CHAPTER 5

Mitochondrial Biogenesis in Yeast

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I. Introduction

Isolated mitochondria of yeast are able to incorporate amino acids into their structural protein but apparently not into specific proteins (Wintersberger, 1965). Such protein synthesis presumably proceeds by way of a DNA–RNA system since these nucleic acids are present in the mitochondrion. The idea of an intrinsic genetic system in the mitochondrion is substantiated by the finding that there is cytoplasmic inheritance of respiratory deficiency (the petite mutation) in *Saccharomyces cerevisiae*. The main points about this mutant are as follows: (1) it is apparently irreversible; (2) in crosses to normal, the deficiency is not inherited among sexual progeny; (3) biochemically there is loss of mitochondrial enzymes including cytochromes *a*, *b* and c_1 and an apparent loss of mitochondrial DNA; (4) there is morphological aberration in that the inner membrane is incomplete and (5) the mutation can be specifically induced by UV light and by acridines (for details and literature see Roodyn and Wilkie, 1967). From these facts it is generally believed that there is an extra-chromosomal genetic unit, assumed to be the mitochondrial DNA, necessary for mitochondrial development and which can be spontaneously lost or specifically eliminated by mutagens.

From electron microscope pictures it would appear that intact mitochondria can be transmitted to daughter cells in growing yeast cultures which are

actively respiring. Intercalary growth and fission of the organelle is also apparent so the perpetuation of the complete mitochondrial system can be visualized by these means. However, there are no detectable mitochondria in the cells of anaerobically-grown *S. cerevisiae* (Linnane *et al.*, 1962), one of many yeasts that are facultative anaerobes. Nonetheless, these cells inherit the necessary information for making mitochondria as can be seen on transferring them to aerobic conditions. It would seem then that it is not necessary to have intact mitochondria in order that the instructions for making the organelle are transmitted at cell division.

II. Studies with Respiratory Mutants

A. UV induction of respiratory deficiency

UV induction curves of the petite mutation (Wilkie, 1963) are linear in anaerobic cells leading to the conclusion that a single copy of the mitochondrial genetic unit survives mitochondrial disintegration under anaerobiosis. This copy has been termed the mitochondrial DNA master template. At each anaerobic cell division the master template replicates and the replica is inherited by the daughter cell. It is further theorized that on transfer to aerobic conditions many replicas will be made independently of cell division with a single replica per new mitochondrion. That the mitochondrial copies can function genetically is shown by the lag in the UV induction of petites in aerobic cells indicating multiple targets (Fig. 1). It is presumed the multiple targets comprise the master unit together with mitochondrial copies. It is clear, however, that the mitochondrial replicas which must be comparatively numerous in fully adapted cells cannot be accorded the heritability or stability of the master otherwise the UV dose required to destroy all copies would be very great indeed. Also it can be seen that the spontaneous mutation rate of the master in anaerobic cells is about 6×10^{-3} so if all copies are equivalent in aerobic cells the spontaneous rate here should be $(6 \times 10^{-3})^n$ where n is the number of genetic units. In fact the two rates differ only by a factor of about 3.

B. The *GI* mutant

The case of mitochondrial transmission of the genetic unit is brought into sharper focus in the *gi* mutant. Unlike normal cells, repression of the mitochondrial system in this mutant either by anoxia or glucose repression, both of which give incompletely formed mitochondria (Yotsuyanagi, 1962), results in the apparent loss of the cytoplasmic genetic information among daughter cells which are petite. These facts were established by studies of cell lineages involving micromanipulation of daughter cells (Wilkie and Negrotti, 1968). In this mutant it thus appears to be necessary to have conditions for mitochondrial production and proliferation in order that the information be

transmitted. Although genetic analysis of the *gi* character has been hampered by low viability of ascospores from crosses involving this mutant, the indications are fairly clear that the character is under the control of a nuclear gene. In terms of the master template, it is feasible that this gene regulates the replication of the unit. Following mutation of the gene and loss of function, the master can no longer replicate and be transmitted in which case genetic continuity of the unit would then depend on the proliferation of the copies in the mitochondria and their transmission in the intact organelle. Alternatively, the master unit may fail to replicate only under conditions of repression. This appears more likely since *gi* cells have a normal respiratory system regarding both content of cytochromes and genetic stability when growing on media containing non-fermentable substrate or sugars other than glucose. The finding of revertants to normal among *gi* populations of cells also tends to favour the alternative hypothesis; restoration of gene function could be seen to restore normal replication of the master unit.

Although it is not clear what the connection is between repression of the respiratory system and loss of genetic information in *gi* cells, this mutant is of major importance in current studies on the genesis and control of the mitochondrial genetic information.

C. INDUCTION OF RESPIRATORY DEFICIENCY WITH ACRIDINES

Acridines in very low concentrations have been found to cause 100% induction of the petite mutation in growing cultures where fermentable substrate is available. No other mutagenic effect is apparent at these levels of the dye (about 1 ppm) so it is many times more effective in destroying the mitochondrial genetic unit compared with its general mutagenic activity. This activity is believed to result from intercalation of the dye molecule into DNA (Lerman, 1964) causing errors in the replication process and depressing the formation of messenger RNA in the transcription process.

The concentration of acriflavin inducing the respiratory deficiency in yeast strains can be accurately determined by observing colony formation and development on a solid medium containing a non-fermentable substrate such as glycerol. Failure of cells to develop into visible colonies indicates 100% petite induction among daughter cells. In most strains of *S. cerevisiae* this is brought about at a concentration of around 0·5 ppm. The differential mutagenic effect of the dye on the respiratory system is emphasized by the fact that the induced petite cells will grow normally in medium containing between 200 and 500 ppm (depending on the strain) so long as fermentable substrate is available. This extreme sensitivity of both the mitochondrial and the postulated master units is indicative of a cytoplasmic location in each case. An analogy can be drawn between this sytem and that of episomes such as the F factor in bacteria. In this case, when the F factor is in the free

cytoplasmic state (F^+), it is highly susceptible to the action of acridine orange which eliminates this genetic unit from cell populations; when the factor is integrated in the bacterial chromosome, however, it is relatively resistant to the dye (Hirota, 1960). In the case of yeast, the cytoplasmic factor is the mitochondrial DNA, the relatively stable units being the nuclear genes.

Spontaneous mutants of *S. cerevisiae* showing resistance to acriflavin ranging from 0·5 to 10 ppm have been isolated from glycerol-dye plates (Thomas and Wilkie, 1967). The demonstration that there is no concomitant increase in resistance on sugar-dye media suggests that this is resistance of the mitochondrial system to mutagenic action and not a mutational change leading to alteration in permeability of the cell to the dye or to a mechanism for inactivation of the mutagen. In one particular case, a 50-fold increase in resistance to induction of respiratory deficiency has been accompanied by an actual 2-fold increase in sensitivity to acriflavin on a sugar-containing medium. Genetic analysis of a few of these mutants provides evidence that changes in nuclear genes form the basis of the resistance and so far cytoplasmic inheritance of resistance, which would reflect a direct heritable change in the mitochondrial DNA itself, has not been observed. Perhaps these mutant genes make an altered mitochondrial-DNA polymerase (see below) which is better able to recognize points of intercalation of the dye along the double helix and deal with these aberrations during replication.

The degree of intercalation may also be a factor and this can be determined by observing changes in thermal transition (melting point) of the DNA brought about by the process (Lerman, 1964). This is currently under investigation in these studies.

In all the foregoing discussion attention has been focused on the transmission and mutagenicity of the mitochondrial DNA, the tacit assumption being made that this DNA is the cytoplasmic genetic factor of the respiratory system of the yeast cell. A number of controversial points have been made not least of which is the theory of a master template. If it exists, its origin is still obscure. However, these speculations serve to put the problem in perspective and provide starting points for discussion and experiment.

At the present time attention is focused more on the isolation and characterization of the mitochondrial DNA in terms of its amount, base composition and function in regulating protein synthesis in the organelle. These aspects will now be considered.

III. Nucleic Acids in Yeast Mitochondria

A. DNA

The mitochondria of all organisms so far examined have been found to contain DNA distinguishable from nuclear and other satellite DNA by

differences in buoyant density and/or thermal transition point (see Nass *et al.*, 1965). Tewari *et al.* (1965) isolated a discrete native DNA from yeast mitochondria of low buoyant density of 1·685 g/cm^3. Moustacchi and Williamson (1966) identified a similar satellite band in their preparations of whole cell DNA of yeast. Both groups of investigators report the absence of the mitochondrial DNA in cytoplasmic petite strains (see also Mounolou *et al.*, 1966) while Moustacchi and Williamson make the further observation that in the early stages of growth of cultures, during which the cells show glucose repression of the respiratory system, there is little synthesis of mitochondrial DNA relative to nuclear DNA. When cells approach the final stages of growth and are fully respiring, this relationship is reversed with the rate of synthesis of the former showing a relative increase over that of the nuclear DNA. These authors suggest that the specific inhibition of the synthesis of mitochondrial DNA may be the cause rather than the consequence of repression of respiratory enzyme synthesis in the presence of glucose. It is likely that repression by anoxia also leads to a relative loss of mitochondrial DNA. Evidence of this has been seen in this laboratory where satellite DNA of the mitochondrial type is greatly reduced in amount in anaerobically-grown cells of *S. cerevisiae* in thermal transition studies (K. Giles and D. Wilkie, unpublished results). These findings are consistent with a theory of breakdown of mitochondrial DNA under conditions of repression and resynthesis from a master DNA template under inducing conditions.

Based on the estimate of Avers *et al.* (1965) of a complement of 50 mitochondria in a stationary phase cell, the average amount of DNA per mitochondrion has been calculated as $1{\cdot}6 \times 10^{-10}$ μg. This amount could code for about 100 proteins of average size. This compares with an estimated 30 proteins encoded in the circular DNA of mouse liver mitochondria (Sinclair and Stevens, 1966).

The finding that isolated yeast mitochondria contain a DNA polymerase with properties similar to the DNA polymerases of bacteria and mammalian cells (Wintersberger, 1966) is evidence that mitochondrial DNA has genetic continuity. This means, in other words, that mitochondrial DNA can and probably does undergo replication under suitable conditions.

B. RNA

As for DNA, the mitochondria of a wide variety of organisms have been found to contain RNA in amounts ranging from about 10–20 μg/mg mitochondrial protein. Contamination with microsomes is a problem in these investigations but a useful feature of mitochondrial RNA is its insensitivity to ribonuclease when it is located in intact, undamaged mitochondria. This allows the cleaning of preparations by treatment with this enzyme so a good deal of contaminant RNA can be removed in this way.

Analysis of yeast mitochondrial RNA has been carried out by Wintersberger (1966) who was able to separate the RNA into three species by ultracentrifugation. These sedimented with coefficients of about 23S, 16S and 4S. The 4S peak corresponds to *S*RNA while the 23S and 16S fractions correspond to the subunits of bacterial ribosomes which have a coefficient of the ribosome of higher cells. Although this implies a close affinity between the bacterial ribosome and the high molecular weight RNA of the mitochondrion, investigators in this field are not in agreement on this point as not all find a clear separation into these sedimenting fractions (see Rifkin *et al.*, 1967). Kroon (1966) tentatively concludes in his analysis of rat liver mitochondria that one of the RNA components has a sedimentation rate corresponding to 23S but that a possible 16S peak was largely masked by the breakdown products of cellular RNA. Nonetheless he expresses the view that these components represent the intact RNA of mitochondrial ribosomes of rat liver. Taken together, the available evidence indicates the presence of ribosomes in mitochondria but whether they are of the bacterial type awaits confirmation. Indirect evidence that they could be akin to the bacterial ribosome is provided by the studies on antibacterial antibiotics and their effects on mitochondrial synthesis described below.

IV. Protein Synthesis in Isolated Mitochondria

Isolated mitochondria of *S. cerevisiae* incorporate amino acids into their protein as demonstrated by Wintersberger (1965). Using ^{14}C-leucine and ^{14}C-phenylalanine, he showed that the incorporation depends on a functional electron transport system and that it is inhibited by puromycin, actinomycin, chloramphenicol and acriflavin but not by ribonuclease. The labelled amino acids were present to a large extent in an insoluble protein fraction containing RNA and only small amounts of radioactivity were detected in the soluble mitochondrial proteins (free enzymes). This is in agreement with the findings of Kadenbach (1967) for rat liver mitochondria in which it was also shown that the soluble proteins of the mitochondria are first synthesized in the cytoplasm by the microsomes (that is, they are coded for by nuclear genes in the usual way) and then pass into the developing organelle (see also Haldar *et al.*, 1966; 1967).

The presence of transfer RNA and amino acid activating enzymes, a DNA-dependent RNA polymerase and probable ribosomes in yeast mitochondria (Wintersberger, 1965; 1966) leaves little doubt that the organelle has its own machinery for synthesizing proteins. At the same time it is clear from the limited amount of DNA present that the mitochondrion itself does not carry sufficient information to specify these components of its protein-synthesizing system and code for mitochondrial proteins as well.

V. Effects of Antibiotics on Mitochondrial Synthesis

There are one or two brief reports in the literature of the inhibition of amino acid incorporation into mitochondrial proteins *in vitro* by the antibacterial antibiotic chloramphenicol. Linnane and his collaborators subsequently demonstrated the inhibition of mitochondrial enzyme synthesis by this antibiotic in the intact yeast cell (Huang *et al.*, 1966). These investigations were extended to include a range of antibacterial drugs such as tetracycline and erythromycin, with similar results (Clark-Walker and Linnane, 1966). The

Table I

Resistance levels of the respiratory system of yeast strains to various antibiotics and of spontaneous resistant mutants of these strains.

Strain	Resistance (mg/ml)*						
	CAP	TC	ER	CA	OL	SP	LI
22–4B	<0·1	0·1	<0·1	<0·5	<2	5	10
22–4B–CAPR	1	1	<0·1	<0·5	<2	5	10
41	0·1	0·1	0·1	0·5	10	2	10
41–CAPR	2	1	0·1	0·5	10	2	10
41–ERR	0·1	0·1	8	0·5	10	2	10
D243–P1	1	0·5	0·1	<0·5	5	<2	2
D243–P1–ERR	1	0·5	8	0·5	20	2	10
D243–F2	<0·1	0·25	0·1	<0·5	<0·5	<2	<2
D243–F2–ERR	1	1	8	<0·5	0·5	2	10
10–19B	1	4	0·5	<0·5	5	<2	10
10–19B–ERR	1	4	8	0·5	10	2	10
10 (diploid)	2	0·5	0·1	<0·5	2	<2	10
10–ERR	2	1	8	0·5	5	2	10
M (diploid)	1	0·5	0·1	0·5	5	10	10
M–CAPR	4	2	0·1	0·5	5	10	10
44C1	0·5	0·5	0·5	<0·5	5	2	5
4C1–TCR	1	2	0·5	<0·5	5	2	5

Abbreviations:
CAP, chloramphenicol; TC, tetracycline; ER, erythromycin; CA, carbomycin; OL, oleandomycin; SP, spiramycin. CAPR, ERR, TCR denote spontaneous resistant mutants to chloramphenicol, erythromycin and tetracycline, respectively.
* Range of concentrations used (mg/ml):
CAP, 0·1–4; TC, 0·1–4; ER, 0·1–8; CA, 0·5; OL, 0·5–20; SP, 2–10; LI, 2–10.
(From Wilkie *et al.*, 1967).

conclusion was drawn that the antibiotics were directly inhibiting the synthesis of mitochondrial enzymes. In more recent studies (Wilkie *et al.*, 1967) it has been established that the level of tolerance to these drugs is strain-dependent (Table I). In a detailed genetic analysis of various spontaneous resistant mutants to erythromycin (resistance is ability of cells to grow and divide

by utilizing non-fermentable substrate in the presence of the drug and selective plating allows the detection and isolation of such cells), both nuclear genes and cytoplasmic factors have been identified in controlling resistance in respective cases (Thomas and Wilkie, 1968). It has been deduced that the cytoplasmic factors for resistance are carried in the mitochondrial DNA since it was found that these factors are lost when the petite mutation is induced in this category of resistant cell. Petite mutation in gene-determined resistance, on the other hand, has no effect on the transmission of resistance.

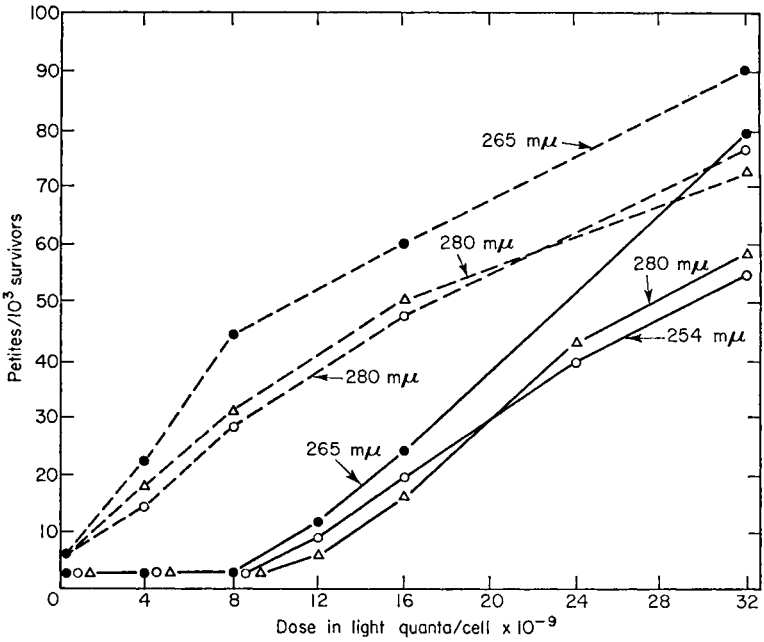

FIG. 1 UV induction curves of the petite mutation in *Saccharomyces cerevisiae*. Solid rule indicates aerobic cells; broken rule indicates anaerobic cells. (From Wilkie, 1963).

From the point of view of mechanism of resistance to erythromycin, it was found in those cases studied that gene mutants, but not mitochondrial DNA mutants, lost the character of resistance after being subjected to a period of anaerobic growth and then put down on the drug. If, however, aerobic growth was permitted once more before exposure to the drug, resistance was restored. Since anaerobic growth results in breakdown of mitochondrial membranes, these results indicate that resistance in gene mutants depends on the prior existence of membranes which have altered components so that they constitute a permeability barrier to the drug. By inference the nuclear genes involved are believed to be specifying these components. Furthermore,

since the outer membrane synthesis is unaffected by the antibiotic (Clark-Walker and Linnane, 1967), these are more likely to be components of the inner membrane. The mechanism of resistance in the mitochondrial DNA mutants may be by alteration in the component of the protein-synthesizing machinery of the mitochondrion which is the site of action of the antibiotic. In the bacterial system this site is the 70S ribosome (see Vazquez, 1966) so it may be assumed that the mitochondrial "ribosome" is likewise the target for drug action. Further evidence for this may come from the study of the binding capacity of antibiotic to RNA fractions of sensitive and resistant mitochondria. If alteration in mitochondrial ribosomes is in fact the mechanism of resistance in mitochondrial DNA mutants (see Cooper *et al.*, 1967, for account of alteration of yeast 80S ribosomes in resistance to the antifungal drug cycloheximide) then, again by inference, mitochondrial DNA is specifying these units.

In summary, the picture that is emerging of mitochondrial biogenesis in yeast is that the organelle has limited auto-reproductive capacity and although possessing intrinsic genetic information, this may function only in specifying components of the organelle's protein-synthesizing system by means of which information of nuclear origin is processed in providing the proteins for assembly of the inner membrane.

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