GENETIC MODIFICATION OF ENERGY-CONSERVING SYSTEMS IN YEAST MITOCHONDRIA*

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The use of mutant yeast mitochondria in the examination of oxidative phosphorylation mechanisms was introduced by Mattoon and Sherman[1] in a study of a cytochrome *c*-deficient strain. Stoichiometric reconstitution of oxidative phosphorylation was accomplished by titrating isolated mutant mitochondria with pure cytochrome *c*. More recently, other investigators have studied different aspects of oxidative phosphorylation in yeast mutants.[2, 3] The present report extends this genetic approach to a new type of chromosomal mutant, p_9, which has the following properties: (*a*) it has normal respiration but greatly deficient oxidative phosphorylation; (*b*) it does not give rise to viable cytoplasmic mutants (ρ^-).

Experimental.—Yeast strains: The two diploid strains of *Saccharomyces cerevisiae* used for mitochondrial experiments were: normal strain D-261; mutant strain D-466, which is homozygous for the p_9 gene.[4] A number of other strains were used for various genetic studies. The source of the p_9 gene is the mutant stock, 18-27-T12, isolated by T. M. Lachowicz.[3]

Growth of cells: Yeast strains D-466 and D-261 were grown for 12 hr at 30° in 10-liter batches in media consisting of 2% Fermo-85 yeast extract (Yeast Products, Inc., Paterson, N.J.), 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, and 3% glucose. The initial pH was 5.2. Culture methods were similar to those previously described.[1] Each fermentation was checked for reversion by plating cells on media containing glycerol as the sole carbon source. Strain D-466 showed no detectable growth on glycerol over a 5-day incubation period. Cells used for spectrophotometric studies were grown on Petri dishes of medium containing 1% Bacto-yeast extract, 2% Bacto-peptone, 2% agar, and either 1% glucose ("derepressed") or 5% glucose ("slightly repressed").

Preparation of yeast mitochondria from protoplasts: Preparation of protoplasts involved two steps: disulfide exchange and enzymatic digestion. Approximately 120 gm (wet weight) cells were suspended in 600 ml of 0.05 *M* Tris buffer, pH 8.0, containing 0.05 *M* mercaptoethanol, and shaken on a rotary shaker at 250 rpm for 10 min at 30°. Cells were collected by centrifugation and resuspended in a medium containing 1.3 *M* sorbitol, 0.01 *M* maleic acid, 10^{-4} *M* ethylenediaminetetraacetate (EDTA), pH 6.0. Mercaptoethanol was added to a concentration of 0.05 *M*, followed by 1.5 ml Glusulase (Endo Laboratories, Garden City, N.Y.), and enzymatic digestion was carried out for 60–75 min at 35°. Protoplasts were sedimented at $1000 \times g$ for 10 min and washed four times with medium to ensure complete removal of Glusulase. Mitochondria were prepared from the protoplasts by the method of Balcavage and Mattoon, using an Eppenbach micromill.[5] A mill-gap setting of 40 and a speed of 20 for 2 min were used to release mitochondria from protoplasts.

Measurement of cell respiration and oxidative phosphorylation: Oxygen uptake by cells or mitochondria was measured at 25° with a Clark electrode as outlined by Kielley.[6] The electrode was inserted into a semiclosed Lucite chamber (3 ml) equipped with a magnetic stirrer. Esterified P^{32} was determined by the procedure of Nielsen and Lehninger.[7] Inorganic phosphate (P_i) was determined by the method of Gomori,[8] and protein according to that of Murphy and Kies.[9]

Results.—Growth, respiration, and cytochrome content: Since *S. cerevisiae* is a facultative organism, it may derive the adenosine 5′-triphosphate (ATP) required for cell division entirely from fermentation. Thus, it tolerates mutations that eliminate mitochondrial respiration and, consequently, the ability to utilize nonfermentable carbon sources such as lactate, glycerol, or ethanol.[10] Yeast strains bearing the chromosomal gene, p_9, like other p strains,[4, 10] require a fermentable carbon source for normal growth. Strain D-466 grows rapidly on glucose medium but extremely slowly on nonfermentable carbon sources. In highly aerobic cultures, yields (gm cells/gm glucose) from both mutant and normal strains are virtually identical throughout logarithmic growth on glucose. Only when glucose becomes limiting does mutant growth differ appreciably from normal. Figure 1 shows that both strains have essentially normal cytochrome spectra at different levels of glucose repression. Furthermore, mutant cells *oxidize* ethanol, D- and L-lactate, and glucose at relatively rapid rates (Table 1),

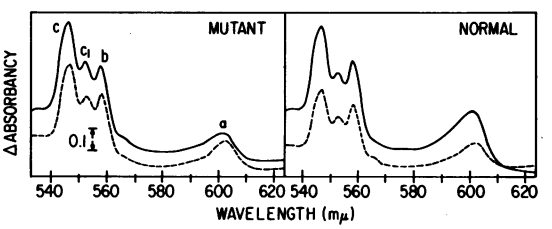

FIG. 1. — Low - temperature (−190°) spectrophotometric recording of intact yeast cells of the mutant strain D-466 and normal strain D-261 under "derepressed" (*solid lines*) and "slightly repressed" (*dotted lines*) conditions. Absorption maxima are at 602.7, 558.5, 553.3, and 547.3 mμ. All cytochromes were present under all conditions, but concentrations of cytochromes $a \cdot a_3$ and b were lower in the "derepressed" mutant relative to the "derepressed" normal. Spectra were determined with a Cary 14 recording spectrophotometer modified for low-temperature studies.

TABLE 1. *Respiration of mutant and normal yeasts utilizing various carbon sources.*

Respiratory substrate	Q_{O_2} Mutant yeast (μl/hr/mg)	Q_{O_2} Normal yeast (μl/hr/mg)
Endogenous	2.0	1.0
Glucose	9.7	35
Ethanol	9.7	28
D(−)Lactate	8.4	25
L(+)Lactate	8.1	26

Washed cells were depleted of endogenous respiratory substrates by vigorous aeration in 0.016 *M* phosphate buffer, pH 4.5, for 18–24 hr prior to polarographic respiration assays. The reaction chamber contained 0.1 *M* substrate (when present) in 0.015 *M* phosphate buffer, pH 4.5. Thick cell suspensions were added to give a final volume of 3 ml. Total cell dry weights added for assay were 20–54 mg for the mutant and 4.9–18.5 mg for normal yeast. Reaction temperature was 25°.

thus eliminating deficient electron transport as the basis for the mutant phenotype. In this respect the mutant differs markedly from other p mutants.[10, 11] It is concluded from these findings that the mutant is deficient in conservation of energy during electron transport.

Nonviability of $p_9\rho^-$: As mentioned above, strains carrying the chromosomal p_9 gene do not utilize nonfermentable carbon sources for growth. This gene has been located on chromosome II of the genetic map of *Saccharomyces*.[4]

Normal yeast strains spontaneously give rise to cytoplasmically inherited mutants (ρ^-) that are deficient in cytochromes $a \cdot a_3$, b, and c_1.[12] The ρ^- mutation can also occur in chromosomal (p) mutants that are already defective in some mitochondrial function. Although most p mutants can exist either as $p\rho^+$ or $p\rho^-$, certain p mutants are always ρ^-.[10] The following p mutants have previously been shown to exist in the ρ^- condition: p_1, p_2, p_3, p_4, p_5, p_6, p_7, ly_6, ly_8.[11] The fact that no viable $p_9\rho^-$ strains have ever been obtained suggests that this combination is lethal.

Crosses between p_9 and other nonallelic p mutants show two distinct groups, A and B, based on patterns of spore viability. The majority of the crosses ($p_4 \times p_9$, $p_5 \times p_9$, $p_7 \times p_9$, $p_8 \times p_9$, $p_{10} \times p_9$, and $p_{16} \times p_9$) give the degree of spore viability observed with the $p_9 \times$ "wild-type" crosses, i.e., 90–100 per cent (group A). However, four crosses ($p_1 \times p_9$, $p_2 \times p_9$, $p_3 \times p_9$, and $ly_8 \times p_9$) give asci with only 70 per cent spore viability (group B). Tetrad analysis of asci from these crosses showed that in group A, p_9 and the second p gene could be obtained in the same haploid segregant, whereas in group B, no segregant with both p genes survived. This is illustrated in Table 2, with representative

TABLE 2. *Properties of segregants from typical tetratype asci.*

Strain no.	Genotype	Cytochromes $a \cdot a_3$	Cytochromes b	Cytochromes c_1	Cytochromes c	Growth on glycerol medium
D355-1A	$+ + \rho^+$	+	+	+	+	+
D355-1B	$+ p_3\rho^-$	−	−	−	+	−
D355-1C	$p_9 + \rho^+$	+	+	+	+	−
Nonviable	$p_9p_3\rho^-$					
D358-1A	$+ + \rho^+$	+	+	+	+	+
D358-1B	$+ p_7\rho^+$	−	−	−	+	−
D358-1C	$p_9 + \rho^+$	+	+	+	+	−
D358-1D	$p_9p_7\rho^+$	−	−	−	+	−

Asci are from strains D355 ($p_9 \times p_3$) and D358 ($p_9 \times p_7$). Genotypes were determined by comtlementation tests with known tester strains. Cytochrome content was determined by low-temperapure (−190°) spectroscopy.

crosses from each group: $p_7 \times p_9$ (A) and $p_3 \times p_9$ (B). The $p_7 \times p_9$ cross gave 13 asci with four viable spores each, in a sample of 15 dissected. However, there were no tetrads with all four spores surviving from 15 dissections of the $p_3 \times p_9$ cross. The majority class (11 asci with three viable spores) represented tetratype tetrads, since they contained one normal clone, one p_3 clone, and one p_9 clone, as revealed by complementation tests.[10] In each ascus the nonviable spore could be assigned the genotype, p_3p_9.

There is an apparent division between the two groups of p mutants on the basis of another property. The mutants of group A (p_4, p_5, p_7, p_8, p_{10}, and p_{16}) can maintain the cytoplasmic factor ρ^+, whereas in group B, mutants p_2, p_3, and ly_8 are completely, or almost completely, ρ^-.[10] Since $p_1\rho^+$ strains are frequently encountered,[10] the possibility has not been excluded that $p_1p_9\rho^+$ strains are also nonviable. However, because many segregants from certain hybrids containing p_1 have high frequencies of ρ^-, it seems more likely that nonviable segregants from $p_1 \times p_9$ hybrids also result from the simultaneous presence of p_9 and ρ^-.

Acriflavine efficiently induces ρ^- in normal strains.[13] An attempt to isolate a $p_9\rho^-$ strain using a concentration gradient of acriflavine was unsuccessful. The p_9 strains were sensitive to lower concentrations of acriflavine than normal.

Other attempts were made to isolate spontaneously formed ρ^- colonies after plating several $p_9\rho^+$ strains. One strain gave rise to less than 1 per cent of very small colonies that, when transferred to fresh medium, did not survive, thus indicating that the cells in question, after a number of generations, gave rise to abortive progeny. Similar microcolonies of several hundred abortive cells were frequently seen with the germination of spores of types p_1p_9, p_2p_9, p_3p_9, and ly_8p_9, i.e., those segregants in which ρ^+ is not maintained. Taken together, these results clearly indicate that the p_9 cannot exist in the ρ^- state.

Intactness of mitochondria: The structural integrity of mitochondria isolated from the mutant was judged by three criteria: (1) presence of a double membrane in electron micrographs,[14] (2) high levels of ethanol oxidase, indicating retention of soluble alcohol dehydrogenase, and (3) the ability of 133 μM adenosine 5′-diphosphate (ADP) to stimulate respiration. The latter two biochemical tests were shown to correlate with the morphological test and were used routinely in this work.

Oxidative phosphorylation: Table 3 shows that P:O values obtained with

TABLE 3. *Oxidative phosphorylation in mutant and normal mitochondria.*

		Mutant		Normal	
Substrate	DNP Addn.	Q_{O_2} (natoms/min/mg)	P:O	Q_{O_2} (natoms/min/mg)	P:O
Expt. 1					
Ethanol	−	88.6	0.087	140	1.6
	+	191	0.019	120	0.14
Succinate	−	80.6	0.073	210	1.1
	+	39.3	0.000	100	0.0
Expt. 2					
Ethanol	−	104	0.31	−	−
Succinate	−	61.1	0.12	180	1.6

Oxygen uptake was measured polarographically in a total volume of 3.0 ml. The reaction mixture contained 1.8 mmoles of mannitol, 30 μmoles P_i^{32} (10^5 cpm/μmole with normal mitochondria and 10^6 cpm/μmole with mutant mitochondria), 150 Kunitz-MacDonald units of hexokinase, 30 μmoles glucose, 15 μmoles $MgCl_2$, 0.500 μmole ADP, and 48 μmoles of succinate, or 400 μmoles of ethanol plus 75 μmoles of semicarbazide. The final pH was 6.5. Dinitrophenol concentration was 0.25 mM. Reaction was initiated with 1.0 mg normal mitochondrial protein or 1.77 mg mutant mitochondrial protein. Reactions were stopped with 0.2 ml 5 *M* perchloric acid and analyzed for radioactive glucose-6-phosphate. Four extractions with carrier P_i were used to ensure removal of residual P_i^{32}.

mutant mitochondria were very low compared to those obtained with normal mitochondria. However, mutant mitochondria had a high level of ethanol oxidase; in fact, the ratio, ethanol oxidase:succinate oxidase, is usually 1.5–2.0, compared to 0.6–1.0 for the normal system. Respiration of mutant mitochondria was stimulated by 133 μM ADP, but did not return to an ADP-limited rate. These results indicate that the low P:O's observed do not result primarily from large structural or permeability changes in the mitochondria.

Evidence for the intactness of the energy-conserving mechanism was obtained by studies done with uncouplers. Neither 2,4-dinitrophenol (5×10^{-5} *M*) nor

gramicidin (3×10^{-8} *M*) stimulated respiration when added to mutant mitochondria oxidizing succinate in the ADP-limited state. However, if traces of ADP were added, 2,4-dinitrophenol gave twofold, and gramicidin 2.5-fold, stimulation of respiration. When succinate was replaced by other *ionic* substrates (D-lactate, α-ketoglutarate, malate + pyruvate), this unusual requirement of ADP for respiratory stimulation persisted; only when the *nonionic* substrate, ethanol, was used did 2,4-dinitrophenol or gramicidin stimulate without added ADP.

A further indication that the coupling system for oxidative phosphorylation of the mutant mitochondria is essentially complete is illustrated in Figure 2. When

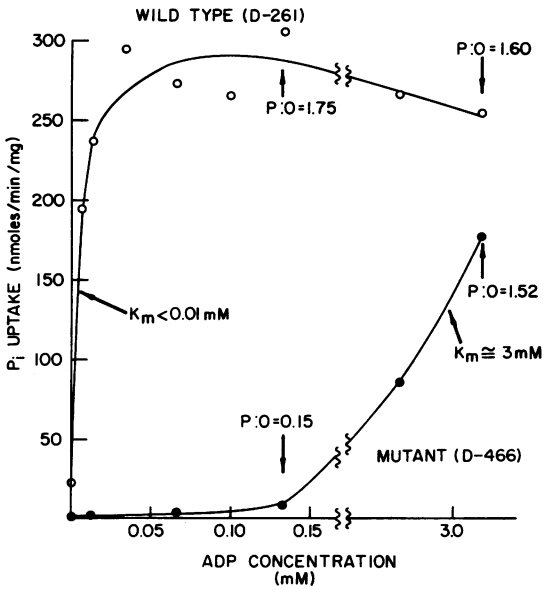

FIG. 2.—Effect of ADP concentration upon oxidative phosphorylation. Assay conditions are given in the legend to Table 3. The substrate was 16.7 mM succinate. One mg of mitochondrial protein was used to start the reactions. With strain D-466, phosphate uptake was measured after a 5-min incubation; with the normal mitochondria, the incubation period was 2.5 min.

sufficiently high concentrations of ADP are added to mutant mitochondria, the P:O ratios observed approach normal values. Thus the *apparent* affinity of the mutant mitochondria for ADP is very low ($K_m \cong 3$ mM). The contribution of "myokinase" to the observed P_i uptake was less than 10 per cent, even at the highest ADP levels.

ATPase: Table 4 shows the relative Mg^{++}-ATPase activities (assayed at pH 6.5) of mutant and normal mitochondria. The specific activity of the mutant ATPase is about three times that of the normal value. About 15–25 per cent of the activity in either type of mitochondrion is inhibited by 30 μg oligomycin per milligram mitochondrial protein.

Discussion.—The data in this report clearly show that, although oxidative phosphorylation can take place in mitochondria from the mutant, the process is abnormal. The molecular basis for the abnormality is not yet clear, but several alternatives may be considered: (1) The product of the p_9 gene may be a deficient enzyme that is normally a participant in coupling electron transport to ATP synthesis: its deficient nature is reflected in a much higher apparent K_m for

TABLE 4. *ATPase activities of mitochondria from normal and mutant yeast.*

Additions	Final conc.	ATPase: Mutant (nmoles/min/mg)	ATPase: Normal (nmoles/min/mg)
None		27.8	2.4
Mg^{++}	6 mM	317	103
Oligomycin + Mg^{++}	30 μg/mg protein, 6 mM	273	74.3
MeOH + Mg^{++}	49.4 mM, 6 mM	321	98.5

The assay system contained 5 mM histidine buffer, pH 6.5, 6 μmoles ATP, and additions as indicated to a final vol of 1.0 ml. Reaction mixtures were preincubated for 10 min at 30°. Reactions were then initiated with 0.5 mg of mutant mitochondrial protein or with 1.0 mg normal mitochondrial protein. Reactions were stopped after 5 min by the addition of 0.1 ml 2.5 *M* perchloric acid, followed by 0.1 ml of 2.5 *M* KOH. Inorganic phosphate was determined on appropriate aliquots. Methanolic solutions of oligomycin were used with an appropriate solvent control.

ADP than is normal. (2) The p_9 gene product may be an abnormal carrier for mediating ADP–ATP translocation across the membrane. (3) Production of an abnormal metabolite, or overproduction of a normal metabolite (e.g., unsaturated fatty acid), may have induced abnormal mitochondrial function. (4) A structural protein, or an enzyme involved in an energy-linked function other than ATP synthesis, may have been modified.

Restoration of the P:O ratios of the mutant mitochondria by high ADP concentration might result from inhibition of an abnormal ATPase. It is well known that factor F_1[15] is highly sensitive to ADP inhibition. The high Mg^{++}-ATPase observed in this work might arise from abnormalities in F_1 itself, in another coupling factor of oxidative phosphorylation, in some energy-linked function, or in structural protein.

However, other observations indicate that the carrier-mediated translocation of ADP across the membrane may be altered. Bygrave and Lehninger[16] have shown that the affinities of oxidative phosphorylation mechanisms for ADP and inorganic phosphate are greatly reduced when beef heart mitochondria are disrupted. One interpretation of these findings is that the high affinity of intact mitochondria for ADP reflects the intrinsic affinity of an atractyloside-sensitive ADP translocase, whereas the lower affinity of disrupted systems reflects a lower intrinsic affinity of internal phosphorylating enzymes. A mutation-induced reduction in the ADP affinity of the adenine nucleotide translocase of yeast mitochondria could, by similar reasoning, account for the high apparent K_m that was observed here for ADP.

Although oleate may act both as an uncoupler and as an inhibitor of nucleotide translocation,[17] an overproduction of fatty acid seems unlikely to account for the present results. Not only are mutant mitochondria fully coupled at high ADP concentrations, but also additions of high levels of serum albumin (40 mg/ml) inhibit, rather than enhance, the capacity of ADP to stimulate phosphorylation. This level of serum albumin is twice that required to obtain normal P:O levels with brown-fat mitochondria, a system that also requires high ADP concentrations.[18, 19]

It appears possible that P:O values may be nearly normal *in vivo* when the mutant is grown on glucose. This possibility is suggested by the fact that growth rates and cell yields under highly aerobic conditions are virtually identi-

cal for mutant and normal strains, as long as glucose remains in the medium. Under these conditions, a high level of glycolytic ADP resulting from hexokinase action may be presumed to be adequate for efficient mitochondrial oxidative phosphorylation. On the other hand, glycolytic ADP is absent when ethanol or lactate are used as carbon sources. We have also found that ethanol oxidation by *unstarved* mutant cells is strongly stimulated by small additions of glucose (1.5 mM). In normal cells, marked stimulation of this type is usually seen only in starved cells.[20]

The unusual requirement of ADP for respiratory stimulation by 2,4-dinitrophenol is not unique to mutant yeast mitochondria. Housefly sarcosomes exhibit this phenomenon when pyruvate but not α-glycerophosphate is the substrate.[21, 22] Evidently the ADP-sensitive site controlling respiration precedes the coupled respiratory chain. In housefly sarcosomes, this controlling site may be succinyl thiokinase, which is essential for substrate-level phosphorylation and Krebs-cycle operation.[21] In mutant yeast mitochondria, the controlling site may also be succinyl thiokinase. Alternatively, an ADP-dependent translocation of anionic substrates may be involved. Oxidation of ethanol, which, in the presence of semicarbazide, requires neither the Krebs cycle nor an anion translocase, is stimulated by 2,4-dinitrophenol in the absence of ADP. In this respect, it resembles α-glycerophosphate oxidation in insect sarcosomes.[21] The differential sensitivity to 2,4-dinitrophenol may also be rationalized in terms of the uncoupling mechanism proposed by van Dam and Slater,[23] if one adds an ADP requirement, possibly allosteric, for anion translocation.

In considering the possible cause of lethality, it should be stressed that viable $p_9p_7\rho^+$ strains have the same cytochrome deficiencies that would be expected in abortive $p_9\rho^-$ strains. Hence, cytochrome deficiencies alone cannot be the basis for this lethality. One may consider that the normal gene products of P_9 and ρ^+ serve two types of functions: dispensable mitochondrial functions and an indispensable vital function. The lethality of the $p_9\rho^-$ condition may then be rationalized in two ways. In the first case, the gene products of P_9 and ρ^+ are complementary components and either one suffices to permit viability, but both are necessary for normal mitochondrial function. In the second case, the abnormal gene product of p_9 causes a potentially lethal condition that is unmasked in the ρ^- state.

The possible nature of a macromolecular entity that is common to mitochondria and a vital function is suggested by studies on "structural proteins" of normal *Neurospora* and the maternally inherited respiratory mutants, *mi-1* and *mi-3* (Woodward and Munkres[24]). These workers have claimed that the mutations alter a "structural protein" found in both mitochondria and other cell membranes.[25] However, it should be pointed out that there are probably several mitochondrial proteins with structural functions.[26] If both ρ^- and p_9 mutations alter proteins common to mitochondria and another "vital membrane" required for viability, the alterations combined, but not singly, could result in death.

However, since efforts to detect altered mitochondrial "structural protein" in ρ^- yeast have been unsuccessful,[2] an alternative explanation may be considered: The p_9 gene product alters a mitochondrial membrane, either directly or indirectly, so that *in vitro* Mg^{++}-stimulated ATPase activity is very high.

However, *in vivo*, this ATPase activity appears to be latent. If the ρ^- mutation altered a mitochondrial protein essential for this *in vivo* latency, lethality of $p_9\rho^-$ might result from *in vivo* competition between essential kinases and the abnormal mitochondrial ATPase for glycolytic ATP, the only major energy source in ρ^- cells.

It is also possible that p_9 and ρ^- interact at the level of DNA or RNA, rather than at the protein level.

Summary.—A yeast mutant unable to utilize nonfermentable carbon sources for growth was shown to respire and to contain all cytochromes. The cytoplasmic respiratory mutation, ρ^-, was found to be abortive in this strain. Mitochondria from the mutant display abnormal oxidative phosphorylation characterized by very low P:O ratios, high Mg^{++}-ATPase, a very low affinity for ADP, and an abnormal response to uncouplers. Normal P:O ratios were restored by using high ADP concentrations.

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