

Integrity of Mitochondria in a Mammalian Cell Mutant Defective in Mitochondrial Protein Synthesis

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ABSTRACT A defect in mitochondrial protein synthesis has previously been identified in the respiration-deficient Chinese hamster lung fibroblast mutant V79-G7. The present work extends the characterization of this mutant. A more sensitive analysis has shown that mutant mitochondria synthesize all mitochondrially encoded peptides, but in significantly reduced amounts. This difference is also seen when isolated mitochondria are tested for in vitro protein synthesis. To distinguish between a defect in the translational machinery and a defect in the transcription of mitochondrial DNA, we investigated the synthesis of the 16S and 12S mitochondrial rRNA species and found them to be made in normal amounts in G7 mitochondria. These rRNA species appear to be assembled into subunits whose sedimentation behavior is virtually indistinguishable from that of the wild-type subunits. We also examined the consequences of the defect in mitochondrial protein synthesis on mutant cells and their mitochondria-utilizing techniques of electron microscopy, two-dimensional gel electrophoresis and immunochemical analysis. G7 mitochondria have a characteristic ultrastructure distinguished by predominantly tubular cristae, but the overall biochemical composition of mitochondrial membrane and matrix fractions appears essentially unaltered except for the absence of a few characteristic peptides. Specifically, we identify the absence of two mitochondrially encoded subunits of cytochrome *c* oxidase on two-dimensional gels and demonstrate a drastic reduction of both cytoplasmically and mitochondrially synthesized subunits of this enzyme in immunoprecipitates of G7 mitochondria.

The characterization of respiration-deficient (res^-) mutants in *Saccharomyces* and *Neurospora* has led to the identification of several mitochondrial and nuclear genes whose products are required to maintain functional mitochondria. The majority of structural proteins and enzymes comprising the mitochondria are encoded by the nuclear genome and translated on cytoplasmic ribosomes. Mitochondrial DNA codes for the mitochondrial rRNAs, for most, if not all, mitochondrial tRNAs, and for about one dozen proteins which are translated on mitochondrial ribosomes in the organelle. In yeast these proteins include three of seven subunits of cytochrome *c* oxidase (complex IV), one of seven subunits in coenzyme QH₂-cytochrome *c* reductase (complex III), and at least two of ten in the oligomycin-sensitive ATPase (complex V) (3, 29). Respiratory complexes III, IV, and V are firmly embedded in the inner membrane. Those of their subunits synthesized in the mitochondria are very hydrophobic and serve to some extent as anchors for the more hydrophilic, cytoplasmically synthesized subunits (21, 27). There is good evidence that the cytoplasmically made subunits of the ATPase are made as precursors

which are processed during assembly into the inner mitochondrial membrane (14).

It is therefore not unexpected that the mitochondria of many cytoplasmic res^- (petite) mutants of yeast are reduced in number and have little internal structure (32). These mutants frequently have large deletions of the mitochondrial genome and are consequently unable to synthesize several or all mitochondrially encoded proteins (24). Such observations suggest that this class of proteins may be critical for maintaining the functional and structural integrity of the organelle in yeast.

The present study examines the morphology, composition, and residual protein and RNA synthesis of mitochondria in a res^- mammalian fibroblast mutant which has properties reminiscent of the petite phenotype in yeast, i.e., a defect in mitochondrial protein synthesis leads to multiple enzyme deficiencies (9). This mutant cell line V79-G7 was isolated as one of a group of 35 res^- mutants in Chinese hamster lung fibroblast lines V79 and CCL16 (8). These mutants fall into seven complementation groups (26), and the biochemical defect has been localized in a number of these (6, 25, 4). The mutant G7 is the

only representative of its complementation group. G7 mitochondria exhibit greatly reduced cytochrome *c* oxidase and oligomycin-sensitive ATPase activities, and spectrophotometric analysis has shown a virtually complete absence of cytochromes *aa₃* and *b* (9). The characterization of the G7 cell line is extended in the present work to show that despite very low levels of mitochondrial protein synthesis, normal amounts of mitochondrial rRNA are synthesized and assembled into subunits whose sedimentation behavior is virtually indistinguishable from that of the wild-type subunits. We also demonstrate that G7 cells maintain large numbers of mitochondria with a distinctly altered morphology but essentially normal biochemical composition.

MATERIALS AND METHODS

Cells and Cell Culture

The parental Chinese hamster lung fibroblast line V79 was kindly provided by Dr. R. Klevecz (City of Hope, Duarte, Calif.). The res⁻ cell line G7 was isolated from V79 cells and maintained as previously described (8).

Electron Microscopy

Cells were trypsinized and collected by centrifugation. The cell pellet was fixed with 2% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, followed by 1% OsO₄ in 0.2 M phosphate buffer. After dehydration in a series of graded ethanol-water mixtures the cells were embedded in Epon A for sectioning. Fixation, sectioning, electron microscopy, and photography were performed by Dr. P. Farnsworth (Department of Biology, University of California, San Diego).

Isolation of Mitochondria and Subfractionation

Mitochondria were isolated by differential centrifugation as described (9). Where indicated, mitochondria were further purified by resuspending the washed pellet from a 10,000 g centrifugation in 200 μ l of homogenizing buffer (250 mM sucrose, 50 mM Tris-HCl, 2 mM EDTA, pH 7.4), layering the mitochondria on top of two layers of sucrose (1.7 and 1.0 M), and centrifuging at 100,000 g for 45 min in an SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The mitochondrial layer that formed between the sucrose layers was removed with a Pasteur pipet and washed several times with homogenizing buffer. The resulting mitochondrial fraction was judged to be essentially free of microsomal contamination by the absence of the microsomal marker enzyme activity NADPH-cytochrome *c* reductase (16).

Mitochondrial membranes were isolated by lysis with digitonin (1.3 mg/10 mg mitochondrial protein) followed by Lubrol treatment (1.0 mg/10 mg mitochondrial protein) and centrifugation at 130,000 g for 60 min (23). Mitochondrial matrix proteins were collected in the 10,000 g supernate of Lubrol-lysed mitoplasts from which intermembrane space proteins had previously been removed by digitonin treatment (23). Purity of membrane fractions was confirmed by the presence of inner membrane marker enzyme cytochrome *c* oxidase (9) and outer membrane marker monoamine oxidase (31), and the absence of intermembrane space marker adenylate kinase (23) as well as the absence of matrix malate dehydrogenase activity (19). Similarly, purity of the matrix fraction was confirmed by the presence of malate dehydrogenase activity and the absence of other marker enzyme activities.

Labeling of Mitochondria with [³⁵S]methionine

Whole cells were labeled with [³⁵S]methionine (sp act >800 Ci/mmol) as described by Ditta et al. (9). When employed, chloramphenicol (CAP) was added at 200 μ g/ml for 5 h before addition of label to ensure effective inhibition of mitochondrial protein synthesis, while emetine-HCl was added at 100 μ g/ml 30 min before labeling when inhibition of cytoplasmic protein synthesis was desired. After the labeling and chase periods, the cells were trypsinized and mitochondria were isolated and fractionated as described above.

Polyacrylamide Gel Electrophoresis

Before one-dimensional SDS polyacrylamide gel electrophoresis, samples were solubilized by heating at 100°C for 5 min in 50 mM Tris HCl, 2% SDS, and 5% β -mercaptoethanol, pH 7.4. Electrophoresis in 12.5% acrylamide (1:16 or 1:30 bis:acrylamide) was performed on 1-mm slab gels by the method of Laemmli

and Favre (12). For two-dimensional gel electrophoresis, mitochondria, mitochondrial membranes, or matrix proteins were solubilized with 2% SDS and 5% β -mercaptoethanol as described above and then prepared for isoelectric focusing according to the method of Ames and Nikaido (1). Two-dimensional nonequilibrium pH gradient gel electrophoresis, employing isoelectric focusing in the first dimension (pH 3.5–10) and SDS polyacrylamide gel electrophoresis (12.5 or 10–12.5% gradient acrylamide) in the second dimension, was performed as described by O'Farrell et al. (20). After electrophoresis, slab gels were fixed in 50% TCA, stained with Coomassie Blue, and destained with 2:3:35 acetic acid:methanol:water. Slab gels were then treated with En³Hance (New England Nuclear, Boston, Mass.) for 1 h, washed with water, and dried under vacuum. Fluorography was performed with preflashed Eastman Kodak X-OMAT (XR-5) x-ray film at -70° for various lengths of time as required. Densitometric scans of one-dimensional fluorograms were performed with a Joyce-Loebl densitometer at 500 nm.

Immunoprecipitation

Anti-cytochrome *c* oxidase antibody against the purified beef heart enzyme was raised in rabbits in the laboratory of Dr. E. Racker and was a generous gift of Drs. R. Carroll and S. J. Singer. Anti-cytochrome *c* oxidase IgG was reacted with V79 mitochondria which had been solubilized in 10 mM sodium phosphate, 100 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Indirect immunoprecipitation with *Staphylococcus aureus* (Pansorb; Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) was performed as described (15). After removal of *S. aureus*, the immunoprecipitates were applied directly to one-dimensional polyacrylamide gels or precipitated in cold 10% TCA, collected on filters, and measured for incorporation of [³⁵S]methionine in a Beckman Scintillation Counter, model LS8000.

Protein Synthesis in Isolated Mitochondria

In vitro protein synthesis employing the method of Lansman and Clayton (13) was performed with mitochondria purified by differential centrifugation. For each time point, 100–200 μ g of mitochondrial protein was added to the described protein-synthesizing buffer which contained in addition 200 μ Ci/ml of [³⁵S]methionine and 100 μ g/ml emetine-HCl. For each time point, the reaction was stopped by the addition of 1 ml of homogenizing buffer containing 40 mM unlabeled methionine. The mitochondria were washed three times, precipitated in cold 10% TCA, collected on filters, and measured for [³⁵S]methionine incorporation.

Labeling and Fractionation of Mitochondrial Ribosomal RNAs and Subunits

Whole cells were preincubated with 0.1 μ g/ml actinomycin D (Act D) and, where appropriate, with 1.0 μ g/ml ethidium bromide (EtBr), then labeled for 6 h with 2 μ Ci/ml [³H]uridine. To display mitochondrial rRNAs, mitochondria were isolated by differential centrifugation, resuspended in buffer containing 10 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl, pH 7.2, and lysed with 1% SDS. The lysates were centrifuged at 30,000 g for 20 min, then sedimented on 15–30% sucrose gradients in a Beckman SW 27 rotor at 25,000 rpm for 25 h. To display mitochondrial ribosomal subunits, the labeled, isolated mitochondria were lysed with 1% NP-40 (in 50 mM Tris-HCl, pH 7.2, 100 mM KCl, 10 mM MgCl₂, and 0.1% diethyl pyrocarbonate), then centrifuged at 30,000 g for 20 min and the supernates were sedimented on 15–30% sucrose gradients as above for 14 h. Fractions collected from these sucrose gradients were precipitated with cold 10% TCA and 50 μ g/ml carrier protein (bovine serum albumin), collected on filters, and measured for [³H]uridine incorporation.

Chemicals and Radioisotopes

Ampholines, pH 3.5–10, were obtained from LKB Instruments, Inc. (Rockville, Md.), and CAP, Act D, and EtBr from Calbiochem-Behring Corp. Emetine-HCl and diethyl pyrocarbonate were purchased from Sigma Chemical Co. (St. Louis, Mo.), and L-[³⁵S]methionine and [³H]uridine from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

In a previous report (9) we showed that very little protein synthesis occurs in G7 mitochondria. This conclusion was based on labeling of mutant and wild-type cells with [³H]-leucine in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis. Mitochondria from labeled cells

were isolated and lysed. Products of mitochondrial protein synthesis were detected after separation on SDS polyacrylamide gels by electrophoresis, and extraction of radioactivity from gel slices. Some residual synthesis of two size classes of peptides was detected by this procedure.

To more thoroughly understand the nature of the G7 defect and its effect on mitochondrial function, mitochondrial protein synthesis in mutant cells was studied on a more sensitive scale, utilizing a more effective inhibitor of protein synthesis (emetine), more intense labeling of cells with [³⁵S]methionine, and fluorographic detection of radioactivity, permitting better resolution of individual peptide bands.

First, the synthesis and incorporation of protein into mitochondria of V79 and G7 cells were measured quantitatively by labeling intact cells with [³⁵S]-methionine and determining the incorporation of radioactivity into TCA-insoluble material in gradient-purified mitochondria. The inhibitors emetine and CAP were used to distinguish mitochondrial and cytoplasmic protein synthesis. Several of these measurements are summarized in Table I.

In the absence of any drug, slightly less [³⁵S]methionine-labeled protein is incorporated into G7 than V79 mitochondria. But, when cytoplasmic protein synthesis is blocked by emetine, the defect in mitochondrial protein synthesis in G7 cells becomes apparent. After subtraction of background levels of precipitable counts in the presence of both emetine and CAP, it is calculated that G7 mitochondria exhibit at least a sevenfold reduction in CAP-sensitive protein synthesis.

Rates of mitochondrial protein synthesis were also measured in vitro in V79 and G7 mitochondria isolated by differential centrifugation. Wild-type mitochondria incorporate [³⁵S]methionine at a constant rate for at least 15–20 min (Fig. 1). Assuming complete equilibration of all methionine pools with the external medium, V79 mitochondria incorporate 0.09 pmol of methionine/min per mg mitochondrial protein. This in vitro rate is only 4% of the in vivo rate of mitochondrial protein synthesis, but the latter was measured at 37°C, while in vitro labeling studies were carried out at 29°C. Under identical in vitro conditions G7 mitochondria do not exhibit a significant CAP-sensitive incorporation of [³⁵S]methionine into acid-precipitable material (Fig. 1).

Supernate from yeast cytoplasm that has been dialyzed and centrifuged at 100,000 g has been reported to stimulate in vitro yeast mitochondrial protein synthesis (22). Attempts to enhance mitochondrial protein synthesis in G7 and V79 mitochondria with wild-type cytoplasm have not been successful.

The [³⁵S]methionine-labeled proteins made and incorporated into mitochondria in vivo in the presence of emetine were further analyzed by electrophoresis on 12.5% polyacrylamide

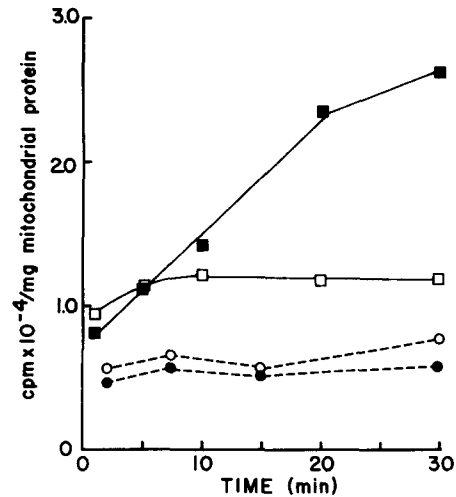


FIGURE 1 Time-course of [³⁵S]methionine incorporation into TCA-precipitable material in the in vitro protein-synthesizing system. These experiments were performed at 29°C. ■, V79 + emetine; □, V79 + emetine + CAP; ●, G7 + emetine; ○, G7 + emetine + CAP.

gels in the presence of SDS. As shown in Fig. 2a, V79 mitochondria synthesize at least 12 major size classes of protein, whose production is sensitive to CAP. A similar fractionation of mitochondria from G7 cells demonstrates at least a 10-fold reduction in the corresponding proteins. However, despite this drastic reduction, all major size classes of CAP-sensitive proteins can be detected in G7 mitochondria, suggesting that the block is general but slightly leaky (Fig. 2c).

Mitochondrial rRNA Metabolism

The defect in mitochondrial protein synthesis in G7 cells might arise from a defect in RNA synthesis. The most abundant and easily detected species of RNA transcribed from the mitochondrial genome are the ribosomal RNAs, which have sedimentation values distinct from those of the corresponding cytoplasmic rRNAs. To examine the transcription of mitochondrial DNA coding for rRNAs, mutant and wild-type cells were labeled with [³H]uridine in the presence of 0.1 μg/ml actinomycin D, an inhibitor of cytoplasmic RNA synthesis. Mitochondria were isolated from the labeled cells, lysed with 1.0% SDS, and sedimented on 15–30% sucrose gradients for 24 h. As shown in Fig. 3A, in both G7 and V79 mitochondria two distinct species of RNA are labeled which migrate more slowly than the 18S cytoplasmic rRNA marker. The sedimentation characteristics of these labeled RNAs are similar to those of 16S, 12S, and 4S mitochondrial rRNAs of HeLa (2), baby hamster kidney, and mouse cells (17). G7 mitochondria synthesize substantial amounts of these rRNAs. Assuming that a single mechanism (RNA polymerase) exists for transcription of the mitochondrial genome, it is suggested by these data that G7 mitochondrial DNA is transcribed normally, localizing the defect to the translation of its RNA.

To examine the mitochondrial ribosomal subunits, G7 and V79 mitochondria labeled with [³H]uridine in the presence of Act D were lysed with 1.0% NP-40 and sedimented on 15–30% sucrose gradients for 14 h. Assay of radioactivity across the gradient reveals labeled RNA associated with two bands at 40S and 30S that sediment as expected for mitochondrial ribosomal subunits. G7 mitochondria appear to assemble normal amounts of these subunits that sediment at rates virtually identical to those of subunits from V79 mitochondria (Fig. 3B). Under a

TABLE I

Synthesis and Incorporation of Protein into Mitochondria of G7 and V79 Cells

| Treatment | V79 | G7 |
|--------------------------------------|------------------------------|-----------------|
| | cpm/μg mitochondrial protein | |
| No inhibitor | 8,400 ± 2,600 (8) | 5,900 ± 850 (4) |
| 100 μg/ml Emetine | 490 ± 170 (8) | 120 ± 47 (3) |
| 100 μg/ml Emetine + 200 μg/ml CAP | 34 ± 6 (3) | 59 ± 44 (4) |

Cells were labeled with [³⁵S]methionine in the presence of the indicated inhibitors as described in Materials and Methods. Mitochondria were gradient purified, and incorporation of labeled amino acid into TCA-precipitable materials was measured. Each value is an average ± one standard deviation with the number of determinations given in parentheses.

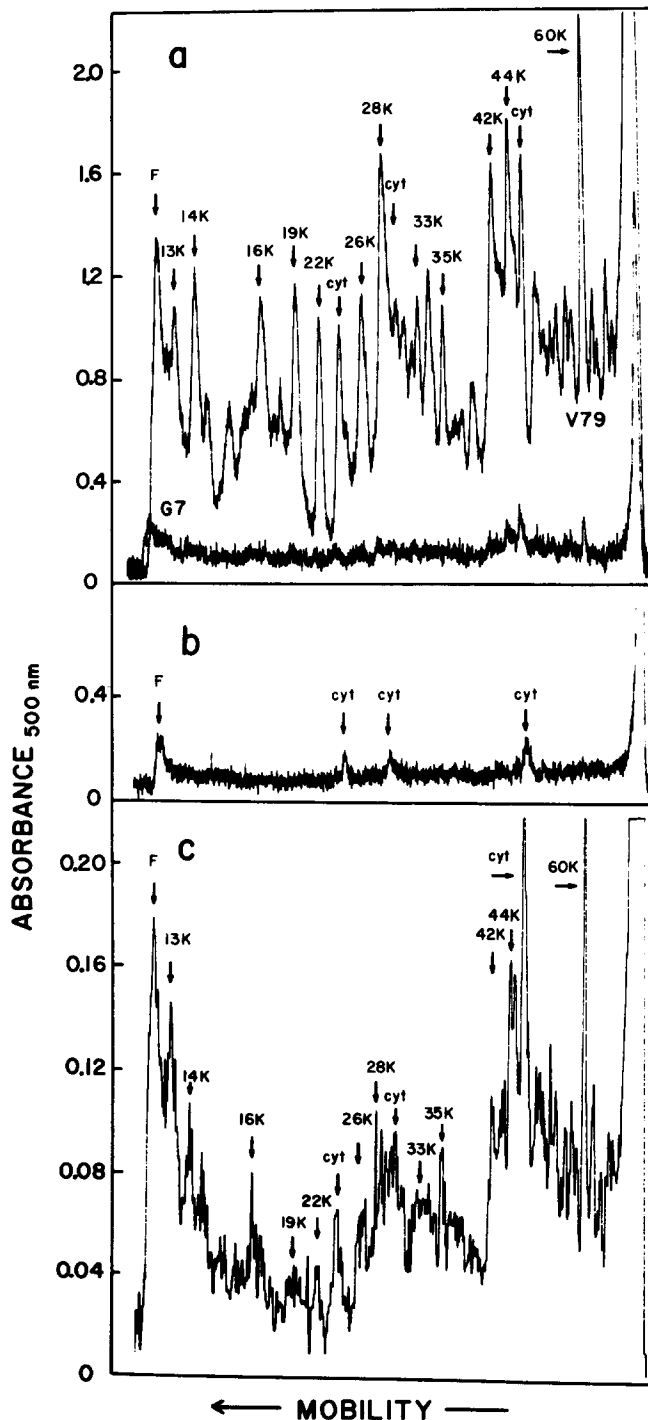


FIGURE 2 Densitometer scans of fluorograms from one-dimensional SDS polyacrylamide gels on which mitochondrial proteins from cells labeled with [³⁵S]methionine in the presence of emetine and/or CAP are fractionated by molecular weight. 60 μg of mitochondrial protein was applied to each gel. Major peaks that are consistently present between independent labelings and are almost completely suppressed in experiments with CAP are designated with approximate molecular weights. Proteins whose synthesis is incompletely suppressed by CAP and may represent residual cytoplasmic protein synthesis are designated by *cyt*. (a) Mitochondria from V79 and G7 cells labeled in the presence of emetine; (b) mitochondria from V79 cells labeled in the presence of emetine and CAP; (c) high sensitivity scan of the same G7 + emetine fluorogram displayed in (a), demonstrating the presence of all those peptides whose synthesis is suppressed by CAP in wild-type cells. *F*, buffer front.

wide range of conditions (variable ionic strengths, several concentrations of Mg²⁺ and Ca²⁺, treatment with puromycin or chloramphenicol, addition of reducing agents), only dissociated ribosomal subunits were detected in gradients of G7 or V79 mitochondrial lysates. However, 80–90% of TCA-precipitable counts applied to the gradients were recovered, indicating that no significant amount of monosomal or polysomal material was lost as a pelleted aggregate.

Incidence and Morphology of G7 Mitochondria

Many proteins synthesized by mitochondria are embedded in the inner membrane. Thus, the G7 cell might be expected to maintain structurally altered mitochondria or reduced numbers of these organelles per cell.

However, two kinds of measurements suggest that mutant cells have comparable numbers of mitochondria per unit volume of cytoplasm, but the mitochondria may have a reduced total mass (Table II). Six electron micrographs from two independently prepared samples of each cell type were scored

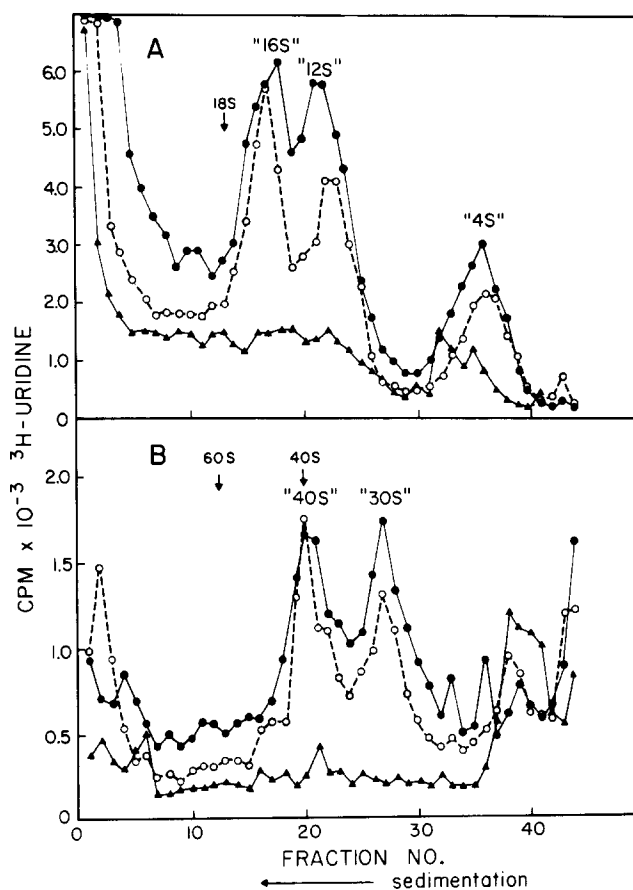


FIGURE 3 Density gradient sedimentation patterns of mitochondrial (A) rRNAs and (B) ribosomal subunits from V79 and G7 cells labeled with [³H]uridine in the presence of actinomycin D. Procedures for labeling, isolation, and lysis of mitochondria are described in Materials and Methods. Indicated sedimentation markers (↓) were assigned from positions of cytoplasmic rRNAs or subunits as detected by absorbance at 260 nm. Approximate sedimentation values assigned to mitochondrial subunits and rRNAs are also indicated within quotation marks. Symbols represent acid-precipitable counts in fractions across the gradients as follows: ●, V79 mitochondria; ○, G7 mitochondria; ▲, V79 mitochondria labeled in the presence of EtBr. The amount of mitochondrial protein treated for application to each gradient was (A) V79, 1.1 mg; G7, 0.8 mg; V79 + EtBr, 0.8 mg; (B) V79, 0.8 mg; G7, 0.7 mg; V79 + EtBr, 0.7 mg.

TABLE II
Incidence of Mitochondria in Wild-Type V79 and
res⁻ G7 Cells

| Measurement | V79 | G7 |
|--|------------|------------|
| μg Mitochondrial protein/10 ⁸ cells | 437 ± 26 | 288 ± 43 |
| Total mg postnuclear (750 g) supernatant protein/10 ⁸ cells | 21.2 ± 1.3 | 38.1 ± 4.2 |
| μg Mitochondrial protein/mg postnuclear supernatant protein (calculated value) | 20.6 | 7.6 |
| Mitochondria/mm ² cytoplasmic section | 102 ± 23 | 139 ± 23 |

Protein determinations were performed on gradient-purified mitochondria. As determined by marker enzyme activity, ~40% of mitochondria in the whole cell is recovered by gradient purification, but no correction is made for recovery in the data above. Number estimates of mitochondria were made from low-power electron micrographs and represent the number of recognizable mitochondria in cross-sectional view of cell cytoplasm in six micrographs of two independently prepared samples. Each value is derived from a minimum of three independent measurements and expressed as the mean value ± one standard deviation.

for the number of mitochondria per unit area of cytoplasm in sections, with comparable results. On the other hand, the total mass (protein) of gradient purified mitochondria recovered per 10⁸ cells or per milligram of postnuclear supernatant protein was consistently less from G7 than from V79 cells. This gradient purification gave ~40% recovery of mitochondria from both mutant and wild-type cells, as monitored by recovery of mitochondrial marker enzyme activities cytochrome *c* oxidase and monoamine oxidase. Therefore, the reduced mass of mitochondria purified from G7 cells cannot be explained by differential breakage of these organelles in the two cell types.

The morphologies of mutant and wild-type mitochondria were compared in high magnification electron micrographs shown in Fig. 4. Mitochondria from parental V79 cells exhibit classical morphology with lamellar cristae. This morphology is essentially independent of the medium in which the cells were grown, with glucose present either in vast excess or in limiting amounts. In contrast, G7 mitochondria have intact inner and outer membranes, but exhibit distinctive tubular cristae with infrequent lamellar shelves.

To determine whether tubular cristae in G7 mitochondria might result from the known defect in mitochondrial protein synthesis, an attempt was made to reproduce this morphology in wild-type cells by growing them for 3 d in the presence of 100 μg/ml CAP. Under these conditions, the mitochondria of V79 cells frequently contain tubular cristae, but are more easily distinguished by bulging or swirling cristae, characteristic of degenerating mitochondria (Fig. 4c). Similar mitochondrial ultrastructure has been observed in chick embryo fibroblasts after long-term culture in the presence of CAP (18). Thus, the structural alteration in G7 mitochondria may arise, at least in part, from the defect in mitochondrial protein synthesis but probably does not reflect a degenerating condition.

Biochemical Composition of Mitochondria

The reduced mass and unusual morphology of G7 mitochondria might arise from alterations in their biochemical composition. High-resolution two-dimensional gel electrophoresis employing isoelectric focusing in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension was used to display and compare the protein compositions of wild-type and mutant mitochondria. The membrane fraction

of gradient-purified mitochondria from cells labeled with [³⁵S]methionine was isolated by solubilization with digitonin and Lubrol, and similar amounts of radioactivity from mutant and wild-type membrane preparations were displayed on two-dimensional gels. As shown by representative fluorograms in Fig. 5a and b, the spot patterns are virtually indistinguishable. Careful comparison of the two patterns does reveal the absence of at least two proteins in G7 mitochondrial membranes: one with an apparent molecular weight of 42,000, pI 6.5, and a second at 28,000, pI 5.3. These spots correspond to those seen in two-dimensional gels of V79 mitochondria labeled in the presence of emetine (Fig. 5d) and are also absent in membranes from V79 cells labeled in the presence of CAP (Fig. 5c). These two spots therefore correspond to peptides made in the mitochondria and, as shown below, represent components of the cytochrome *c* oxidase (complex IV). The other products of mitochondrial protein synthesis identified in Fig. 2 cannot be detected in these two-dimensional gels, probably because of their hydrophobic nature and their inability to enter the first-dimension, isoelectric focusing gel. They may also be too weakly labeled relative to other proteins.

In separate studies (Fig. 5e and f) mitochondrial matrix proteins from G7 and V79 mitochondria were compared on two-dimensional gels and no consistent difference between them was observed. Thus, the defect in mitochondrial protein synthesis does not lead to significant alterations in the overall biochemical composition of mitochondrial matrix and membranes. Only a few peptides synthesized by the mitochondria can be shown to be absent.

Cytochrome *c* Oxidase

Although most mitochondrial membrane proteins are properly assembled into G7 mitochondria, respiratory complexes III, IV, and V which contain both cytoplasmically and mitochondrially synthesized subunits may present special problems of assembly. Cytoplasmically synthesized subunits may or may not continue to be imported and partially inserted into the mitochondrial membrane.

In most organisms that have been studied, complex IV (cytochrome *c* oxidase) is composed of 6–7 subunits, the three largest of which are synthesized by the mitochondrial machinery. Previous studies (9) had shown a very low level of cytochrome *c* oxidase activity and the absence of an absorption band corresponding to cytochromes *aa*₃ in G7 cells. The availability of an antibody raised against beef heart cytochrome *c* oxidase made possible an immunochemical search for the presence of cytoplasmically synthesized subunits of this complex in G7 mitochondria.

The anti-cytochrome *c* oxidase IgG raised in rabbits against the beef heart enzyme cross-reacts with the hamster enzyme from solubilized V79 mitochondria. The antibody specifically precipitates seven polypeptides which on SDS polyacrylamide gels have apparent molecular weights of 55,000–60,000, 42,000, 28,000, 15,000, 11,000, 10,000, and 7,000 (Fig. 6a). From mitochondria of V79 cells labeled in the presence of emetine, at least two of the three largest cytochrome *c* oxidase subunits (42,000 and 28,000) can be immunoprecipitated by the antibody (Fig. 6b). These two subunits correspond to two of the most intensely labeled bands in one-dimensional gels of complete hamster cytochrome *c* oxidase and total mitochondrially synthesized polypeptides. In addition, the 28,000 and 42,000 polypeptides correspond to the same peptides that are absent from total G7 mitochondrial membrane protein as displayed

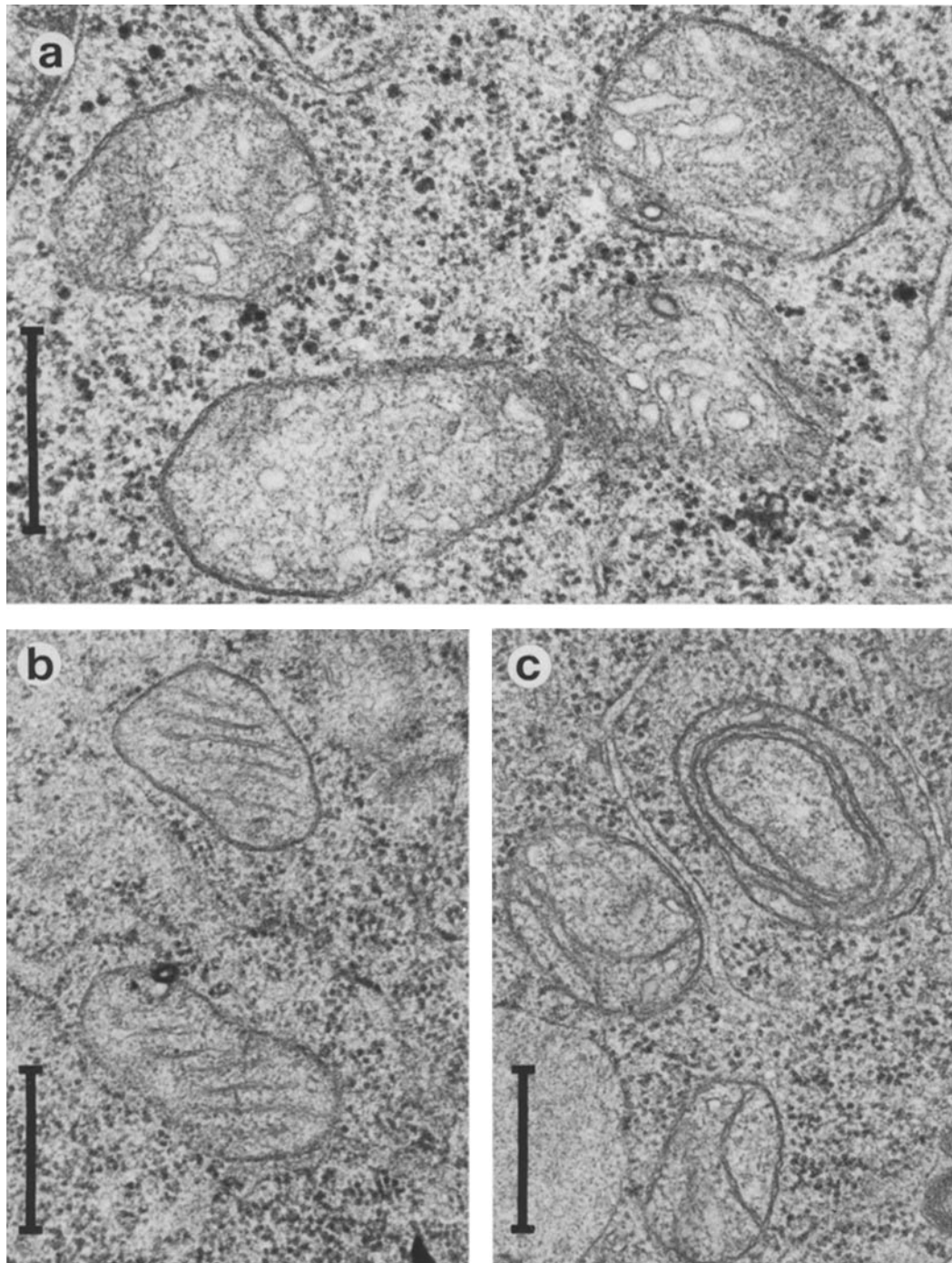


FIGURE 4 Electron micrographs of representative mitochondria from Chinese hamster lung fibroblasts. (a) G7 mitochondria. $\times 63,000$. (b) Mitochondria of V79 cells maintained in high glucose. $\times 57,000$. (c) Mitochondria of V79 cells treated for 3 d with 100 $\mu\text{g}/\text{ml}$ CAP. $\times 50,000$. Bars, 0.5 μm .

on two-dimensional gels (indicated by arrows 1 and 2 in Fig. 5d).

When anti-cytochrome *c* oxidase IgG is reacted with equal amounts of protein from [^{35}S]methionine-labeled mitochondria and values are normalized to equal amounts of initial mitochondrial radioactivity, 10 times more TCA-precipitable counts are recovered from V79 compared to G7 mitochondria (Table III). Furthermore, immunoprecipitation of G7 or V79 mitochondria labeled in the presence of emetine and/or CAP reveals greatly decreased amounts of immunologically recognizable cytochrome *c* oxidase subunits of both mitochondrial

and cytoplasmic origin (Table III). Cytoplasmically synthesized cytochrome *c* oxidase subunits or precursors are either not accumulating in the mutant mitochondria or not recognized by the antibody. The antibody also fails to detect significant amounts of a labeled precursor(s) for cytochrome *c* oxidase subunits in the 100,000 *g* cytoplasmic supernate of G7 or V79 cells.

DISCUSSION

Among a large number of *res*⁻ Chinese hamster cell mutants studied in our laboratory, only the G7 cell line exhibits pleio-

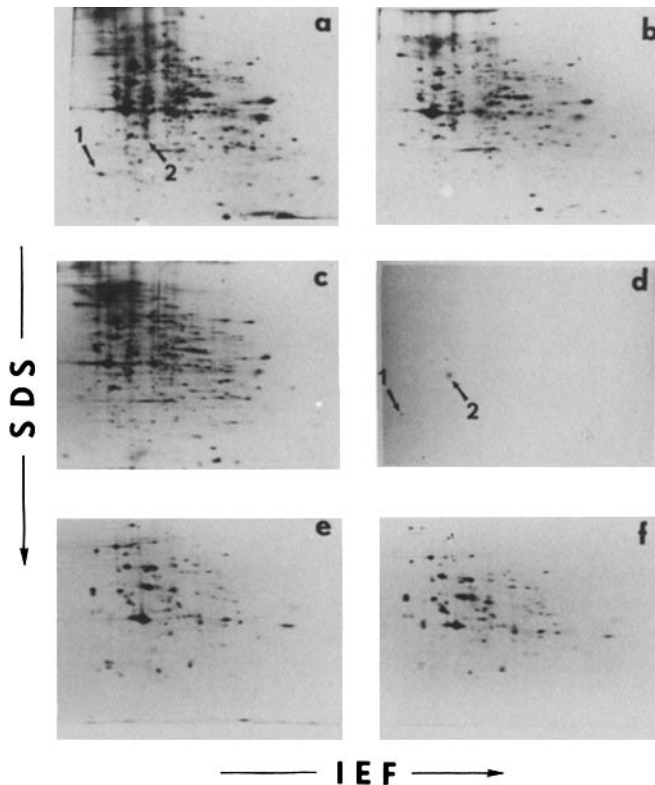


FIGURE 5 Fluorograms of two-dimensional gels displaying components of mitochondrial membranes from [³⁵S]methionine-labeled cells. Approx. 20 µg of mitochondrial protein was applied to each gel. Isoelectric focusing (IEF) with pH 3.5–10 ampholines was performed in the horizontal direction in all gels. SDS gel electrophoresis was in the vertical direction, using a gradient of 10–12.5% polyacrylamide for a–d and 12.5% polyacrylamide for e and f. (a) V79 mitochondrial membrane proteins; (b) G7 mitochondrial membrane proteins; (c) V79 + 100 µg/ml CAP mitochondrial membrane proteins; (d) V79 + 100 µg/ml emetine mitochondrial membrane proteins; (e) V79 matrix proteins; (f) G7 matrix proteins. 1 and 2 indicate spots present in the wild-type pattern that are absent in fluorograms of G7 and V79 + CAP membranes. Two additional spots in d result from residual cytoplasmic protein synthesis.

tropic enzyme defects resulting from very low levels of mitochondrial protein synthesis. Phenotypically, this *res⁻* mutant resembles many petite mutants of yeast, but its defect has been localized to a nuclear gene, and its revertibility suggests that it is caused by a point mutation (26).

There has also been one other report of mammalian cell mutants defective in mitochondrial protein synthesis (30). These mutants suffer from the same enzyme deficiencies as reported here, but in contrast to the situation in G7 cells, the mutation appears to be cytoplasmically inherited, as was intended from the selection scheme employed. These mutants may have an alteration in their mitochondrial ribosomes, as the cells are resistant to CAP.

The primary defect in the G7 mutant remains to be precisely defined. The mutation appears to be leaky and G7 mitochondria show the capability to correctly translate (and process) a specific set of proteins at a very low level (Fig. 2c). The 16S and 12S species of mitochondrial rRNA are synthesized in normal amounts and are assembled into 40S and 30S subunits. If a single mechanism exists for transcription of all mitochondrial DNA, then synthesis of ribosomal RNAs indicates normal transcription of the entire mitochondrial genome. These studies

indicate that the primary defect may lie in the translation process, involving (a) defective ribosomal proteins, (b) defective initiation or elongation factors, or (c) defective transport of cytoplasmic factors involved in mitochondrial protein synthesis.

The present study addresses the consequences of severely repressed mitochondrial protein synthesis in a mammalian cell. The data show that the total number of mitochondria per unit volume of cytoplasm is not changed significantly even though the mitochondrial mass per milligram cytoplasm may be reduced as much as twofold. G7 mitochondria have a characteristic ultrastructure distinguished by predominantly tubular cristae, but the overall biochemical composition of mitochondrial membrane and matrix fractions appears virtually unaltered, except for the absence of a few characteristic peptides.

Physical, biochemical, and immunochemical experiments reported here and in an earlier paper (9) show a severe shortage of mitochondrially synthesized peptides, an absence of cytochrome *b* and *aa₃* absorption bands, and much reduced mitochondrial ATPase (complex V) and cytochrome *c* oxidase (complex IV) activities in G7 cells. These results confirm and strengthen the conclusion that the contribution of the mitochondrial genome to functional mitochondria in mammalian cells is very similar to the situation in yeast (3, 28). Specifically,

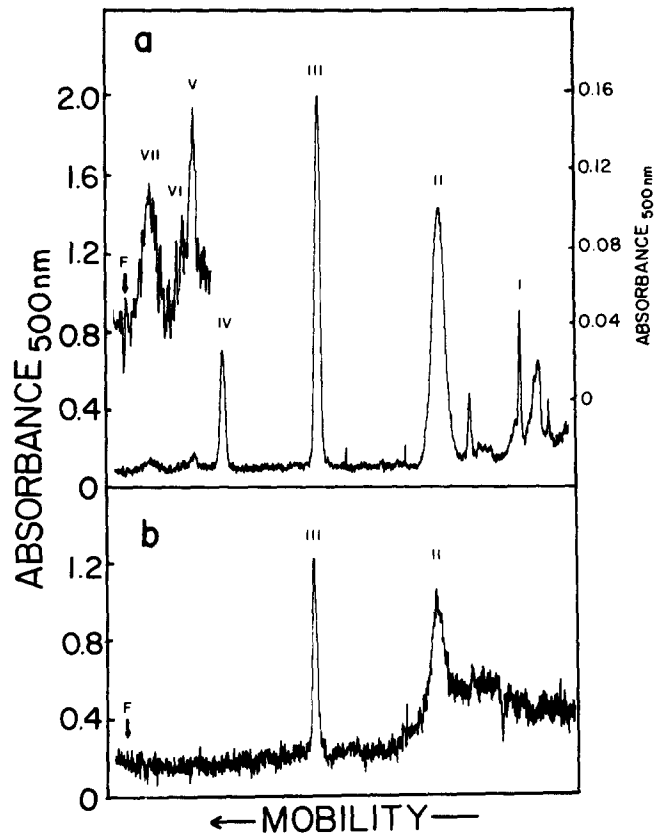


FIGURE 6 Densitometer scans of fluorograms from one-dimensional SDS polyacrylamide gels displaying immunoprecipitates of [³⁵S]methionine-labeled V79 mitochondria reacted with antiserum to cytochrome *c* oxidase. Each well contains the immunoprecipitate from 200 µg of mitochondrial protein. (a) Immunoprecipitate from V79 mitochondria labeled in the absence of drug. (b) Immunoprecipitate from V79 mitochondria labeled in the presence of emetine. Subunit molecular weights were determined from molecular weight markers run in parallel on each gel. Calculated subunit molecular weights are: I, 55,000–60,000; II, 42,000; III, 28,000; IV, 15,000; V, 11,000; VI, 10,000; and VII, 7,000.

TABLE III
Immunoprecipitation of Cytochrome *c* Oxidase from
[³⁵S]methionine-Labeled Mutant and Wild-type Mitochondria

| Treatment | V79 | G7 |
|---------------------|---|--|
| | cpm precipitated/ 1.2 mg mt protein | cpm precipitated/ 1.5 mg mt protein |
| No inhibitor | 26,000 | 2,000 |
| + 100 µg/ml Emetine | 15,000 | 620 |
| + 200 µg/ml CAP | 5,900 | Not detectable |

For each immunoprecipitation, 29 µg of V79 mitochondrial (mt) protein or 290 µg of G7 mitochondrial protein was reacted with 10 µl of anti-cytochrome *c* oxidase IgG. Specific radioactivities of mitochondrial protein labeled in the absence of protein synthesis inhibitors are 8,300 cpm/µg V79 mitochondrial protein and 6,700 cpm/µg G7 mitochondrial protein. Thus, 10⁷ TCA-precipitable cpm correspond to 1.2 mg of V79 mitochondrial protein and 1.5 mg of G7 mitochondrial protein. Values given are averaged from two independent experiments and rounded to two significant figures.

at least two peptides of molecular weights (42,000 and 28,000) of the cytochrome *c* oxidase complex are made in normal Chinese hamster mitochondria and are almost absent in G7 mitochondria. Similarly, Breen and Scheffler (5) have recently demonstrated that the oligomycin-sensitive ATPase of Chinese hamster mitochondria also contains at least one mitochondrially encoded subunit.

The immunochemical studies of cytochrome *c* oxidase suggest an almost complete absence of cytoplasmically synthesized subunits of this complex in G7 mitochondria, in addition to the absence of mitochondrially synthesized peptides. However, two-dimensional gel patterns of wild-type and mutant mitochondrial membranes are very similar (Fig. 5). This seeming disparity could be explained if most components of complexes IV and V are (a) too insoluble to enter the first-dimensional isoelectric focusing gel, (b) too small to be separated on the SDS gel system of the second dimension, or (c) too lightly labeled with [³⁵S]methionine to be detected on fluorograms.

The tubular cristae observed in G7 mitochondria are also found in mitochondria of mammalian adrenal cortex cells and certain protozoans (10). However, tubular morphology is not associated with the res⁻ phenotype of another Chinese hamster cell mutant in our collection which is defective in complex I of the electron-transport chain (7). The mitochondrial ultrastructure of CAP-treated V79 cells suggests a correlation of the tubular arrangement with suppression of mitochondrial protein synthesis. The inner mitochondrial membrane is quite rich in proteins, of which complexes IV and V constitute a considerable proportion. As a result of its severe deficiency in these complexes, the G7 mutant may be able to assemble only a small amount of inner membrane per mitochondrion, producing cristae in tube forms rather than the expansive lamellar sheets in wild-type mitochondria. Alternatively, studies by Green and co-workers (11) have suggested that Krebs cycle substrates, electron-transport chain inhibitors, and uncouplers of oxidative phosphorylation can alter crista structure in isolated mitochondria. Thus, tubular crista structure may be a secondary expression of altered proton-gradient formation across the inner membrane, or an unusual conformational state of respiratory complexes in G7 mitochondria.

Based upon initial data demonstrating the extreme deficiency of mitochondrially synthesized peptides in the G7 cell line, it was not expected that this res⁻ mutant would maintain large numbers of recognizable mitochondria with virtually normal biochemical composition. Current investigation of the G7 mutant suggests that these mitochondria might be critically nec-

essary to some mammalian cell function other than respiration, possibly involving the mitochondrial ATPase. Thus, the G7 cell line may well prove to be useful in defining and understanding the role of mitochondria beyond oxidative phosphorylation and energy metabolism.

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