JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 209-212

Vol. 118, No. 1
Printed in U.S.A.

New Chromatographic Form of Phenylalanine Transfer Ribonucleic Acid from *Escherichia coli* Growing Exponentially in a Low-Phosphate Medium

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Received for publication 19 November 1973

Reversed-phase chromatography has been used to detect the presence of a new form of phenylalanyl-transfer ribonucleic acid (Phe-tRNA) from *Escherichia coli* growing exponentially in media containing low but nonlimiting levels of inorganic phosphate. The amount of this extra Phe-tRNA form is greatest in slowly growing cells (0.8 generations/h), and becomes negligible in media supporting a rapid growth rate (2.14 generations/h).

A variety of physiological influences can induce alterations in the nature of cellular transfer ribonucleic acid (tRNA) in bacteria. Some of these conditions include treatment of cells with chloramphenicol (7, 11), deprivation of iron (13), and growth into stationary phase (5). The topic has been recently reviewed by Littauer and Inouye (6). Radiophosphate labeling of cells in medium containing low concentrations of inorganic phosphate has been used widely for studies on RNA metabolism and for determination of RNA nucleotide sequence. In this study (a portion of a dissertation submitted by M.B.M. to the Johns Hopkins University Graduate Board in partial fulfillment of a Ph.D. degree, 1973), we investigated possible effects of such growth conditions on phenylalanine (Phe)-tRNA in *Escherichia coli*. We found that low levels of inorganic phosphate in conjunction with growth rates of less than 1.4 generations per h can give rise to a second chromatographic peak of Phe-tRNA detectable by reversed-phase chromatography. The significance of this finding points to possible artifacts inherent in the methods presently employed in the preparation of ^{32}P-labeled tRNA for nucleotide sequence determination.

MATERIALS AND METHODS

The bacterial strain used in this study was *E. coli* THU requiring thymine, histidine, and uracil. Culture media are described in Table 1. L-[^{14}C]phenylalanine (255 mCi/mmol) was from Schwarz/Mann, and L-[^{3}H]phenylalanine (12.8 Ci/mmol) was from New England Nuclear Corp.

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Growth of cells. Growth was measured by the change in absorbancy of the culture at 650 nm with a Coleman Jr. spectrophotometer. Cultures of 170 ml were grown in 500-ml side-arm flasks. An optical density at 650 nm of 0.1 corresponded to 4×10^8 cells/ml. Viable cell count was made after appropriate dilution on brain heart infusion agar plates. Cells were harvested by centrifugation.

Preparation of tRNA. Cell paste, suspended in buffer [0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5; 0.05 M sodium ethylenediaminetetraacetic acid (Na_2-EDTA); 2% sodium dodecyl sulfate, 0.2% bentonite; and 0.02 M β-mercaptoethanol] and mixed with glass beads (10 μm diameter), was treated for 30 min in a Raytheon sonic oscillator (model Df 101). The extract was extracted once with phenol. The aqueous layer was made 2% in potassium acetate (pH 5) and precipitated with 2 volumes of 95% ethanol for 1 h at -20 C. The RNA was collected by centrifugation and dissolved in water.

Aminoacylation of RNA with radiolabeled phenylalanine was carried out as described by Waters and Novelli (12). The reaction (total volume 250 μliters) contained (per milliliter): Tris-hydrochloride buffer (pH 7.5), 100 μmol; adenosine triphosphate, 4 μmol; magnesium acetate, 40 μmol; KCl, 5 μmol; β-mercaptoethanol, 1 μmol; RNA, less than 2 mg; labeled phenylalanine, 5 μCi for ^{3}H, and 2.5 μCi for ^{14}C; and 150 μg of a crude aminoacyl tRNA synthetase from *E. coli* B (General Biochemical Corp.) prepared by the method of Meunch and Berg (8) up to the step of hydroxyapatite chromatography. Charged RNA was purified by diethylaminoethyl-cellulose chromatography by the method of Waters, as described by Yang and Novelli (14).

Reversed-phase column chromatography system five (RPC-5). Chromatography on the RPC-5 column was carried out by method B of Pearson et al. (9). The column was 0.9 by 25 cm and was operated at 250 to 350 lb/inch^2. RNA was eluted with a linear NaCl gradient in buffer containing 10 mM magnesium

TABLE 1. *Growth rate of E. coli in various media*

Composition of medium	Growth rate (generations/h)
1. BHI[a]-P_i[b] supplement	2.14
2. Tris[c]-glucose-P_i supplement	1.36
3. Tris-glycerol-P_i supplement	1.10
4. Tris-succinate-1 mM P_i	0.90
5. Tris-glycerol-1 mM P_i; no Casamino Acids	0.81

[a] BHI, Brain heart infusion (Difco). It contains 20 mM inorganic orthophosphate (P_i) as supplied commercially. BHI was dephosphated to a level of 0.2 mM (100-fold decrease) by making standard BHI 0.05 M in $MgCl_2$, bringing the pH to 9.0 with concentrated NH_4OH, heating the solution to boiling, and suction filtering in a Buchner funnel apparatus. The filtrate was neutralized with concentrated HCl to pH 7.4 and autoclaved before use. BHI (1 mM P_i) was prepared from "dephosphated" BHI by supplementing with potassium dihydrogen phosphate to the appropriate concentration. Phosphate levels in all media used were determined by the method of Fiske and Subbarow (3).

[b] P_i, Inorganic orthophosphate ion supplied as potassium dihydrogen phosphate. The extent of supplementation is indicated in figure legends.

[c] Tris, Tris-buffered minimal medium containing per liter: Tris base, 12 g; potassium chloride, 2 g; ammonium chloride, 2 g; magnesium chloride hexahydrate, 0.5 g; sodium sulfate, 20 mg; and sufficient concentrated HCl to bring the pH to 7.2. To this was added L-histidine, 20 μg/ml; thymidine, 10 μg/ml; uracil, 20 μg/ml; and 0.02% vitamin-free casein hydrolysate (General Biochemicals Corp.). Concentrations of carbon source were 0.5, 0.5, and 0.6% of glucose monohydrate, glycerol, and sodium succinate, respectively.

acetate (pH 5.0) and 1 mM EDTA. Radioactivity in the eluted column fractions was determined by mixing 1 ml from each fraction with 10 ml of scintillation fluor composed of 1 liter of toluene, 0.5 liter of Triton X-100, 8.25 g of 2,5-diphenyloxazole, and 0.25 g of 1,4-bis[2-(4 methyl-5-phenyloxazolyl)] benzene.

RESULTS

Phe-tRNA from *E. coli* normally migrates during chromatography as a single component. The material has been shown to have a unique primary nucleotide sequence (1). It is eluted from reversed-phase chromatography (RPC-5) at 0.80 M NaCl (Fig. 1). Treatment of *E. coli* with high levels (>50 μg/ml) of chloramphenicol induces two additional forms of Phe-tRNA (11). One was eluted from RPC-5 at 0.62 M NaCl (form I) and the other was eluted at 0.68 M NaCl (form II) (7). One of these inducible forms, namely, form II, is shown to arise also in exponentially growing cells, under commonly employed laboratory conditions. The conditions for the expression of this effect depend on moderately slow rate of growth and low but nonlimiting levels of inorganic phosphate. Chromatography of radiolabeled Phe-tRNA from cells growing exponentially in a glucose-supplemented, minimal salts medium containing a low level of phosphate revealed the presence of normal Phe-tRNA as well as a single additional component that was chromatographically indistinguishable from the form II generated in chloramphenicol-treated cells. This additional form of Phe-tRNA will be referred to in

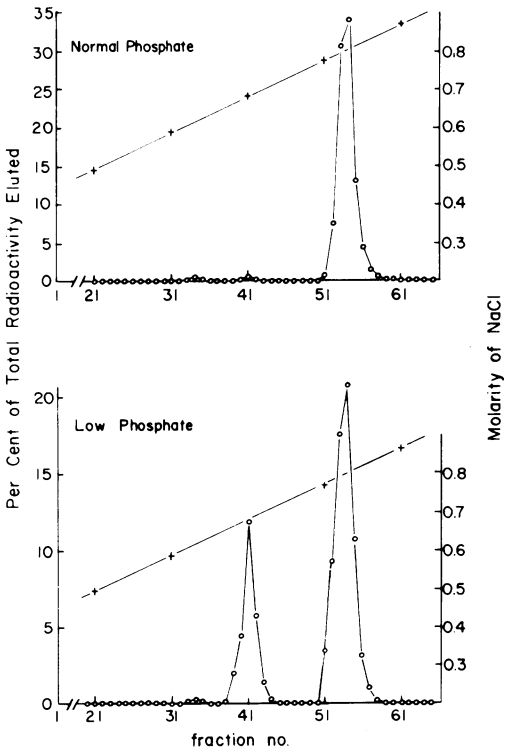

FIG. 1. *Chromatographic elution behavior of phenylalanyl-tRNA from E. coli grown in normal and low-phosphate medium. The RNA from 170-ml cultures (5 × 10⁸ cells/ml) of fully adapted, exponential-phase cells grown at 37 C was extracted, charged with either [¹⁴C]- or [³H]phenylalanine, and chromatographed on RPC-5 as described in Materials and Methods. Cells were grown in a glycerol-supplemented minimal medium (Table 1, entry 3) containing either 20 mM (upper panel) or 0.2 mM (lower panel) potassium dihydrogen phosphate. Cells were thoroughly adapted by repeated subculturing for seven generations prior to extraction of RNA. Cultures were not allowed to exceed 5 × 10⁸ cells/ml. For media containing 0.2 mM phosphate, this level of growth corresponded to a phosphate consumption of 50% as determined by radiophosphate uptake.*

this report as form II, although its absolute identity with the chloramphenicol-induced species remains to be demonstrated.

The amount of form II relative to normal Phe-tRNA was determined in a series of cultures grown in three types of media, each supporting growth at a different rate. Each of the three was tested at three different concentrations of inorganic phosphate, so that, in all, nine culture conditions were examined. Table 1 (entries 1, 2, and 3) lists the composition of these three media and the growth rate supported by each. The levels of inorganic phosphate were 20, 1, and 0.2 mM. The concentration of phosphate was saturating in all cases and did not influence culture doubling time. Figure 2 gives the level of form II in each of the cultures, expressed as a percentage of total phenylalanine acceptor activity. The level is seen to increase as the phosphate level is decreased. The extent of the effect diminishes as the growth rate increases, and disappears altogether in the medium supporting the fastest doubling time. If we assume that form II can be aminoacylated with the same efficiency as normal tRNA, then these values are representative of the relative molar quantities of the two forms present.

Figure 3 shows the change in the level of form II with growth rate. The five different growth rates were obtained with the five media listed in Table 1. A single phosphate concentration of 1 mM was used. The results show that the level of form II decreases with increasing growth rates.

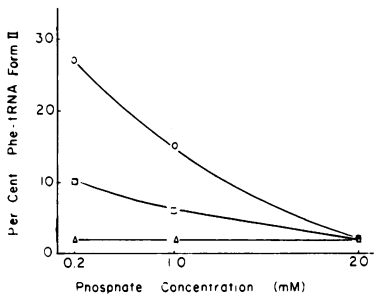

FIG. 2. *Dependence of the level of Phe-tRNA (form II) in exponentially growing cultures of E. coli upon growth rate and phosphate concentration. The RNA from various cultures of E. coli was analyzed for Phe-tRNA content as described in Fig. 1. The radioactivity found with form II was determined and expressed as a percentage of the total radioactivity eluted from the column. The results are given for three different media at three different phosphate concentrations. The composition of the media is given in Table 1. Symbols: △, medium 1, 2.14 generations per h; □, medium 2, 1.36 generations per h; ○, medium 3, 1.10 generations per h.*

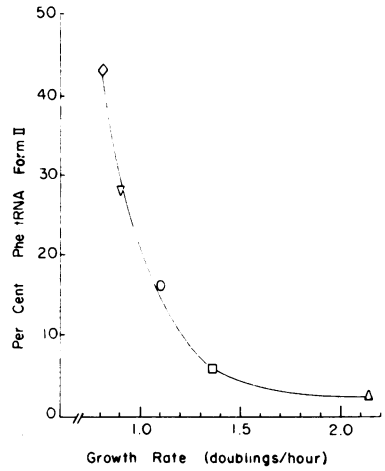

FIG. 3. *Dependence of the level of Phe-tRNA (form II) on growth rate. The RNA from various cultures were processed and analyzed as described in Fig. 1. The media are described in Table 1 and the phosphate concentration was 1 mM. Symbols: △, medium 1; □, medium 2; ○, medium 3; ▽, medium 4; ◇, medium 5.*

DISCUSSION

In this study, it is shown that the appearance of a new chromatographic form of Phe-tRNA (form II) occurs in exponentially growing *E. coli* cells given the combined conditions of slow growth and low phosphate concentration. Stated another way, the formation of form II is suppressed by either rapid cell growth or high levels of phosphate. Results not shown suggest that form II arises de novo rather than by conversion from the normal form, but the exact mechanism of this phenomenon is presently unknown.

Form II may be abnormal with respect to the extent of modification of the 2-methylthio-N^6-(Δ^2-isopentenyl) adenosine (2ms6iA) adjacent to the 3′ side of the anticodon. The biosynthesis of this minor nucleoside component in tRNA is known to be impaired by a variety of factors. Gefter and Russell (4) showed that the 2ms6iA of *E. coli* suppressor tyrosine tRNA was undermodified in cells infected with the transducing phage ϕ80 psu$_{III}$. Chromatographically separable forms of the tRNA were shown to be identical save for the degree of modification of the 2ms6iA residue. One abnormal form contained a partially modified N^6-(Δ^2-isopentenyl) adenosine (6iA); the other contained a totally unmodified adenosine.

Bartz et al. (2) found that bulk tRNA from *E. coli* in late log phase had elevated levels of 6iA in place of the normal 2ms6iA present in exponential-phase cells.

Finally, Rosenberg and Gefter (10) demonstrated that tyrosine tRNA from *E. coli* grown in iron-poor medium was again deficient in 2-methylthiolation. They showed further that, in iron-poor medium, all of the *E. coli* tRNA species containing 2ms6iA, including Phe-tRNA, gave chromatographic profiles qualitatively resembling that of tyrosyl-tRNA. The profiles showed the appearance of a new tRNA peak eluted well ahead of the normal form. By comparison, it is conceivable that the new form of Phe-tRNA reported here is a 2-methythio-deficient variety. This view is strengthened by the fact that the iron-supplemented controls in Rosenberg and Gefter's study (10) revealed considerable amounts of the early-eluting abnormal tRNA form for several species of tRNA including Phe-tRNA. The medium employed in that study was a modified M-9 medium containing 0.162 mM phosphate, and, as shown in this study (Fig. 2), would be expected to give rise to the observed early eluting material analogous to form II.

Should the dependence of tRNA modification upon physiological factors prove to be a general phenomenon, then certain commonly used conditions for cell culturing may not be suitable for experiments in which the production of only native tRNA molecules is desired. Moreover, depending on which minor nucleoside is affected, a defect in modification may go undetected by the methods of separation currently available.

In view of the fact that tRNA species produced under conditions of low phosphate supply may be undermodified, caution should be exercised when interpreting data obtained from nucleotide sequence analyses of tRNA labeled with [^{32}P]phosphate in low-phosphate medium. In this regard, we find that labeling carried out in "dephosphated" brain heart infusion affords good incorporation of label and prevents the formation of extra tRNA forms (Fig. 2). The exact nature of inducible, chromatographically novel forms of tRNA is being examined in light of these findings.

ACKNOWLEDGMENTS

This work is supported by grants from the American Cancer Society (P-661) and the National Science Foundation (GB-24662). Michael B. Mann is the recipient of a Public Health Service predoctoral fellowship (F01GM45709) from the National Institute of General Medical Sciences.

We wish to acknowledge also the excellent laboratory assistance provided by Patricia Kropkowski.

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