MITOCHONDRIAL DNA FROM YEAST "PETITE" MUTANTS : SPECIFIC CHANGES OF BUOYANT DENSITY CORRESPONDING TO DIFFERENT CYTOPLASMIC MUTATIONS.

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One of the major unsolved problems of cell heredity is the molecular nature and informational role of cytoplasmic hereditary determinants. In yeast, the formation of respiratory enzymes and mitochondrial structures is under the hereditary control of both chromosomal genes and cytoplasmic genetic determinants (Ephrussi, 1953 ; Slonimski, 1953 ; Yotsuyanagi, 1962 ; Sherman and Slonimski, 1964). There is chemical (Schatz et al, 1964) and cytological (Yotsuyanagi, 1966 ; Yotsuyanagi and Guerrier, 1965) evidence that yeast mitochondria contain DNA. We have asked the question : is the mitochondrial DNA modified in cytoplasmic "petite" mutants ?

DESIGN OF EXPERIMENT

A comparison of properties of mitDNA* that will bear on the molecular nature of cytoplasmic hereditary determinants has to fullfill simultaneously the following genetic and physiological criteria :

a) Strains that differ by the presence or absence of the ρ^+ cytoplasmic determinant have to be as closely isogenic as possible in respect to chromosomal genome. This is easy to realize because acriflavine induced or spontaneous cytoplasmic "petite" mutations do not modify nuclear information (Ephrussi, 1953).

b) Strains that differ by the presence or absence of one of the chromosomal wild type alleles, P_x, controlling respiratory enzymes have to be as closely isogenic as possible in respect to chromosomal and cytoplasmic genomes. This can be realized by comparing a p_x mutant with its back mutant, P_x ("grande").

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* mitDNA = mitochondrial DNA, ρ^+ = cytoplasmic hereditary determinant of mitochondrial respiration and cytochrome oxidase synthesis, ρ^- = absence of the active determinant, P_x = wild type alleles of chromosomal genes controlling mitochondrial respiration, p_x = corresponding mutated inactive alleles.

c) Comparison has to be made not only between a "grande" and a "petite" strain, but between two "petite" strains,one cytoplasmic, the other chromosomal of identical metabolic phenotypes. This control is of paramount importance because it permits to test for the presence of the genetic determinant ρ^+ under metabolically gratuitous conditions. If this control is missing, and if a difference in mitochondrial DNA content is found between a "grande" and a "petite" strain, a wrong conclusion may be drawn. Indeed, mitochondrial development is greatly influenced by the respiration and the fermentation of the cell (Slonimski, 1956, 1958 ; Ephrussi et al, 1956; Yotsuyanagi, 1962), which are, of course, completely different in $\underline{P}_x\ \rho^+$ and $\underline{P}_x\ \rho^-$ cells. In such a case, the effect due to differences in cell metabolism may be misinterpreted as being the genetic cause of these differences.

Therefore the amount and/or molecular properties of mitDNA have to be compared in a set of four cellular genotypes ($\underline{P}_x\ \rho^+$, $p_x\ \rho^+$, $p_x\ \rho^-$, $\underline{P}_x\ \rho^-$), the three latter leading to "petite" phenotypes. If one finds that mitDNA differs in the first genotype from the three other genotypes, it is clear that this difference reflects changes in respiratory metabolism and is irrelevant to changes in cytoplasmic genome. If, on the other hand, one finds that mitDNA is identical in the first two genotypes and different from the two others it is clear that this difference is not due to changes in respiratory metabolism and reflects changes in the cytoplasmic genome.

The necessity of this experimental design was not realized by Corneo et al, 1966 and Tewari et al, 1966, have drawn a logically wrong conclusion from their experimental results. Moustacchi and Williamson, 1966, have compared a chromosomal mutant with a cytoplasmic one, but unfortunately the strain used DP1-1C ($cy_{1-1}\ \underline{P}\ \rho^+$) is not a respiratory deficient one and synthesizes normal cytochromes a, a_3 and b (cf. Slonimski et al, 1963).

d) Cytoplasmic "petites" belong to several hereditary classes that can be distinguished by the respiratory phenotype of the diploïds issued from the cross of "petite" by normal "grande". In the case of a "neutral petite" (ρ_n^-), the great majority of diploïds respire and the "petite" character behaves therefore as a recessive one. In the opposite case, i.e. "suppressive petite" (ρ_s^-), the great majority of diploïds do not respire and the "petite" character shows a dominant behaviour (Ephrussi et al, 1955 ; Ephrussi and Grandchamp, 1965). The question has to be asked whether mitDNA is identical or not in different hereditary classes of cytoplasmic "petites".

e) The development of mitochondrial enzymes and structures is repressed by an intense fermentative metabolism of C and energy source (Slonimski, 1956, 1958 ; Ephrussi et al, 1956 ; Yotsuyanagi, 1962; Utter et al, 1965). This metabolic repression can be made inoperative in two ways : by growing yeast on a non fermentable energy source (eg. ethanol lactate) or by growing it on a fermentable one (eg. glucose) but under conditions where glucose is the sole limiting factor of the rate of growth. The first way cannot be applied to "petites" which, by definition, do not grow on non fermentable energy sources.

In the present work, all strains are grown aerobically at 28° C in a chemostat ; the medium contains 1% Difco yeast extract, 0,12% $(NH_4)_2SO_4$, 0,12% KH_2PO_4 and glucose (5% in the reservoir) is delivered at the washing-out rate $\frac{w}{V}$ = 0,11 hr^{-1}. (cf. Novick and Szilard, 1950). The cell density is maintained between 2,2 and 3 mg dry weight per ml for various "petite" strains.

In the present work, comparison is made between four strains :

- D243-2B ($p_7\rho^+$, Sherman and Slonimski, 1964) a chromosomal, single gene mutant deficient in cytochromes a, a_3, b and having no respiration. It carries the cytoplasmic determinant ρ^+. Cultures of this strain contain generally less than 20% of ρ^- cells (tested by complementation with C-982-19dA_1, cf. Jakob, 1965) although this proportion may show some fluctuations.
- D243-2B-R_1 ($\underline{P}_7\ \rho^+$) a respiratory sufficient strain isolated from the previous one. This reversion has been shown not to be due to an unlinked suppressor mutation. Cultures of this strain contain less than 5% of ρ^- cells.
- D243-2B-g ($p_7\ \rho_n^-$) a cytoplasmic neutral mutant derived from the first strain and metabolically identical with it. Cultures of D-243-2B-g contain 100% of ρ^- cells. The degree of suppressiveness (cf. Ephrussi and Grandchamp, 1965 ; Sherman and Ephrussi, 1962) as determined with C-982-19d ($\underline{P}\ \rho^+$) tester is very low (less than 5%).
- D243-2B-13 ($p_7\ \rho_s^-$), a cytoplasmic suppressive mutant derived from the first strain and metabolically identical with it. Cultures of D243-2B-13 contain 100% of ρ^- cells. The degree of suppressiveness is very high ($\geq$ 90%).

METHODS

Cells are suspended in sucrose 0.44 M, Tris pH = 7, 0.02 M, EDTA 0.001 M (cf. Manler et al, 1964) and broken down in a refrigerated Nossal shaker with glass beads (30 sec.). Intact cells and debris are eliminated at 1000 g in 10 min. Two methods of isolation of DNA have been used :

1) Intact cells or mitochondria rich fractions are frozen down at -25° C and thawed back in Tris 0.01 M, EDTA 0.01 M. Duponol (1%) is added and the mixture incubated for 3hrs at 37° C. DNA is purified by successive treatments with pronase, T_1 and pancreatic RNAases, three deproteinisations by Sevag procedure and precipitations with ethanol and isopropanol (Smith and Halvorson, 1966).

2) Mitochondria rich fraction is treated with phenol in the presence of EDTA 0.01 M and Duponol 1%. DNA is purified by successive treatments with RNAases and ethanol precipitation (cf. Marmur, 1961).

Analysis of DNA is carried out by CsCl gradient (Meselson et al, 1957). The density marker is Micrococcus lysodeiticus (kindly supplied by Dr. L. Hirschbein) DNA taken as 1.731 g cm^{-3} (Schildkraut et al, 1962).

RESULTS

DNA isolated from whole cells of a "grande" ($\underline{P}_{\chi}\ \rho^{+}$) shows three components : a major band (1.701 g cm^{-3}) and two shoulders, one lighter (1.687 g cm^{-3}) and one heavier (1.705 g cm^{-3}). This result is in good agreement with recent data (Tewari et al, 1965 ; Corneo et al, 1966 ; Moustacchi and Williamson, 1966 ; Guérineau et al, 1966). Fig. 1 shows the tracing of DNA isolated from the mitochondria rich fraction of the same strain : now the main band corresponds in density to the light satellite (1.687 g cm^{-3}) of the bulk DNA. The 1.687 g cm^{-3} band shows a typical absorption spectrum of nucleic acids and disappears after DNAase treatment. Similar results have been recently reported by Smith and Halvorson (1966).

Fig. 1

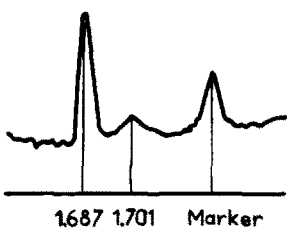

Fig. 2

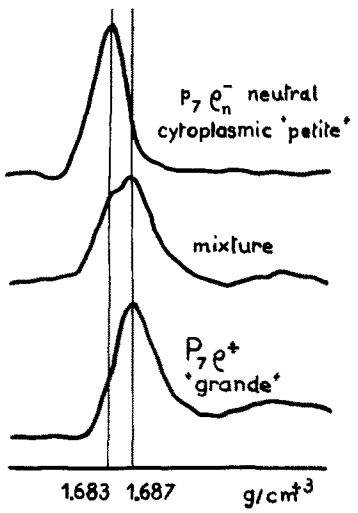

Fig. 1. - Microdensitometer tracing of mitDNA from a "grande" centrifuged to equilibrium in a CsCl gradient. The major peak has a different density (1.687) from bulk DNA of whole cells (1.701).

Fig. 2. - Differences in density between mitDNA from a cytoplasmic "petite" and a "grande". Artificial mixture of these two DNA's clearly shows the existence of two components of different density.

Table 1 summarizes the main features of DNA's isolated from whole cells and from the mitochondria rich fractions of various strains. DNA of nuclear origin shows no difference in density. Mitochondrial DNA presents remarkable differences according to the cytoplasmic genome : it is lighter in cytoplasmic neutral "petite" (1.683) and heavier in cytoplasmic suppressive "petite" (1.695), both being different from the "grande" (1.687). In view of the design of experiment it is clear that these changes in the density of mitDNA are specific to cytoplasmic mutations and do not reflect neither the chromosomal mutation nor changes in mitochondrial metabolism. Fig. 2 shows that differences in density of mitDNA although small are significant because an artificial mixture of purified mitDNA's

MAIN FEATURES OF NUCLEAR AND MITOCHONDRIAL DNA'S PREPARED
FROM CHROMOSOMAL AND CYTOPLASMIC MUTANTS AFFECTING RESPIRATORY CHAIN ENZYMES

STRAIN	NATURE OF GENETIC DETERMINANTS		Q_{O_2}	CYTOCHROMES			DNA			NUMBER	
	Chromosomal	Cytoplasmic		$(a+a_3)$	(b)	(c)	Nuclear (density)	Mitochondrial (density)	Mitochondrial % total	Runs	Cultures
Normal "Grande" $\underline{P}_7\ \rho^+$ D243-2B-R_1	normal	normal	100	+	+	+	1.701 ± 0.002	$\underline{1.687}$ ± 0.002	ca 14	7	3
Chromosomal "petite", $p_7\ \rho^+$ D243-2B	mutated recessive gene	normal	0	-	-	+	"	$\underline{1.687}$ ± 0.002	ca 10	8	3
Neutral "petite" $p_7\ \rho^-_n$ D243-2B-g	- " -	mutated recessive	0	-	-	+	"	$\underline{1.683}$ ± 0.003	ca 14	6	2
Suppressive "petite" $p_7\ \rho^-_s$ D243-2B-13	- " -	mutated dominant	0	-	-	+	1.702 ± 0.001	$\underline{1.695}$ ± 0.001	ca 10	6	3

All strains are isogenic except for differences that are specified. Every culture corresponds to an independent chemostat (glucose limited, mean division time ca 5 hours, cells harvested after at least 8 divisions that have occured in the chemostat. Mitochondria prepared by mechanical breakage of cells in 0.44 M sucrose, centrifugation at 25kg 30 min. DNA purified by successive treatments with Duponol 1%, Sevag (3 times), pronase digestion, RNAse digestion (pancreatic and T_1) precipitation with ethanol and isopropanol and analysed in CsCl density gradient ($g.cm^{-3}$). Several analytical runs per culture performed.

can be resolved by CsCl gradient into its original components. Furthermore the amount of mitDNA synthesized in different strains, "grandes" or "petites", is of the same order of magnitude (table 1). This amount (ca 12% of total DNA) is much higher in chemostat, glucose-limited, grown cells than in ordinary glucose grown cultures. This may be due to a greater extent of fermentative derepression and/or to a slower growth rate. It should be added that : 1) whatever the method of isolation of mitDNA, the differences in density resulting from cytoplasmic mutations are constantly observed, 2) "Grande" strains of different chromosomal genotypes show the same mitDNA of 1.687 density, but in various amounts.

It can be concluded that the cytoplasmic "petite" mutation is not due to a lo of mitDNA but to a change in its buoyant density. Furthermore, two extreme genetic states of the cytoplasmic, hereditary determinants, the recessive and the dominant one, differ in the density of their mitDNA. The mitDNA may be identified therefore with the ρ genetic determinant and the mechanism of hereditary changes of its buoyant density raises several questions : does it correspond to changes in the base-composition, to differences in the extent of methylation or to the presence of unknown components ?

Do different cytoplasmic mutations result from an induced change in the density of a single molecular species of mitDNA or from a selection, that could be either intermitochondrial or intramitochondrial, among a population of non-identical mitDNA molecules originally present in the wild type cell ?

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