Modolell, J., and Davis, B. D. (1968), *Proc. Nat. Acad. Sci. U. S. 61,* 1279.
Monro, R. E., and Vazquez, D. (1967), *J. Mol. Biol. 28,* 161.
Nomura, M., and Engbaek, R. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1526.
Oleinick, N. L. (1974), *in* Antibiotics III—Mechanism of Action of Antimicrobial and Antitumor Agents, Corcoran, J. W., and F. E. Hahn, Ed., Springer-Verlag (in press).
Oleinick, N. L., and Corcoran, J. W. (1969), *J. Biol. Chem. 244,* 727.
Oleinick, N. L., and Corcoran, J. W. (1970), *Progr. Antimicrob. Anticancer Chemother., Proc. Int. Congr. Chemother. 6th, 1969,* 202.
Otaka, E., Teraoka, H., Tamaki, M., Tanaka, K., and Osawa, S. (1970), *J. Mol. Biol. 48,* 499.
Pestka, S. (1971), *Annu. Rev. Microbiol. 25,* 487.
Pestka, S. (1972a), *Proc. Nat. Acad. Sci. U. S. 69,* 624.
Pestka, S. (1972b), *J. Biol. Chem. 247,* 4669.
Pestka, S. (1974), *Antimicrob. Agents Chemother. 5,* 255.
Tai, P.-C., Wallace, B. J., and Davis, B. D. (1973a), *Biochemistry 12,* 616.
Tai, P.-C., Wallace, B. J., Herzog, E. L., and Davis, B. D. (1973b), *Biochemistry 12,* 609.
Tanaka, N., Kinoshita, T., and Masakawa, H. (1969), *J. Biochem. (Tokyo) 65,* 459.
Tanaka, S., Otaka, T., and Kaji, A. (1973), *Biochim. Biophys. Acta 331,* 128.
Tanaka, K., and Teraoka, H. (1966), *Biochim. Biophys. Acta 114,* 204.
Tanaka, K., and Teraoka, H. (1968), *J. Biochem. (Tokyo) 64,* 635.
Tanaka, K., Teraoka, H., and Tamaki, M. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13,* 65.
Taubman, S. B., Jones, N. R., Young, F. E., and Corcoran, J. W. (1966), *Biochim. Biophys. Acta 123,* 438.
Teraoka, H. (1970), *J. Mol. Biol. 48,* 511.
Vogel, Z., Vogel, T., and Elson, D. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 15,* 249.
Wallace, B. J., and Davis, B. D. (1973), *J. Mol. Biol. 75,* 377.
Wallace, B. J., Tai, P.-C., and Davis, B. D. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 1634.
Wallace, B. J., Tai, P.-C., Herzog, E. L., and Davis, B. D. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1234.
Wilhelm, J. M., and Corcoran, J. W. (1967), *Biochemistry 6,* 2578.

Histidyl-tRNAs and Histidyl-tRNA Synthetases in Wild Type and Cytoplasmic Petite Mutants of *Saccharomyces cerevisiae*†

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ABSTRACT: *Saccharomyces cerevisiae* has two distinguishable species of $tRNA^{His}$. They can be separated from each other by sequential chromatography on DEAE-Sephadex and a modified reversed phase column. One of these tRNAs seems to be coded by a mitochondrial gene because it is absent in a mutant lacking mtDNA and it hybridizes specifically to mtDNA. This mitochondrial $tRNA^{His}$ can be charged by the histidyl-tRNA synthetase isolated from *Salmonella typhimurium,* whereas the cytoplasmic $tRNA^{His}$ cannot be acylated under the same conditions. There are also two different histidyl-tRNA synthetases in yeast. One of these accounts for 10% of the total histidyl-tRNA synthetase activity in the cell and cosediments with mitochondria in sucrose gradients; the rest of the activity is in the soluble fraction. Although both enzymes behave identically on a DEAE-cellulose column and show similar molecular weights (100,000), they can be distinguished on the basis of their affinities for tRNA. The mitochondrial synthetase fails to charge bulk *Escherichia coli* tRNA, whereas the cytoplasmic enzyme charges it efficiently. The cytoplasmic enzyme has a 10–20-fold greater affinity for both cytoplasmic and mitochondrial $tRNA^{His}$. The mitochondrial synthetase seems to be under the control of a nuclear gene, because it is found in mutants lacking mtDNA.

Histidyl-tRNA synthetase and $tRNA^{His}$ have been implicated in the repression control of the histidine pathway in *Salmonella typhimurium* (Brenner and Ames, 1971; Goldberger, 1974). For this reason it seemed worthwhile to investigate the properties of the activating enzyme and $tRNA^{His}$ in a eucaryote, *Saccharomyces cerevisiae,* as a preliminary step toward elucidation of regulatory processes in this organism.

Yeast cells contain two distinct systems which can synthesize protein, one in the cytoplasm and one in the mitochondrion. Mitochondria of several organisms have been shown to contain their own activating enzymes (Barnett *et al.,* 1967; Buck and Nass, 1969; Kislev and Eisenstadt, 1972) and tRNA species (Barnett *et al.,* 1967; Buck and Nass, 1969; Casey *et al.* 1972). Hybridization experiments

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with wild type and ρ^- mutants have shown that many tRNAs are coded for by mtDNA. The location of genes for the mitochondrial synthetases is less clear. Studies on the *leu5* cistron in *Neurospora* indicate that this gene is involved in specifying both the cytoplasmic and mitochondrial leucyl-tRNA synthetases, despite the physical and functional differences between them (Weeks and Gross, 1971). In this report we present evidence that yeast mitochondria possess their own components of the histidine activating system (aminoacyl-tRNA synthetase and $tRNA^{His}$) and show that these components differ from the ones present in the cytoplasmic fraction. Mitochondrial $tRNA^{His}$ is coded for by the mitochondrial genome, whereas the mitochondrial and cytoplasmic activating enzymes as well as cytoplasmic $tRNA^{His}$ are not.

Experimental Section

Yeast Strains. Haploid strains of *Saccharomyces cerevisiae* were used throughout this study. Our normal respiratory competent (ρ^+) yeast strain S288C was obtained from Dr. Robert K. Mortimer. An isogenic petite mutant (ρ^-), *EB2*, was obtained from this strain by treatment with ethidium bromide for 48 hr according to the procedure of Goldring *et al.* (1970). Strain *A664a/18A* and its ethidium bromide induced derivative, *III-1-7* (containing no detectable mtDNA, Goldring *et al.*, 1970), were obtained from Dr. Julius Marmur, Albert Einstein College of Medicine, New York, N.Y.

Growth Medium. A slightly modified complete medium was used for growing yeast strains (Sherman *et al.*, 1972). The medium contained 2% peptone, 1% yeast extract, and 0.8% glucose instead of the usual 2%. The glucose concentration was lower than usual in order to avoid glucose repression of mitochondria (Criddle and Schatz, 1969).

Chemicals. [^{14}C]-L-Histidine (specific activity 250–280 Ci/mol) and [^{3}H]-L-histidine (54 Ci/mmol) were purchased from New England Nuclear Corp. These radioactive amino acids were purified on a Dowex 50-X8 column according to the procedure of Moore and Stein (1951). Glusulase (snail juice) was obtained from Endo Laboratories, Inc., Garden City, N.Y. Bulk Brewer's yeast tRNA and *Escherichia coli* K12 tRNA were obtained from Schwartz Bioresearch Inc., Orangeburg, N.Y.

Preparation and Partial Purification of Cytoplasmic and Mitochondrial $tRNA^{His}$. Bulk yeast tRNA was prepared from approximately 1 kg of cells from either strain A664a/18A or III-1-7 according to the method of Silbert *et al.* (1966). Approximately 1 g of these tRNAs or 1 g of commercial Brewer's yeast tRNA was fractionated on a DEAE-Sephadex A50 column and then a reverse phase chromatography column (RPC) according to the procedure of Takeishi *et al.* (1967). The procedure was modified in that the RPC4 system of Kelmers *et al.* (1971) was substituted for the RPC1 system used by Takeishi *et al.* (1967). In addition, histidyl-tRNA synthetase from *Salmonella typhimurium* rather than from *E. coli* was used to distinguish between different fractions of $tRNA^{His}$.

Isolation of DNA. nDNA was isolated from spheroplasts of strains A664a/18A or III-1-7 by the method of Marmur (1961). It was further purified by either poly(lysine) kieselguhr chromatography (Finkelstein *et al.*, 1972) or by isopycnic centrifugation on a CsCl gradient (Flamm *et al.*, 1966). mtDNA was obtained from spheroplasts of A664a/18A and further purified by poly(lysine) kieselguhr chromatography.

Both n- and mtDNA preparations were analyzed in the Model E ultracentrifuge and revealed bands with buoyant densities of 1.699 and 1.683 g/cm^3, respectively.

Hybridization. The hybridization system of Halbreich and Rabinowitz (1971) was used. Retention of radiolabeled DNA to filters was monitored in preliminary experiments. Under hybridization conditions, more than 90% of the input DNA was retained on the filter at the end of the procedure.

Determination of Histidyl-tRNA Synthetase Activity. Synthetase activity was measured by following the aminoacylation of bulk tRNA with [^{14}C]histidine. Unless otherwise stated, each assay tube contained in a total volume of 0.25 ml: 0.08 M potassium cacodylate (pH 7.0), 4 mM ATP, 8 mM $MgCl_2$, 2 μM [^{14}C]histidine, 100 μg of gelatin, 5.7 mM 2-mercaptoethanol, and 250 μg of bulk yeast tRNA. The reaction was initiated by the addition of enzyme (usually 2–5 μg of protein or less) and terminated at the desired time intervals by the addition of 3 ml of cold 5% trichloroacetic acid. The reaction tubes were cooled in ice and the precipitate was collected by filtration on glass fiber filters (Gelman Instrument Company, Type A). Filters were washed three times with 3 ml of 5% trichloroacetic acid, dried, and monitored for radioactivity in a toluene cocktail of 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 4 g of 2,5-diphenyloxazole per liter with a Nuclear Chicago Mark I liquid scintillation counter at an efficiency of 80% or better. The counts were corrected by subtracting a zero-time blank. Unless otherwise specified the reaction was carried out at 23°.

One unit of histidyl-tRNA synthetase activity is defined as that amount of enzyme esterifying 1 pmol of histidine in 1 min under the above conditions. Specific activity is expressed in units per milligram of protein.

Preparation of Mitochondria. GLASS BEADS TECHNIQUE. The procedure of Criddle and Schatz (1969) was employed except that the mannitol concentration in the buffer containing mannitol–20 mM Tris-SO_4 (pH 7.4)–1 mM EDTA (MTE[1] buffer) was increased to 0.6 M. Crude mitochondria obtained in this way were purified on a preparative scale by isopycnic centrifugation in a 20–70% sucrose gradient containing 20 mM Tris-SO_4 (pH 7.4) and 1 mM EDTA.

NADH oxidase activity was used as a mitochondrial marker (Dr. G. Schatz, personal communication) and was measured spectrophotometrically by adding aliquots of each fraction to a cuvet containing 0.1 mg of NADH in 1 ml of 1 M Tris-HCl (pH 8.0). The reaction was carried out at room temperature and the activity expressed as a decrease in absorbancy at 340 nm per min per 10-μl aliquot. Fractions containing most of the NADH oxidase activity were pooled and diluted 1:1 with MTE, and the mitochondria were pelleted by centrifugation. The pellet was homogenized gently by hand in an all glass homogenizer and the volume adjusted as needed with MTE. This fraction was called the purified mitochondrial preparation.

SPHEROPLAST TECHNIQUE. This technique was used to prepare defective mitochondria from petite mutants and also mitochondria from wild type when comparisons were made between different strains. The procedure of Kovac *et al.* (1972) was adopted. This method involves the use of Glusulase (snail juice) to convert yeast cells into sphero-

[1] Abbreviations used are: SSC, 0.15 M sodium chloride–0.015 M trisodium citrate; MTE buffer, mannitol–20 mM Tris-SO_4 (pH 7.4)–1 mM EDTA.

plasts. The spheroplasts are then lysed and the mitochondria isolated and purified. Modifications in the procedure included the use of MTE buffer instead of a mannitol-EDTA-bovine serum albumin solution for lysing the spheroplasts and omitting a 12,000*g* centrifugation. Instead, a centrifugation for 20 min at 40,000*g* in the SW 25.2 rotor of a Spinco centrifuge was used to pellet the mitochondria. The mitochondrial pellet was suspended in MTE, centrifuged again, resuspended in MTE, homogenized, and purified by isopycnic centrifugation as described above. Treatment of gradient fractions leading to the purified mitochondrial preparation was identical with that described for the glass beads procedure.

Preparation of Cytoplasmic Histidyl-tRNA Synthetase. CRUDE PREPARATION. Yeast strains were grown at 30° in a New Brunswick rotary shaker until late-logarithmic phase ($5-6 \times 10^7$ cells/ml). The cells were harvested by centrifugation at 4000*g* for 5 min and treated as described in Preparation of Mitochondria. A general preparative scheme is presented in Figure 1.

The homogenate, from which unbroken cells and debris had been removed by centrifugation at 60,000*g*, was then centrifuged for 20 min at 120,000*g* in a Spinco ultracentrifuge in the SW 25.1 or SW 25.2 rotor. The supernatant solution was then recentrifuged for 1 hr at 103,000*g* in a Type 65 fixed angle rotor in a Spinco ultracentrifuge. The pellet was discarded together with a loose layer just above it, and the clear yellow supernatant solution was collected. This solution was called the soluble cytoplasmic fraction.

AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate was added slowly with stirring to the soluble cytoplasmic fraction. The protein precipitate resulting from this addition was removed by centrifugation at 27,000*g* for 15 min, and more solid ammonium sulfate was added to the supernatant in order to bring it to 60% saturation. The protein precipitating at 45–60% ammonium sulfate was collected by centrifugation as above and suspended in MTE. This solution was then desalted on a Sephadex G-25 (Pharmacia, Sweden) column and equilibrated with 1 mM potassium phosphate buffer (pH 7.4). The eluate was further purified by DEAE-cellulose chromatography.

DEAE-CELLULOSE CHROMATOGRAPHY. Chromatography on DEAE-cellulose (standard grade, Schleicher and Schuel, Inc., Keene, N.H.) was performed essentially in the manner described by Von Tigerstrom and Tener (1967) except for the following modifications. The ammonium carbonate wash was omitted, and the column was equilibrated with 0.01 M potassium phosphate buffer (pH 7.4) containing 35% (v/v) propylene glycol. A linear gradient of 0.04–0.30 M potassium phosphate (pH 7.4) containing 35% (v/v) propylene glycol was employed for the elution of histidyl-tRNA synthetase activity. The most active fractions were pooled and used in all of our experiments as the source of cytoplasmic enzyme. This preparation was stored at −20° for over 15 months without significant loss of activity. Freezing was prevented by the presence of 35% propylene glycol.

Preparation of Mitochondrial Histidyl-tRNA Synthetase. Purified mitochondria obtained from spheroplasts were treated in the following way: 15-ml suspensions of the purified mitochondrial preparation were disrupted at the maximum output using a Branson Sonifier (Model S125). Four 15-sec treatments were given allowing sufficient time between treatments for cooling of the samples. Material which had been disrupted by sonification was designated

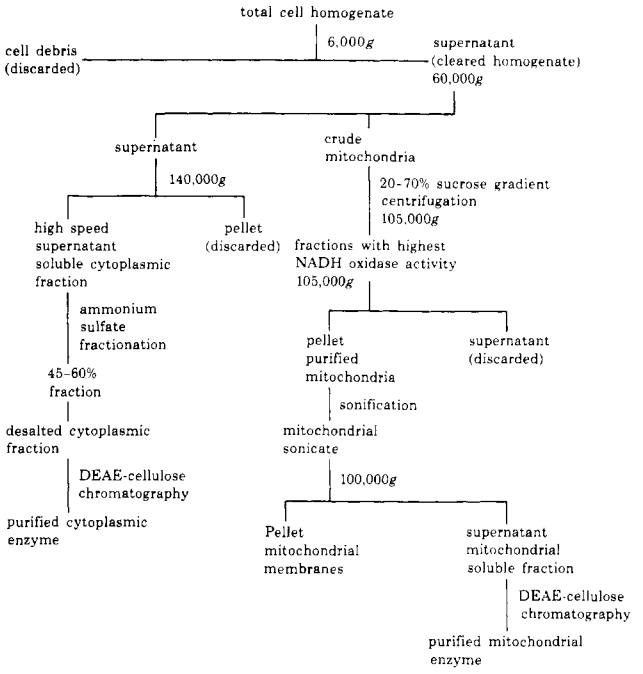

FIGURE 1: Purification scheme for cytoplasmic and mitochondrial histidyl-tRNA synthetases.

"mitochondrial sonicate." This fraction was then centrifuged in the SW 65 rotor of a Spinco ultracentrifuge for 2 hr at 75,000*g*. The resulting clear supernatant solution contained most of the synthetase activity. The pellet, containing mitochondrial membranes and very little activity, was discarded.

The soluble mitochondrial fraction was chromatographed on a DEAE-cellulose column under conditions identical with those employed for the cytoplasmic enzyme except that a 0.04–0.5 M potassium phosphate gradient was used. The fractions containing most of the activity were pooled, designated "mitochondrial enzyme," and stored at −20°.

Preparation and Partial Purification of Histidyl-tRNA Synthetase from Salmonella typhimurium. A purification technique suggested by Dr. B. N. Ames (personal commu-

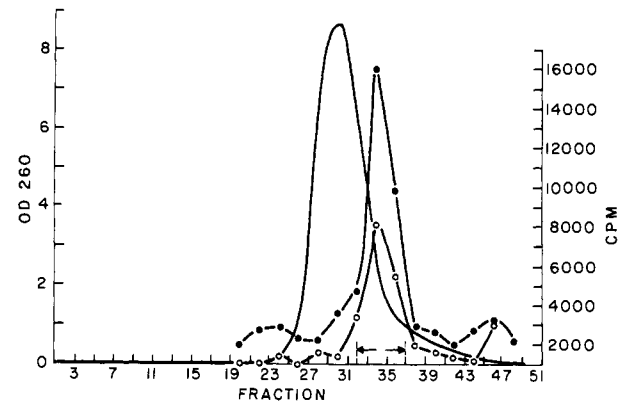

FIGURE 2: DEAE-Sephadex column chromatography of bulk tRNA isolated from ρ^+ yeast. About 750 mg of bulk tRNA from A664a/18A was applied in starting buffer to a column (3 × 105 cm) of DEAE-Sephadex. Elution was performed with a 4-l. linear gradient. One reservoir contained 2 l. of 0.02 M Tris-HCl (pH 7.5), 0.008 M $MgCl_2$, and 0.375 M NaCl. The other contained 2 l. of 0.02 M Tris-HCl (pH 7.5), 0.016 M $MgCl_2$, and 0.525 M NaCl. The flow rate was 60 ml/hr, and 10-ml fractions were collected and monitored for OD_{260} (—). Samples of 200 μl were assayed with partially purified yeast cytoplasmic histidyl-tRNA synthetase (○) and partially purified enzyme from *S. typhimurium* (●). Arrows indicate fractions pooled for further analysis.

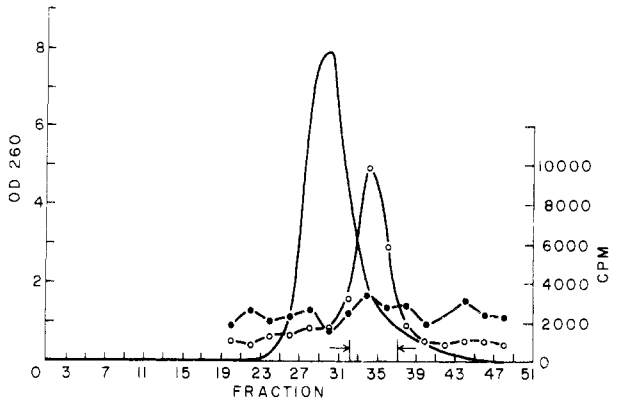

FIGURE 3: DEAE-Sephadex column chromatography of bulk tRNA isolated from ρ^- yeast. About 750 mg of bulk tRNA from III-1-7 was processed as described in Figure 2.

nication) was used. In this procedure, 12 g wet weight of *S. typhimurium* strain LT2 was suspended in 0.03 M Tris-HCl (pH 7.5) and disrupted with a Branson Sonifier, Model S125, by treatment for about 2 min at setting 7. The resulting suspension was centrifuged for 15 min at 27,000*g* and then for 90 min at 100,000*g* in the Spinco ultracentrifuge. The clear supernatant solution was applied to a 32-ml (bed volume) phosphocellulose (Whatman) column equilibrated with 0.03 M Tris-HCl (pH 7.5). The column was washed with the same buffer until absorption at 280 nm dropped below 0.05. At this point the buffer containing 0.1 M KCl was applied and elution continued until absorption at 280 nm was about 0.05. The final buffer, containing 0.25 M KCl, eluted the histidyl-tRNA synthetase as a very sharp peak.

Other Procedures. Molecular weight determinations were carried out according to the method of Martin and Ames (1961) with the modification that a 10–30% sucrose gradient was employed. Beef liver catalase (Worthington Biochemical Corp., Freehold, N.J.) was used as a standard and assayed spectrophotometrically as described in the Worthington Enzyme Manual (1972). Protein was measured by the method of Lowry *et al.* (1951) using insulin (Mann) as a standard, or in more purified preparations by the absorption at 280 nm.

Results

Cytoplasmic and Mitochondrial tRNA^{His}. Bulk tRNA from both A664a/18A and its ρ^- derivative III-1-7 was examined for its histidine-accepting activity. No significant differences between the tRNAs were apparent when either cytoplasmic or mitochondrial enzyme from yeast was utilized for the assay. However, enzyme preparations from *Salmonella typhimurium* acylated tRNA from the ρ^+ strain tenfold more than it acylated tRNA from the ρ^- strain.

In order to determine the basis for the differential charging of tRNA from the ρ^+ strain, bulk tRNA from both ρ^+ and ρ^- strains was chromatographed on a DEAE-Sephadex column. Fractions were assayed with yeast cytoplasmic enzyme or the *Salmonella* enzyme. The tRNA^{His} of A664a/18A was somewhat displaced from the main peak of tRNA, and the components charged by the yeast and the *Salmonella* enzyme chromatographed together (Figure 2). The tRNA^{His} of III-1-7 eluted in the same portion of the chromatogram as that of A664a/18A, but there was no evidence in the mutant tRNA of a component that could be charged by the *Salmonella* enzyme (Figure 3).

Fractions from the DEAE step containing tRNA^{His} were pooled, concentrated, and chromatographed on a reverse phase column. Chromatography of tRNA from strain A664a/18A revealed two peaks for tRNA^{His}, one charged by yeast cytoplasmic enzyme and the other charged by *Salmonella* enzyme (Figure 4). It appears, however, that under the appropriate conditions the yeast enzyme can actually charge both species of tRNA^{His}. The inability to charge one species is apparently a result of the high salt concentration employed in the elution because both species are charged by the yeast enzyme when the column fractions are desalted. No other peaks of tRNA^{His} appeared when individual column fractions were desalted prior to their assay.

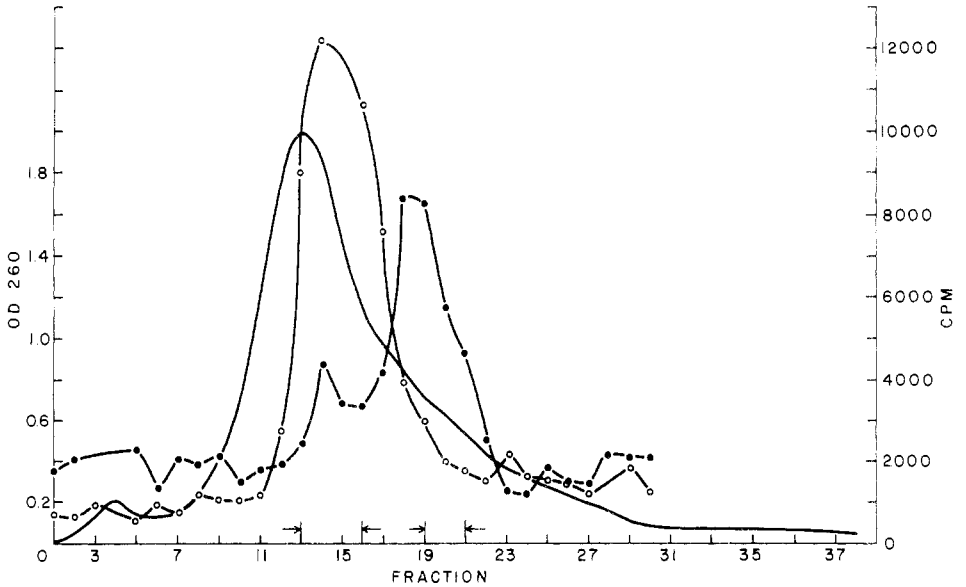

FIGURE 4: Reverse phase column chromatography of histidyl-tRNA-rich fraction from ρ^+ yeast. The fractions containing histidyl-RNA were pooled, as indicated in Figure 2, precipitated with 2 volumes of ethanol, and resuspended in starting buffer. A sample containing about 3 mg of tRNA was applied to a column containing a 10-ml bed volume of Chromosorb W. Elution was performed with 140 ml of a linear gradient from 0.02 M Tris-HCl (pH 7.5)–0.45 M NaCl to 0.02 M Tris-HCl (pH 7.5)–0.006 M $MgCl_2$–0.65 M NaCl. The flow rate was 24 ml/hr, and 3-ml fractions were collected and monitored for OD_{260} (—). A 200-μl sample of each fraction was assayed with partially purified yeast cytoplasmic histidyl-tRNA synthetase (○) and partially purified enzyme from *S. typhimurium* (●)

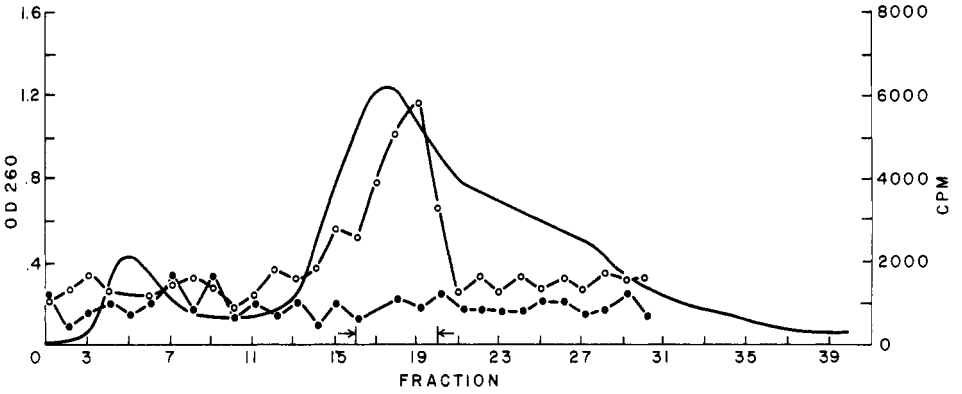

FIGURE 5: Reverse phase column chromatography of histidyl-tRNA-rich fraction from ρ^- yeast. The fractions containing histidyl-tRNA from the DEAE-Sephadex chromatography were pooled, as indicated in Figure 3, and processed as described in the legend to Figure 4.

Chromatography of tRNA from strain III-1-7 revealed only the $tRNA^{His}$ charged by the yeast enzyme (Figure 5).

In order to distinguish these tRNAs in more detail, hybridization experiments were performed with both n- and mtDNA. Each species of $tRNA^{His}$ was resolved on a preparative RPC4 column (Figure 6), acylated with [^{3}H]histidine, and then hybridized to DNA. The $tRNA^{His}$ which is charged by *Salmonella* enzyme and which is absent from strain III-1-7 hybridized specifically to mtDNA (Figures 7 and 8) and thus is called mitochondrial $tRNA^{His}$. The other species, apparently cytoplasmic $tRNA^{His}$, hybridized specifically to nDNA (Figures 7 and 8).

Identification of Mitochondrial Histidyl-tRNA Synthetase. Preliminary experiments showed that mitochondria from wild type yeast cells contain histidyl-tRNA synthetase activity. Thus, it was of particular interest to compare mitochondria from wild type ρ^+ and the isogenic ρ^- strains for the presence of the enzyme. Small samples of crude mitochondrial preparations from strains S288C, EB2, A664a/18A, and III-1-7 were applied to separate 10-ml sucrose gradients and centrifuged for 2 hr at 25,000 rpm in the SW 41 rotor of the Spinco ultracentrifuge. After centrifugation, the gradients were photographed and then fractionated into 0.38-ml fractions. Twenty-nine fractions were collected from each gradient and assayed for NADH oxidase and for synthetase activities with the expectation that if the mitochondria contain histidyl-tRNA activating enzyme, the two activities should coincide on the gradient. The results are presented in Figure 9 and show in the wild type (S288C) preparation a sharp peak of NADH oxidase activity cosedimenting with a peak of the synthetase activity. Another synthetase peak appears near the top of the gradient and probably results from either mitochondrial breakage or cytoplasmic contamination. By contrast, the petite (EB2) preparation shows very little or no NADH oxidase activity. There is, however, a definite broad peak of synthetase associated with the band of defective mitochondria. The peak at the top of the gradient is essentially unchanged in comparison to S288C. Examination of Figure 10 (photograph) reveals that the S288C preparation yields one relatively homogeneous major band about two-thirds down the length of the gradient and one small band of lipoprotein on top. The preparation from EB2 does not give clear-cut bands in the sucrose gradient. The major band can be seen as two zones. There is considerable smearing throughout the length of the gradient, and an additional band appears near the top. This diffuse pattern probably results from the fragility of defective mitochondrial particles in EB2. In another experiment (not shown), it was found that EB2 mitochondria contain only about 1% of the total synthetase activity of the cell as compared to 10% in mitochondria from the wild type.

Unexpected results were obtained when mitochondria from A664a/18A and III-1-7 strains were extracted and

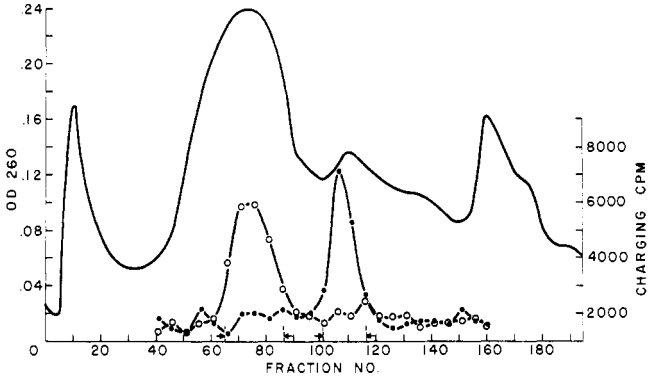

FIGURE 6: Preparative reverse phase chromatography of cytoplasmic and mitochondrial $tRNA^{His}$. About 8 mg of a histidine tRNA-rich fraction was applied to a column (1.6 × 71.5 cm) of Chromosorb W. Elution was performed with a 4-l. linear gradient from 0.02 M Tris-HCl (pH 7.5)–0.45 M NaCl to 0.02 Tris-HCl (pH 7.5)–0.006 M $MgCl_2$–0.65 M NaCl. The flow rate was 60 ml/hr, and 10-ml fractions were collected and monitored for OD_{260} (—). 200-μl samples of each fraction were assayed with partially purified yeast cytoplasmic histidyl-tRNA synthetase (○) and partially purified enzyme from *S. typhimurium* (●). Arrows indicate fractions pooled for further analysis.

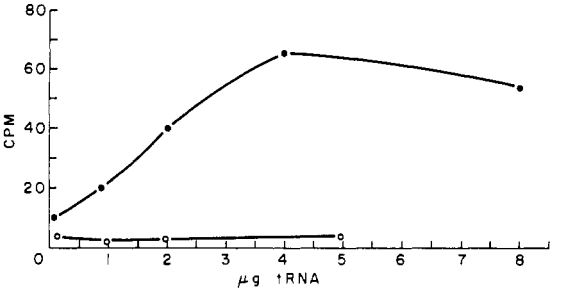

FIGURE 7: Hybridization of cytoplasmic (●) or mitochondrial (○) $tRNA^{His}$ to nDNA. nDNA isolated from the ρ^- strain was alkaline denatured and immobilized on nitrocellulose filters. Discs, each containing 80 μg of DNA, were punched out from the filter, and incubated for 2 hr in 300 μl of 33% formamide in 2 × SSC with varying concentrations of [^{3}H]histidine tRNA. Cytoplasmic $tRNA^{His}$, representing fractions 65–86 in Figure 6, was fully acylated with [^{3}H]histidine by yeast cytoplasmic enzyme, treated with phenol, and purified from small molecules by passage through a Sephadex G-100 column. Mitochondrial $tRNA^{His}$, representing fractions 101–116 in Figure 6, was fully acylated with [^{3}H]histidine by *S. typhimurium* enzyme and purified identically to cytoplasmic $tRNA^{His}$.

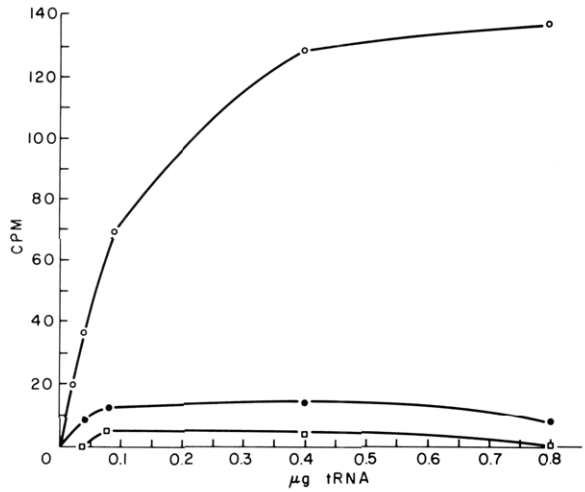

FIGURE 8: Hybridization of cytoplasmic (●) or mitochondrial (○) $tRNA^{His}$ to mtDNA. Filters without DNA (□) were incubated with mitochondrial $tRNA^{His}$ as a control. mtDNA was immobilized on nitrocellulose filters. Discs with 4 μg of mtDNA were incubated as in Figure 7 with varying concentrations of cytoplasmic (●) or mitochondrial (○) [^{3}H]histidine $tRNA^{His}$.

sedimented in a parallel experiment. Mitochondria in both the normal strain and its ρ^- derivative gave heterogeneous density patterns. The wild type preparation from A664a/18A resembles EB2 rather than S288C. The main mitochondrial band is fragmented in both these strains. The petite member of the pair, strain III-1-7, shows pronounced smearing, a very wide main band, and two (as compared to one in EB2) minor bands near the top. Two important conclusions can be drawn from this experiment. First of all, the presence of the synthetase in defective mitochondrial particles of EB2 and III-1-7 indicates that this enzyme is not coded for by the mitochondrial genome. Secondly, only isogenic strains should be used if comparisons of the kind described are to be meaningful. The heterogeneity of mitochondria from A664a/18A indicates that there might exist differences in the distribution of activities in the gradient. However, when the gradient was fractionated and the fractions were assayed, the pattern of activities was not different from that of S288C (Figure 9). The resolution seen in the photographs was probably lost during fractionation.

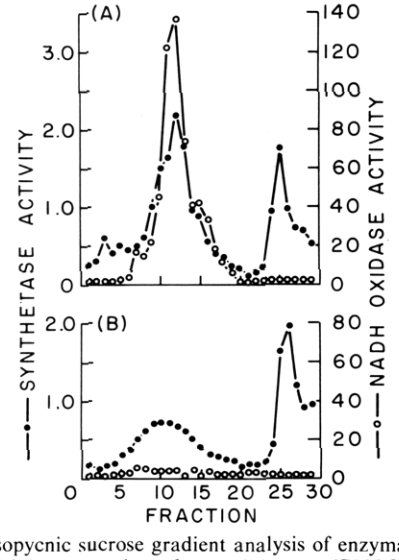

FIGURE 9: Isopycnic sucrose gradient analysis of enzymatic activities in mitochondrial preparations from wild type (S288C) and petite (EB2) strains. Sucrose gradients and mitochondrial preparations were as described in Figure 10. Histidyl-tRNA synthetase (●) and NADH oxidase (○) activities were measured as described in Materials and Methods. Panel on top, S288C; panel on bottom, EB2.

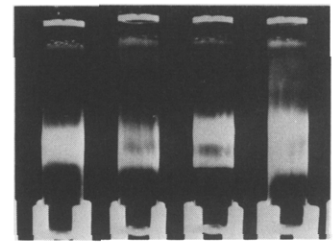

FIGURE 10: Isopycnic sucrose gradient banding of mitochondria obtained from normal and petite strains of yeast. Crude mitochondrial preparations from different yeast strains were centrifuged in 20-70% sucrose. 1-ml samples were applied to 10-ml gradients and centrifuged as described in the text. From the left to the right are shown: S288C (5.2 mg of mitochondrial protein), EB2 (3.9 mg), A664a/18A (4.0 mg), and III-1-7 (5.4 mg).

General Properties of Cytoplasmic and Mitochondrial Histidine Activating Enzymes. No differences have been detected in the chromatographic behavior of the synthetases on DEAE-cellulose columns. Both enzymes eluted at the same ionic strength (0.08–0.15 potassium phosphate). Molecular weight estimates (data not shown) gave a value of about 100,000 for both cytoplasmic and mitochondrial synthetases. Michaelis constants (K_m) for histidine and ATP are also very similar for both partially purified enzymes, regardless of the strain used (Table I). Both synthetases are inactivated rapidly at 37° and are protected from this inactivation by histidine and to a lesser extent, bulk tRNA (data not shown).

Charging specificities have been utilized in determining subcellular localization of a number of synthetases (Barnett *et al.*, 1967; Buck and Nass, 1969; Gross *et al.*, 1968; Reger *et al.*, 1970). In testing the histidine activating enzymes of yeast for possible differences in the aminoacylation reaction, cytoplasmic and mitochondrial tRNA preparations enriched for $tRNA^{His}$ were used. The $tRNA^{His}$ content of each preparation was determined by acylating small aliquots of tRNA with histidine until a plateau was reached. K_m determinations for both cytoplasmic tRNA and mitochondrial tRNA were performed using cytoplasmic and mitochondrial enzyme preparations from either S288C or EB2 strains. The results of these determinations are shown in Table II. It is apparent that the mitochondrial enzyme from either strain has 10–20 times higher affinity for both tRNA species than the cytoplasmic enzyme. However, both enzymes charged both tRNAs.

Since no difference in specificity was observed with either

TABLE I: Affinity of Cytoplasmic and Mitochondrial Synthetases for Histidine and ATP.

Strain	Enzyme Source	K_m Values (M) Histidine	ATP
Wild Type (S288C)	Cytoplasm	1.8×10^{-6}	1.4×10^{-4}
	Mitochondria	0.9×10^{-6}	0.8×10^{-4}
Petite mutant (EB2)	Cytoplasm	1.0×10^{-6}	*a*
	Mitochondria	0.6×10^{-6}	*a*

[a] Not determined.

TABLE II: Affinity of Cytoplasmic and Mitochondrial Synthetases for Cytoplasmic $tRNA^{His}$ and Mitochondrial $tRNA^{His}$.

Strain	Enzyme	K_m Values (M $\times 10^{-7}$)[a] Cytoplasmic tRNA	K_m Values (M $\times 10^{-7}$)[a] Mitochondrial tRNA
Wild type (S288C)	Cytoplasmic	6.7 ± 0.2 (4)	6.5 ± 0.12 (4)
	Mitochondrial	0.66 ± 0.15 (4)	0.3 ± 0.11 (3)
Petite mutant (EB2)	Cytoplasmic	6.7 ± 0.1 (4)	4.2 (1)
	Mitochondrial	0.24 ± 0.01 (2)	0.24 ± 0.01 (2)

[a] The number in parentheses shows the number of determinations. A ± sign shows the standard deviation from the mean.

$tRNA^{His}$, both enzymes were tested for their ability to charge *E. coli* tRNA. The results of an experiment with enzymes isolated from S228C are presented in Figure 11. Cytoplasmic enzyme charges bulk *E. coli* tRNA, while the mitochondrial enzyme is almost inactive (at most 10% of the extent of the cytoplasmic enzyme) regardless of the temperature employed. Yeast tRNA is charged by both enzymes to the same extent (Figure 11c). The rate of charging of yeast tRNA is higher in the case of the cytoplasmic enzyme, since its specific activity is about 10 times higher than that of the mitochondrial enzyme. Thus, *E. coli* tRNA is charged specifically by the cytoplasmic synthetase but only very poorly by the mitochondrial synthetase of yeast. Very similar results were obtained with synthetases from EB2: cytoplasmic enzyme charged *E. coli* tRNA, whereas the mitochondrial enzyme did not.

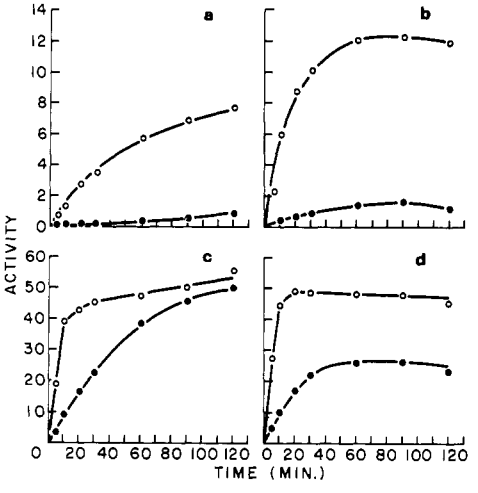

FIGURE 11: Charging of yeast and *E. coli* tRNA by the cytoplasmic and mitochondrial synthetase. Charging of *E. coli* and yeast tRNA (panels a–b and c–d, respectively) was performed at 26° (panels a and c) and at 37° (panels b and d). Cytoplasmic (○) and mitochondrial (●) preparations were used as indicated (cytoplasmic enzyme, 1.9 μg of protein, specific activity 1492 units/ml; mitochondrial enzyme, 3.6 μg of protein, specific activity 158 units/mg). 100 μg of *E. coli* tRNA and 200 μg of bulk yeast tRNA were used per assay. The fact that at 37° (Figure 11d) mitochondrial enzyme does not reach the same level of charging of yeast tRNA as the cytoplasmic enzyme can be explained by inactivation of the mitochondrial enzyme at that temperature. Inactivation is not observed with the cytoplasmic enzyme, because the amount of cytoplasmic enzyme added is sufficient to aminoacylate all available tRNA within 20 min. Increasing the concentration of the mitochondrial enzyme fivefold did not lead to an increase in the charging of *E. coli* tRNA.

To test for the possible presence in the mitochondrial extract of an inhibitor of *E. coli* tRNA-specific synthetase activity, the cytoplasmic enzyme was mixed with the mitochondrial enzyme, the mixture was diluted 1:1 with 35% propylene glycol, and charging of yeast and *E. coli* tRNA was determined. Corrections for dilution were made. The results are shown in Table III. The activity of the yeast cytoplasmic enzyme is not affected by the presence of the mitochondrial enzyme. Therefore, the charging of *E. coli* tRNA by the cytoplasmic enzyme and lack of charging by the mitochondrial enzyme are not artifacts and reflect the differential specificity of these enzymes.

Discussion

The results presented here show that yeast cells possess, in addition of two histidyl-tRNA synthetases, two distinct species of $tRNA^{His}$. These species differ with respect to chromatographic properties and the ability to be charged by a heterologous enzyme system. The absence of one of the species from defective mitochondria of certain petite strains and the hybridization of that species to mtDNA suggest that the mitochondrial $tRNA^{His}$ is coded by mtDNA. The cytoplasmic $tRNA^{His}$ is probably encoded in the nDNA. The conclusion from studies on charging of mitochondrial

TABLE III: Charging of Bulk Yeast and *E. coli* tRNA by Cytoplasmic Enzyme in the Presence and Absence of Mitochondrial Enzyme.[a]

Enzyme	[^{14}C]Histidine esterified (pmol): Yeast tRNA (200 μg)	[^{14}C]Histidine esterified (pmol): *E. coli* tRNA (100 μg)
Cytoplasmic	2.5	0.5
Cytoplasmic + mitochondrial (1:3 v/v)[b]	2.8	0.5

[a] Charging of yeast tRNA was carried out at 23°, and charging of *E. coli* tRNA at 37°. [b] Cytoplasmic preparation (1 ml, 0.19 mg of protein, specific activity 1492 units/mg) was mixed with 3 ml of mitochondrial enzyme (0.36 mg of protein, specific activity 158 units/mg). The mixture was diluted 1:1 with 35% (v/v) propylene glycol, and the charging of yeast and *E. coli* tRNA was determined.

and cytoplasmic $tRNA^{His}$ is that at least *in vitro* these tRNAs can substitute for each other as substrates for the synthetase.

In the hybridization experiments reported here, nDNA was obtained from the strain lacking mtDNA. If this precaution were not taken, it is likely that mitochondrial tRNA would show hybridization to nDNA, because of low level contamination of nDNA by mtDNA. Another interpretation, however, is that nDNA of respiratory competent yeast cells contains sequences not found in the nDNA of an isogenic ρ^- strain.

Takeishi *et al.* (1972) and Singh *et al.* (1973) have found four and six species, respectively, of $tRNA^{His}$ in *Saccharomyces cerevisiae*. Some of the minor species are also charged preferentially by the *E. coli* synthetase. Although our fractionation procedures were similar to those used earlier, only two species were found. The multiple species seen by the other authors may be attributed to the fact that tRNA was extracted from commercially grown cells. Uncontrolled variables such as the purity of a strain, the ploidy of a strain, the growth phase of cells, etc., may influence the number of iso-accepting tRNAs. In work reported here, all strains were genetically defined haploids grown to mid-logarithmic phase to eliminate those variables.

In addition to the cytoplasmic and mitochondrial $tRNA^{His}$, two histidine activating enzymes can be resolved. One species of the enzyme is present in the soluble portion of the total homogenate. The other synthetase representing less than 10% of the total cell activity is associated with the mitochondrial fraction. The presence of the activating enzyme in this fraction does not seem to be due to contamination of the mitochondria with the cytoplasmic enzyme.

The mitochondrial synthetase differs from its cytoplasmic counterpart in that it has 10–20-fold higher affinity for both cytoplasmic and mitochondrial $tRNA^{His}$. This lack of specificity in charging is not surprising since most (though not all) mitochondrial synthetases described for other organisms (Barnett *et al.*, 1967; Buck and Nass, 1969) are also capable of charging both cytoplasmic and organellar tRNAs. Differences in the specificity of recognition are apparent in the inability of the mitochondrial synthetase to charge bulk *E. coli* tRNA, while the cytoplasmic enzyme acylates this tRNA efficiently.

One important result of the present study is the finding that defective mitochondrial particles of petite strains of yeast contain histidyl-tRNA synthetase. The properties of this enzyme are indistinguishable from the properties of mitochondrial synthetase from a respiratory-competent strain. Since petite strains used in this work were obtained by a procedure known to remove all detectable mtDNA, it may be assumed that the mitochondrial genome does not code for the mitochondrial synthetase. Other proteins involved in mitochondrial protein synthesis have been found to be coded for by the nucleus. For example, Richter (1971) found mitochondrial peptide chain elongation factors which had properties identical with the factors from the normal mitochondria. Moreover, iso-1-cytochrome *c* is a mitochondrial protein coded for by a nuclear gene (Sherman *et al.*, 1966). In summary, all of our data suggest that both the mitochondrial and cytoplasmic histidyl-tRNA synthetases are encoded in the nucleus. Weeks and Gross (1971) reached a similar conclusion for the leucyl-tRNA synthetases of *Neuospora*. In addition these authors concluded that the two synthetases were under the control of the same gene. The *leu5* mutant has an altered cytoplasmic synthetase and barely detectable mitochondrial synthetase. Revertants have restored mitochondrial activity and a cytoplasmic leucyl-tRNA synthetase with kinetic properties from both the *leu5* mutant and wild type. It is possible that the differences in the properties of the two enzymes result from post-translational modifications of a common polypeptide.

The absence of histidyl-tRNA synthetase mutants in yeast makes it difficult to decide whether there is a single gene coding for both the cytoplasmic and mitochondrial histidyl-tRNA synthetases or whether two or more genes code for these enzymes. These synthetases differ markedly in their affinities for yeast tRNA. Most strikingly the mitochondrial synthetase is unable to acylate *E. coli* tRNA. In addition, our data suggest slight differences in temperature sensitivity and salt sensitivity. These features are compatible with the idea that one gene controls the synthesis of both enzymes, the differences in their properties resulting from post-translational modifications. On the other hand, it is just as likely that more than one gene is involved in the synthesis of the activating enzymes, one for the cytoplasmic and one for the mitochondrial enzyme. Neither of these possibilities can be excluded *a priori*. It is possible that the mitochondrial and cytoplasmic enzymes are functionally interchangeable. If the two gene hypothesis were correct, a mutational alteration in either of the genes would leave the other one intact. This hypothesis predicts in effect that there are duplicate synthetase genes. Evidence for a duplicated glycyl-tTNA synthetase gene of *E. coli* has been presented by Folk and Berg (1971). The structural gene-modifying gene hypothesis predicts that a mutation in the structural gene could alter both enzymes simultaneously, whereas a mutation in the modifying gene would affect only one of the synthetases. Further work on the biochemical genetics of the synthetase should help to decide between these two alternatives.

References

Barnett, W. E., Brown, D. H., and Epler, J. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 1775.

Brenner, M., and Ames, B. N. (1971), *in* Metabolic Pathways, Vol. V, Vogel, H. J., Ed., New York, N.Y., Academic , Press.

Buck, C. A., and Nass, M. M. K. (1969), *J. Mol. Biol. 41,* 67.

Casey, J., Cohen, M., Rabinowitz, M., Fukuhara, H., and Getz, G. S. (1972), *J. Mol. Biol. 63,* 431.

Criddle, R. S., and Schatz, G. (1969), *Biochemistry 8,* 322.

Finkelstein, D. B., Blamire, J., and Marmur, J. (1972), *Biochemistry 11,* 4853.

Flamm, W. C., Bond, H. E., and Burr, H. E. (1966), *Biochim. Biophys. Acta 129,* 310.

Folk, W. R., and Berg, P. (1971), *J. Mol. Biol. 58,* 595.

Goldberger, R. (1974), *Science 183,* 810.

Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R., and Marmur, J. (1970), *J. Mol. Biol. 52,* 323.

Gross, S. R., McCoy, M. T., and Gilmore, E. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61,* 253.

Halbreich, A., and Rabinowitz, M. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 294.

Kelmers, A. D., Weeren, H. O., Weiss, J. F., Pearson, R. L., Stulberg, M. P., and Novelli, G. (1971), *Methods Enzymol. 20,* 9–34.

Kislev, N., and Eisenstadt, J. M. (1972), *Eur. J. Biochem. 31,* 226.

Kovac, L., Groot, G. S. P., and Racker, E. (1972), *Biochim.*

Biophys. Acta 256, 55.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Marmur, J. (1961), *J. Mol. Biol. 3,* 208.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Moore, S., and Stein, W. H. (1951), *J. Biol. Chem. 192,* 663.
Reger, B. J., Fairfield, S. A., Epler, J. L., and Barnett, W. E. (1970), *Proc. Nat. Acad. Sci. U. S. 67,* 1207.
Richter, D. (1971), *Biochemistry 10,* 4422.
Sherman, F., Fink, G. R., and Lawrence, C. W. (1972), Methods in Yeast Genetics, Laboratory Manual, Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory for Quantitative Biology.
Sherman, F., Stewart, J. W., Margoliash, E., Parker, J., and Campbell, W. (1966), *Proc. Nat. Acad. Sci. U. S. 55,* 1498.
Silbert, D. F., Fink, G. R., and Ames, B. N. (1966), *J. Mol. Biol. 22,* 335.
Singh, H., Rector, E., Waters, B., and Vadasz, J. (1973), *Biochim. Biophys. Acta 312,* 276.
Takeishi, K., Nishimura, S., and Ukita, T. (1967), *Biochim. Biophys. Acta 145,* 605.
Takeishi, K., Nomoto, A., and Ukita, T. (1972), *Biochim. Biophys. Acta 262,* 262.
Von Tigerstorm, M. and Tener, G. (1967), *Can. J. Biochem. 45,* 1067.
Weeks, C. O., and Gross, S. R. (1971), *Biochem. Genet. 5,* 505.
Worthington Enzyme Manual (1972), Worthington Biochemical Corp., Freehold, N.J., p 41.