique or normal $tRNA^{Phe}$ precharged with [^{14}C]phenylalanine was added to a poly(U)-directed amino acid incorporating system. At the times indicated 100-μl portions of the reaction mixture were sampled to measure the amount of [^{14}C]polyphenylalanine formed. Composition of the cell-free reaction mixture, incubation conditions, and the procedures used to measure amino acid incorporation into protein are described under Methods. Incorporation in the absence of poly(U) template was also determined.

the rate of incorporation of phenylalanine was lower with the unique $tRNA^{Phe}$ and that only 60% as much protein was formed when translation ceased. At all levels of $tRNA^{Phe}$ tested, up to 30 pmol, from 30 to 50% less phenylalanine was incorporated into protein by the unique $tRNA^{Phe}$ than with the normal species. Virtually no incorporation occurred in the absence of poly(U).

Because the same result could be realized with tRNAs that are equally efficient in protein synthesis but differ in the rate at which they become deacylated during the course of the incorporation assay, the rates of deacylation of the unique and normal species were also compared. Reaction mixtures lacking only the poly(U) template were sampled with time and the aliquots were tested for content of [^{14}C]phenylalanyl-tRNA. The results, shown in Figure 6, indicate that the unique and normal species are deacylated at the same rate and that the half-life of the hydrolytic reaction was about 22 min.

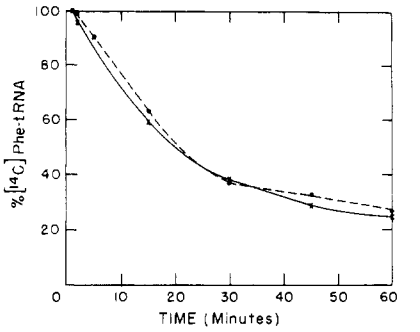

FIGURE 6: Rate of deacylation of unique and normal species of phenylalanyl-tRNA during synthesis of polyphenylalanine. Equal amounts (8.5 pmol) of unique and normal $tRNA^{Phe}$ aminoacylated with [^{14}C]phenylalanine were added to separate reaction mixtures containing all the components for polyphenylalanine synthesis except poly(U). The composition of the reaction mixtures and the incubation conditions were the same as those used in the experiment presented in Figure 5 and are described under Methods. At the times indicated, 100-μl portions were removed from the reaction mixture and precipitated with trichloroacetic acid, and the acid-insoluble material collected on Millipore filters. The extent of deacylation was determined by liquid scintillation counting. The results are expressed as the proportion of input [^{14}C]phenylalanyl-tRNA remaining

The apparent termination of [^{14}C]phenylalanine incorporation in the kinetic study shown in Figure 5 corresponds well with the release of amino acid from precharged tRNA by deacylation. The differences in extent of protein synthesis with the two species of $tRNA^{Phe}$ are probably due, in fact, only to the differences in the rate of incorporation. The differences in the rates observed show the unique species to be about 60% as efficient as the normal $tRNA^{Phe}$ in supporting protein synthesis.

Because the levels of aminoacyl-tRNA synthetase and tRNA are relatively high in the S-30 supernatant, the possibility existed that some of the [^{14}C]phenylalanine incorporation observed may have been promoted by endogenous $tRNA^{Phe}$. Labeled phenylalanine released from the test tRNA by deacylation could have been incorporated into endogenous $tRNA^{Phe}$ and then into protein. If so, the actual efficiency of the unique species could be even less than that observed. To rule on this point, charged tRNA was isolated from a poly(U)-free reaction mixture after a 30-min incubation and the distribution of [^{14}C]phenylalanine in $tRNA^{Phe}$ analyzed by RPC-5 chromatography. The unique tRNA was the test RNA. Column analysis showed that all the radioactivity was associated with the unique species. Thus, the incorporation observed in Figure 5 was due only to the activity of the $tRNA^{Phe}$ species being tested.

Discussion

The results reported here demonstrate that the major unique isoacceptor species of phenylalanine tRNA produced in *rel*$^-$ *E. coli* during leucine starvation is derived from a DNA sequence closely related to, if not identical with, that which codes for the normal species of $tRNA^{Phe}$. The hybridization results also suggest that there may be only one gene for $tRNA^{Phe}$ in the *E. coli* chromosome. These results, taken with the finding that unique species can be converted to chromatographically normal forms, indicate that these isoacceptors have a precursor–product relationship, differing only in the extent to which they have undergone secondary modification. Of course, final proof of the indicated relationship must come from direct sequence analysis, (see note added in proof).

Results from base compositional analyses of ^{32}P- and ^{35}S-

labeled unique and normal $tRNA^{Phe}$ indicate that the major unique species is deficient in dihydrouridine, pseudouridine, and 3-(3-amino-3-carboxypropyl)uridine (relative to the normal isoacceptor) and contains isopentenyladenosine (i^6A) in place of 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A). Chromatographic results obtained with phenoxyacetylated $tRNA^{Phe}$ revealed that the unique species of $tRNA^{Phe}$ contains subspecies devoid of 3-(3-amino-3-carboxypropyl)uridine (Webb et al., 1975; Kitchingman and Fournier, 1975). Except for the differences just noted, no minor nucleotides have been found in the unique species which do not occur in the normal isoacceptor. Taken together, the results support the hypothesis that both unique phenylalanine tRNAs produced under this condition are undermodified precursors to the normal species. Some of the enzymes involved in the post-transcriptional modification of precursor tRNAs may be short-lived and thus must be synthesized continually; in the absence of protein synthesis modification-deficient tRNAs accumulate.

While the results from the ^{32}P pulse-chase experiment demonstrate that unique species are converted to normal forms upon restoration of protein synthesis, the conversion occurs only slowly and the labeling pattern suggests the possibility that the unique PAHS-derived subspecies U2 may not be converted at all. This view is based on the observation that the relative amount of labeled U2 remains essentially constant over the recovery period. Alternatively, of course, the rates of formation and conversion of U2 could be equivalent. The apparent lack of accumulation of subspecies N1 could reflect a rapid conversion rate for that species. Together the results suggest that, while the unique species may be undermodified forms of the normal $tRNA^{Phe}$, they may not be the normal precursors to the mature form. Some of the base modification reactions may normally occur in a somewhat specific sequence, while others could occur at any time. If the modification sequence is violated and certain of the reactions occur out of order, it is possible that the resulting products will be poor substrates for the reactions which did not occur. Thus, when normal growth is restored, the conversion of some of the undermodified forms may proceed slowly or not at all.

Because species U1 and N1 do not react with PAHS, it is assumed that each is devoid of $4abu^3U$. Subspecies U2 and N2 are derivatizable and presumably contain this nucleotide. Based on this consideration alone, U1 could be converted to U2 or N2, but U2 should not be converted to U1 or N1.

The presence of i^6A in the unique species in place of ms^2i^6A probably accounts for the altered chromatographic behavior. This assumption is based on the observation of Gefter and Russell (1969) that an undermodified form of su_3^+ tyrosine tRNA which lacks only the *S*-methyl moiety of ms^2i^6A elutes much earlier on BD-cellulose than the fully modified isoacceptor. Additional support comes from analyses of $tRNA^{Phe}$ from iron-starved cells, a condition which blocks the conversion of i^6A to ms^2i^6A (Rosenberg and Gefter, 1969). A unique isoacceptor of $tRNA^{Phe}$ occurs in such cells which elutes earlier than the normal species on BD-cellulose (Rosenberg and Gefter, 1969; Juarez et al., 1975) and RPC-5 chromatography (Webb and Fournier, unpublished results). We have observed that the major unique $tRNA^{Phe}$ from iron-starved cells elutes slightly before the major unique $tRNA^{Phe}$ from leucine-starved cells. Thus, while direct sequence analyses will be required to show that the $tRNA^{Phe}$ from iron-starved cells lacks only the thiomethyl modification, it seems reasonable to assume that this particular deficiency is probably responsible for the major shift in chromatographic position of the unique species of *rel*⁻ $tRNA^{Phe}$. Thus, the conversion of subspecies U1 and U2 to a normal form probably involves, at least, the formation of ms^2i^6A.

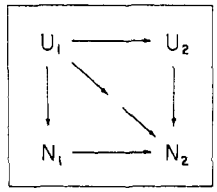

FIGURE 7: Possible scheme for the conversion of $tRNA^{Phe}$ subspecies U1, U2, and N1 to mature $tRNA^{Phe}$ (N2).

Considering only the deficiencies in $4abu^3U$ and ms^2i^6A, the conversion of subspecies U1, U2, and N1 and N2 could proceed by the pathways shown in Figure 7. U1 and N1 could be converted to U2 and N2, respectively, by the formation of $4abu^3U$; U1 and U2 could be converted to N1 and N2, respectively, by the conversion of i^6A to ms^2i^6A. Finally, the conversion of U1 to N2 must involve the formation of both $4abu^3U$ and ms^2i^6A. Because U2 appeared to persist for several hours after the starved cells were allowed to recover, it can be suggested that ms^2i^6A is normally formed before $4abu^3U$. Of course, this scheme must be considered conjectural until detailed information about the structures of each subspecies is available. Sequence analyses are currently in progress.

In addition to demonstrating that the multiple forms of phenylalanine tRNA have a common origin, the results reported here show that the major unique species is functionally inferior to the normal $tRNA^{Phe}$. The unique species is aminoacylated at a lower rate than the normal species and is only 60% as efficient in supporting the cell-free synthesis of polyphenylalanine. Although the rates of charging were estimated to differ by about fivefold, this value can only be considered an approximation because of the nature of the comparative assay used. Rigorous kinetic analyses must be performed in order to obtain a more precise value. These analyses were not undertaken after discovering that the unique species contained subspecies deficient in 3-(3-amino-3-carboxypropyl)uridine.

We do not yet know which particular modified base or bases in $tRNA^{Phe}$ are responsible for the functional differences observed. Normal *E. coli* $tRNA^{Phe}$, shown in Figure 8, contains a total of 10 modified bases of which 7 are unique (Barrell and Sanger, 1969; Ohashi et al., 1974). These bases are: pseudouridine (three), dihydrouridine (two), 4-thiouridine (one), 2-thiomethyl-N^6-(Δ^2-isopentenyl)adenosine (one), 7-methylguanosine (one), 3-(3-amino-3-carboxypropyl)uridine (the "X" base-one), and ribothymidine (one).

Of these bases, one is an especially attractive candidate to focus on in attempting to explain one of the altered properties of the unique $tRNA^{Phe}$. The hypermodified base, 2-thiomethyl-N^6-(Δ^2-isopentenyl)adenosine (ms^2i^6A), has been shown to play a role in ribosome binding in *E. coli* tyrosine tRNA (Gefter and Russell, 1969). $tRNA^{Tyr}$ deficient in this base binds to ribosomes with much less efficiency than fully modified tRNA and is accordingly less efficient in supporting protein synthesis. This base occurs in all *E. coli* tRNAs in the U-codon class and is located immediately adjacent to the first base in the anticodon (Peterkofsky and Jesensky, 1969). Thus, it is reasonable to suggest that the absence of the thiomethyl moiety of the ms^2i^6A in the unique $tRNA^{Phe}$ is the basis for the decreased efficiency in supporting poly(phenylalanine) biosynthesis.

The basis for the altered rate of aminoacylation of the unique $tRNA^{Phe}$ is more difficult to explain. To date there has been no report of an involvement of dihydrouridine or pseu-

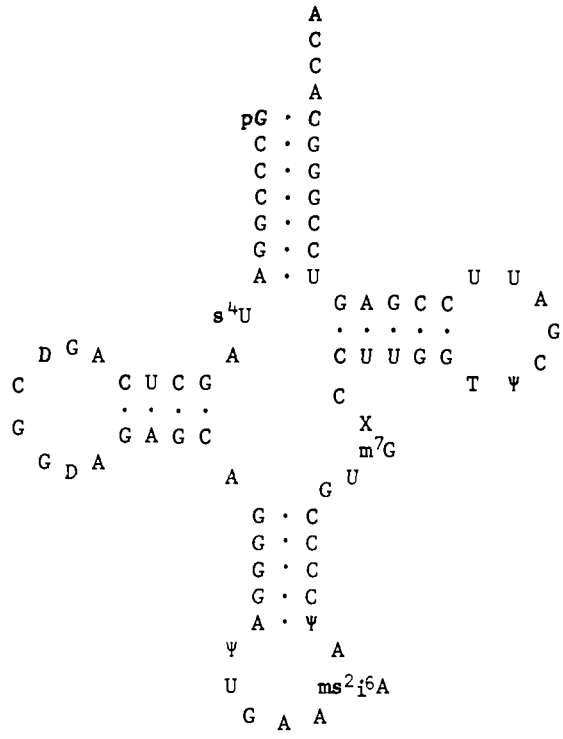

FIGURE 8: Structure of *E. coli* phenylalanine tRNA (Barrell and Sanger, 1969). The base designated "X" has recently been identified as 3-(3-amino-3-carboxypropyl)uridine (Ohashi et al., 1974).

douridine in the aminoacylation of any *E. coli* tRNA. In the case of ms^2i^6A, there is evidence that this base has no role in the charging of tyrosine tRNA. Gefter and Russell (1969) found that $tRNA^{Tyr}$ isoacceptor species containing an unmodified A or i^6A in place of ms^2i^6A are charged at a rate close to that of the fully modified species. Of course, these results do not preclude the possibility that ms^2i^6A is involved in the aminoacylation of phenylalanine tRNA, especially in view of the evidence for involvement of the anticodon region in tRNA:synthetase interaction in the cases of $tRNA^{Glu}$, $tRNA^{Gln}$ (Seno et al., 1974), $tRNA^{fMet}$ (Schulman and Goddard, 1973; Dube, 1973), $tRNA^{Ile}$ (Schimmel et al., 1972; Harada and Nishimura, 1974; Budzik et al., 1975), $tRNA^{Gly}$ (Squires and Carbon, 1971), $tRNA^{Tyr}$ (Schoemaker and Schimmel, 1974), and su_7^+ tRNA (Crothers et al., 1972) from *E. coli*, and $tRNA^{Phe}$ (Thiebe and Zachau, 1968; Schoemaker et al., 1975) and $tRNA^{Val}$ (Mirzabekov et al., 1971; Chambers et al., 1973) from yeast.

It has been known for some time that unique species of tRNA produced by methionine starvation can also differ functionally from the fully modified species (Shugart et al., 1968; Stern et al., 1970; Capra and Peterkofsky, 1968). Of particular interest to the work described here are the findings that the unique species of $tRNA^{Phe}$ are aminoacylated at a lower rate (Shugart et al., 1968) and interact less efficiently with synthetic codons (Stern et al., 1970). Although it seems clear that the unique species examined in those studies were deficient in methylated nucleosides and probably contained i^6A in place of ms^2i^6A (Isham and Stulberg, 1974), it is not known what deficiencies were responsible for the functional differences observed. Indeed, it appears that tRNA derived from *rel*$^-$ cells starved of methionine is also deficient in dihydrouridine and 4-thiouridine (Jacobson and Hedgcoth, 1970; Waters et al., 1973). Thus, it is possible that methyl deficiency may not be the biochemical basis for the effects reported.

Although Stern et al. (1970) found the codon–anticodon interaction to be less efficient for methyl-deficient $tRNA^{Phe}$ these workers did not detect any difference between the normal and methyl-poor species in a cell-free phenylalanine-incorporating system directed by poly(U). This finding is in contrast with that reported here for undermodified $tRNA^{Phe}$ derived by leucine deprivation and can be taken as evidence that the deficiencies produced by the two conditions are different.

While it seems clear that the unique tRNA species which accumulate when protein synthesis is blocked are undermodified precursors, the biological consequences of this phenomenon are not clear. Do these undermodified tRNAs play a regulatory role in the cell? There is precedence for such a role from the elegant work of Ames and his collaborators who have demonstrated that the defective regulation of histidine biosynthesis by certain mutants of *Salmonella typhimurium* is related to the absence of two specific pseudouridine residues in the anticodon loop and stem of histidine tRNA (Singer et al., 1972). Of interest, too, is the question of whether or not the unique tRNAs observed by us are normally occurring intermediates in the tRNA biosynthetic process. Because many of the base modification reactions can now be performed in vitro, it should be possible to use these undermodified species to determine if tRNA base modification reactions occur in an ordered or random manner. These species should prove to be especially valuable in studying the effect of base modification on tRNA structure and function.

Added in Proof

Results from nucleotide sequence analyses have revealed that the major unique $tRNA^{Phe}$ differs from normal $tRNA^{Phe}$ at positions 16, 37, 39, and 47 from the 5′ terminus. The unique $tRNA^{Phe}$ contains uridine (U) in place of dihydrouridine-16, a mixture of U and pseudouridine in position 39, a mixture of U and 3-(3-amino-3-carboxypropyl)uridine at position 47, and isopentenyladenosine in place of 2-thiomethyl-N^6-(Δ^2-isopentenyl)adenosine-37.

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