Biochimica et Biophysica Acta, 292 (1973) 92–104

BBA 46447

FREEZE–FRACTURE STUDIES ON MITOCHONDRIA FROM WILD-TYPE AND RESPIRATORY-DEFICIENT YEASTS

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(Received August 25th, 1972)

SUMMARY

The phenomenon of respiratory deficiency in yeasts brought about by loss or alteration of mitochondrial DNA results in functionally-deficient membranes lacking oligomycin-sensitive ATPase, several respiratory enzyme activities, and the spectral components of cytochromes a, a_3, b and c_1. Since all of the activities catalyzed by these components are associated with high molecular weight membrane protein complexes, it was important to determine how loss of these functions affects membrane structure.

Mitochondria from respiratory-deficient yeast without mitochondrial DNA and from wild-type cells have been compared with respect to enzyme activity, fatty acid composition and membrane structure. Respiratory deficiency and loss of oligomycin-sensitive ATPase were not accompanied by any significant change in fatty acid composition. Examination of isolated mitochondrial preparations by freeze–fracture electron microscopy revealed: (a) the isolated preparations from wild type or mutants without DNA were intact; (b) the fracture faces and structural organization of yeast membranes are similar to those seen in mammalian liver and heart mitochondria; (c) the organization of the membranes in the mutant without DNA and in the wild-type cells did not differ significantly as determined by quantitative evaluation of the size, clustering or distribution of membrane particles.

It must therefore be concluded that the severe loss of membrane-associated enzyme activities is very likely not accompanied by a corresponding loss of structural components or by an alteration in the way they are organized in the membrane.

INTRODUCTION

Yeasts have received much attention in the study of mitochondrial biogenesis since cells with altered mitochondrial development can be obtained in a variety of different metabolic or genetic states. When yeast cells are grown either under conditions of glucose repression or in the absence of oxygen, functional respiring mitochondria are lost and only promitochondrial forms may be observed. It has now been well established that these promitochondria have many of the membrane

proteins, mitochondrial enzymes and mitochondrial nucleic acids of the fully developed mitochondria[1–3]. These promitochondrial forms do not carry out appreciable respiration, however, and they lack detectable amounts of the spectral components characteristic of cytochromes *a*, a_3, *b* and c_1, as well as other enzyme activities[1]. Electron microscopic examination of such promitochondria has demonstrated that these organelles have, at least at a superficial level, most of the morphological features typical of mitochondria from aerobic or derepressed cells, although the amount of inner membrane structures is diminished[3].

The petite mutants of yeast which have been studied have many phenotypic characteristics similar to those of anaerobically-grown cells[4]. Such mutants are typically characterized by their lack of spectrally detectable levels of cytochromes *a*, a_3, *b* and c_1 and their corresponding inability to carry out respiration. Cytochrome *c* is often present in these cells at normal or even elevated levels[5]. In many cases, the petite mutation is cytoplasmically inherited with major alterations observed in the mitochondrial DNA (mtDNA), a fact which suggests a relationship between the alteration of the mtDNA and enzymic composition of the mitochondrial structures[6].

Electron microscopy of freeze–fracture preparations of mitochondria allows detailed examination of particle number and particle size distribution within well-characterized fracture faces of mitochondrial membranes[7–9]. This method has therefore been employed in a comparative study of mitochondria from wild-type and petite yeast in an attempt to relate structural differences to the measured enzymic changes in the mitochondria from the petite strains. One of the two mutants studied has been shown to be a deletion mutant retaining only 50% of the normal mtDNA sequences of the wild type[10]. The other mutant completely lacks mtDNA[11]. Thus, in this case, all mitochondrial components present must necessarily be genetically of nuclear origin.

MATERIALS AND METHODS

Yeast strains

The respiratory competent (wild type) strain of *Saccharomyces cerevisiae* used was D243-4A (a, ade_1, lys_2, trp_1) obtained from Dr Fred Sherman. The petite strains were an ethidium bromide induced petite, D243-4A/8, lacking mitochondrial DNA[11], and a spontaneous petite, D243-2B-R_1-6 containing 50% of the normal sequences found in wild type mitochondrial DNAs[10].

Preparation of mitochondria

Cells were grown to early stationary phase in a medium containing 2% glucose, 1% Difco yeast extract and 1% Difco peptone extract except in certain conditions where noted. Mitochondria from wild-type and petite cells were prepared from yeast spheroplasts obtained following digestion of the cell walls with glusulase (Endo Laboratories) using the method of Kováč *et al.*[12]. Mitochondria were then isolated as described by Criddle and Schatz[1].

Following enzymic digestion of the cell walls, the suspension was centrifuged at $5000 \times g$ for 10 min to sediment the cells. The supernatant fraction was saved for recovery of the glusulase enzymes. The supernatant fraction was brought to 65% saturation with $(NH_4)_2SO_4$, and the slurry was stored at 5 °C. The enzyme activity

was stable for at least 6 months. Before use, the slurry was centrifuged at low speed and the precipitate dissolved in digestion buffer for use in spheroplast formation. This procedure of enzyme recovery and reuse was carried out as many as six times over a 6-month period.

Enzyme assays

Enzyme activities were assayed in the mitochondria from wild-type and petite cells by the following procedures: ATPase, Pullman *et al.*[13]; succinate–ferricyanide reductase, Schatz[14]; cytochrome *c* oxidase, Wharton and Tzagoloff[15]; succinate–cytochrome *c* reductase, Schatz and Klima[16]; succinate dehydrogenase, Criddle and Schatz[1].

Spectral analysis

Difference spectra of the isolated mitochondria were measured in a Cary Model 14 spectrophotometer at liquid-nitrogen temperature. Mitochondria were suspended in 0.25 M mannitol, 20 mM Tris–sulfate at pH 7.4, 2 mM EDTA, 50% glycerol at a protein concentration of 5.2 mg/ml. Spectra were recorded as the difference spectra of oxidized *vs* sodium dithionite reduced mitochondrial preparations. Protein was measured by the method of Lowry *et al.*[17] in the presence of 0.33% sodium deoxycholate to solubilize particulate protein.

Freeze–fracture and electron microscopy

Mitochondria were suspended in 25% glycerol, centrifuged, and a drop of the pelleted mitochondria was rapidly frozen in Freon 22 and replicas obtained according to Wrigglesworth *et al.*[18] on a Balzers freeze–etching apparatus. A stage which accepts four different samples was used; specimens were fractured and immediately replicated. Replicas were examined with either a Siemens Elmiskop Ia or a RCA EMU-3H electron microscope. All photographs presented are positives, *i.e.* were processed so as to show shadows in white and deposition of platinum and carbon in black. Shadow direction and magnification are indicated on the figures.

Fatty acid analyses

Lipids were extracted from mitochondrial suspensions with 20 vol. chloroform–methanol (2:1, v/v) with 0.5 mg hydroquinone added. The extract was filtered and washed and the fatty acids methylated and analyzed by gas–liquid chromatography[19,20].

RESULTS

Enzyme analyses

Mitochondria isolated from either the petite strain lacking one-half of the normal mtDNA still possess measurable levels of ATPase, succinic dehydrogenase and succinate–ferricyanide reductase, all of which are characteristic mitochondrial enzymes (Table I). These mitochondria are unable to carry out respiration, however, as is evident from the absence of measurable cytochrome oxidase activity. In this respect, they resemble mitochondria from glucose repressed or anaerobically-grown cells.

TABLE I

ENZYME CONTENT OF *S. CEREVISIAE* MITOCHONDRIA

Enzyme	*Specific activity (μmoles substrate transformed per min per mg protein)*		
	Wild type	*Petite, 1/2 mtDNA*	*Petite, no mtDNA*
ATPase	3.72*	2.85**	1.45**
Succinate dehydrogenase	0.202	0.085	0.107
Succinate–ferricyanide reductase	0.182	0.0086	0.0072
Cytochrome *c* oxidase	0.980	≈0.001	≈0.001
Succinate–cytochrome *c* reductase	0.082	≈0.001	≈0.001

* Oligomycin-sensitive ATPase.
** Total ATPase activity.

Furthermore, the spectra shown in Fig. 1 indicate a complete absence of absorption bands characteristic of cytochromes *a* and a_3 in the preparations of the mitochondria without mtDNA. Absorption bands characteristic of cytochromes *b* and c_1 (with the exception of a component with limited absorbance in the region of b_1) are also absent. In contrast, cytochrome *c* is present at nearly normal levels. This

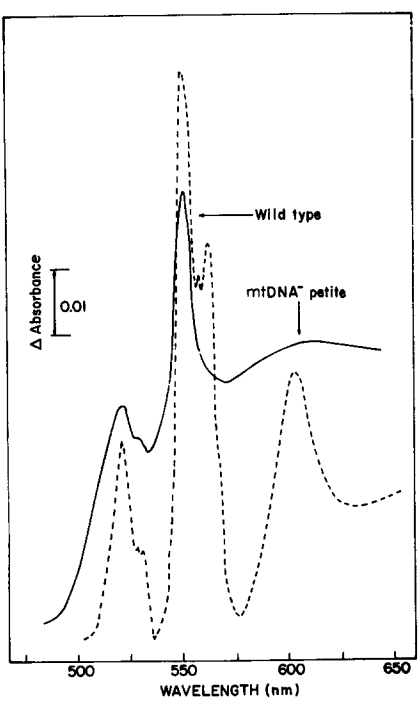

Fig. 1. Oxidized-*minus*-reduced difference spectra of mitochondria from wild-type yeast and mutant yeast without mtDNA.

high yield of cytochrome *c* is an indication that the petite mitochondria were isolated in a reasonably intact form[21], a conclusion supported further by the electron micrographs. The spectra for mitochondria from the strain lacking one-half of the mtDNA sequences were indistinguishable from the mutant without mtDNA. These data confirm the generally-accepted view that the mitochondria from petite cells still contain characteristic mitochondrial enzymes but completely lack some of the functional and spectral components directly involved in the electron transport process.

Fatty acid composition

Fatty acid analyses of the mitochondrial lipids were also done (Table II) to determine whether the lipids from the mitochondria lacking DNA would contain a lower proportion of unsaturated fatty acids than would mitochondria from the wild-type cells. A lower proportion of unsaturated fatty acids might be expected in the mitochondria without DNA since in many respects, mitochondria from petite mutants resemble those of wild-type cells grown anaerobically in which the proportion of unsaturated fatty acids is drastically reduced, if the cells are grown anaerobically without unsaturated fatty acid supplementation[1,2]. The data in Table II, however, show clearly that the mutant and wild-type strains were remarkably similar in their mitochondrial fatty acid composition.

TABLE II

FATTY ACID COMPOSITION OF *S. CEREVISIAE* MITOCHONDRIA

Fatty acid	*Mitochondrial fatty acids (weight %)*	
	Wild type	*No mtDNA*
<12:0	0.2	0.2
12:0	0.8	0.6
Unknown	—	0.1
14:0	0.9	0.9
Unknown	0.5	0.2
16:0	12.9	17.7
16:1	46.7	44.0
Unknown	1.1	0.6
18:0	6.5	4.0
18:1	28.3	30.6
18:2	0.4	0.1
>18:2	1.5	1.0
Σ 16:1+18:1+18:2	75.4	74.7
Σ Other fatty acids	24.6	25.3

Membrane particles

Low magnification, freeze–fracture electron micrographs of the suspensions of wild-type mitochondria and those without mtDNA are shown in Figs 2A–B and 3A–B, respectively. These preparations generally show numerous mitochondrial profiles in fracture faces and also in cross sections.

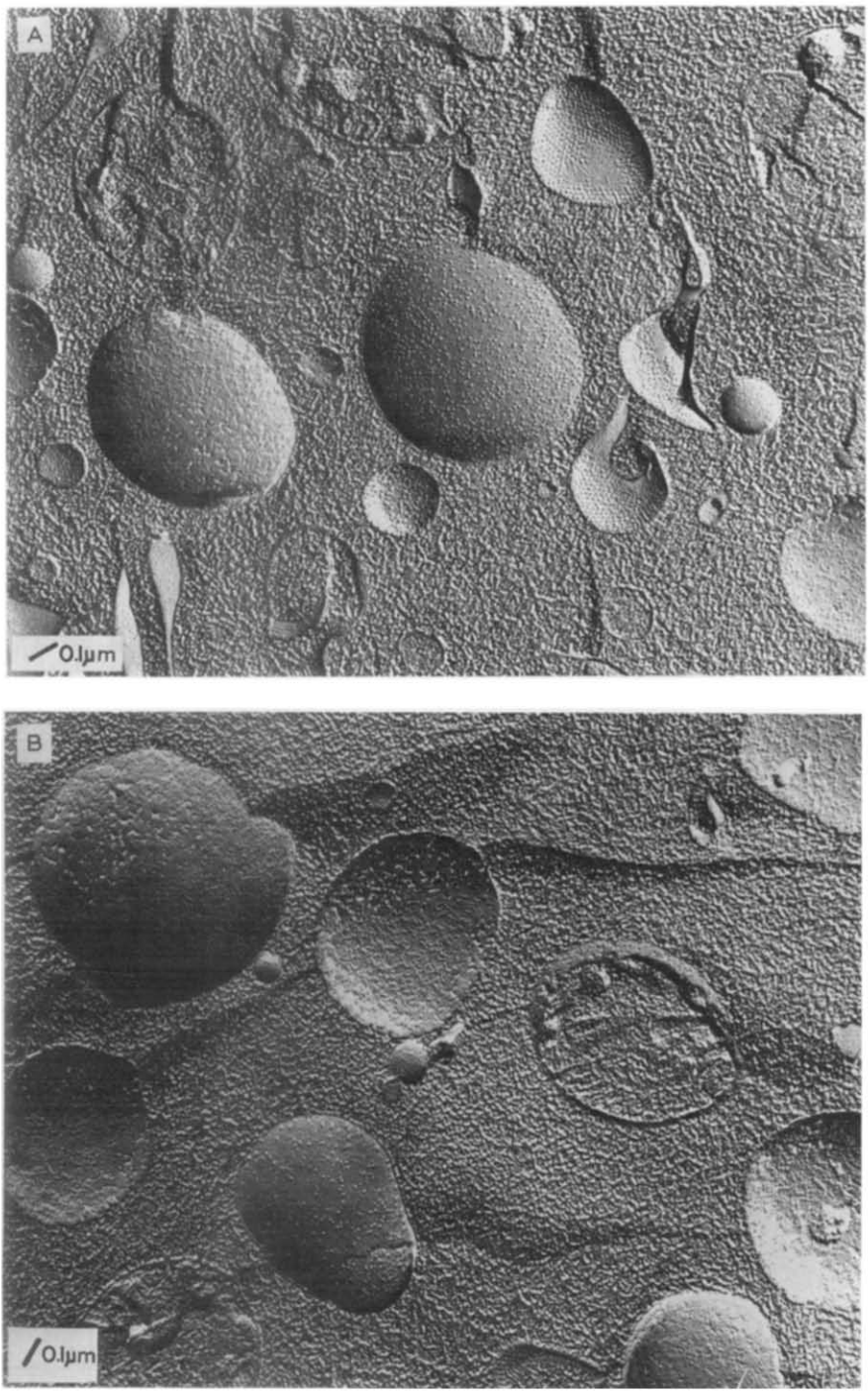

Fig. 2. Low magnification electron micrographs of freeze–fracture preparations of mitochondria isolated from wild-type yeast.

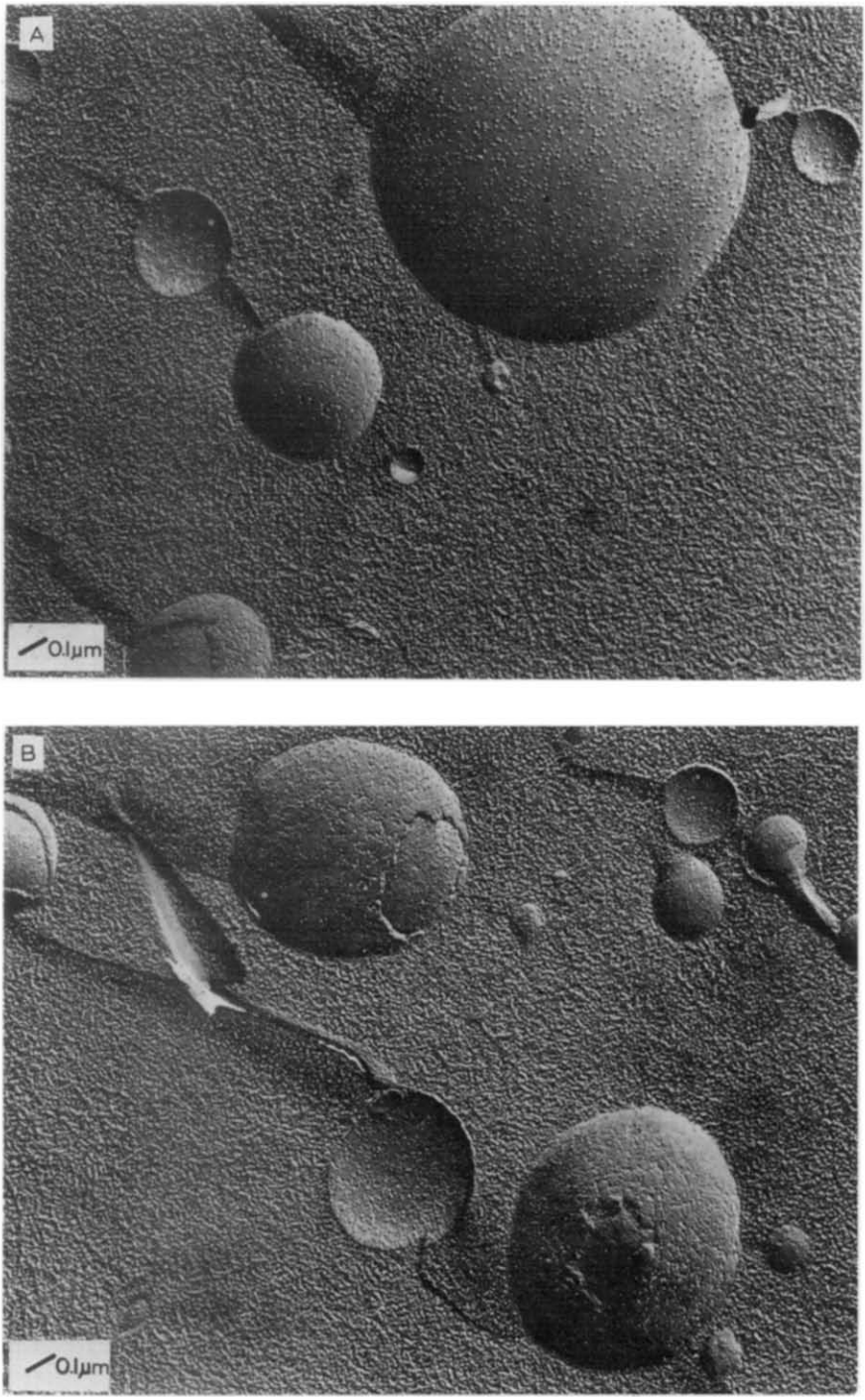

Fig. 3. Low magnification electron micrographs of freeze–fracture preparations of mitochondria isolated from mutant yeast without mtDNA.

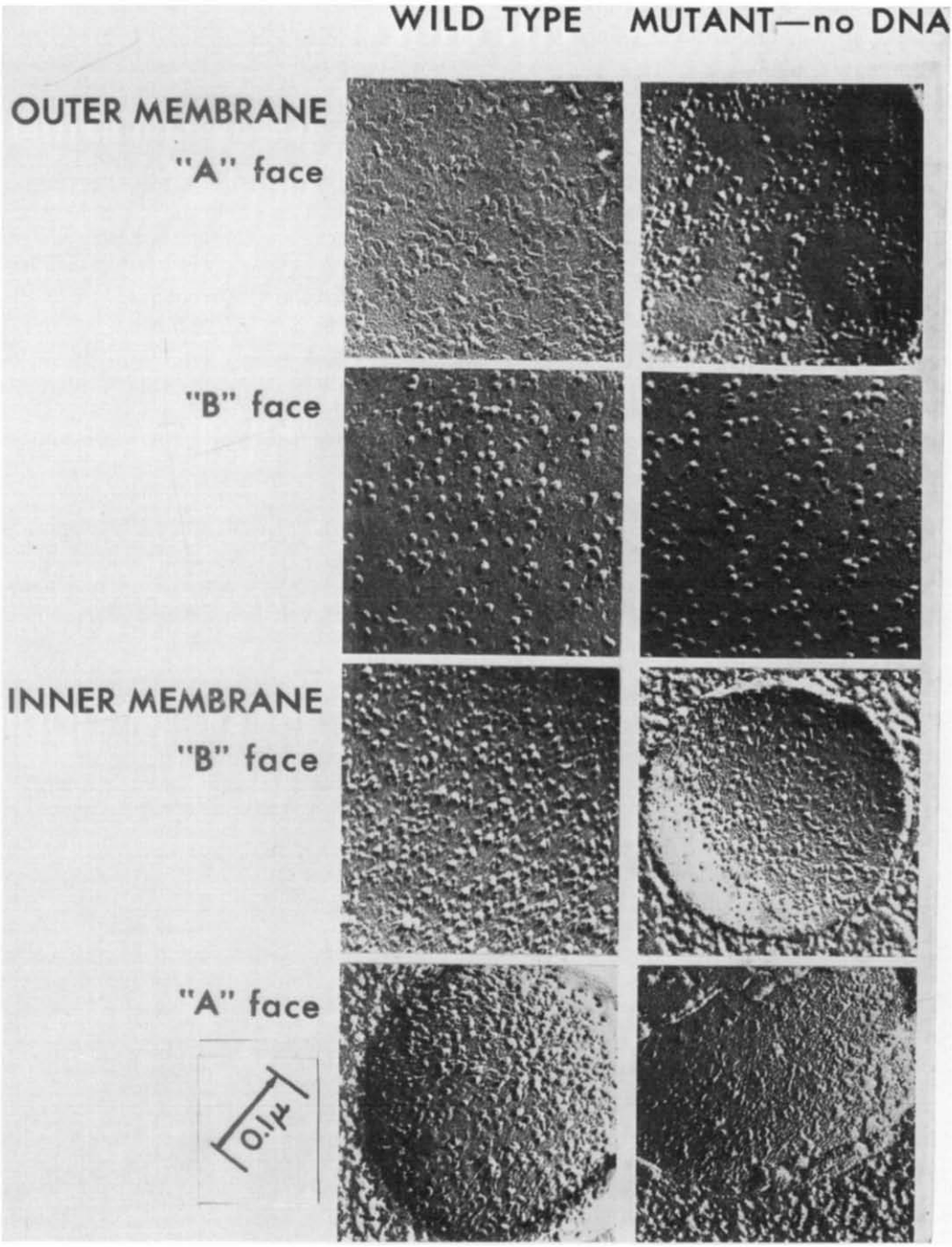

Fig. 4. Characteristic fracture faces of outer and inner membranes from mitochondria isolated from wild-type yeast or from mutant yeast without mtDNA.

Low magnification electron micrographs show the presence of material with a regular fibrous-like pattern (Fig. 2A, upper portion). This apparently non-mitochondrial material is thought to be remnants of cell walls which contaminate all the mitochondrial preparations.

Mitochondrial membranes, even at low magnification, show characteristic fracture faces. For example, Figs 2A and 3A show clearly the particle distributions of the convex fracture face associated with the outer membrane. The interpretation of the different half-membranes and their particle distributions is made from examination of replicas in which the fracture faces of two membranes are present (*cf.* Fig. 2B and Fig. 3B). If we assume that the mitochondrion is intact and that the fracture occurs through the hydrophobic center to split the membrane into two "half-membranes", then the two double fracture faces in Figs 2B and 3B represent mitochondrial outer membrane and inner membrane, respectively. The half membranes, conventionally designated A and B faces, are the particle-dense (A face) and the particle-sparse (B face) half-membranes that arise after freeze–fracture.

The double fracture faces in Figs 2B and 3B show an outer B and an inner A face. The double fracture face shown in Fig. 3B of the petite mutant shows an outer B face of the mitochondrion with holes where particles from the outer A face of the mitochondrion have apparently been torn away (*cf.* Fig. 4, outer A face particle distribution).

In the manner described above, the characteristic structures of the outer and inner membranes can be recognized in freeze–fracture preparations of intact mitochondria in which the two membrane fracture faces appear in the same mito-

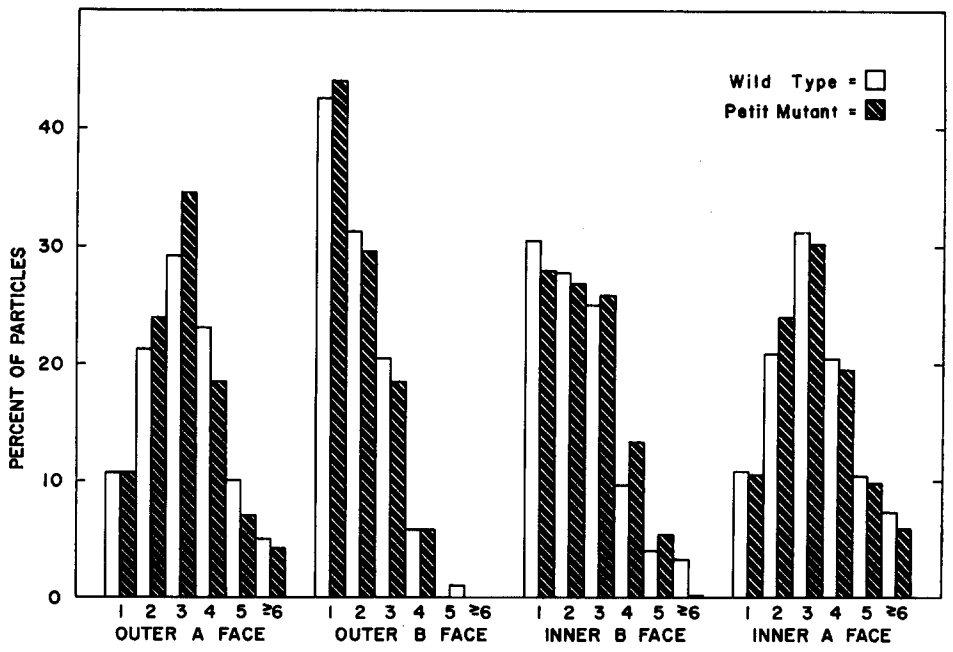

Fig. 5. Histogram of distribution of particle clusters in fracture faces of mitochondria from wild-type yeast and mutant yeast without mtDNA.

chondrion[7–9]. Alternatively, these structures have been recognized by examining isolated inner and outer membrane preparations as shown by Melnick and Packer[22]. Fig. 4 shows typical membrane fracture faces of isolated mitochondria from wild-type cells and cells without mtDNA. The particles in concave and convex fracture faces show characteristic distributions and densities in A and B faces.

Fig. 5 shows that the distribution of particle aggregates or clusters is similar in the four half-membranes of the wild-type yeast and the mutant without mtDNA. The particle size (Figs 6A and 6B) and particle density (Table III) in the fracture faces of both the outer and inner mitochondrial membranes are also similar in these two strains. Table III summarizes the particle distributions and densities in the A and B faces of membranes from two experiments. No major differences in particle densities are seen in the mitochondria of wild-type cells, those completely lacking mtDNA, or those with one-half the normal amount of mtDNA. The A/B ratio (*i.e.* the ratio of the particle density in the A face to that in the B face) is an additional measure of intrinsic membrane organization reflecting interactions of membrane components. These ratios for the wild type and the mutants show little difference.

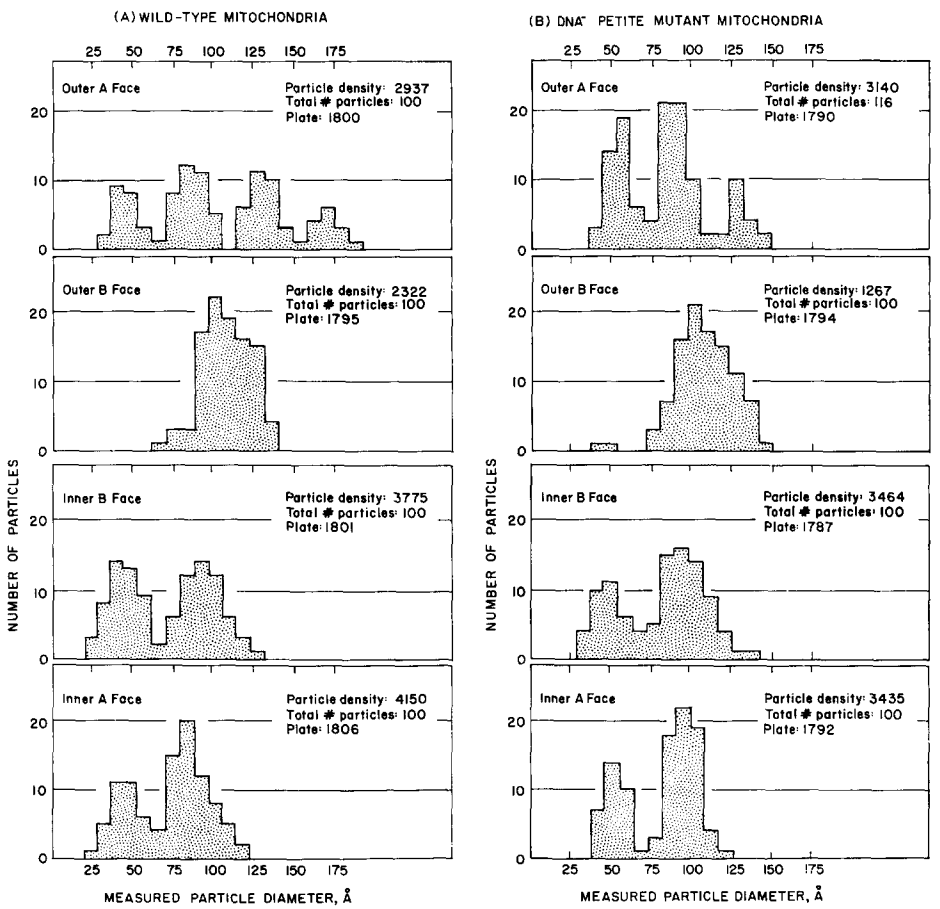

Fig. 6. Histogram of particle size (diameter) distribution in fracture faces of mitochondria from (A) wild-type yeast and (B) mutant yeast without mtDNA.

TABLE III

ANALYSIS OF MEMBRANE-PARTICLE DISTRIBUTION IN FRACTURE FACES OF MITOCHONDRIA ISOLATED FROM WILD-TYPE *S. CEREVISIAE* AND FROM MUTANTS LACKING mtDNA

Mitochondrial fracture face	*Expt FE 94*					
	Wild type	*Ratio A/B*	*1/2 mtDNA*	*Ratio A/B*	*No mtDNA*	*Ratio A/B*
Outer A	3270	2.06	3334	2.50	2909	2.48
Outer B	1589		1330		1175	
Inner B	3660	1.12	3433	1.18	4120	1.06
Inner A	4100		4050		4380	
	Expt FE 147				*Ratio FE 94/FE 147*	
	Wild type	*Ratio A/B*	*No mtDNA*	*Ratio A/B*	*Wild type*	*No mtDNA*
Outer A	2937	2.31	3140	2.59	1.11	0.93
Outer B	1273		1262		1.25	0.93
Inner B	3775	1.10	3464	0.99	0.97	1.19
Inner A	4150		3425		0.99	1.28

Mutants of *Saccharomyces pombe* have also been examined in collaboration with Dr A. Goffeau (University of Louvain, Laboratory of Enzymology). Strain $972h^{-}$-COB_2 showed spectra characteristic of cytochromes *b*, *c* and c_1, but lacked cytochromes $a+a_3$. ATPase activity was also reduced in this strain. Strain pet B showed spectra characteristic of cytochrome *c*, but lacked $a+a_3$, *b* and c_1. Examination of freeze–fracture replicas of these yeast cells and those of the wild type (strain $972h^{-}$) showed the characteristic fracture faces seen in the mitochondria isolated from *Saccharomyces cerevisiae* (*cf.* Figs 2–4). Table IV summarizes the particle densities found upon analysis of the fracture faces of the *S. pombe* strains.

TABLE IV

ANALYSIS OF MEMBRANE PARTICLE DISTRIBUTION IN MITOCHONDRIAL FRACTURE FACES OF WILD-TYPE AND PETITE *S. POMBE* CELLS

Mitochondrial fracture face	*Wild type*		*Petite B*	
	Particles per μm²	*Ratio A/B*	*Particles per μm²*	*Ratio A/B*
Outer A	2167	3.10	2038	2.87
Outer B	696		709	
Inner B	2370	1.84	2780	1.56
Inner A	4366		4350	

DISCUSSION

The existence of DNA located in the mitochondrion and distinct from nuclear DNA is well established[23], but the identity of specific protein products of the mtDNA has not yet, in any instance, been definitely established. Although the entire apparatus for protein synthesis occurs in mitochondria and the biosynthesis of membrane proteins has been demonstrated *in vitro*[24], the role of mtDNA in the assembly and growth of new mitochondria, especially in relation to the question of membrane formation, is largely unknown. Tzagoloff and Meagher[25] have presented evidence for the mitochondrial synthesis of some components of the mitochondrial ATPase in yeast. However, although these proteins appear to be synthesized by the mitochondrion, it still has not been determined whether the mitochondrial RNA which directs this synthesis has been transcribed from nuclear or mitochondrial DNA[26]. Similar conclusions have been suggested for cytochrome oxidase synthesis[27].

The mitochondria isolated from the respiratory-deficient strains of yeast in this study have been shown to be greatly altered in their enzymatic and spectral properties. The fatty acid composition, however, shows no major differences in comparison with that of the wild type. In addition, the striking deficiencies in functional components which accompany mutations in mtDNA are not accompanied by any significant changes in the morphology of mitochondrial membranes, as detected by techniques of electron microscopy currently available.

It seems somewhat surprising that such major changes in functional properties of the mitochondria are not reflected in some measurable alteration in membrane structure at the resolution studied. The fact that no such change is seen suggests that loss of respiratory components may not be accompanied by loss of major enzyme complexes of the size normally attributed to the electron transport complex, cytochrome oxidase, the cytochrome b–c_1 complex, or ATPase. However, if respiratory deficiency resulted only from loss of certain (possibly small) catalytic subunits or small components from the complex, then one would not necessarily expect to find structural changes detectable by electron microscope examination.

This would be consistent with the findings of Schatz *et al.*[27] that most of the apoprotein portion of cytochrome oxidase and the oligomycin-insensitive mitochondrial ATPase remain associated with the mitochondrial membranes even under conditions where normally functional enzyme is not present. These findings would lead one to predict further that other complexes of the respiratory transport system behave in a similar fashion. For example, the b–c_1 complex probably also has such an apoprotein portion present in non-functional mitochondria lacking only certain catalytic units or heme proteins for full activity. Such a proposal would necessarily also suggest that the production of active enzyme complexes requires the normal functioning of mitochondrial DNA for protein synthesis in conjunction with additional protein synthesis in the cytoplasm to produce such active respiratory enzymes.

ACKNOWLEDGEMENTS

The authors would like to express their appreciation to Lou Worthington and Susan L. Tinsley for assistance with freeze–fracture electron microscopy, and Kim Burchiel for preparation of mitochondria. This research was aided by grants from

the U.S. Public Health Service (HD-05875-01, AM-12024, GM-10017) and the Veterans Administration.

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