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Genetic Analysis of Mitochondrial Biogenesis and Function in *Saccharomyces cerevisiae*

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Introduction

For an understanding of complex functions like oxidative phosphorylation or electron transport, the structural components must be identified and their stoichiometry and three-dimensional arrangement determined. The identification also includes the exploration of the genetic origin of these macromolecules, the regulation of their synthesis, the site of translation, and the mechanism of assembly. The past decade has demonstrated that mutants and genetic methods are invaluable tools to explore cellular functions. They should be especially useful in the case of mitochondria and chloroplasts, as these two organelles have the peculiarity of possessing their own DNA and, hence, their own genetic systems. However, as the majority of mitochondrial proteins are derived from nuclear genes, complex mitochondrial functions will be the result of an interaction of macromolecules from two different genetic systems. Both nuclear and mitochondrial mutations are needed to elucidate these interactions. This article will mainly concentrate on mutations of mitochondrial DNA. With its limited informational content, mitochondrial DNA represents a relatively simple system and therefore has attracted considerable interest. The field of organelle genetics has been rapidly expanding during the past few years and has become a science with its own terminology, largely devoted to pure genetic problems such as the mechanism of recombination.

In view of several recent extensive reviews bearing on organelle genetics and biogenesis, we shall not try to present a compilation of data, but rather concentrate on the methodology and strategy of mitochondrial genetics, as far as it is of direct interest to bioenergetics. We shall

first describe mitochondrial mutations, their isolation and characterization, using a minimum of genetic terminology. This will be followed by a short description of mutations conferring resistance to inhibitors of mitochondrial protein synthesis, and by a more detailed description of mutations conferring resistance to oligomycin. This last category implicates mitochondrial ATPase, which is of central interest to bioenergetics; therefore, functional information to be gained from the use of oligomycin-resistant mutants for the mechanism of oxidative phosphorylation will be outlined in some detail.

For a complete survey of the literature, the reader is referred to several recent books [1–3], symposia [4–7], and review articles [8–15].

Mutations of Mitochondrial DNA (mtDNA)

Two classes of mutations have been found in the mitochondrial genome of yeast: the ρ^- petite mutations and point mutations.

Petite (ρ^-) mutations. These mutations occur spontaneously with a high frequency of about 1–10% (in certain cases even 90%) and are caused by large deletions or the complete loss of mtDNA. Because of the loss of DNA sequences, they cannot revert to wild type cells. The residual DNA fragment of a mtDNA-containing petite mutant is amplified to give about the same cellular amount of mtDNA as the wild type cells, and probably also molecules of the same size of 25 μm [16–18]. These mutants are very useful for enriching and purifying special parts of the mitochondrial genome [16]. Owing to the large deletions, most ρ^- mutants have lost the genes coding for the mitochondrial ribosome (Table I), and consequently, mitochondrial protein synthesis is absent. As a result, certain components of the electron transport and energy conserving system are missing in the mitochondria of ρ^- cells (cf. [12]); therefore any mitochondrial protein still present must be due to cytoplasmic, not mitochondrial ribosomes. Interesting recent examples are the occurrence in ρ^- mitochondria of cytochrome c_1 [19], and recombination of mitochondrial DNA [20], which had formerly been considered to be absent; their presence now demonstrates their independence of mitochondrial protein synthesis.

Point mutations. Because of their pleiotropicity of phenotype, ρ^- petite mutants are not suitable tools for defining which mitochondrial genes code for which proteins. This purpose is better achieved by correlating a point mutation with a single change in a protein sequence. The operational definition of a point mutation is its low frequency (about 10^{-7}–10^{-8} cells), and the occurrence of reversions. In procaryotes, point mutations have been shown to be of three types, the nonsense, frame-shift, or missense type. At the protein level, a nonsense mutation will stop elongation of the polypeptide chain at the nonsense

TABLE I. Mitochondrial mutations in *Saccharomyces cerevisiae*

Locus	Phenotype		Presumably due to mutation		Presumable gene product	Mitochondrial protein synthesis	Respiration	Ref.
			Point	Deletion				
RIB1	Growth on oxidizable carbon source, Yes	Resistant to chloramphenicol	+		Ribosome	Yes	Yes	38, 13
RIB2	Yes	Resistant to erythromycin and/or spiramycin	+		Ribosome	Yes	Yes	38, 13
RIB3	Yes	Resistant to erythromycin and/or spiramycin and/or chloramphenicol	+		23S RNA	Yes	Yes	38, 13
PAR1	Yes	Resistant to paromomycin	+		Ribosome	Yes	Yes	40, 41
MIK1	Yes	Resistant to mikamycin	+		?	Yes	Yes	42
OLI1	Yes	Resistant to oligomycin and venturicidin	+		ATPase complex (oligomycin resistant)	Yes	Yes	43, 44
OLI2	Yes	Resistant to oligomycin	+		ATPase complex (oligomycin resistant)	Yes	Yes	43, 44
OLI3	Yes	Resistant to oligomycin and venturicidin	+		ATPase complex (oligomycin resistant)	Yes	Yes	44
OLI4	Yes	Resistant to oligomycin	+		? (ATPase complex oligomycin sensitive)	Yes	Yes	45
TSM8	Yes	Temperature sensitive	+		?	Yes	Yes	46
RD73/1	No	Pleiotropic	+		?	Yes	No	27
E3-19	No		+		Cytochrome oxidase	Yes	No	31
M3-9	No				?	Yes	No	31
ρ^-	No	Pleiotropic		+		No	No	13, 16, 2

codon position, thus producing protein fragments. Frame-shift mutations are caused by the insertion or deletion of a base pair, which therefore changes the reading frame. A completely changed amino acid sequence will be the result. Missense mutations will cause a change of amino acid within the respective polypeptide chain. Depending on the position and the amino acid, such a change results in a reduced, temperature-sensitive, drug-resistant, or completely inactive function. It is reasonable to assume that such mutations also occur in mtDNA of yeast, and a series of mitochondrial point mutations have been isolated, but no correlative change in a polypeptide chain has yet been demonstrated.

One difficulty might be encountered by this kind of approach. Many of the missense mutations will be characterized as pleiotropic, since the parent and its mutant derivatives sometimes differ not by only one but by many aspects of their phenotype. Such pleiotropy is to be expected because of the multiple interactions between mitochondrial membrane components. Some nuclear point mutations are already known that cause the disappearance of several mitochondrial proteins [21–24]. On the other hand, the lack of one protein in a mitochondrial mutant might not be a sufficient criterion to identify the respective gene product. Therefore, mutations causing not a loss but a change of functions are preferable for demonstrating sequence changes in a protein. Notwithstanding these difficulties, the pleiotropy of a well-characterized mutation can give interesting insights into the interactions of the respective gene products.

Isolation of Mitochondrial Mutants

A spontaneous point mutant usually occurs with a low frequency in the order of 1 in 10^7 to 10^8 cells. As only about 10^9 yeast cells can be seeded onto an agar plate, it is useful to increase the mutation rate by mutagenic agents. Preference should be given to mutagens like *N*-methyl-*N*-nitro-nitrosoguanidine or ethyl-methane-sulfonate, which are known to increase point mutations. Agents inducing cytoplasmic petite mutants, such as ethidium bromide, might be less useful, as they cause large deletions of mtDNA. The mutagenic action of Mn^{++} might be of special interest: It has been shown to increase specifically mitochondrial antibiotic resistant mutants, although numerous ρ^- mutants were also induced [25].

Mutagenesis can be made more effective during cycloheximide treatment; In these conditions, nuclear DNA replication will be arrested while mtDNA replication will continue for some time [17, 26, 27].

Mitochondrial mutants should, of course, be isolated only in a yeast strain carrying appropriate nuclear markers to permit further genetic manipulations. Genetic crosses can be done at once if the mutations have

been isolated in a haploid strain. Diploid strains have the advantage of favoring the appearance of mitochondrial mutants, as recessive nuclear mutations of the same phenotype will not be expressed. On the other hand, diploids have to be sporulated before further genetic manipulation.

A mitochondrial mutation occurring in one of the many mtDNA molecules of the yeast cell will probably not be expressed at the cellular level. Intracellular selection seems to be necessary to isolate cells with pure mtDNA. Such strong selection is facilitated in facultative aerobes like *S. cerevisiae,* as mitochondrial function is dispensable on glucose medium and indispensable on nonfermentable carbon sources. Antibiotics, like mikamycin, paromomycin, erythromycin, chloramphenicol, spiramycin, oligomycin, and venturicidin, which inhibit growth of yeast on nonfermentable carbon sources only, are specific inhibitors of mitochondrial functions [9]. They have been successfully used to select the respective mitochondrial resistance mutants on glycerol plates containing an appropriate concentration of one of these antibiotics [1, 2, 4, 5]. Sometimes the uptake of an antibiotic or a drug by yeast cells will not occur, or is restricted to a special pH of the medium. The pH of the growth medium should therefore be adjusted for optimal inhibition by the drug [28]. Another limitation of this selection procedure can often be the unspecificity of a so-called specific inhibitor. It is evident that a drug affecting several mitochondrial or cellular functions will most probably give rise to permeability mutants.

While antibiotic-resistant mutants can be detected very easily, no comparable selection is available for growth-defective mutants. It would be of tremendous importance to have an enriching technique for such negative mutants. Amphotericin B and nystatin seem to preferentially kill growing yeast cells [29. 30], thus enriching for the mutant phenotype under conditions where the mutant cannot grow. However, this selection technique is more difficult to control in various yeast strains than the analogous penicillin selection method in bacteria. Using amphotericin B, Flury et al. [27] have isolated a mitochondrial point mutant that might be due to enrichment by this agent. This mutant cannot grow on glycerol, has nevertheless retained the capacity for mitochondrial protein synthesis, reverts, and exhibits a pleiotropic phenotype.

Another possibility for obtaining mitochondrial mutants is to investigate, by a fast biochemical assay, a large number of cell clones for the presence or absence of a function such as mitochondrial protein synthesis. If a single gene specifies the function to be investigated, the probability of finding such a defective mutant would be about 1 in 10^7 cells. This means that at least 10^7 clones have to be tested in order to find the desired spontaneous mutant. Mutagenesis will increase the probability say by a factor of 100. In case the function is affected not by a single gene but by ten genes, for example, the respective mutant

phenotype might be increased again by a factor of 10 to occur with a frequency of 10^{-4}. The detection of mutants by such an approach will usually be very laborious; nevertheless, it has been recently used by Tzagoloff [31] to isolate mitochondrial point mutants that cannot grow on glycerol but have retained the capacity for mitochondrial protein synthesis. In two such mutants the cytochrome oxidase complex seems to be affected.

Genetic Characterization of Mitochondrial Mutants

Mitotic Segregation

The mutants to be investigated should be crossed with a wild type strain of opposite mating type containing the appropriate nuclear markers. The resulting zygotes contain mixtures of mutated and nonmutated mtDNA molecules. The two different types of mtDNA molecules are sorted out between the mother cell and the bud at each cell division. Such segregation leads eventually to the formation of pure and stable cells containing either the wild type DNA or the mutated DNA. In yeast the segregation into the two pure types of diploid cells is usually completed after 20–40 cellular generations. The occurrence of two classes of diploid cells, a phenomenon called mitotic segregation, is strong evidence for localizing the mutation on extranuclear DNA. It should be mentioned that for this kind of analysis the two phenotypes (mutant and wild type cells) have to be clearly distinguishable. Some aspects of mitotic segregation might be mimicked if, for example, pure sensitive diploid colonies are replica plated on plates containing insufficient antibiotic to stop growth completely. On such plates, a variety of colonies might be observed, some of them showing little or no growth, whereas some other colonies have grown very rapidly.

Meiotic Segregation

When mitotic segregation has occurred, the two types of diploid colonies should be isolated and purified further. The purified diploid cells contain only one type of mtDNA, either mutated or wild type molecules. It is evident that, after meiosis, all four spores will receive the same molecules and therefore the same phenotype as the diploid cell from which they originate: A homogeneous population of mtDNA cannot segregate during meiosis. In contrast, two homologous chromosomes of a diploid organism will be separated from each other during meiosis. If they differ, for example, by one mutation, two spores will receive, while two spores will not receive, this mutation: A single nuclear gene segregates during meiosis in the ratio 1:1.

Both phenomena, mitotic segregation, and meiotic nonsegregation, have been used to identify mitochondrial mutations in yeast. However, it should be stressed that both methods yield a negative result, characterizing the mutations as not being localized on the chromosomes of the nucleus. All extrachromosomal mutations carried by viral, episomal, mitochondrial, and chloroplast DNA can segregate in the same way. In this respect, it is of considerable interest that, even in the simple eucaryotic yeast cell, extrachromosomal mutations occur that definitively cannot be attributed to changes in mtDNA [32, 33].

Loss of Mitochondrial DNA Induced by Ethidium Bromide

When yeast strains carrying a certain point mutation are grown in the presence of ethidium bromide, cytoplasmic petite mutants will arise that have either diminished amounts of mitochondrial genetic information or none at all [16, 34–37].

In the first case, a random fragment of mtDNA is kept and amplified to give about the same mass as in wild type cells. Most of these mutants have lost the initially carried mitochondrial point mutation. Some petite mutants, however, do exist that have kept a DNA fragment carrying this mutation. If another mitochondrial marker is frequently retained at the same time, both have to be very close to each other on the mitochondrial genome. Such analysis of petite mutants will not only allow one to localize mutations on mtDNA, but furthermore establishes their linkage.

In the second case, all mtDNA will be lost, which occurs particularly after growth in the presence of high ethidium bromide concentrations. Of course, no mitochondrial markers will be present in such mutants.

Cross Against a Petite Mutant Completely Lacking mtDNA

Sometimes it might be necessary to subdivide a large number of mutants, having all the same phenotype (i.e., all are, for instance, oligomycin resistant) into mutants of nuclear or mitochondrial origin. In such a case the haploid mutants should be crossed with a cytoplasmic petite mutant of opposite mating type having lost all mtDNA. The function of a nuclear mutation will be complemented in the diploid cell by the nuclear DNA of the cytoplasmic petite mutant. No such restoration of function is conceivable for a mitochondrial mutation, as the petite mutant tester strain lacks all mtDNA.

Linkage with Known Mitochondrial Mutations

The most straightforward procedure for characterizing mitochondrial mutants would be to cross them with one or several tester strains containing most or all of the known mitochondrial mutations. If one of

these mitochondrial mutations and the mutation to be tested are due to a change in the same base pair of mtDNA, they are called allelic and there is no possibility of obtaining even a single wild type molecule by recombination. The probability of obtaining wild type molecules increases regularly with an increasing distance between the two mutations. Linkage of two mutations is demonstrated by a very low percentage of recombinant wild type and double mutant cells. The demonstration of linkage with known mitochondrial mutations is positive and unequivocal evidence for localizing a mutation onto mtDNA. Simultaneously, linkage studies yield data to establish a linear arrangement of mutations [38]. For future work in mitochondrial genetics this method will become the most important one and therefore will be the method of choice for mapping genes on mtDNA. A general model for segregation and recombination of mitochondrial genes in yeast has been recently developed, and predictions based on this model have been found consistent with the experimental data [47].

Biochemical Characterization of Mitochondrial Mutants

Apart from the presumable gene products indicated in Table I, it has been shown that most, if not all, mitochondrial tRNA species [39] and the two ribosomal RNAs (cf [10, 48]) are coded by mtDNA.

It will be noted that the mutations listed in Table I have been separated into two groups, according to growth, or nongrowth, on purely oxidizable carbon sources (in fact, glycerol was used, which is not fermented in yeast). It is generally considered that mutations with a restricted phenotypic effect still allowing growth on an oxidizable carbone source are the best candidates for point mutations, especially if the selection is based on resistance to a specific substance like an antibiotic. However, recently two types of mutations have been described which do not allow growth on glycerol and nevertheless seem to be point mutations [27, 31]. In our following discussion of the biogenetic and functional implications of the mutations involving mtDNA, we are not mentioning these very recently described mutations where the correlation between genetic and biochemical data has not yet been fully worked out. We shall limit ourselves to the "ribosomal" and "oligomycin" mutations, where a discussion of the results seems to be appropriate at the present time.

Mutations Conferring Resistance to Inhibitors of Mitochondrial Protein Synthesis [28, 49, 50, 40, 51, 38, 41]

These mutations are of particular interest if they can be shown to concern structural genes coding for either mitochondrial ribosomal

protein or mitochondrial ribosomal RNA. Although there is good correlation between the antibiotic resistance of whole cells and of mitochondrial ribosomes *in vitro* [52], no protein alterations in mitochondrial ribosomal proteins could be detected by one-dimensional acrylamide gel electrophoresis. More resolutive techniques will have to be applied before a definitive conclusion can be reached as to an eventual change in mitochondrial ribosomal protein.

As regards mitochondrial ribosomal RNA, there is evidence that mutations conferring erythromycin and/or spiramycin resistance, clustered in a genetic locus named RIB3 [38], are very closely linked or possibly inside the 23S RNA gene [53, 54], while the gene coding for the 16S RNA is in the vicinity of the PAR1 locus [55]. The fact that the RIB3 and PAR1 regions are not near to each other [41] on the presumably unique mitochondrial DNA molecule species indicates that there is no common precursor unit to the two mitochondrial ribosomal RNAs, contrarily to what was found in *Neurospora* and HeLa cells (unless there is very long spacer distance covering the whole region between RIB3 and PAR1).

Mutations Conferring Resistance to Oligomycin

The Mutations Due to Nuclear Genes

Before developing in some detail the oligomycin resistance determined by the mitochondrial genome, another class of mutants resistant to oligomycin should be mentioned. These mutants show cross resistance to a variety of functionally unlinked inhibitors [56–59]. Although nuclear genes are clearly involved, some of these mutants exhibit unusual genetic properties suggesting both chromosomal and episomal location. Correlation between occurrence of small 2-μm circular DNA and oligomycin resistance was found in one case [59].

For these mutants, mitochondrial ATPase exhibited unchanged oligomycin sensitivity [56, 59, 60], and no further biochemical studies have yet been published.

In another instance, a nuclear gene determined not oligomycin resistance but complete absence of the mitochondrial ATPase; however, in this case there was a simultaneous strong decrease in the amounts of cytochromes a–a_3 and b [23].

Mutations Due to Genes Located on Mitochondrial DNA [61, 62, 57, 63, 43]

The mutations due to genes located in mtDNA are presently arranged on four loci: OLI1 is closely linked to OLI3 [44], OLI2 is closely linked to OLI4 [45]. OLI1–OLI3 is not linked to OLI2–OLI4. All four mutations,

at the cellular level, are specific for oligomycin and, in certain cases, show cross resistance to venturidicin, another inhibitor of oxidative phosphorylation [44].

A mutation, which is supposed to map also at the OLI2 locus on mtDNA, not only confers oligomycin resistance but shows an interesting nucleocytoplasmic relation: It suppresses nuclear venturicidin resistance [56].

ATPase, in its mitochondrially integrated, or in highly purified form, shows different levels of oligomycin resistance, characteristic of each mitochondrial mutation. This has been established by comparing the different mitochondrial mutations in the same nuclear genetic background [64, 65]. The comparison in such "isonuclear" strains is, of course, the only way to ensure that eventual differences in oligomycin sensitivity will be due only to the respective mitochondrial DNA mutation and not to differences in the nuclear genome. The biochemical method of quantification of oligomycin sensitivity is described in [65].

Significance for Biogenesis

What part of the mitochondrial ATPase complex is coded by the OLI loci? It is known from Tzagoloff et al. [11] that four subunits of the "membrane factor" of the mitochondrial ATPase complex are synthesized on mitoribosomes (by the criterion of cycloheximide resistance and chloramphenicol sensitivity of synthesis) while F_1 [66] and OSCP (the oligomycin-sensitivity conferring protein) [67] are synthesized on cytoplasmic ribosomes. Reconstitution experiments with F_1 + OSCP on the one hand, and "membrane factor" on the other hand, have shown that the mitochondrially determined oligomycin resistance resides in the "membrane factor" [68, 64].

SDS–polyacrylamide gel electrophoresis has shown [64, 69], that none of the subunits is missing or differently migrating when mutant (OLI1 and OLI2) and wild type purified ATPase complexes [70] were analyzed. It should be noted that this method of purification retains the "membrane factor", and the resulting highly purified ATPase complexes from wild type and mutant strains show the same differences in oligomycin sensitivity as the mitochondrially integrated ATPase. More refined techniques, particularly suited for revealing slight changes in hydrophobic proteins, will be needed before it can be concluded whether the four OLI loci correspond to four modified subunits, or whether the two groups of linked loci specify different modifications on two subunits.

Readers familiar with bioenergetics are well aware of the fact that phospholipids attached to the mitochondrial ATPase complex [11] are indispensable for the expression of oligomycin sensitivity [71]. Although this is not a very attractive possibility, it cannot be excluded at

the present time that one of the mutations corresponds to a change in phospholipids associated with the ATPase, owing to a mutation influencing an enzyme implicated in lipid metabolism. Because of the growing interest in lipids in membrane biochemistry, we would like to briefly discuss some biogenetic implications related to the presence of phospholipids in the ATPase complex.

It should be noted that mere analysis of phospholipids associated with the purified ATPase complex could provide no formal proof that the mutation did, or did not, concern an enzyme of phospholipid metabolism instead of a protein subunit of the ATPase: Any difference in phospholipid composition might in fact be a secondary consequence of a change in the hydrophobic, lipid-carrying protein subunit of the ATPase. Attacking the problem from the lipid angle would yield conclusive results by (a) splitting phospholipids off the purified complex (resulting in disappearance of oligomycin-sensitivity), (b) adding back known amounts of well-defined phospholipid species and recovering partial oligomycin sensitivity. If the different oligomycin sensitivities observed in the mutants are restored under these conditions, it might be said that the initial difference in oligomycin sensitivity was due to a mutational change in a protein subunit of the ATPase complex. Although this approach has not yet been carried out, somewhat related experiments with Phospholipase A or very low amounts of Triton X 100 have shown [72] that oligomycin sensitivity in the mitochondrially integrated, or in the highly purified ATPase complex, is limited by a permeability barrier (i.e., these reagents produce a more than tenfold increase in the apparent affinity for oligomycin). However, it is *not this barrier that is coded for* by the OLI1 and OLI2 genes, because the genetically determined differences between strains subsists even when the oligomycin sensitivity is greatly increased by these treatments.

Significance for Function

In ρ^- respiratory deficient cells it is well known that the mitochondrial ATPase is *fully oligomycin resistant* [73] owing to pleiotropy including the absence of mitochondrially synthesized ATPase "membrane factor" subunits. In the case of the point mutations of mtDNA conferring oligomycin resistance, it was initially thought that the ensuing *partial oligomycin resistance* produced no energetic drawback; in fact, the mutants and the wild type had the same growth rate, and the same growth yield, on natural medium supplemented with an oxidizable carbon source. However, it was found quite recently, with one mutated allele at each locus [74], that on synthetic medium, and only with an oxidizable carbon source, the growth rate of the mutants is reduced in proportion to the increase of oligomycin resistance of the mitochondrial ATPase:

Oligomycin resistance of ATPase	wild type	$\leqslant$ OLI4	$<$ OLI2	$<$ OLI1
Mean generation time (h)	5	5	6	7

At the level of mitochondria isolated from the mutant at locus OLI1, it was found that state-3 respiration showed increased oligomycin resistance, while P/O ratios and respiratory control values were significantly lowered as compared to the isonuclear wild type [74]. These "energy conservation parameters" are confined to the isolated mitochondrion [75] and cannot be transposed in any simple way to explain the reduced growth rate of the oligomycin-resistant mutants. Nevertheless, these parameters do indicate that in the mitochondria from these mutants the coupling membrane shows an altered efficiency or fragility associated to a genetically determined change in the ATPase complex. The study of proton transport through the mitochondrial membrane will probably be rewarding in this respect, especially if it is possible to dissociate electron transport, driven proton ejection, and the return flow of protons during state-3 respiration. Insofar as, according to Mitchell's hypothesis [76], the backflow occurs through the mitochondrial ATPase, and more particularly the "membrane factor" of the ATPase, a part of the H^+ backflow should be oligomycin sensitive in the wild type, and oligomycin resistant in the mutants. One of the most challenging problems of oxidative phosphorylation would thus become amenable to experimentation.

Concluding Remarks

Different mitochondrial mutants have been isolated that affect mitochondrial ribosome function. These mutants were used to establish most of the known methods and principles of mitochondrial genetics in yeast. Another class of mitochondrial mutants have been shown to affect mitochondrial ATPase and, more specifically, the "membrane factor" of mitochondrial ATPase. These mutants might be very useful in studying the energy-conserving function, and the interaction between the hydrophobic and hydrophylic parts, of the ATPase complex. New types of mitochondrial point mutations, concerning cytochrome a–a_3 or b, will soon open up new fields of investigation.

The biochemical and genetic analysis of numerous mutants belonging to that category and recently obtained [31] is being currently pursued in Tzagoloff's and Slonimski's laboratories.

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