RECOMBINATION IN YEAST MITOCHONDRIAL DNA

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When haploid yeast strains containing mitochondrial DNAs (mtDNAs) of different buoyant densities are mated, the resulting zygotes contain a mixed population of mitochondria and mitochondrial DNAs. During vegetative growth of diploid cells formed from such a cross between a petite strain with mtDNA of density 1.677 g cm^{-3} and a respiratory competent strain with mtDNA of density 1.684 g cm^{-3}, mtDNAs with intermediate buoyant densities are obtained. Virtually all newly synthesized mtDNA in diploid ρ^- progeny has the intermediate buoyant density. Therefore, within 2 generations of growth of the diploid cells, the intermediate buoyant density species predominate. In crosses between a respiratory competent strain and other petite strains with different values of genetic suppressiveness, it was found that the amount of recombination yielding mtDNAs of intermediate buoyant densities roughly parallels the degree of suppressiveness. Individual clones of respiratory deficient cells from such crosses were also isolated to confirm that stable mtDNAs with intermediate buoyant densities were obtained. Thus, it is apparent that some form of recombination takes place within the mtDNAs of yeast cells that results in stable mtDNA species.

1. INTRODUCTION

The findings that mitochondria contain unique DNA molecules which function in directing specific RNA synthesis and that mitochondria also contain all of the components required to allow them to carry out a rapid protein synthesis, have raised the question of the degree of genetic independence of the function of mitochondria (1). The early observations by Ephrussi *et al.* (2), describing suppressiveness as a genetic determinant characteristic of respiratory deficient petite (ρ^-) mutants of yeast, together with the observations linking changes in mtDNA composition to the formation of certain petites (3), raised the possibilities of a functional mitochondrial genetic system. More recently, workers in several laboratories have prepared and investigated antibiotic resistant mutants of yeast in which the mutations appear associated with mitochondrial function (4-6). Many such mutants show a non-Mendelain inheritance of the resistance characteristics which has been attributed to their location on mtDNA.

It has also been demonstrated in crosses among such resistant strains that a genetic recombination or complimentation can occur and that such events take place with reproducible and measurable frequencies. Thus, the possibility of studying mitochondrial genetics has been suggested and a preliminary mapping of certain resistance markers on the mtDNA has been produced (6).

Evidence that mtDNA may truly undergo a physical recombination has come from the work of Carnevali *et al.*, who have recovered from a respiratory competent (ρ^+) X respiratory defficient (ρ^-) cross, 2 ρ^- strains in which the

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density of the "satellite DNA" is different from the density of the parent ρ^- strain (7). In the present study, we have further investigated recombination among mtDNAs by analyzing the total distribution of mtDNAs in the diploid cells resulting from a large scale cross between ρ^+ and ρ^- cells having different mtDNA buoyant densities. Evidence for a large scale recombination has been obtained and the appearance of mtDNAs with buoyant densities between those of the parental strains has been tentatively correlated to the genetic suppressiveness of the ρ^- strains used in the cross.

2. METHODS

The following strains of *Saccharomyces cerevisiae* were used in these studies:

D243-4A: ρ^+; a,ade_1,lys_2; obtained from F. Sherman.

D243-4A/9: 25% suppressive petite; ethidium bromide induced; buoyant density of mtDNA 1.679 $g \cdot cm^{-3}$; (8).

D243-4A/13: 94% suppressive petite; ethidium bromide induced; buoyant density of mtDNA 1.674 $g \cdot cm^{-3}$; (8).

D243-4A/15: 93% suppressive petite; ethidium bromide induced; buoyant density of mtDNA 1.677 $g \cdot cm^{-3}$; (8).

X979-2A: ρ^+; α,try_1,his_3; buoyant density of mtDNA 1.684 $g \cdot cm^{-3}$; obtained from R. Snow.

Petite mutants were mated with ρ^+ strain X979-2A using the procedure of Jakob except that aeration of zygotes after centrifugation was omitted (9). In each case, 2 g of the ρ^- strain were mixed with 2 g of ρ^+ strain X979-2A for mating. After mating, the entire cell mixture was suspended in 2 liters of minimal medium (9) containing 2% glucose as carbon source and the zygote cells grown with rapid shaking in a Fernbach flask at ambient temperature. The mean doubling time of cells under these conditions was 6.5 hr. Cells were maintained in logarithmic growth throughout the experiment by removal of aliquots containing 2 g wet weight of cells and transferring to fresh media as required.

Aliquots of cells were removed at various time intervals following mating and whole cell DNA was prepared from the yeast by the procedure of Bernardi *et al.* (10). Only one hydroxyapatite column chromatography step was used, however, with the DNA being applied to the column in 0.2 M sodium phosphate (NaPi) buffer (pH 7.0) and washed with 0.2 M NaPi to remove RNA. The DNA was eluted in one step with 0.5 M NaPi (pH 7.0).

Buoyant densities of the whole cell DNA preparations were measured by equilibrium centrifugation in CsCl gradients in a Beckman Model E analytical ultracentrifuge equipped with a dual beam scanner using the procedure of Schildkraut *et al.* (11). *Micrococcus lysodeikticus* DNA was used as a density marker and solutions prepared as described by Michaelis *et al.* (8). Several diploid petite progeny of a cross between D243-4A/15 and X979-2A were selected by growing the cells in minimal medium plus 2% glucose for 7 generations following mating, then plating colonies on 2% agar containing this same medium. Selected clones were then grown on 1% Difco peptone, 1% Difco yeast extract and 2% glucose solution, harvested and the DNA extracted as above for determinations of the individual mtDNA densities.

Genetic suppressiveness of the various petite mutants used in these studies was calculated by the method of Ephrussi and Grandchamp (12). Ethidium bromide petites were prepared as described by Michaelis *et al.* (8).

3. RESULTS

Crosses between D243-4A petites and the wild type X979-2A using the conditions described, routinely gave rise to nearly 20% mating. The resultant zygotes, when grown on minimal medium had a mean doubling time of 6.5 hr. Thus, following 13 hr or 2 generations of growth of the diploid cells, the

residual nongrowing haploid cells would represent about 50% of the total cells in the culture. After 5 generations, the haploid cells (if stable) would still represent nearly 12% of the total but at later times their relative numbers would become negligible. Thus, extracts of DNA from the total cell cultures shortly after mating would be expected to have appreciable amounts of mtDNA from the haploid parent cells. Only at later times would this decrease to where its contribution to the total DNA extract would be very small.

In Fig. 1 are shown the density distributions of the mtDNAs extracted from the total cell cultures at the indicated times after mating with high suppressive petite 15. The distribution of mass of the mtDNA, banded at equilibrium in a CsCl density gradient, indicates that within 5 generations after mating a band with density intermediate between the parent types predominates in the pattern. Fifteen generations after mating, when the contribution to the pattern due to haploid parent cell mtDNA is gone, most of the mtDNA in the cultures is found with a surprisingly narrow distribution of buoyant densities with a maximum near 1.679 $g{\cdot}cm^{-3}$. Little or no mtDNA remains with buoyant density corresponding to that of the ρ^- parent, but a definite shoulder with buoyant density corresponding closely to that of the wild type mtDNA is observed.

Since petite 15 used in these studies is 93% suppressive when tested against X979-2A, one would expect that 7% of the progeny of this cross should retain their ρ^+ character and therefore presumably also the mtDNA buoyant density of the ρ^+ parent. The exact amount of DNA in the 15 generation sample with ρ^+ density is difficult to calculate from these distributions because of the probable combined size and density heterogeneity present. However, it is

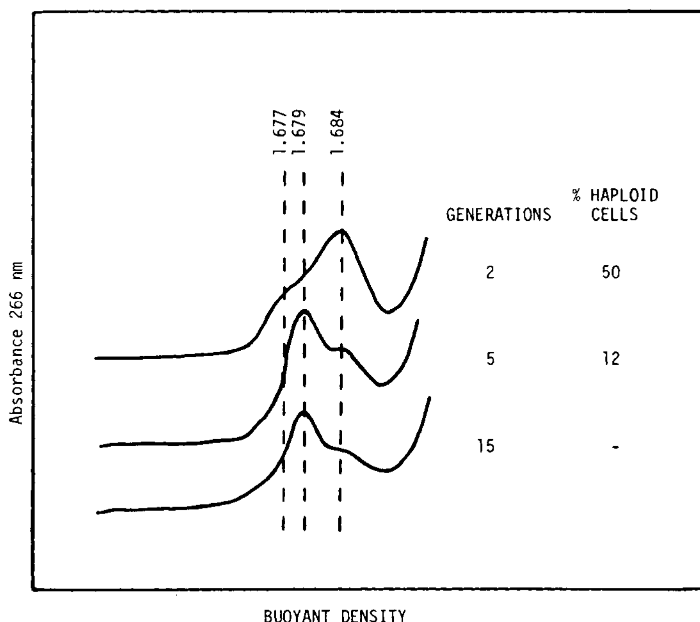

Fig. 1. CsCl density gradient banding of mtDNAs from the cross between the high suppressive (93%) petite 15 and wild type X979-2A. Total DNA was isolated from aliquots of the culture (which included both haploid and diploid cells) at 2, 5 and 15 generations after mating. The DNAs were centrifuged at 40,000 rpm for 24 hr at 25°. The distribution of DNAs was measured by the absorbance at 266 nm. Only the mitochondrial density region is illustrated; the nuclear DNA bands at 1.699.

obvious that most of the DNA has an intermediate buoyant density and only a small fraction has the parent ρ^+ density. Moreover, it might be expected that growth of cells with ρ^+ phenotype would be somewhat enhanced giving a shorter doubling time than the culture as a whole. Thus, the relative fraction of DNA with ρ^+ density might also be somewhat enhanced in the final preparation. Still it is readily apparent that mtDNA with intermediate buoyant density predominates in this cross between the high suppressive petite and ρ^+ cells, and we estimate that only somewhere near 10% of the total mtDNA has the wild type density.

When ρ^- clones from the cross between petite 15 and X979-2A were randomly selected, cultured and analyzed, buoyant densities of mtDNAs were obtained as shown in Table 1. These buoyant densities were intermediate between those of the parental strains as expected from the results of the total cell culture studies.

TABLE 1

Buoyant densities of mtDNA isolated from individual ρ^- clones selected following a cross between petite 15 and wild type X979-2A

Clone	Buoyant density
15d	1.680
15e	1.679
15f	1.678
15g	1.678
15i	1.679
15j	1.678
15k	1.679
X979-2A wild type	1.684
Petite	1.677

Fig. 2 illustrates a similar experiment performed by mating the low suppressive petite 9 with the respiratory competent X979-2A. Again it is apparent that the buoyant density peak corresponding to ρ^- parent disappears from the pattern with time and that a component with intermediate buoyant density is obtained. In this case, however, even after 15 generations the predominant DNA band is centered at 1.684 $g \cdot cm^{-3}$, the density of ρ^+ mtDNA.

Since petite 9 is 25% suppressive in such a cross, it would be expected that 75% of the diploid cells would have the ρ^+ phenotype and thus the ρ^+ mtDNA buoyant density. Again, the multiple variables of size and density heterogeneity prevent accurate analysis of density distributions among the various components. The shoulder component with an intermediate density on the mtDNA density distribution curve must amount to greater than 10% of the mass in order to be observable. On the other hand this would appear to amount to less than 30 to 40% of the total. Thus, a rough parallel exists in these cases between the degree of suppressiveness and the fraction of mtDNA obtained with intermediate buoyant density.

If a relation exists, as suggested, between suppressiveness and formation of intermediate buoyant density mtDNAs, it would be expected that any substance which interferes with one of these phenomenon should also correspondingly interfere with the other. When suppressiveness measurements are carried out as described in Methods, but with all solutions containing 10% glucose, the normally high suppressive petite 15 now is measured as low suppressive (Table 2). Thus, highly repressive glucose concentrations interfere in some

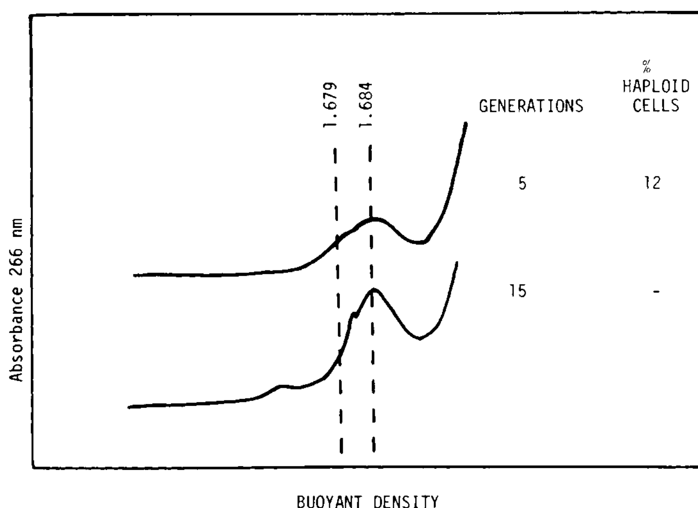

Fig. 2. CsCl density gradient banding of mtDNAs from the cross between the low suppressive (25%) petite 9 and wild type X979-2A. Experimental conditions were as described in the legend for Fig. 1.

TABLE 2

Suppressiveness of petite 15 tested against X979-2A in conditions of high and low glucose

	% Suppressiveness				
Experiment	1	2	3	4	5
Low glucose (2%)	91.5	93	91	96	93
High glucose (10%)	3.6	4.3	9.1	0	0

fashion with the suppressiveness of petite 15. It is seen from Fig. 3 that high glucose also inhibits the formation of intermediate buoyant density species in petite 13 crosses with X979-2A. The rate of formation of buoyant density intermediates is slowed, and the final density distribution is altered relative to the 2% glucose experiments.

4. DISCUSSION

Two major conclusions may be drawn from these studies. First, following the mating between petite and wild type cells, vegetative growth of the zygotes yields mtDNA density distributions in which essentially all of the mtDNA with ρ^- parent density has disappeared by the fifth generation, and mtDNA species with a new density intermediate between ρ^+ and ρ^- densities predominate. Therefore ρ^- mtDNAs with the density of the parental ρ^- do not continue to be produced and the numerous ρ^- diploids formed have a narrow range of intermediate buoyant densities.

Second, the relative amounts of intermediate buoyant density species formed generally parallel suppressiveness. It is not implied that those species which

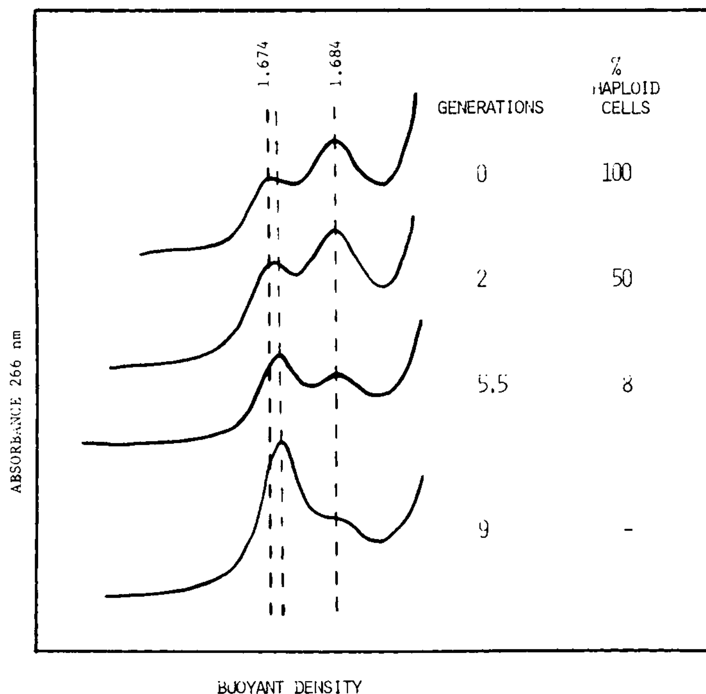

Fig. 3. CsCl density gradient banding of mtDNAs from a cross between the high suppressive (94%) petite 13 and wild type X979. Experimental conditions were as described in the legend for Fig. 1 except that 10% glucose was present in all solutions for mating and growth.

do not shift to the intermediate buoyant density also do not undergo recombination. On the contrary, to be consistant with genetic observations on transfer of resistance markers from petites into ρ^+ cells, one must assume that such recombination into the ρ^+ mtDNA is frequent (6). It would be more consistant to postulate at this time that recombination does take place among all mtDNA species but in the case of low suppressive petites, most such events result in the regeneration of mtDNA with normal ρ^+ density and structure.

If we assume that any major alterations of mtDNA, particularly those sufficient to yield buoyant density differences, can give rise to the petite phenotype, then it is logical to expect that any "non-ρ^+" mtDNA density species would correspond to ρ^- progeny. Therefore, it should be no surprise that the fraction of ρ^+ mtDNA in the preparation should be representative of the fraction of ρ^+ cells in the culture. This should lead to a simple correlation between suppressiveness and ρ^+ mtDNA concentration in the total cell culture. What is surprising, however, is the near quantitative decrease in ρ^- mtDNA parental density species. It appears that some form of recombination events must take place between essentially all of the ρ^- mtDNAs and the ρ^+ mtDNAs to yield intermediate density species. It appears that this recombination event is a major causative factor in suppressiveness and the amount of recombination therefore correlates with the degree of suppressiveness.

One of the common models offered to explain genetic suppressiveness has been the suggestion that the petite mtDNA, because of replicative advantage, predominates in the progeny cells. This would result in selective advantage for production of cells of petite phenotype. The studies reported here are

clearly not in agreement with this simple model. Virtually no ρ^- parental type mtDNA is produced by the zygotes. Thus, a major event in bringing about suppressiveness must be a very early recombination between the normal ρ^+ mtDNA and the greatly altered ρ^- mtDNA, with both recombinant mtDNAs being deficient in normal sequences. This may be followed by selective replication or more frequent replication of certain recombinants so that cells with distinct mtDNA molecules are segregated out. Such an explanation of suppressiveness based on recombination also provides a model which can explain how a single mutational event in one molecule of mtDNA in a cell can result in the formation of a petite with all mtDNAs altered.

The distribution of mtDNA densities in the vegetative diploid cultures after 10 to 15 generations, while difficult to analyze in detail, is obviously very narrow and compares favorably with mtDNA preparations from individual ρ^- strains. This narrow distribution suggested from the whole culture studies is substantiated by the examination of mtDNA densities in individual ρ^- clones selected from the cross between petite 15 and X979-2A. Density values obtained for petite mtDNA from such a cross give intermediate buoyant densities in the range of 1.678 to 1.680 $g \cdot cm^{-3}$, the approximate density for the recombinant mtDNA in the whole cell studies. These results indicate that not only is recombination taking place, but also that it is occurring in a very specific fashion which depends critically upon the nature of the ρ^- mtDNA.

These results also suggest that mitochondrial recombination observed in the transfer of genetic markers is acting at the mtDNA level rather than resulting from mtDNA mixtures within one organelle or organelle mixtures within one cell.

The mechanism by which mtDNAs of intermediate buoyant densities are generated cannot be clearly defined from these studies. The major possibilities to be examined, however, would include a model in which strand breakage and recombination can occur directly between parental DNAs to yield a mixture of hybrid molecules that are subsequently selected and replicated (13). Alternatively, the recombination could take place by a copy choice mechanism in which replication proceeds by a partial transcription of one parental molecule followed by a crossing over phenomenon which results in further transcription using another parental molecule as template (14). Experiments to examine these possible mechanisms by direct procedures have as yet been unsuccessful. However, analysis of the time course of the appearance of intermediate buoyant density forms does indicate that this is a continuing process over several generations. A specific direct parental strand recombination, occurring early after zygote formation, followed by selective replication of certain recombinants could provide such kinetics. A copy choice mechanism which would generate such a narrow distribution of progeny densities could, however, also be envisioned if a very specific alignment of ρ^- fragments with ρ^+ DNA allowed reproducible crossing over at the specific site of the ρ^- DNA.

One interesting aspect of these observations stems from the apparent ease and frequency with which mtDNAs within a yeast cell can mix. No matter what mechanism is proposed for the recombination, the observations which demonstrate in this case a rapid and frequent recombination, and the genetic observations which indicate a corresponding recombination of certain resistance markers, together require that the various mtDNAs be brought into close proximity. Therefore it is suggested that the contents of yeast mitochondria are rapidly mixed, probably by a process of fusion and division of the mitochondrial membranes. In such a case the various aspects of mitochondrial function, stability, etc. must be examined in terms of the mitochondria as a fraction rather than as individual entities.

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