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Qualitative and Comparative Nature of Mitochondrial Translation Products in Mammalian Cells†

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ABSTRACT: A method has been described for the efficient incorporation of [^{35}S]methionine into isolated mitochondrial particles from various mammalian tissues. The method involves the incubation of digitonin-treated mitochondrial particles (mitoplasts) in a low sucrose medium. Electrophoretic analysis of ^{35}S-labeled products on sodium dodecyl sulfate–polyacrylamide gels under reducing conditions shows that mitoplasts from Ehrlich ascites cells, mouse liver, and rat liver synthesize 19–24 polypeptide species including some high molecular weight components in the size range of 1.0×10^5. The polypeptide species synthesized in the mitoplast system resemble the cycloheximide-resistant products synthesized in the intact cells with respect to size distribution and total number, although significant quantitative differences between the two systems are observed. Experiments on pulse–chase analysis of ^{35}S-labeled mitochondrial products and the effects of protease inhibitors on the electrophoretic profiles suggest no significant proteolytic degradation during the incubation or analysis. Further, control experiments with nuclease-treated mitoplasts and use of specific protein synthesis inhibitors show that all of the labeled polypeptides are the intramitochondrial translation products. Extensive comparison between the products synthesized in Ehrlich ascites and mouse and rat liver mitochondria, using one- and two-dimensional gels under denaturing conditions, shows striking variations, suggesting possible heterogeneity.

Since the first demonstration by McLean et al. (1958) that rat liver mt[1] can actively incorporate radioactive amino acids into proteins, there have been numerous studies on the characterization of this unique translation system [for details, see O'Brien (1976) and Buetow & Wood (1978)] as well as the nature of mt translation products (Schatz & Mason, 1974; Avadhani et al., 1975) in various cell types. Many of the early studies on the analysis of translation products indicated that mt from varied unicellular eukaryotes as well as from the animal sources may synthesize about eight polypeptides [see Beattie (1971), Schatz & Mason (1974), Sebald et al. (1968), Ibrahim et al. (1973), Coote & Work (1971), Schatz et al. (1972), Lederman & Attardi (1973), and Lansman & Clayton (1975)] in the size range of 6×10^3–5.5×10^4 daltons. In yeast and *Neurospora* it was also shown that the electrophoretic patterns of polypeptides synthesized in the whole cells

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[1] Abbreviations: mt, mitochondria; $NaDodSO_4$, sodium dodecyl sulfate; LES, Lettre Ehrlich ascites cells; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; CHX, cycloheximide; CAP, chloramphenicol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate.

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in the presence of cycloheximide closely resemble the products formed in isolated mt particles (Ibrahim et al., 1973; Sebald et al., 1969). In addition, detailed characterization of the mt inner-membrane fraction with special reference to the complexes of electron transfer chain and oxidative phosphorylation in yeast showed definite functional roles to six intramitochondrially synthesized polypeptides (Schatz & Mason, 1974; Tzagoloff et al., 1979). These studies have given rise to the general concept that irrespective of the genome size and source, the function of mt translation system is to contribute to about eight polypeptides of comparable size distribution and possibly function. In contrast, recent DNA sequence analysis studies show the presence of at least 13 potential coding regions in the human and mouse mt genome (Anderson et al., 1981; Montoya et al., 1981; Bibb et al., 1981). Similarly, by use of a high-resolution electrophoresis system it was shown that yeast mt may synthesize as many as 22 polypeptides including the variant forms (Douglas & Butow, 1976). In addition, studies showing distinct translation sites for the dicyclohexylcarbodiimide binding proteolipid in yeast and *Neurospora* suggest significant species-specific variations in the products of mt translation (Tzagoloff et al., 1979).

Significant tissue-specific variations in the polypeptide patterns of mt inner-membrane and matrix fractions in different animal cell systems are now well documented (Schnaitman, 1966; Melnick et al., 1976; Keenes, 1969; Henslee & Srere, 1979). Similarly, there is also increasing evidence on the organ-specific functional variations in animal cell mitochondria with associated variations in the specific enzyme contents (Coty & Pederson, 1975; Clarke, 1976; Hall et al., 1979; Iyengar & Iyengar, 1980; Niranjan & Avadhani, 1980). In spite of these observations showing possible mt heterogeneity, studies on the comparison of mt translation products in animal systems have been limited, mainly due to the difficulty in high specific activity labeling of mt translation products.

In this paper we have described procedures to label mt translation products with [^{35}S]methionine in the isolated mt particles from mouse and rat liver and also from the ascites tumors. Analysis of these translation products using both one- and two-dimensional systems reveals that mouse liver, mouse LES, and rat liver mt synthesize about 19–24 products, including some unusually large polypeptides ranging up to 1.0 $\times$ 10^5 daltons. The translation products of mouse liver mt show significant variation from those of mouse ascites mt with respect to total number and size.

Materials and Methods

Chemicals. All the chemicals used were of analytical grade. Almost all of the enzymes including pancreatic RNase, pancreatic DNase, creatine phosphokinase, and collagenase were obtained from Worthington Biochemical Corp. Nucleotide triphosphates, creatine phosphate, chloramphenicol, cycloheximide, mannitol, bovine serum albumin, and digitonin were purchased from Sigma Chemical Co. RNase-free sucrose was from Schwarz/Mann. Tissue culture media, vitamin solutions, and other tissue culture reagents were the products of Gibco. Electrophoresis-grade acrylamide, bis(acrylamide), tetramethylethylenediamine, $NaDodSO_4$, and urea were from Bio-Rad. [^{35}S]Methionine was purchased from Amersham Radiochemicals Corp. Royal X-omatic SB5 X-ray films and other photography chemicals were purchased from Eastman Kodak Co.

General. All the solutions used for mt isolation and for various incubations were sterilized by membrane filtration. The glassware used was acid cleaned and heat sterilized. As far as possible, sterile conditions were maintained throughout the cell fractionation and labeling procedures.

Cell and Tissues. The cell types used in these studies include Lettre Ehrlich ascites cells (LES), mouse and rat liver, and mouse embryonic liver. Maintenance and growth of LES in the peritoneal cavity of Swiss mice were as described before (Chun et al., 1969). Swiss mice (25–30 g) and male Sprague-Dawley rats (150–200 g) were used as the source of liver.

Isolation of Mitochondria. Mitochondria were isolated essentially as described before (Avadhani et al., 1974; Lewis et al., 1976) except that the sucrose–mannitol buffer system (Pedersen et al., 1978) was used to obtain optimal incorporation rates. Freshly harvested LES cells from animals bearing 7-day-old tumors and mouse as well as rat livers washed free of blood clots were used for mt isolation. Cells or tissue slices were suspended in 2 volumes of mt isolation buffer [2 mM Hepes (pH 7.5), 0.22 M D-mannitol, 0.07 M sucrose, 1 mM EDTA, and 0.5 mg/mL BSA] and homogenized with a Teflon homogenizer (0.004-cm clearance for LES cells and 0.02-cm clearance for liver slices). Mitochondria were pelleted by differential centrifugation (Avadhani et al., 1974), and the mitoplasts were prepared by the digitonin method adopted from Greenawalt (1974) as described before (Niranjan & Avadhani, 1980). The mitoplasts were washed once with mt isolation buffer and once with a buffer containing 0.25 M sucrose, 30 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $Mg(CH_3COO)_2$, 7 mM 2-mercaptoethanol, and 5 mM potassium phosphate and used for protein synthesis.

Protein Synthesis with Mitoplasts. The method was a modification of the procedure of Fukamachi et al. (1972). The mitoplasts were suspended in a medium containing 15 mM Tris-HCl (pH 7.4), 60 mM KCl, 6 mM $Mg(CH_3COO)_2$, 4 mM 2-mercaptoethanol, and 3 mM potassium phosphate buffer (pH 7.4) and 0.14 M sucrose at a concentration of 10 mg of protein/mL and supplemented with 2 mM ATP, 2 mM GTP, 5 mM creatine phosphate, 4 mM pyruvate, 0.2 mg/mL creatine phosphokinase, and 100 μM each of the 19 L-amino acids except methionine. The mixture was preincubated by gentle shaking for 5 min at 35 °C with or without added inhibitors (CHX or CAP). At the end of this preincubation 150 μCi/mL [^{35}S]methionine (600 Ci/mmol) was added, and the incubation was continued for 60 min or as required. Aliquots of 10–20 μL were removed and used for determining the extent of ^{35}S incorporation by the hot CCl_3COOH method. At the end of the incubation, mitoplasts were pelleted at 10000*g* for 10 min, washed once with mt isolation buffer without added BSA, and kept frozen at −70 °C in the presence of 3 μg each of leupeptin and pepstatin as protease inhibitors. Unless otherwise stated, 300 μg/mL CHX was present throughout the incubation and the preincubation period. When added, CAP was at 500 μg/mL.

Protein Synthesis in Whole Cells. LES cells were labeled with 100 μCi/mL [^{35}S]methionine essentially as described earlier (Aroskar et al., 1980). Cells were suspended in Hank's balanced salt medium (containing Ca^{2+} and Mg^{2+}), supplemented with 5 mM malate and 50 μM each of the 19 L-amino acids (excepting methionine) at a density of about 3 $\times$ 10^6 cells/mL, and preincubated in a shaker water bath at 37 °C for 15 min, with or without added inhibitors (CHX or CAP). CHX when added was at 300 μg/mL, and CAP was at 1.0 mg/mL. At the end of the preincubation, 100 μCi/mL [^{35}S]methionine was added, and the incubation was continued for 2 h. At intervals, 20-μL aliquots were withdrawn for determining the amount of hot CCl_3COOH insoluble cpm.

The cells were pelleted, washed twice with Hank's medium, and stored at −70 °C in the presence of 5 μg each of leupeptin and pepstatin.

Analysis of Labeled Proteins on One- and Two-Dimensional Systems. Samples (mt or whole cells) were sonicated in 100–200 μL of buffer containing 20 mM Tris–acetate (pH 7.5), 5 mM $Mg(CH_3COO)_2$, and 3 μg each of leupeptin and pepstatin for 20 s at setting 40 of a Branson sonifier and incubated for 5 min at 35 °C with 20 μg each of pancreatic RNase and DNase. Samples were then freeze-dried and used for analysis in the gradient polyacrylamide gel electrophoresis or in the two-dimensional system of O'Farrell (1975). For electrophoretic analysis on the gradient gels, dried samples were dissolved in 50–100 μL of Laemmli sample buffer containing 4% $NaDodSO_4$ and 10% 2-mercaptoethanol (Laemmli, 1970), and proteins were dissociated by heating at 90 °C for 3 min. The buffer and the gel systems used were essentially as described by Laemmli (1970). Slab gels (14 × 16 × 0.15 cm) of 8–16% gradient polyacrylamide were used. Both the gel and the buffer system contained 0.2% $NaDodSO_4$. Samples containing 200–300 μg of protein were electrophoresed at 20 mA for 15 h. β-Galactosidase, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and cytochrome *c* were used as markers. The mt proteins were also resolved on 12% polyacrylamide gels containing 7 M urea by a procedure modified from Shapiro et al. (1967). The buffer system for urea gel analysis consisted of 0.1 M sodium phosphate (pH 7.2) and 0.2% $NaDodSO_4$. Before electrophoresis, mt proteins were dissociated in 10 mM sodium phosphate (pH 7.2), 7 M urea, 4% $NaDodSO_4$, and 5% 2-mercaptoethanol by heating at 90 °C for 3 min. Electrophoresis was carried out overnight at 3.5 V/cm. For two-dimensional analysis, the samples (50–200 μg of protein and about 1 × 10^6 cpm) were dissolved in 50 μL of O'Farrell's lysis buffer containing 9 M urea and 5% 2-mercaptoethanol and resolved on 0.3 × 11 cm polyacrylamide–urea gels by isoelectric focussing (O'Farrell, 1975). The isoelectric focussing gels were equilibrated with a buffer containing 125 mM Tris-HCl (pH 6.8), 2.3% $NaDodSO_4$, 5% 2-mercaptoethanol, and 10% glycerol (O'Farrell, 1975) and overlaid on top of 8–16% gradient polyacrylamide gels containing 0.2% $NaDodSO_4$ for electrophoresis in the second dimension (Aroskar et al., 1980). The gels were stained with 0.2% Coomassie blue, destained (O'Farrell, 1975), and processed for fluorography as described by Bonner & Laskey (1974).

Other Procedures. For determination of hot CCl_3COOH insoluble radioactivity, aliquots of 10–20 μL were precipitated with 5 mL of 10% CCl_3COOH containing 1 mM unlabeled methionine. The precipitates were heated at 90 °C for 15 min, filtered through membrane filter disks (0.2 nm, Amicon Corp.), and washed with 15–20 mL of 10% CCl_3COOH. The filter disks were air-dried and counted with 10 mL of Cab-o-sil scintillation mixture in an Intertechnique SL-4000 counter. The protein content was estimated by the differential absorbance at 280 and 310 nm as described by Clarke (1976). This procedure gives values very close to those obtained with standard procedure (Lowry et al., 1951) and is applicable to mt preparations from diverse mammalian tissues.

Results

Nature of the Mitoplast System. A number of procedures have been developed for the incorporation of radioactive amino acids into isolated mt particles (Coote & Work, 1971; Ibrahim et al., 1973; Poyton & Kavanaugh, 1976). None of these procedures were directly applicable to rat liver or LES mt systems for obtaining high specific activity labeling. The modification of the hypoosmotic procedure of Fukamachi et al. (1972) as described in this paper yielded the highest stimulation. The method involves the incubation of digitonin-stripped mt particles in a buffer system containing low sucrose (0.14 M). Addition of high levels of GTP (2 mM) and malate or pyruvate may be responsible for the observed high incorporation in our system. The stimulatory effects of GTP on the incorporation of labeled amino acids by yeast mt have been documented (Ohashi & Schatz, 1980). The kinetic patterns of [^{35}S]methionine incorporation in rat liver mt in this system have been presented in Figure 1. As can be seen, the system is highly sensitive (>95%) to CAP, a specific inhibitor of the mitochondrial ribosomal system. Treatment of mt with digitonin is an important step for obtaining the high rate of incorporation. As seen in Figure 2, crude mt without digitonin treatment yield only about 50% activity, while a digitonin concentration of 50 μg/mg of protein yields the highest incorporation. At higher digitonin concentrations, however, a substantial reduction in the activity is observed (Figure 2). Although not shown here, this procedure is applicable to mt particles from different tissues such as brain, heart, kidney, spleen, etc. The extent of incorporation, however, varied significantly among the various tissues. For example, mt preparations from LES and beef heart show the highest activity [about (1.0–1.4) × 10^6 cpm/mg] while rat liver and rat brain, as well as the mouse liver mt, show moderately high activity in the range of (5–8) × 10^5 cpm/mg and mt particles from kidney and spleen are the least active in this system [(0.8–1.0) × 10^5 cpm/mg].

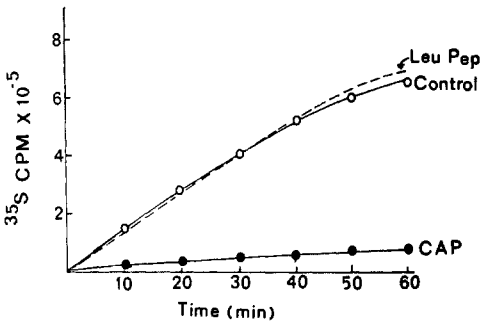

FIGURE 1: Kinetics of amino acid incorporation by rat liver mitoplasts. Mitoplasts were prepared by digitonin treatment (0.05 mg/mg of protein) and used for protein synthesis as described under Materials and Methods. Leupeptin was added at 2 μg/mL and CAP was at 500 μg/mL. All the assay tubes contained 300 μg/mL CHX. After a 5-min preincubation, the protein synthesis was initiated with 150 μCi/mL [^{35}S]methionine (600 Ci/mmol). Other details were as described under Materials and Methods.

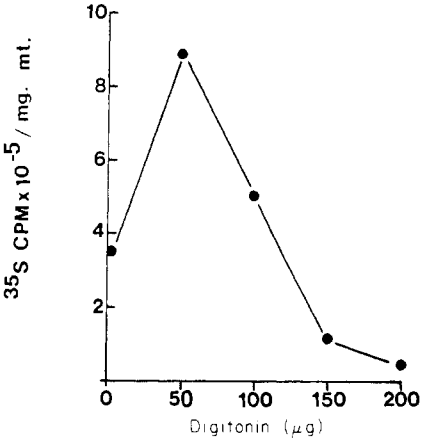

FIGURE 2: Effects of digitonin concentration (μg/mg of protein) on mt protein synthesis. Either untreated mt or mt preparations treated with varied digitonin concentrations were used for protein synthesis as described in Figure 1. Incubation was carried out for 60 min.

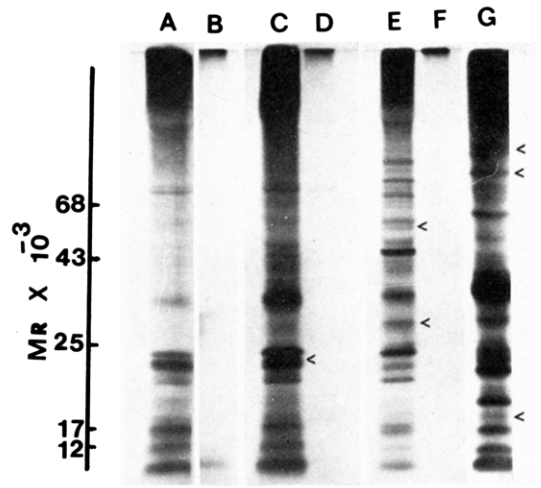

FIGURE 3: Electrophoretic analysis of mt translation products. Protein synthesis with LES and mouse and rat liver mitoplasts was carried out for 60 min as described in Figure 1. Samples containing 200 μg of protein in each case were solubilized in 50 μL of buffer containing 120 mM Tris-HCl (pH 6.8), 4% (w/v) $NaDodSO_4$, 10% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol by heating at 90 °C for 3 min and electrophoresed on 8–16% gradient polyacrylamide gels as described under Materials and Methods. Lane A is rat liver mt pretreated with RNase. For RNase treatment, mitoplasts were suspended in mt isolation buffer containing 2 mM $MgCl_2$ (no EDTA) at a concentration of 10 mg of protein/mL and incubated at 30 °C for 10 min with 20 μg/mL pancreatic RNase. Mitoplasts were washed twice with mt isolation buffer and used for protein synthesis. Lanes C, E, and G are control rat liver, mouse liver, and LES mt, respectively. Lanes B, D, and F are mitoplasts labeled in the presence of 500 μg/mL CAP. Arrowheads in lanes C, E, and G represent the variant forms.

The presence of protease activity in the rat liver mt preparations has been demonstrated in several studies, although the precise location and the nature of these activities remain unclear (Gear et al., 1974; Manjunath et al., 1979). There are also indications that the labeled products in rat liver mt may be rapidly degraded due to the proteolytic activity (Wheeldon & Lehinger, 1966; Wheeldon et al., 1974). Recently, however, it was shown that peptide inhibitors such as leupeptin and pepstatin inhibit mt protease activity (Kalnov et al., 1979). So that the extent of protease activity could be determined, [^{35}S]methionine incorporation was carried out in the presence and absence of leupeptin. The results of the experiments (see Figure 1) show that the rate as well as the extent of incorporation is nearly the same in the incubation system with and without added leupeptin, suggesting minimal proteolytic degradation during incubation.

Electrophoretic Analysis of mt Translation Products. Although the protein synthesis system is applicable to mt particles from various tissues, in order to permit easy presentation and comparison with mt products synthesized in whole cells, we have chosen the mouse LES, mouse liver, and rat liver mt systems for analysis. The autoradiogram presented in Figure 3 shows the electrophoretic patterns of ^{35}S-labeled products synthesized in the mt particles. It is seen that the products of rat liver, mouse liver, and also mouse LES are resolved into several discrete components ranging from about 6×10^3 to 1.0×10^5 daltons. Comparison of patterns C and E suggests marked species-specific variations between rat and mouse liver systems as indicated by the arrows. Similarly, the ^{35}S-labeled products synthesized in LES mt (lane G) show significant quantitative and qualitative differences from mouse liver mt products (lane E). As indicated in Figure 1, the translation is highly sensitive to CAP, and almost all of the products seen in control samples (Figure 3, lanes A, C, E, and G) are uniformly inhibited by this specific inhibitor of mt ribosomal activity (Figure 3, lanes B, D, and F). Also, predigestion of mt with RNase has no effect on the extent of labeling and the polypeptide patterns (Figure 3A), suggesting that mRNA coding for these polypeptides is indeed localized within the inner-membrane compartment. This observation was further substantiated in a control experiment showing that over 95% of the ^{35}S-labeled nascent chains synthesized in the isolated mt particles are indeed associated with mt ribosomes. As shown in Table I, the release of ^{35}S-labeled nascent chains by puromycin is inhibited by pretreatment with CAP but unaffected by CHX.

Table I: Effects of Specific Inhibitors on the Release of ^{35}S-Labeled Nascent Chains from Polysomes[a]

inhibitor added after 10-min pulse	^{35}S cpm in polysomes ($\times 10^{-3}$)	^{35}S cpm released by puromycin ($\times 10^{-3}$)	% cpm released
chloramphenicol	416.6	9.9	2.4
cycloheximide	501.4	446.7	89.0

[a] LES mitoplasts were labeled with [^{35}S]methionine in duplicate flasks (40 mg each) as described in Figure 1 in the absence of inhibitors. After 10 min of labeling, 500 μg/mL CAP was added to the first flask and 300 μg/mL CHX to the second. Incubation was continued for 5 min, and mt particles were pelleted and washed twice with mt isolation buffer. Each pellet was mixed with 40 mg of unlabeled mitoplasts and used for isolating the polysomes as described before (Lewis et al., 1976). The polysome pellets were suspended in 3 mL of buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 80 mM KCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, and 1% sodium deoxycholate. The clear suspension was incubated with 100 μg/mL puromycin for 15 min at 30 °C, and the ribosomal fraction was pelleted by centrifugation at 215000*g* for 90 min. Other details were as described under Materials and Methods.

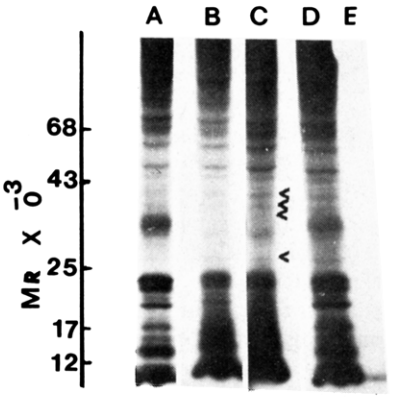

FIGURE 4: Pulse–chase characteristics of mt translation products. LES mitoplasts were pulse labeled with [^{35}S]methionine for 5 min as described under Materials and Methods and either chased with a 10^3-fold excess of cold methionine or resuspended in unlabeled medium and chased for 50 min. (Lane A) LES mt labeled for 60 min; (lane B) LES mt pulse labeled for 5 min; (lane C) LES mt pulse labeled for 5 min and chased for 50 min with a 10^3 excess of unlabeled methionine; (lane D) LES mt pulse labeled for 5 min and chased for 50 min in unlabeled medium; (lane E) LES mt pulse labeled for 5 min and chased for 50 min in the presence of CAP. In each case, 200 μg of mt protein was used for the electrophoresis. The lower intensity of bands in lane C indicated by the arrowheads suggests faster turnover rates.

In view of the large number of products seen in our experiment and also because of the presence of some unusually large molecular weight products, it was thought that some of these polypeptides may represent the precursors. For verification of this possibility, a pulse–chase experiment was carried out with the LES mt system (Figure 4). Mitoplasts pulse labeled for 5 min (lane B) were either chased with a 10^3-fold excess of unlabeled methionine (lane C) or resuspended in cold

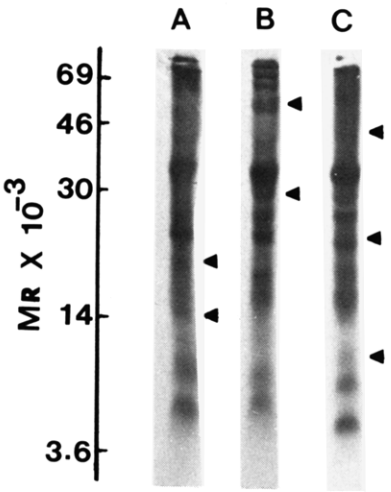

FIGURE 5: Electrophoretic analysis of mt translation products in urea–polyacrylamide gels. Mitoplasts labeled with [^{35}S]methionine (150 μg of protein in each case) were dissociated by heating at 90 °C for 3 min in the presence of 10 mM sodium phosphate buffer (pH 7.2), 4% NaDodSO$_4$, 5% 2-mercaptoethanol, and 7 M urea and electrophoresed on 12% polyacrylamide slab gels containing 7 M urea as described under Materials and Methods. (Lane A) Rat liver mt; (lane B) mouse liver mt; (lane C) LES mt. Arrowheads in lanes A–C indicate the variant translation products.

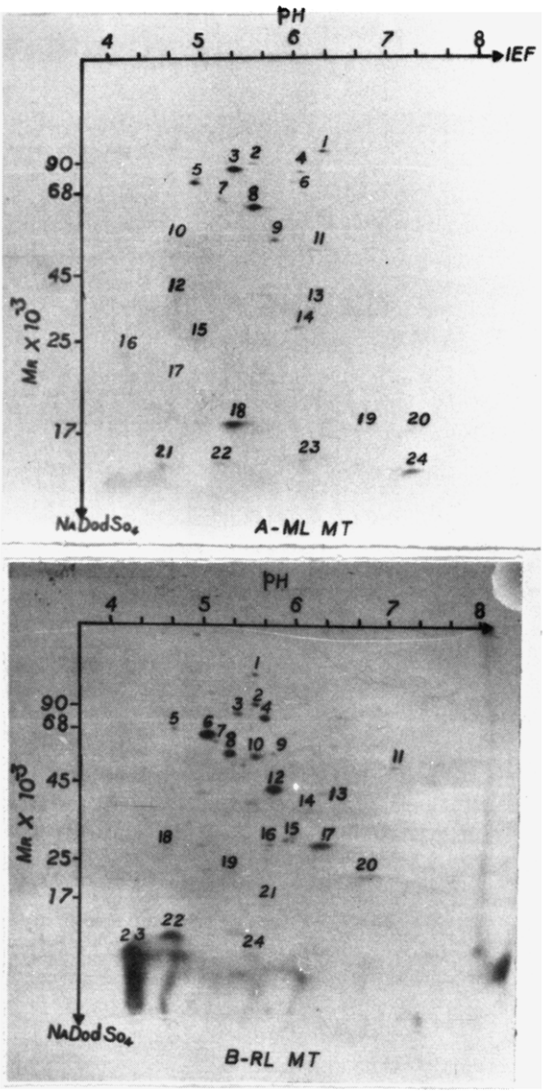

FIGURE 6: Two-dimensional analysis of mt translation products. (A) Mouse liver mt and (B) rat liver mt labeled with [^{35}S]methionine. Details of labeling and two-dimensional analysis were as described under Materials and Methods. In each case about 250 μg of protein was used for analysis.

medium after pelleting and chased for 50 min (lane D). As shown in Figure 4, the majority of the polypeptides labeled during the short pulse of 5 min (lane B) are visible even after a 50-min chase, although some quantitative differences are apparent. The significantly lower intensity of some of the polypeptides indicated by the arrows in Figure 4 (lane C) suggests faster turnover rates for these mt translation products. Similarly, some polypeptides in the region of $<2.5 \times 10^4$ daltons show steady accumulation, suggesting slower turnover rates. Although not shown here, control experiments using protease inhibitors such as leupeptin and pepstatin show that these polypeptides are not degradation products. Further, all of these low molecular weight polypeptides are detectable by staining with Coomassie blue even in freshly isolated mt preparations. The results of this experiment (Figure 4), however, show that a major polypeptide of about 3.8×10^4 daltons, which is not significantly labeled during the pulse, accumulates at a longer chase time, suggesting that this protein is possibly derived from a larger polypeptide. In general, however, most of the species seen during the 5-min pulse labeling appear to lack the properties of short-lived precursors.

The hydrophobic nature of mt translation products (Beattie, 1971) and the problems of aggregation during electrophoresis (Schatz & Mason, 1974; Moorman & Grivell, 1976; Downer et al., 1976) are now well documented. A number of methods including pretreatment with alkali (Tzagoloff, 1972) and electrophoresis in the presence of urea, 2-mercaptoethanol, EDTA, etc. have been proposed to minimize aggregation. We have used a combination of high-temperature (90 °C), highly reducing conditions (10% 2-mercaptoethanol) and high concentrations of NaDodSO$_4$ (4%) to dissociate the mt membrane proteins. Similar conditions have been successfully used in the past to analyze mt translation products in varied systems (Douglas & Butow, 1976; Douglas et al., 1979; Attardi & Ching, 1979; Cabral & Schatz, 1979). In order to further minimize aggregation, we dissociated the mt translation products in the presence of high concentrations of NaDodSO$_4$ (4%), 2-mercaptoethanol (5%), and urea (7 M) at 90 °C and electrophoresed them under denaturing conditions in the presence of 7 M urea. As shown in Figure 5, on a gel system containing 12% polyacrylamide and 7 M urea, the rat liver, mouse liver, and LES translation products are resolved into 15–18 species of 5.0×10^3 to about 6.9×10^4 daltons. In agreement with the results of gradient gel analysis (Figure 3) the mt translation products in the three systems show significant variations as indicated in columns A–C of Figure 5. Further, high molecular weight components in the range of 7.0×10^4–1.2×10^5 observed in the gradient gel system (Figures 3 and 4) can also be resolved in 8% gels containing 7 M urea (results not presented). In spite of this, however, it is possible that some of these high molecular weight species may represent nonspecific aggregates.

Finally, the molecular complexity and nature of mt translation products were verified by the two-dimensional system similar to that described by O'Farrell (1975). In this system, the translation products are first resolved by isoelectric focusing in the presence of 9 M urea under reducing conditions and then electrophoresed in the presence of 5% 2-mercaptoethanol and 2.3% NaDodSO$_4$. As shown in Figure 6, the mouse liver (A) and rat liver (B) mt translation products resolve into about 24 products in the two-dimensional system. Further, as shown in Table II, a majority of the ^{35}S-labeled products in the mouse and rat liver mt systems exhibit comparable size distribution,

Table II: Size Distribution of Radioactive Polypeptides Synthesized in Mouse and Rat Liver Mitoplasts[a]

spot no.	size ($M_r \times 10^{-3}$) mouse liver	rat liver
1	105	109
2	90	90
3	85	84
4	80	81
5	75	74
6	74	73
7	65*	65.6*
8	58*	58*
9	51*	57
10	43*	56
11	39*	52*
12	35.5*	42*
13	30*	39*
14	26*	36*
15	25*	29.5*
16	24	27
17	22	26.5*
18	19	25*
19	18.5*	20
20	18	19
21	<17	<17
22	<17	<17
23	<17	<17
24	<17	<17

[a] The molecular weight of individual spots (1–24) from the two-dimensional gels in Figure 5 was determined for comparison. Because of the limitations of this gel system, the molecular weights of spots 21–24 (<17 000) could not be determined with reasonable accuracy. The 10 spots marked with asterisks in the rat and mouse liver systems resemble the polypeptide species predicted in the human mt system (Anderson et al., 1981) with respect to size distribution.

although a significant variation in the isoelectric points of these proteins is observed (Figure 6). Furthermore, a number of products in these two systems show significant differences in size (Table II). It should also be noted that both mouse and rat liver mt synthesize about 6 products in the size range of 0.7×10^5–1.0×10^5 daltons, 14 components in the size range of 0.18×10^5–0.66×10^5 daltons, and 4 components smaller than 0.17×10^5 daltons.

mt Protein Synthesis in Whole Cells. In order to determine if the results obtained with isolated mt particles represent the in vivo situation, we have carried out [^{35}S]methionine incorporation in intact cells. The LES cells were labeled in the presence of 300 μg/mL CHX to suppress cytoplasmic translation. The kinetic pattern of [^{35}S]methionine incorporation presented in Figure 7 shows that CHX inhibits the activity by about 90–93% while CAP and CHX together cause inhibition in the range of 97–99%.

The patterns of CHX-resistant translation products in intact cells and the ^{35}S-labeled components synthesized in isolated mitoplasts have been compared in Figure 8. A large number of polypeptides synthesized in intact LES cells (column A) are inhibited by CHX (column B). The CHX-resistant products in column B are severely inhibited by CAP (column C). Further, there is a close qualitative resemblance between the CHX-resistant polypeptides synthesized in intact cells (columns B and D) and the products synthesized in the isolated mitoplasts (column E). The qualitative difference indicated by marked differences in the relative intensities of many of the bands may be due to inherent differences between the in vivo and in vitro systems including amino acid pools and protein synthetic as well as degradation rates. A comparison of the pattern in column E with the schematic presentation in column D indeed suggests that almost all of the polypeptides

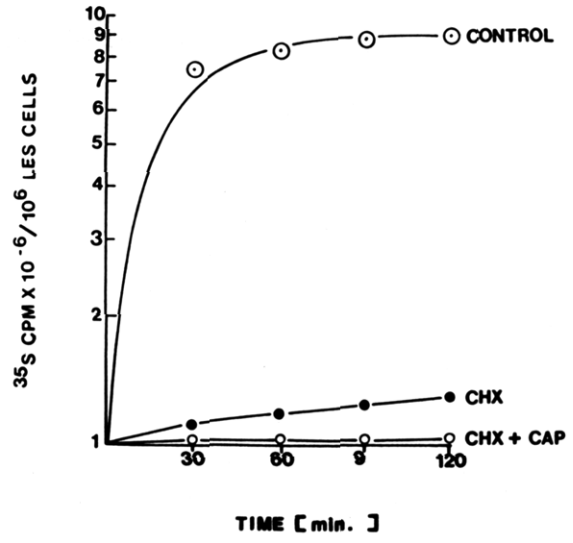

FIGURE 7: Kinetic patterns of [^{35}S]methionine incorporation by intact LES cells. Cells (3×10^6/mL) were labeled with 100 μCi/mL [^{35}S]methionine (600 Ci/mmol) as described under Materials and Methods. Control LES cells labeled in the absence of inhibitors (⊙); LES cells labeled in the presence of 300 μg/mL CHX (●); LES cells labeled in the presence of 300 μg/mL CHX and 500 μg/mL CAP (○). Aliquots (10 μL) were used for determining the hot CCl_3COOH insoluble cpm.

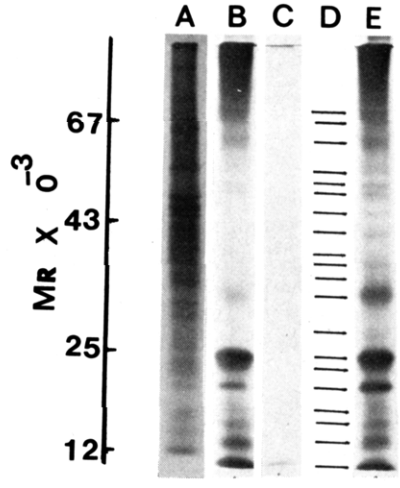

FIGURE 8: Labeling patterns of mt translation products in intact cells and in isolated mitoplasts. Whole cells were labeled with [^{35}S]-methionine for 120 min as described in Figure 7. Labeling of LES mitoplasts with [^{35}S]methionine was for 60 min as described in Figure 1. (Lane A) Cells labeled in the absence of inhibitors; (lane B) cells labeled in the presence of 300 μg/mL CHX; (lane C) cells labeled in the presence of 300 μg/mL CHX and 500 μg/mL CAP; (lane D) schematic representation of bands visualized in lane B; (lane E) LES mitoplasts. About 50 μg of protein in lane A and 150–200 μg of protein in lanes B, C, and E were used. Other details were as described under Materials and Methods.

synthesized in isolated mitoplasts are true mt translation products. Previous attempts to characterize mt translation products in the rat hepatocytes (Gellerfors et al., 1979; Wilson et al., 1981) resulted in the labeling of only six to eight polypeptides. In this respect the >19 CHX-resistant and CAP-sensitive products obtained in the present experiments are probably due to higher incorporation efficiency.

Discussion

Although there have been attempts at identifying mt translation products from mammalian cells (Rascati & Parsons, 1979; Gellerfors et al., 1979), in general the progress has been hampered because of the low levels of incorporation.

Poyton & Kavanaugh (1976) described a procedure to label yeast mt particles in the presence of added cytoplasmic S-100 fraction. This procedure appears to provide an excellent method for high specific activity labeling of yeast mt, and it has been successfully used for the translation of cytochrome oxidase subunits (Poyton & Kavanaugh, 1976). This procedure, however, is only marginally active with mt preparations from LES cells and also from mouse and rat livers. The use of digitonin-stripped particles in an incubation mixture containing low sucrose (0.14 M) and high GTP (2 mM) as described under Materials and Methods yields high enough incorporation to permit further analysis. The higher activity in digitonin particles may be due to the removal of the lysosomal fraction and associated hydrolytic enzymes, or alternately, the removal of the outer membrane may permit partial swelling of mt particles, facilitating higher incorporation.

The presence of protease activity in mt preparations from the animal sources (Wheeldon et al., 1974) is a major concern that can undermine the usefulness of isolated mt particles in the analysis and characterization of translation products. We have, therefore, used two different experimental approaches to determine the extent of such activity in the present system. It has been shown that leupeptin, an inhibitor of cathepsin-like activities, inhibits mt protease activity (Kalnov et al., 1979). Almost identical kinetics of [^{35}S]methionine incorporation (Figure 1) and electrophoretic patterns (results not presented) in the presence and absence of leupeptin strongly suggest that there may not be extensive proteolysis of the products under our experimental conditions. Similarly, most of the largest translation products [(0.8–1.2) $\times 10^5$ daltons] labeled during the 5-min pulse are seen even after a 50-min chase (Figure 4), suggesting negligible proteolytic degradation during the incubation period. It is possible that most of the protease activity in the mammalian mt may be of lysosomal origin (Manjunath et al., 1979) and that the digitonin treatment and subsequent washings used in these experiments may have substantially reduced the level. The residual activity may be inhibited by the phosphate buffer included in the incubation mixture [see Manjunath et al. (1979)].

Detailed analyses of mt translation products in yeast and *Neurospora* have ascribed functional roles for six of the mt products (Schatz & Mason, 1974; Tzagoloff et al., 1979) although additional products of unknown function have been detected in these systems (Douglas & Butow, 1976). In view of this, the large molecular weight products seen in our studies suggest the following possibilities.

(1) Although the pulse–chase experiment indicates that all the ^{35}S-labeled products of $>5 \times 10^4$ daltons may have a relatively long half-life, a precursor role for some of these products cannot be excluded.

(2) Another possibility is that a number of these radiolabeled products may represent the activity of contaminating cytoplasmic polysomes. This appears to be an unlikely possibility for the following reasons. First, the system used for mt translation would be the least favorable for the translation of cytoplasmic polysomes. Results in our laboratory show that the optimum conditions for cytoplasmic polysome translation include 1.8–2.0 mM Mg^{2+} and 120–150 mM KCl (Aroskar et al., 1980). Under the salt conditions (6 mM Mg^{2+} and 60 mM KCl) used in these experiments, cytoplasmic polysomes, even if present, would show only 3–6% activity. Second, digestion of mitoplasts with RNase has no significant effect on the electrophoretic pattern in general and also the labeling of high molecular weight components (Figure 3). Furthermore, the release of ^{35}S-labeled nascent chains from the mt polysome fraction by puromycin is completely inhibited by CAP treatment, suggesting that almost all of the products formed in our mt system are translated on mt-specific ribosomes. Finally, the labeling of polypeptides is refractory to the action of CHX but is highly sensitive to CAP.

(3) Although the results of electrophoretic analysis under highly reducing and denaturing conditions as presented in Figures 3–6 suggest that all of the ^{35}S-labeled components may be true mt translation products, in view of the hydrophobic nature of mt proteins and reports showing covalent joining of mt translation products in yeast (Beattie & Clejan, 1980), it is likely that some of the high molecular weight components in the range of (0.9–1.2) $\times 10^5$ may consist of aggregates. Nevertheless, the synthesis of high molecular weight components in the range of (0.8–1.0) $\times 10^5$ has been shown to occur in other mt systems (Beattie et al., 1979; Alziari et al., 1981).

All the three systems studied synthesize about 24 products in the size range of 6.0×10^3 to about 1.0×10^5 daltons. Furthermore, the products synthesized in isolated mitoplasts closely resemble the in vivo products qualitatively (Figure 8), although there is a significant quantitative difference between the two patterns. This difference may reflect upon the inherent properties of the in vivo and in vitro systems. There is also significant difference between each of the systems studied. In general there is a closer similarity between the two liver systems as compared with the mouse LES tumor system (Figure 3). About 16 out of 24 products in both mouse and rat liver mt have comparable size (Table II). The observed difference in the isoelectric points of several products may reflect sequence variations in the mt DNA from these two cell types. A surprising observation in the present experiments is the synthesis of several additional products in the LES tumor mt. These results, therefore, suggest possible heterogeneity in mt translation products in varied cell types.

Recent DNA and RNA sequence studies suggest that there may be approximately 13 reading frames coding for as many polypeptides in the range of 0.79×10^4–6.6×10^4 daltons in the human mt DNA (Anderson et al., 1981; Montoya et al., 1981). Evidence has also been presented showing that the overall structural organization of mt genome in the bovine and mouse systems may be almost identical (Barrell et al., 1979; Van Etten et al., 1980; Anderson et al., 1981; Bibb et al., 1981). The results presented in Table II show that both rat liver and mouse liver mt synthesize all the 10 polypeptides of 1.8×10^4–6.6×10^4 daltons predicted by the sequence data (Anderson et al., 1981; Bibb et al., 1981). Although not shown in this table, the remaining three smaller polypeptides of 0.79×10^4, 1.0×10^4, and 1.3×10^4 daltons predicted by Anderson et al. (1981) are among the translation products in our system (see Figure 5). To our knowledge, the system described in this paper is the only one so far that is capable of synthesizing all of the 13 polypeptides predicted by the sequence data. The additional mt products seen in the present experiments and also in other reported instances (Attardi & Ching, 1979), therefore, raise several possibilities. It is possible that the higher than expected level of complexity of mt translation products may be due to heterogeneity in mt DNA molecules. The intraspecies polymorphism of mt DNA has been shown to exist in rat liver (Hayashi et al., 1981), *Drosophila* (Reilly & Thomas, 1980), and human mt systems (Brown, 1980). It has also been shown that mt DNAs from different tissues of the same animal may vary with respect to sequence properties (Coote et al., 1979). Second, and a more likely possibility, is that some of the mt polypeptides seen in our experiments may be coded for by mRNAs imported from the cytoplasm.

Experiments are under way to determine the genetic origin of mt translation products not accounted for by the DNA sequence data.

In summary, our results suggest that animal mt systems synthesize 16–24 discrete polypeptides including some unusually large products. The results also show significant physiological and species-specific variations in mammalian mt translation products. The method described for labeling may provide a useful system for characterizing mt translation products in animal cells.

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Kinetics of Acyl Transfer Ribonucleic Acid Complexes of *Escherichia coli* Phenylalanyl-tRNA Synthetase. A Conformational Change Is Rate Limiting in Catalysis†

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ABSTRACT: Kinetics of complex formation between phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} have been measured by the stopped-flow technique. Either the protein intrinsic fluorescence or the fluorescence of the added indicator 6-(*p*-toluidinyl)naphthalene-2-sulfonate was observed. Identical results were obtained with each method. Acyl-tRNAs with variable structures of the acyl and tRNA moieties were examined. Kinetics were measured as a function of pH and at different ionic strengths. Kinetic constants were compared with those of enzymatic phenylalanylation of tRNA^{Phe}. The results are as follows. (1) Phe-tRNA^{Phe} binds to phenylalanyl-tRNA synthetase in two, mutually exclusive types of complexes, one at the tRNA-specific binding site of the enzyme and the other in a region which involves the Phe-specific binding site of the enzyme [Holler, E. (1980) *Biochemistry 19*, 1397–1402]. The Phe site directed association includes a conformational change of the complex that is rate limiting. (2) The conformational change and catalytic tRNA aminoacylation follow similar values of rate constants irrespectively of pH and ionic strength. It is concluded that aminoacylation is limited by the kinetics of a conformational change of the nascent enzyme·Phe-tRNA^{Phe} complex. (3) The nature of Phe site directed binding was probed by variation of the structure of Phe-tRNA^{Phe}. Both the acyl and the tRNA moieties are recognized by the enzyme. Of the acyl moiety, only the phenyl ring but not the amino group is essential for binding. The amino group can be acetylated or replaced by a hydroxyl group. Protonation of the amino group results in loss of Phe site directed binding. It gives a pK_a = 6.9, which is close to that for protonation of a phenylalanine ester. (4) Rate constants were only slightly affected by addition of 200 mM NaCl at pH 7.5, indicating that the contribution by electrostatic forces was probably minimal. Mg^{2+} ions were essential for Phe site directed binding. Complexation of enzyme, Phe-tRNA^{Phe}, and Mg^{2+} either was random or was at preequilibrium with the conformational change. (5) Binding of Phe-tRNA^{Phe} at the tRNA-specific site of the enzyme was studied in the presence of in situ synthesized phenylalanyl adenylate. The reaction was bimolecular with rate constants of 50 μM^{-1} s^{-1} and 15 s^{-1} for association and dissociation, respectively.

The knowledge of the rate-limiting reaction of tRNA aminoacylation is one of the goals directed toward an understanding of the catalytic mechanism. A search for this reaction can be traced back to work by Yarus & Berg (1969) and subsequently to that by Schimmel and co-workers (Schimmel, 1973). Using classical radioactive techniques, they found evidence that dissociation of the enzyme·aminoacyl-tRNA complex was rate limiting. Employment of rapid sampling techniques revealed later that the aminoacyl transfer from AMP to tRNA was the slowest reaction (Pingoud et al., 1973; Bartmann et al., 1975a; Fersht & Kaethner, 1976; Fasiolo & Fersht, 1978).

We have reported in a preliminary note that a complex between phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} [1] exhibits kinetics which are reminiscent of those for tRNA aminoacylation (Holler, 1976). Equilibrium experiments have revealed that the reaction between the enzyme and Phe-tRNA^{Phe} is complex and involves at least two binding sites (Güntner & Holler, 1979). The kinetics, which show similar properties as in the case of tRNA aminoacylation, were found to belong to a type of complex that involved the Phe-specific binding site of the enzyme (Holler, 1976, 1980; Holler et al., 1981a). In the present paper, we attribute these kinetics to a conformational change of this particular enzyme·Phe-tRNA^{Phe} complex. We confirm and extend previous results concerning the structural requirements of an acyl-tRNA and the effects of salt and pH of the reaction mixture.

Materials and Methods

Chemicals. L-[^{14}C]Phenylalanine was a product of Amersham Buchler. TNS was obtained from Serva (Heidelberg). ATP, tRNA^{Phe} from *Escherichia coli* and from brewers yeast

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TNS, 6-(*p*-toluidinyl)-naphthalene-2-sulfonate; Phe-tRNA^{Phe}, L-phenylalanyl ester of tRNA^{Phe}; Phe-tRNA$^{Phe}_{HNO_2}$, L-phenylalanyl ester of tRNA$^{Phe}_{HNO_2}$; Phe(OH)-tRNA$^{Phe}_{HNO_2}$, DL-β-phenyllactyl ester of tRNA$^{Phe}_{HNO_2}$; *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$, *N*-acetylphenylalanyl ester of tRNA$^{Phe}_{Ac}$; Phe-tRNA^{Phe}2′NH, phenylalanyl ester of tRNA^{Phe}2′NH$_2$; Phe-tRNA^{Phe}3′NH, phenylalanyl ester of tRNA^{Phe}3′NH$_2$; Ile-tRNA^{Phe}, isoleucyl ester of tRNA^{Phe}; Phe-tRNA$^{Val}_1$, phenylalanyl ester of tRNA$^{Val}_1$; Phe-tRNA$^{Phe}_{hy}$, phenylalanyl ester of tRNA$^{Phe}_{hy}$; tRNA$^{Phe}_{HNO_2}$, tRNA^{Phe} after treatment with nitrous acid; tRNA$^{Phe}_{Ac}$, tRNA^{Phe} after acetylation of base X; tRNA$^{Phe}_{Ac}$, tRNA^{Phe} cross-linked between positions 8 and 13; tRNA^{Phe}2′NH$_2$, tRNA^{Phe} with the 2′-OH of the terminal adenosine substituted by NH$_2$; tRNA^{Phe}3′NH$_2$, tRNA^{Phe} with the 3′-OH of the terminal adenosine substituted by NH$_2$.

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