Site of Synthesis of the Mitochondrial Cytochromes in Hepatocyte Cultures*

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The biosynthesis of mammalian mitochondrial cytochromes was explored in primary hepatocyte cultures. When these were pulsed with [35S]methionine in the presence of cycloheximide, eight discrete mitochondrial polypeptides were detected by fluorography after their resolution under denaturing conditions by polyacrylamide gel electrophoresis. Since the pulse labeling of the polypeptides was sensitive to chloramphenicol, an inhibitor of mitochondrial translation, they must be translated on mitochondrial ribosomes. Three were identified as the largest subunits of cytochrome oxidase by their immunoprecipitation with antibody directed against purified rat liver cytochrome oxidase. Another (Mr = 28,000) was identified as one of eight subunits of purified rat liver cytochrome b-ε complex by its immunoprecipitation with antibody directed against bovine heart b-ε complex. Since cytochrome b apoprotein is the only product of the mitochondrial genome in the yeast cytochrome b-ε complex (Kriek, J., Bechmann, H., van Hemert, F. J., Schweyam, R. J., Boer, P. H., Kaudewitz, F., and Groot, G. S. P. (1979) Eur. J. Biochem. 101, 607-617), the results strongly suggest that the M r = 28,000 subunit of liver b-ε complex is cytochrome b apoprotein. Thus the contribution of the mitochondrial translation system to the cytochrome complexes in liver is identical to that of yeast and Neurospora, and there appears to be no deletion or transfer to the nuclear genome of structural genes for mitochondrial synthezised cytochromes during eukaryotic evolution.

The mitochondrial inner membrane is a mosaic of both nuclear and mitochondrial gene products (1). Cytochrome oxidase, cytochrome b-ε complex, and adenosine triphosphatase complex are multisubunit mitochondrial membrane enzymes which have been shown in yeast to have one or more mitochondrially translated polypeptides in addition to cytoplasmically translated polypeptides. Since the growth restraints, differentiation, and phylogeny of mammalian cells differ from yeast, the biogenesis of mitochondria in these cells may have a uniqueness that differentiates it from that of lower eukaryotes. Moreover, the size of the mammalian mitochondrial genome is one-fifth that of yeast (3) and this poses the possibility that some mitochondrial structural genes may be deleted or transferred to the nuclear genome during mammalian evolution. To address these points we have begun to study the synthesis and metabolism of the polypeptide components of the mitochondrial inner membrane in primary cultures of rat liver hepatocytes. These cells remain viable for up to 1 week in culture conditions, do not divide, but retain their ability to synthesize proteins and respond to natural metabolic stimuli (4). In addition, they have metabolic processes which approximate those of cells in vivo but are amenable to labeling procedures that can identify the site of synthesis and define the maturation pathways of mitochondrial polypeptides.

Only the site of synthesis of cytochrome oxidase has been studied in mammalian systems. The larger subunits of rat liver cytochrome oxidase were found to be translated in isolated mitochondria (5, 6). The use of isolated mitochondria for study of membrane synthesis, however, has a number of limitations (1). Moreover, inadequate electrophoretic resolution of cytochrome oxidase subunits in one of these reports on the rat liver enzyme (6) and another on the Xenopus enzyme (7) has confused the precise identity and number of those subunits synthesized on mitochondrial ribosomes. Hare et al. (8) recently identified the largest three subunits of human cytochrome oxidase as three of 16-19 mitochondrial translation products in HeLa cells by co-purifying cytochrome oxidase from cycloheximide blocked HeLa cells and from human placenta mitochondria. In this report we detect eight polypeptides that are synthesized on mitochondrial ribosomes in primary hepatocyte cultures. We identify three of these as the largest subunits of cytochrome oxidase and another as a subunit of the cytochrome b-ε complex. The remaining subunits of these enzymes are cytoplasmically translated since their synthesis is resistant to chloramphenicol but sensitive to cycloheximide.

EXPERIMENTAL PROCEDURES

Preparation of Cytochrome Oxidase—All steps were performed at 0-4 °C. Mitochondria were prepared from 200- to 250-g rats and stored frozen at -20 °C. Cytochrome oxidase was prepared as described by others (7, 10, 11) but with the following modifications. Since extraction of mitochondria with Triton X-114 (10) did not produce, in our hands, a good separation of cytochromes, a Triton X-100 separation was used instead. Washed mitochondria were resuspended in 0.25 M sucrose, 0.02 M KPO4, pH 7.0 (SP buffer), to 25 mg of protein/ml as determined by the biuret assay (12). Solid KCl and 20% (w/v) Triton X-100 were added stepwise to 1.0 M and 1 mg/ml of protein, respectively. After centrifugation at 105,000 × g for 45 min, the red supernatant was removed, and the green residue was washed once by homogenization in SP buffer and solubilized in 25 ml of 5% Triton X-100, 0.1 M KPO4 (pH 7.0) for each 1 g of membrane protein processed. The solubilized enzyme was diluted with 3 volumes of cold distilled water and chromatographed on a DEAE-Sephadex column as described (7). The eluate containing the enzyme was fractionated with ammonium sulfate in the presence of 3% potassium cholate and 1% Triton X-100 (7) and subjected to gel permeation chromatography.

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on Sepharose 6B (11). Fractions equal to one-half the peak fraction absorbance at 423 nm were collected, pooled, and concentrated by removal of the carrier ampholyte and addition of 0.5 M NaSO4 (11).

Preparation of Cytochrome b-5 Complex—This enzyme was prepared from either 1 g of frozen bovine heart mitochondria (13) for antisera production or 0.5–2.0 g of rat liver mitochondria (9) by method b of the procedure of von Jagow et al. (14). Since the final product obtained from rat liver mitochondria with this procedure was only 60% pure by spectral determination (Table II), an additional step was included. The b-5, eluting from the hydroxylapatite column with 0.15 M NaPO4, 0.05% Triton X-100 was made 3% in K-cholate and 43% saturated in ammonium sulfate by addition of 20% recrystallized K-cholate (pH 8.0) and saturated, neutralized, enzyme grade ammonium sulfate. After standing at 0°C for 30 min, the suspension was centrifuged at 25,000 × g for 15 min. The recovered floating red pellet was resuspended in a minimal volume of 0.05 M Tris-HCl (pH 8.0).

Preparation of Rabbit Immunoglobulins—Two 1-kg rabbits were bled before injections to obtain preimmune sera. Rabbits received either 1.5 mg of purified rat liver cytochrome oxidase or 2 mg of purified bovine heart cytochrome b-5, each emulsified in 1 volume of Freund’s complete adjuvant, in several intradermal sites in the back and intramuscularly in each hind leg. Each rabbit then received a 1-mg injection of appropriate enzyme in 1 volume of Freund’s incomplete adjuvant on the fourth week and again on the fifth week. Blood was collected on the sixth week. Serum was prepared from clotted blood and the immunoglobulin fraction was prepared by 33% saturated ammonium sulfate fractionation (15). Two out of three rabbits responded to cytochrome oxidase while one of two responded to cytochrome b-5.

Cell Isolation and Culture—Hepatocytes were isolated from livers of 180- to 200-g male rats (16). Isolated cells were at least 80% viable as determined by trypsin blue exclusion. Cells were plated in Falcon tissue culture dishes (60 × 15 mm) to a concentration of 5 × 10⁶ cells/plate in L-15 media supplemented with l-glutamine (0.3 g/l), 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), insulin (0.05 µg/ml), and dextrose (1.5 mg/ml). Cells were incubated at 37°C in humidified air. The media was replaced after 4 h to remove unattached cells and at 24-h periods thereafter. Fifty per cent of the cells attach in the initial 4 h and 80-90% flatten within the first 24 h. All labeling experiments were done on cells between 24 and 48 h of culture.

Cell Labeling—Four or five plates of cells (2.7 × 10⁶ cells/plate) were exposed to cycloheximide (200 µg/ml) or chloramphenicol (50 µg/ml) separately or in combination 1 h before addition of label. Plates were then washed twice with methionine-deficient L-15 media in the absence of serum, and the appropriate inhibitors were added, followed by [35S]methionine (90 µCi/plate). Cells were labeled for 2 h, washed 3 times with complete medium, and chased for 2 h in complete medium. After two washes with cold saline, the cells were harvested and suspended in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4 (ST buffer), to 2.5 × 10⁶ cells/ml.

Preparation of Cell Lysate and Membrane—Harvested cells were sonicated (Heat Systems Model W-22F) at output setting 2.5 for 1 min in four 15-s bursts and the mitochondria fraction was prepared by differential centrifugation (17). For immunoprecipitation of cytochrome oxidase, the mitochondria were solubilized in 0.4 ml of 5% (w/v) Triton X-100, 5 mM EDTA, 1 M KCl, and 0.05 M Tris-HCl (pH 7.4), diluted to 2 ml with 0.05 M Tris-HCl (pH 7.4), and centrifuged at 81,000 × g for 30 min. For immunoprecipitation of cytochrome b-5, the mitochondria in 1.0 ml of ST buffer were treated with 10 µl of antymycin A/mg of protein, diluted in 0.5 ml of 10% (w/v) Triton X-100, 0.5 M NaCl, 0.05 M Tris-HCl (pH 7.4), and centrifuged at 81,000 × g for 30 min. To identify the total mitochondrial translation products, the crude mitochondria fraction was suspended in ST buffer to 4.0 ml, sonicated for 2 min at output setting 4, and centrifuged at 25,000 × g for 15 min, and the membrane fraction was pelleted at 105,000 × g for 1 h and dissolved in 0.05 ml of SDS-urea dissociating buffer (18).

Immunoprecipitation—Preimmune immunoglobulin (1 mg) was added to 2 ml of lysate and incubated 8 h at 0°C. After centrifugation for 5 min in an Eppendorf microfuge (Brinkmann), 1.0 mg (cytochrome oxidase) or 10 mg (b-5) of immunoglobulin was added and incubated at 0°C for 36 h. The immunoprecipitates were removed by microfuge centrifugation, washed twice with 1% Triton X-100, 0.2 M KCl, 0.05 M Tris-HCl (pH 7.4), washed once in H2O, and dissolved in 0.05 ml of SDS-urea dissociating buffer (18).

The abbreviation used is: SDS, sodium dodecyl sulfate.

**Table I**

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<th>Membrane</th>
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<th>5’ Triton X-100 extract</th>
<th>DEAE-Sephalose peak fractions</th>
<th>38% saturated ammonium sulfate-insoluble cytochrome oxidase</th>
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1. The abbreviation used is: SDS, sodium dodecyl sulfate.
Fig. 1. SDS-urea gel electrophoresis of immunoprecipitated cytochrome oxidase. Cytochrome oxidase was immunoprecipitated from 1.5 ml of a 1% Triton X-100 extract of rat liver mitochondria as described under "Experimental Procedures" and subjected to SDS-urea gel electrophoresis and staining with Coomassie blue (c). Other samples include 150 µg of washed rat liver mitochondrial membrane protein (a), 50 µg of purified rat liver cytochrome oxidase (b and d), 25 µg of bovine heart cytochrome oxidase (c), and 53 µg of immunoglobulin (f). Subunits are numbered according to Downer et al. (18); HC, immunoglobulin heavy chain; LC, immunoglobulin light chain. The apparent molecular weights of the rat liver subunits are as follows: I, 43,000; II, 23,000; III, 18,000; IV, 13,500; V, 9,200; VI, 7,400; VII, 5,800. Molecular weight markers used were as follows: bovine albumin, 68,000; ovalbumin, 45,500; chymotrypsinogen A, 23,600; bovine globin, 16,000; horse cytochrome c, 13,400; insulin A chain, 2,900; and insulin B chain, 3,400.

Fig. 2. SDS-urea gel electrophoresis of isolated and immunoprecipitated rat liver and bovine heart cytochrome b-c1 complex. All samples were stained with Coomassie blue. A, 54 µg of purified bovine heart b-c1; B, 50 µg of purified rat liver b-c1 complex; C, rat liver b-c1 complex immunoprecipitated from 2.0 ml of a 0.5% Triton X-100 extract of rat liver mitochondria. HC, immunoglobulin heavy chain; LC, immunoglobulin light chain.

shown in Fig. 1. Purified enzyme (lanes b and d) contained five distinct subunits (I, II, IV, V, VI), a trace of another (III), and three small (Mr ≈ 5,800) polypeptides which we designate collectively as VII in accord with the numbering system of Downer et al. (18). Apparent Mr of each of these subunits is given in the legend to Fig. 1. Purified beef heart cytochrome oxidase used as a reference (Fig. 1, lane e) showed the seven major subunits (I-VII) described by Downer et al. (18). Bovine heart subunits I, II, and III co-migrate with the three largest rat liver subunits. Coomassie blue-stained, immunoprecipitated rat liver enzyme (Fig. 1, lane c) showed subunits I, II, IV, V, VI, and VII in addition to increased amounts of subunit III and an additional polypeptide migrating just ahead of subunit VI.

Properties and Subunit Structure of Cytochrome b-c1—Cytochrome b-c1 complex was prepared from beef heart mitochondria with a yield and purity identical to that described (14). Application of combined peak fractions from hydroxyapatite chromatography to a column of Sepharose 6B in 0.5% Triton X-100, 0.1 M NaPO4 (pH 7.4) failed to improve the specific heme b or c1 content or reduce the number of component polypeptides seen upon SDS-urea polyacrylamide gel electrophoresis. Application of the same isolation methodology to rat liver mitochondria resulted in a lower yield of b and c1 cytochromes than found with beef heart (Table II). Approximate one-third of the heme b was eluted from the hydroxyapatite column in 0.085 M NaPO4, 0.5% Triton X-100, 0.05 M NaCl (pH 7.2) and this had an α-peak at 558 nm. The remaining heme b (α-peak = 562 nm) eluted with 0.15 M NaPO4, 0.05% Triton X-100 (pH 7.2). Ammonium sulfate fractionation of the latter material produced a preparation of high specific heme b and c1 content (Table II).

Analysis of the subunit composition of rat liver and bovine heart b-c1 preparations is shown in Fig. 2. Isolated bovine heart b-c1 (Fig. 2A) showed six polypeptides having apparent Mr of 47,000, 46,000, 30,000, 16,000, 12,000, and 8,000. Rat liver b-c1 (Fig. 2B) showed an additional slowly migrating component (Mr = 50,000) and two polypeptides (Mr = 32,000 and 28,000) in place of the Mr = 30,000 bovine heart polypeptide. The Mr = 50,000 polypeptide appears to be a contaminant since it is not present in the immunoprecipitated enzyme (Fig. 2C; Fig. 4). Covalently attached cytochrome c1 migrated with the Mr = 32,000 subunit as determined by marking its position in unstained gels with ink and proceeding with staining and destaining.

Anti-b-c1 immunoglobulin precipitated only 1/3–1/2 of rat liver cytochromes as b and c1. The immunoprecipitates were resolved into only b-c1 subunits and heavy and light chain γ-globulin by SDS-urea polyacrylamide gel electrophoresis (Fig. 2C). In the case of both the bovine heart and rat liver enzymes, addition of antimycin to mitochondria before their extraction appears to be essential to stabilize the complex during the precipitation reaction.
Cytochrome Synthesis in Hepatocytes

Fig. 3. Site of synthesis of rat liver cytochrome oxidase subunits. The 24-h hepatocyte cultures were labeled with $[^35]S$-methionine as described under "Experimental Procedures" in the presence of cycloheximide (CHI), chloramphenicol (CAP), no inhibitors (none), or both (CHI, CAP). Mitochondria were isolated from the cells, lysed, and cytochrome oxidase immunoprecipitated, and washed immunoprecipitates were analyzed on SDS-urea polyacrylamide gels. Pulse-labeled polypeptides were visualized by fluorography and photographed (A) or scanned with white light in a densitometer (B). I–VII, cytochrome oxidase subunits in the numbering system of Downer et al. (18). a ($M_r = 55,000$), b ($M_r = 47,000$) and c ($M_r = 38,000$), methionine-labeled polypeptides which, e also precipitated by cytochrome oxidase antibody but are resolved from authentic enzyme subunits. Lanes labeled 22 none (22-day exposure) and 4 none (4-day exposure) are identical except for exposure times.

Site of Synthesis of Cytochrome Oxidase Subunits—The 24-h hepatocyte cultures were labeled in the presence or absence of cycloheximide and/or chloramphenicol, and cytochrome oxidase was immunoprecipitated from mitochondria of these cells. Approximately half of the chloramphenicol-sensitive, cycloheximide-resistant radioactivity in the mitochondria fraction was immunoprecipitated by cytochrome oxidase antibody. Labeled polypeptides of the immunoprecipitates were resolved by SDS-urea polyacrylamide gel electrophoresis and visualized by fluorography (Fig. 3). The results show that subunits I, II, and III are labeled in the presence of cycloheximide and thus synthesized on mitochondrial ribosomes. Subunits IV, V, VI, and VII are labeled in the presence of chloramphenicol and are thus synthesized on cytoplasmic ribosomes. This result agrees with those obtained with HeLa cells (8) and isolated rat liver mitochondria (5). A small fraction of subunit II translation appears resistant to inhibition by chloramphenicol. Most likely this represents leak through the chloramphenicol block, but it cannot be excluded that a minor antigenic component is immunoprecipitated with enzyme subunits. Heavily labeled component b as well as components a and c are also immunoprecipitated by the antibody and shown to be translated cytoplasmically by their appearance in chloramphenicol blocked cells but not cycloheximide blocked cells. These same polypeptides are present in cells labeled in the absence of inhibitors (Fig. 3, 22 none). That component b is not identical to subunit I is apparent by its absence in cycloheximide-blocked cells and slightly lower mobility ($M_r = 47,000$) than subunit I ($M_r = 43,000$). The mobility difference is more apparent when the gel is exposed for a shorter period of time (Fig. 3, 4 none). Since a cytoplas- mically translated polypeptide has been proposed to serve as a precursor to cytochrome oxidase subunits IV–VII in yeast (26, 27) and rat liver (11), we attempted to determine if components a, b, or c could be pulse-labeled and chased into the low molecular weight cytochrome oxidase subunits. Label appeared simultaneously in components a, b, and c and subunits IV–VII when cells were pulsed for 30–120 min with $[^35]S$-methionine in the absence of translation inhibitors. A 4-h cold chase after 120 min of labeling did not result in the presence of additional radioactivity in subunits IV–VII, although components a, b, and c were no longer detected. Immunoprecipitates from the microsome fraction of pulse-labeled hepatocytes contained a polypeptide that co-migrated with component b but also proportional amounts of mature cytochrome oxidase subunits I–VII. These experiments indicate

Fig. 4. Site of synthesis of rat liver cytochrome b–c$_1$ subunits. The 24-h hepatocyte cultures were labeled, extracted, and immunoprecipitated as described under "Experimental Procedures." Stained polyacrylamide gel and fluorograms were scanned at 600 nm in a densitometer. A, 50 $\mu$g of Coomassie blue-stained, isolated rat liver cytochrome b–c$_1$ complex; B and C, fluorographed, immunoprecipitated rat liver cytochrome b–c$_1$ complex prepared from hepatocyte cultures labeled in the presence of chloramphenicol (B) or cycloheximide (C).
that, although components a, b, and c are not stable end products of labeling, they also do not appear to serve as intermediates in the synthesis of low M, cytochrome oxidase subunits. Since these components are also not authentic cytochrome oxidase subunits, we believe them to be contaminants which co-immunoprecipitate with cytochrome oxidase subunits.

Site of Synthesis of Cytochrome b-c1 Subunits—The 24-h hepatocyte cultures were labeled in a manner identical to that described above for cytochrome oxidase. The mitochondrial lysates were prepared and cytochrome b-c1-immunoprecipitated with specific immunoglobulin, and the polypeptides in the immunoprecipitates were visualized by fluorography. Densitometer tracings of the results (Fig. 4) show that only the M, = 28,000 subunit is translated in the presence of cycloheximide. The M, = 47,000, 46,000, 32,000, 16,000, and 12,000 subunits are resistant to chloramphenicol inhibition but sensitive to cycloheximide inhibition, showing their cytoplasmic site of translation. The M, = 50,000 and 8,000 subunits were not visible by fluorography in the presence of either inhibitor, indicating they either had a low methionine content or were not immunoprecipitated by antibody.

Number of Mitochondrial Translation Products—When hepatocyte cultures were pulse-labeled with [35S]methionine, most (90%) of the incorporated radioactivity was sensitive to cycloheximide while an additional 5% was inhibited by chloramphenicol. After 2 h or more of labeling, cycloheximideresistant incorporated radioactivity was twice that resistant to both cycloheximide and chloramphenicol, the difference representing mitochondrial translation products. Although a number of labeled bands with M, > 50,000 appeared in cells labeled in the presence of cycloheximide, traces of these components were also present in cycloheximide plus chloramphenicol blocked cells (Fig. 5) and, thus, appear to be a result of leak through the cycloheximide block. Since we have not been able to eliminate this background labeling by employing longer chase periods, more radioactivity, or additional cytoplasmic translation inhibitors, the labeled polypeptides above M, = 50,000 appear as a necessary consequence of the sluggish rate of mitochondrial translation in non-growing cells, where incorporated radioactivity is only twice that of inhibitor-resistant, background labeling. The eight discrete bands of M, = 50,000 or less were present in cells labeled in the presence of cycloheximide but were absent from cells labeled in the presence of both cycloheximide and chloramphenicol (Fig. 5). When immunoprecipitated cytochrome oxidase and cytochrome b-c1 from cells labeled in the presence of cycloheximide were run on an adjacent lane to mitochondrial membranes obtained from similarly labeled cells, those mitochondrial translation products were readily identified and are labeled in Fig. 5. We designate the remaining four mitochondrial translation products by their apparent M,.

**Discussion**

Although they are not in metabolic steady state, primary hepatocyte cultures approximate normalcy in cellular function and structure (4). We are finding these cells to be a convenient system to study the synthesis of the mitochondrial membrane and its regulation by metabolic and hormonal influences. The unusually low concentration of respiratory components in most secondary cell lines hinders isolation and metabolic study of mitochondrial polypeptides in these cells. By contrast, the concentration of respiratory chain enzymes in 24-h hepatocyte cultures is no less than that in isolated cells.2 Nondividing primary hepatocyte cultures, however, incorpo-

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rate radioactive amino acids into mitochondrial protein at a lower rate than found in HeLa cells and a hepatoma cell line we have examined. Despite this handicap, eight chloramphenicol-sensitive bands are seen by fluorography after one-dimen-
sional resolution of mitochondrial membrane proteins labeled in the presence of cycloheximide. The number (8) and size range (M_r = 50,000-14,000) of these components agree with those numbers found in yeast (12 polypeptides, M_r = 52,000-7,500; Ref. 28). Neurosora (9 polypeptides, M_r = 60,000-
11,000, Ref. 29), Xenopus (9 polypeptides, M_r = 44,000-17,500; Ref. 7), isolated rat liver mitochondria (9 polypeptides, M_r = 50,000-10,000; Ref. 30), and HTC cells (9 polypeptides, M_r = 50,000-10,000; Ref. 30). In the yeast system, 20 polypeptides whose synthesis is inhibited by chloramphenicol, but not by cycloheximide, were seen in high resolution polyacrylamide gradient gels (31). Attardi and Ching (32) found 18-19 chloramphenicol-sensitive polypeptides synthesized in the presence of cycloheximide in HeLa cells growing in suspension culture. The reason for the difference between our finding only 8 cycloheximide-resistant polypeptides and those of the latter investigators is not readily apparent. Since we have used the same gel electrophoresis system as Attardi and Ching (32), it seems unlikely the detection systems account for the observed differences. It is doubtful that many more structural genes are present in HeLa than rat liver mitochondria since little difference has been noted in the complexity of rodent and human mitochondrial DNA (3). Since we find these same eight cyclo-
heximide-resistant protein products in a hepatoma cell line, it seems unlikely that differences between tumor and non-
tumor cells account for the discrepancy. Proteolysis of poly-
peptides during labeling or isolation could have produced additional components of high mobility in HeLa cell products. Lastly, the possibility of precursor or post-translationally modified forms of mitochondrial translated polypeptides appearing on denaturing gels is a possibility since the 18-19 polypeptides seen by Attardi and Ching (32) were found in cells pulsed with [35S]methionine but not chased with cold methionine. Whether the number of stable mitochondrionally translated polypeptides is in the range of 8-9 or 18-20 is a question which should be resolved before the identification of specific polypeptides can proceed.

Our finding that three of the mitochondrially translated polypeptides are cytochrome oxidase subunits I, II, and III confirms results obtained with isolated rat liver mitochondria (5) and HeLa cells (8) as well as yeast and Neurosora. It is most surprising that in hepatocytes more than 50% of cyclo-
heximide-resistant incorporation can be found in cytochrome oxidase. These results cannot be explained by differences in the methionine content among the mitochondrially translated polypeptides since the methionine content of bovine heart cytochrome oxidase subunit I and II is not unusually high (33). The low content of radioactivity in pulse labeled subunit III is explained partly by its decreased methionine content (33) and partly by difficulties in its solubilization in prepara-
tion for gel electrophoresis (18).

Our finding that only one mitochondrially translated polypeptide is present in cytochrome b-6-1 complex agrees with results in yeast (34) and Neurosora (35). Since a M_r = 31,000 polypeptide of yeast b-6-1 complex was shown genetically (36) and biochemically (35) to be cytochrome b apoprotein, the cycloheximide-resistant, chloramphenicol-sensitive M_r = 28,000 polypeptide of hepatocyte mitochondria b-6-1 complex is undoubtedly apocytochrome b. This result means that the structural gene for apocytochrome b is not a casualty of deletion of 80% of the lower eukaryotic mitochondrial genome during evolution of mammals. The identification of a specific

rat liver polypeptide as apocytochrome b is also significant in that the identity of the polypeptide associated with heme b in preparations of bovine heart b-c_1 is still uncertain (37). Two bovine heart polypeptides of M_r = 30,000 and 28,000 have been tentatively identified as cytochrome c_1 and b apoproteins, respectively, on the basis of fractionation studies (38). The two rat liver cytochrome apoproteins are easily resolved into M_r = 32,000 and 28,000 components of which the former is cytochrome c_1 and the latter apocytochrome b.

Two investigators have described the presence of both cytoplasmic and mitochondrial pools of an M_r = 48,000-55,000 putative polypeptide precursor to the cytoplasmically translated polypeptides of cytochrome oxidase (11, 26, 27). Two recent reports (39, 40), however, provide strong evidence against the existence of a polypeptide precursor to yeast cytoplasmically translated polypeptides in favor of smaller, cytoplasmically located, discrete precursors to all four sub-
units. We detected three mitochondrial polypeptides which were precipitated by and in the same size range as yeast and rat liver high M_r putative precursors but were not mature enzyme subunits. Although none of these components were stable end products of labeling, their appearance in the mem-
brane did not precede the appearance of cytochrome oxidase subunits IV-VII as would be expected if any fulfilled a pre-
cursor role. Also, we found no evidence for polypeptides antigenically related to cytochrome b-c_1 which were not ma-
ture subunits of this enzyme. For these reasons our work does not support the existence of large pools of precursors to mature subunits of respiratory cytochromes in mammalian mitochondrial membranes as proposed by others (11, 26, 27).

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