JOURNAL OF BACTERIOLOGY, Apr. 1975, p. 7-18

Vol. 122, No. 1
Printed in U.S.A.

Biogenesis of Mitochondria: Analysis of Deletion of Mitochondrial Antibiotic Resistance Markers in Petite Mutants of *Saccharomyces cerevisiae*[1]

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Received for publication 31 December 1974

Yeast strains carrying markers in several mitochondrial antibiotic resistance loci have been employed in a study of the retention and deletion of mitochondrial genes in cytoplasmic petite mutants. An assessment is made of the results in terms of the probable arrangement and linkage of mitochondrial genetic markers. The results are indicative of the retention of continuous stretches of the mitochondrial genome in most petite mutants, and it is therefore possible to propose a gene order based on co-retention of different markers. The order *par*, *mik1*, *oli1* is suggested from the petite studies in the case of three markers not previously assigned an unambiguous order by analysis of mitochondrial gene recombination. The frequency of separation of markers by deletion in petites was of an order similar to that obtained by recombination in polar crosses, except in the case of the *ery1* and *cap1* loci, which were rarely separated in petite mutants. The deletion or retention of the locus determining polarity of recombination (ω) was also demonstrated and shown to coincide with deletion or retention of the *ery1*, *cap1* region of the mitochondrial genome. Petites retaining this region, when crossed with *rho*$^+$ strains, display features of polarity of recombination and transmission similar to the parent *rho*$^+$ strain. By contrast a petite determined to have lost the ω^+ locus did not show normal polarity of marker transmission. Differences were observed in the relative frequency of retention of markers in a number of strains and also when comparing petites derived spontaneously with those obtained after ultraviolet light mutagenesis. By contrast, a similar pattern of marker retention was seen when comparing spontaneous with ethidium bromide-induced petites.

In spite of extensive studies of the cytoplasmic petite mutation of yeast (*rho*$^-$ mutation), there still remains little understanding of this unique mutation (for review, see reference 20). Most previous studies have been concerned with the effects of a wide variety of mutagens, physiological treatments, and genetic manipulation on the frequency of the *rho*$^-$ mutation. More recently, a new means of analyzing this mutation has become available with the identification of several antibiotic resistance markers on mitochondrial deoxyribonucleic acid (DNA). Thus, it was first demonstrated that the petite mutation may result in deletion of segments of mitochondrial DNA by the characterization of *rho*$^-$ mutants that had lost a mitochondrial genetic marker (e.g., the locus coding for erythromycin resistance, *ery1* [10, 27]). Loss or retention of mitochondrial resistance markers other than [*ery1-r*] had shown that petites vary in the segments deleted (7, 21, 28, 29) and, indeed, the complete loss of mitochondrial DNA (*rho-O*) has been observed (11, 24). In addition, analysis of the susceptibility of resistance markers to elimination by ethidium bromide indicated that genes retained in petites may be reiterated (23). These concepts have been reinforced by a variety of physical studies on *rho*$^-$ mitochondrial DNA (5, 8) and, more recently, hybridization experiments have directly shown the retention or loss of mitochondrial transfer (6, 9) and ribosomal (9, 25) ribonucleic acid genes and their amplification in certain petite isolates.

The petite mutation may also provide a useful approach to the study of linkage relationships between mitochondrial loci by an analysis of the retention of different markers in petites isolated from multiply marked strains. The arrangement of different loci on the mitochondrial genome has thus far been determined from crosses between *rho*$^+$ strains, the distinguishing features of polarity of recombination and transmission being employed to construct maps of the mitochondrial genome (1, 17). However, it has proven difficult to order some markers

[1] Publication no. 37 in this series.

(oligomycin, mikamycin, and paromomycin) by this means (14, 30). Previous studies from several laboratories (7, 9, 10, 21, 28, 29) have demonstrated the loss and retention of antibiotic resistance markers in petite mutants and have shown that markers may be lost separately or in combination with each other. However, it is not established whether the petite mutation results in a random deletion of markers or, conversely, whether there is some ordered sequence(s) in this process leading to a limited number of marker combinations. In addition, co-retention of markers might be analyzed in terms of possible functional circularity of the mitochondrial genome, and frequencies of separation of markers might be compared with recombination frequencies as a means of establishing linkage relationships between genes. Recent studies by Suda and Uchida (28, 29) and Deutsch et al. (7) utilized the petite mutation to examine the relationship between mitochondrial loci determining resistance to oligomycin, erythromycin, and chloramphenicol.

This paper presents a study of gene retention in petites isolated from strains carrying five antibiotic resistance markers (mikamycin, *mik1*; oligomycin, *oli1*; erythromycin, *ery1*; chloramphenicol, *cap1*; and paromomycin, *par*) as well as an examination of the loss and retention of the locus determining polarity of recombination (ω). The arrangement and linkage of these six loci on the mitochondrial genome as deduced from analysis of marker retention in petites is compared with the results obtained from analysis of recombination in *rho*$^+$ strains. The analysis of marker retention in petite isolates is extended to strains of different genetic backgrounds and to petites isolated after ethidium bromide or ultraviolet light (UV) mutagenesis. The relative frequency of marker retention is shown to be dependant upon both the strain used and the method of mutagenesis. The ability of some petite strains to display normal characteristics of mitochondrial gene recombination is demonstrated in crosses between stable purified petites and *rho*$^+$ strains.

MATERIALS AND METHODS

Strains. The strains used and their genotypes are listed in Table 1. The isolation of mutants and nomenclature of markers have been described elsewhere along with their genetic and biochemical characterization (4, 14, 18, 22). A paromomycin-resistant mutant was generously provided by R. A. Kleese (16). Haploid strains 761-7A, 432-31, 770-7B, 413-16, and 829-5B were constructed by selection of recombinant diploids from appropriate crosses followed by sporulation and tetrad dissection.

Media. Complex media consisted of 1% yeast extract and 2% peptone with either 2% glucose (YEPD), 2% ethanol (YEPE), or 0.1% glucose and 2% ethanol (YEPDE) as the carbon source. Antibiotic media were prepared from YEPE medium with antibiotics added to the following concentrations: oligomycin, 3 μg/ml; mikamycin, 7.5 μg/ml; erythromycin, 1.5 mg/ml; chloramphenicol, 2.5 mg/ml; paromomycin, 1.5 mg/ml. With the exception of chloramphenicol, antibiotics were added to media after autoclaving. Minimal medium for prototrophic selection of diploids consisted of 0.67% Difco yeast nitrogen base and 2% glucose supplemented where necessary with lysine or tryptophan (50 μg/ml). Presporulation and sporulation media have been described elsewhere (15).

Isolation of petites. Spontaneous petites were isolated from an ethanol-grown culture by plating on the differential YEPDE medium and selecting, after 4 to 6 days of growth, the small colonies unable to use

TABLE 1. *Table of strains*[a]

Strain	Nuclear genotype	Mitochondrial genotype
L2000	*α ade1 trp1*	Antibiotic sensitive, ω^+
L2200	*a ade1 lys2 trp1*	Antibiotic sensitive, ω^+
L2300	*α ade1 lys2 trp1*	Antibiotic sensitive, ω^+
D253-3C	*α his1 trp1*	Antibiotic sensitive, ω^-
D253-9C	*a lys2 trp1*	Antibiotic sensitive, ω^-
L410	*α ura his*	Antibiotic sensitive, ω^+
761-7A	*a ura lys2 arg4-17*	ω^+, *oli1-r*, *ery1-r*, *cap1-r*
432-31	*a ura trp1*	ω^+ *mik1-r*, *oli1-r*, *ery1-r*
770-7B	*a ura lys2 trp1*	ω^+ *mik1-r*, *oli1-r*, *ery1-r*, *cap1-r*
413-16	*a ade1 lys2*	ω^- *oli1-r*, *ery1-r*, *cap1-r*
829-5B	*α lys1*	ω^+ *par-r*, *oli1-r*, *ery1-r*, *cap1-r*

[a] The antibiotic resistance determinants carried by strains 761-7A, 432-31, 770-7B, and 829-5B are all derived by selection of recombinants in a series of crosses from the same primary isolates. The *cap1-r* allele carried by strain 413-16 is the same as in strains 761-7A, 770-7B, and 829-5B, whereas the *oli1-r* and *ery1-r* alleles are independent isolates shown to be inseparable by recombination from the markers carried by strains 761-7A, 770-7B, 432-31, and 829-5B.

the nonfermentable substrate. Petites induced by ethidium bromide were isolated by transferring an inoculum from a YEPE culture to YEPD medium containing ethidium bromide (2 or 5 μg/ml). After growth for 3 to 5 h, samples were washed twice before plating on YEPDE plates. Isolation of petites induced by UV irradiation was carried out by plating cells from a YEPE culture onto YEPD plates to give a final cell count of about 100 per plate after irradiation. Irradiation dosage was varied to give survival between 5 and 50%, with the petite frequency ranging from 5 to 60%. The petite types obtained were found to be independent of the UV dose. White colonies were selected as presumptive petites after 4 days of growth and tested for their ability to grow on YEPE medium. Petites were always isolated from cultures grown on nonfermentable substrate (YEPE), so that all petites isolated were newly arisen or, at most, a couple of generations old.

Test for marker retention. To determine loss or retention of a marker in a petite strain, cells of the strain were crossed in YEPD medium for 4 to 5 h to an antibiotic-sensitive (*rho*$^+$) tester strain of similar polarity type, and samples of the mating mixture were then applied to minimal plates. After 2 days of growth, the patch of diploid progeny was replica-plated onto YEPE agar and appropriate antibiotic plates, which were then scored after 3 days of growth. Resistance markers retained in the petite are expressed in the respiratory competent diploid progeny as a result of recombination between the petite and grande genomes. A high level of transmission of resistance markers from the petite to diploid progeny results in confluent growth on antibiotic plates, whereas lower levels of transmission result in growth as multiple papillae. Generally, marker retention was easily scored by this test, but as a result of inherent instability in some petite isolates (10) only a very low level of retention could be observed. Transmission of resistance to about 1 in 10^4 of the diploid progeny of a particular petite cross was scored as positive for retention of the marker. Doubtful crosses, yielding only a few resistant papillae, were repeated to check for the presence of resistant mutants arising in the sensitive tester or diploid progeny. The spontaneous frequency of mutation to erythromycin resistance, for example, is of the order of 1 in 5×10^7 cells.

RESULTS

Frequencies of retention of mik1, oli1, ery1, and cap1 loci in petite isolates. The retention of five different markers has been studied in petites isolated from several grande strains carrying different combinations of these markers. For the analysis of the retention of the four markers mik1, oli1, ery1, and cap1, the parent strains 770-7B (*mik1-r*, *oli1-r*, *ery1-r*, *cap1-r*), 761-7A (*oli1-r*, *ery1-r*, *cap1-r*), and 432-31 (*mik1-r*, *oli1-r*, *ery1-r*) were employed. Tables 2, 3, and 4, respectively, present the frequencies of petite clones of different genotypes found among spontaneous petites from these strains, as well as their subclones and among petites isolated after ethidium bromide or UV mutagenesis. The proportions of different petite types derived from the three parent strains varied, but a number of general features were observed. Approximately half of the spontaneous petites failed to retain any of the markers, whereas 10 to 30% retained all loci examined. Variation in the overall level of marker retention in these and a number of other strains could not be correlated with any other property of the parental strain. However, the most striking feature of the spontaneous petites was that

TABLE 2. *Frequency of petite types from strain 770-7B*

Markers retained in petite[a]				Primary petites[b]			Subclones of spontaneous petites[c]			
							Parental petite genotype			
mik1	*oli1*	*ery1*	*cap1*	Spontaneous	Ethidium induced	UV induced	*mik1-r oli1-r ery1-r cap1-r*	*mik1-r oli1-r ery1-O cap1-O*	*mik1-O oli1-r ery1-r cap1-r*	*mik1-O oli1-O ery1-r cap1-r*
r	*r*	*r*	*r*	9.2	26	37	47			
r	*r*	*O*	*O*	7.6	11	0.6	2.4	51		
O	*O*	*r*	*r*	11.5	8.5	13	12		6.7	84
O	*r*	*r*	*r*	5.6	4.0	13	2.4		56	
r	*O*	*O*	*O*	0.7	2.6	0	1.4	2.1		
O	*r*	*O*	*O*	0.3	2.6	0.2	1.4	2.8	10	
r	*O*	*r*	*r*	0	1.1	0.2	0			
O	*O*	*O*	*O*	65	45	36	33	44	27	16
				(303)[d]	(352)	(521)	(288)	(144)	(180)	(108)

[a] The letter *r* denotes retention of a resistance marker; *O* denotes deletion of a marker.

[b] Frequencies expressed as a percentage of the petites analyzed.

[c] Primary spontaneous petites retaining different marker combinations were selected, and suspensions of cells were diluted and spread on YEPD plates. Individual subclones were then tested for marker retention.

[d] Number of petites analyzed.

TABLE 3. *Frequency of petite types from strain 761-7A*[a]

Markers retained in petite			Primary petites			Subclones of spontaneous petites	
						Parental petite genotype	
oli1	*ery1*	*cap1*	Spontaneous	Ethidium induced	UV induced	*oli1-r ery1-r cap1-r*	*oli1-O ery1-r cap1-r*
r	*r*	*r*	35	60	53	28	
r	*O*	*O*	8.9	12	2.1	7	
O	*r*	*r*	8.0	8.9	14	10.5	59
O	*r*	*O*	0	0.3	0	0.2	0
O	*O*	*r*	0	0	0.4	0	0.8
O	*O*	*O*	48	20	30	54	40
			(226)	(292)	(670)	(658)	(262)

[a] See Table 2 footnotes.

TABLE 4. *Frequency of petite types from strain 432-31*[a]

Markers retained in petite			Primary petites			Subclones of spontaneous petites		
						Parental petite genotype		
mik1	*oli1*	*ery1*	Spontaneous	Ethidium induced	UV induced	*mik1-r oli1-r ery1-r*	*mik1-O oli1-r ery1-r*	*mik1-r oli1-r ery1-O*
r	*r*	*r*	25	28	54	49		•
r	*r*	*O*	16	17	4.4	11		46
O	*O*	*r*	8.5	6.0	10	4.8	6.8	
O	*r*	*r*	2.2	3.7	5.2	1.7	61	
r	*O*	*O*	0.4	16	0.8	5.3		10
O	*r*	*O*	1.3	0.9	0.4	2.0	17	9
r	*O*	*r*	0.4	2.3	1.2	0		
O	*O*	*O*	46	26	24	26	15	35
			(225)	(218)	(248)	(356)	(252)	(432)

[a] See Table 2 footnotes.

in all cases the *cap1* and *ery1* loci were either retained or lost concomitantly, whereas the *mik1* and *oli1* loci were readily separable from each other and from the *ery1*, *cap1* pair of loci.

For strain 770-7B the most frequently occurring petite types, where a separation of markers was observed, were: *mik1-r*, *oli1-r*, *ery1-O*, *cap1-O*; *mik1-O*, *oli1-O*, *ery1-r*, *cap1-r*; and *mik1-O*, *oli1-r*, *ery1-r*, *cap1-r*. The corresponding genotypes were also the most frequently found among spontaneous petites from the two triply marked strains. The *mik1-r*, *oli1-r*, *ery1-O*, *cap1-O* and *mik1-O*, *oli1-O*, *ery1-r*, *cap1-r* types occur with approximately equal frequency in strain 770-7B. The separation of the *oli1* and *mik1* loci was less common than separation of either locus from the *ery1* and *cap1* loci and, where separation was seen, the genotype *mik1-O*, *oli1-r*, *ery1-r*, *cap1-r* was the major type. Petites retaining only the *mik1* or *oli1* locus were found, but in much smaller numbers. Among spontaneous and induced petites, a few clones of the type *mik1-r*, *oli1-O*, *ery1-r*, *cap1-r* were found, but since co-transmission of the markers to diploids was not observed the clones were presumably mixed for cells of the types *mik1-r*, *oli1-O*, *ery1-O*, *cap1-O* and *mik1-O*, *oli1-O*, *ery1-r*, *cap1-r*.

The variation between these three related strains with regard to retention of the different markers can be seen by comparing the retention of *oli1-r* and *ery1-r*, the two markers common to each of the three parent strains. Thus, the ratios of *oli1-O*, *ery1-r*/*oli1-r*, *ery1-O* for strains 770-7B, 432-31, and 761-7A were 1.5, 0.5, and 0.9, respectively, indicating that different regions of the genome may be preferentially retained in different strains. This variation in

relative retention of *ery1* and *oli1* loci was more clearly seen when comparing unrelated strains. Thus, for strain 413-16, the ratio of petite types *oli1-O*, *ery1-r/oli1-r*, *ery1-O* was 0.14; this strain is of ω^- polarity type, whereas the previously considered strains are all closely related ω^+ strains. A preponderance of the *oli-r*, *ery-O* petite type has also been observed by Suda and Uchida (28) and by Deutsch et al. (7). Experiments are being conducted to determine whether such strain differences in relative marker retention are mitochondrially determined.

When primary petite isolates were subcloned and the subclones were tested for retention of markers, instability in the mutant was generally encountered as evidenced by the number of subclones that had lost some or all of the markers present in the primary isolate. The instability may continue through many subcloning steps. When petites of different genotype were subcloned, the general pattern of marker retention in subclones was similar to that seen in the primary spontaneous petite isolates. Again, the *ery1* and *cap1* pair of loci were readily separable from *oli1* and *mik1*, and only rarely from each other, but one subclone carrying only the *ery1* locus and two carrying the *cap1* locus were obtained (Table 3). Although still a rare occurrence, separation of the *ery1* and *cap1* loci appears to occur more readily among subclones than in primary petite isolates. Petites retaining *oli1* or *mik1* alone were also more frequently encountered during subcloning; for instance, petites retaining only *oli1* comprised 17% of subclones from original isolates of the type *mik1-O*, *oli1-r*, *ery1-r* and likewise *mik1*-retaining petites were 10% of subclones from *mik1-r*, *oli1-r*, *ery1-O*, *cap1-O* petites (Table 4). Petites retaining only an individual marker seem, in general, more stable than petites carrying multiple markers. Thus, the low frequency of singly marked petites among primary isolates may indicate that two or more deletion events are required for their formation.

For comparison with spontaneous petites, the patterns of marker retention in petite isolates generated by treatment with ethidium bromide or UV light have been determined. Induction of petites by ethidium bromide under the growth conditions employed generated petite frequencies of 65, 51, and 30%, respectively, for strains 770-7B, 432-31, and 761-7A. The overall level of retention of markers in these induced petites was higher than among spontaneous isolates. This greater marker retention in ethidium bromide-induced petites may derive from most petites being newly arisen (first generation), whereas spontaneous petites isolated from an ethanol-grown culture may have persisted in the culture for some time and may have undergone a few divisions utilizing residual mitochondrial functions. Segregation of different mitochondrial DNA molecules from parent petite cells during this period could account for the lower overall level of marker retention observed in spontaneous petites.

Another major difference observed between ethidium bromide-induced and spontaneous petites was the greater frequency of clones retaining only *mik1-r* or *oli1-r* markers. These two genotypes arose with equal frequency (2.6%) among ethidium-induced petites from strain 770-7B (Table 2), although there was a predominance of the *mik1-r*, *oli1-O*, *ery1-O* genotype in petites derived from strain 432-31 (Table 4). The increase in frequency of petites retaining either the *mik1* or *oli1* locus after ethidium bromide treatment is consistent with the hypothesis that more than one event is required for their formation. Presumably the ethidium bromide treatment used here produces several deletion events, while the probability of detecting such multiple events among spontaneous petite isolates is considerably lower.

In the case of petite mutants induced by UV light, a marked effect on the distribution of petite genotypes was observed in contrast to the results with ethidium bromide. Petites from all three strains showed an increase in the retention of the *ery1*, *cap1* region relative to the *oli1*, *mik1* region. The ratio of the petite types *oli1-O*, *ery1-r/oli1-r*, *ery1-O* was increased relative to spontaneous petites by factors of twelve, four, and seven, respectively, for strains 770-7B, 432-31, and 761-7A. In general, there was a greater level of marker retention in UV-induced petites; for strain 770-7B there was a marked increase in the frequency of petites of genotype *mik1-O*, *oli1-r*, *ery1-r*, *cap1-r* (Table 2). A similar increase was seen in the frequency of the *mik1-O*, *oli1-r*, *ery1-r* petite genotype in strain 432-31 (Table 4).

It is of interest to compare the effect of UV irradiation on the frequency of different petite types with its effects on recombination, as reported by Slonimski and co-workers (1, 2). These authors found that irradiation of a ω^+ strain prior to mating with a ω^- strain results in a decreased transmission of ω^+ markers, the decrease being greatest for markers furthest removed from the origin of transmission. The three parent strains in this study are of L2200 or ω^+ polarity type. Calculated from the data of Table 2, the frequencies of retention of loci after

UV irradiation were: *eryl*, *capl*, 63%; *olil*, 51%; and *mikl*, 38%. The lower frequency of retention of *olil* relative to *eryl* and *capl* parallels the relative transmission of these markers in wild-type crosses after UV irradiation of the ω^+ parent (2). These similarities are, however, not a general feature of the system, since another ω^+ strain (829-5B, see below) did not give the same shift in the pattern of petite types after UV irradiation.

Frequencies of retention of par-r, olil-r, eryl-r, and capl-r markers in petite isolates. The relationship of the *par* locus to other mitochondrial loci was studied with strain 829-5B. Previous studies of mitochondrial gene recombination have been unable to position the *par* locus definitively relative to other loci (16, 30). In strain 829-5B, the *par* locus was separated with high frequency by petite mutation from the *olil*, *eryl*, and *capl* loci, the most common petite genotype being *par-O*, *olil-r*, *eryl-r*, *capl-r* (Table 5). Some 40% of the petites that retained the *olil* locus but had lost the *eryl* and *capl* loci retained the *par* locus, whereas 87% of petites that had lost the *olil* locus but retained the *eryl* and *capl* loci also retained the *par* locus. This suggests a closer linkage of the *par* locus to the *eryl* and *capl* loci than to *olil*. Such an interpretation must be regarded with caution, however, since differential retention of regions of mitochondrial DNA or stability of particular petite types could also account for the difference observed.

Proportions of petite types isolated after ethidium bromide treatment of strain 829-5B were similar to those seen among spontaneous mutants, although an increase in the proportion of petites retaining all markers was again observed. One petite clone of genotype *par-r*, *olil-O*, *eryl-O*, *capl-r* was isolated, but this clone was found to consist of cells retaining one or the other marker only. In strain 829-5B the pattern of marker retention after UV treatment was inconsistent with results from the three strains previously employed in that the increase in *eryl* and *capl* retention relative to *olil* retention was not observed. The ratio of *olil-O*, *eryl-r/olil-r*, *eryl-O* petite types actually decreased threefold, from 0.36 in spontaneous petites to 0.12 in petites isolated after UV irradiation.

Effect of the petite mutation on retention of the polarity determinant (ω^+/ω^-). A locus (ω) has been postulated to determine the polarity of recombination between genes on mitochondrial DNA (2). When strains carrying opposite alleles (ω^+/ω^-) are crossed, the ratio of reciprocal recombinants is found to differ significantly from one, the value observed in homozygous ($\omega^+ \times \omega^+$; $\omega^- \times \omega^-$) crosses. Studies in our own laboratory have also indicated the existence of these two extreme classes of crosses with respect to polarity or recombination but, in addition, the existence of other factors modulating polarity was indicated (15, 17). In our stocks, strains related to L2200 and D253 most closely correspond to ω^+ and ω^- strains, respectively. The cytoplasmic inheritance of the polarity determinant in these strains has been reported (13, 17), and its expression appears identical to that reported for ω by Bolotin et al. (2).

It has been proposed by Slonimski and colleagues that all recombinants from a heterozygous ($\omega^+ \times \omega^-$) cross carry the ω^+ allele (2). On the basis of this proposal, it is possible to test petite mutants for retention of this determinant. Thus, if a petite from a ω^+ strain retains the locus, recombinant diploids formed in a cross between the petite and an ω^- *rho*$^+$ strain should acquire the ω^+ allele. Back-crosses of

TABLE 5. *Frequency of petite types from strain 829-5B*[a]

Markers retained in petite				Spontaneous	Ethidium induced	UV induced
par	*olil*	*eryl*	*capl*			
r	*r*	*r*	*r*	32	46	60
O	*r*	*r*	*r*	37	29	19
r	*O*	*O*	*O*	5.8	3.7	4.1
r	*r*	*O*	*O*	5.3	6.3	5.7
O	*O*	*r*	*r*	0.6	0.9	0.3
r	*O*	*r*	*r*	4.1	2.8	1.0
O	*r*	*O*	*O*	8.0	5.7	4.8
r	*O*	*O*	*r*	0	0.3	0
O	*O*	*O*	*r*	0.3	0	0
O	*O*	*O*	*O*	6.8	4.8	5.1
				(340)	(351)	(316)

[a] See Table 2 footnotes.

spores from such recombinant diploids can then be used to test whether the ω^+ allele was retained in the petite. It should be noted that some rare minority recombinants carrying the ω^- allele can be found (13), but their occurrence is so infrequent that they can be discounted for the purpose of this analysis. Petite isolates from strains 761-7A, 432-31, and 770-7B (all of L2200 or ω^+ polarity type) were tested in this manner by selecting recombinant diploids from crosses to strain D253-3C (ω^-) and crossing spore segregants from the diploids to ω^+ and ω^- tester strains (Table 6).

The results of these experiments indicate that petite mutants retaining the *eryl*, *capl* region of the genome retain the ω^+ allele, whereas petites deleted in this region do not transmit the ω^+ allele in test crosses. Thus, petites of the genotypes *oli1-O*, *eryl-r*, *capl-r* and *mik1-O*, *oli1-O*, *eryl-r*, *capl-r* all transmitted the polarity determinant to the *rho*$^+$ recombinant diploids, as demonstrated by the non- or low-polar nature of test crosses to strains of L2200 (ω^+) background and the strong polarity seen in similar crosses to strains of the D253 (ω^-) background. This is consistent with the localization of the polarity determinant in this region. Conversely, petites retaining either or both of the *mik1* and *oli1* loci, but not the *eryl* and *capl* loci, failed to transmit the ω^+ determinant to recombinant diploids.

TABLE 6. *Transmission of polarity determinant by petites*[a]

Markers			Polarity of recombination with tester	
Retained	Lost	Pair followed in cross	ω^+	ω^-
eryl, capl	*oli1*	*eryl, capl*	1.6	131/0[b]
			1.7	23/0[b]
			4	97/0[b]
			1.0	70[b]
				78/0[b]
				108[b]
eryl, capl	*mik1, oli1*	*eryl, capl*	1.0	49[b]
			4.5	44[b]
				53[b]
				54[b]
			1.6	58[b]
			1.0	63/0[b]
			1.2	94/0[b]
eryl	*oli1, capl*	*oli1, capl*	0/208	1.2[c]
capl	*oli1, eryl*	*oli1, eryl*	0.11	0.8[d]
oli1, mik1	*eryl*	*eryl, capl*	0.004	[e]
			0.005	[e]
			0.01	[e]
			0.003	[e]
	eryl, capl	*eryl, capl*	0.01	3.1[e]
oli1	*mik1, eryl*	*eryl, capl*	0.005	[e]
			0.003	[e]
			0.005	[e]
oli1	*mik1, eryl, capl*	*eryl, capl*	0.007	0.96[e]
			0.004	1.1[e]
mik1	*oli1, eryl, capl*	*eryl, capl*	0.013	1.6[e]

[a] Each line shows the results of test crosses of a spore derived from the cross of an independent petite isolate to D253-3C. The spore analyzed was selected for the presence of all markers retained in the petite. A number of strains and marker combinations were used in test crosses. The tester strains and the ratios of genotypes employed in the polarity determinations are given.

[b] *capl-r*, *eryl-s/capl-s*, *eryl-r*, L2200, L2300 (ω^+); D253-3C, D253-9C (ω^-).

[c] *capl-s*, *oli1-r/capl-r*, *oli1-s*, 761-7A (ω^+); 413-16 (ω^-).

[d] *eryl-s*, *oli1-r/eryl-r*, *oli1-s*, 761-7A (ω^+); 413-16 (ω^-).

[e] *capl-s*, *eryl-r/capl-r*, *eryl-s*, 761-7A, 770-7B (ω^+); 413-16 (ω^-).

Spores derived from crosses involving these petites showed a high polarity of recombination typical of strains of D253 (ω^-) polarity type in test crosses to L2200 (ω^+) strains. Two petites retaining only part of the *eryl*, *capl* region of genotypes *oli1-O*, *eryl-r*, *capl-O* and *oli1-O*, *eryl-O*, *capl-r* showed a similar loss of the polarity determinant. Spores from recombinant diploids from these petites showed little polarity with D253 (ω^-) tester strains and were polar in crosses to L2200 (ω^+) tester strains (208/0 for *capl*, *oli1* polarity for *eryl-r* strain and 9.4 for *eryl*, *oli1* polarity for *capl-r* strain). That deletion of either *capl* or *eryl* causes loss of the ω determinant is suggestive of a location of ω between the *eryl* and *capl* loci, since loss of ω coincident with loss of *eryl* would not be expected if ω is proximal to the *capl* locus.

Characteristics of recombination in rho⁻ by rho⁺ crosses. Studies in several laboratories of crosses involving two *rho*$^+$ strains have established the major features of mitochondrial recombination, in particular the phenomena of polarity of transmission and recombination of mitochondrial genes. However, the occurrence of similar phenomena in crosses of *rho*$^+$ strains with *rho*$^-$ strains having altered mitochondrial DNA has not yet been reported.

Crosses of this nature have been performed with stable petite strains obtained by repeated subcloning of primary isolates. The petite clones studied were derived from three *rho*$^+$ strains: 770-7B (*mik1-r*, *oli1-r*, *eryl-r*, *capl-r*) and 761-7A (*oli1-r*, *eryl-r*, *capl-r*), both of the ω^+ (L2200) polarity type; and 413-16 (*oli1-r*, *eryl-r*, *capl-r*), of the ω^- (D253) polarity type. The behavior of stable petites from these three parent strains was analyzed in crosses to *rho*$^+$-sensitive strains from both L2200 and D253 backgrounds.

Two petites retaining the *eryl* and *capl* loci, U7 and P1, were obtained from strains 770-7B and 761-7A, respectively. Analysis of the *rho*$^+$ diploids from crosses of these two petites showed that both behaved in a manner very similar to their parental strains. Thus, in crosses to a D253 (ω^-) tester strain the petites showed a high polarity of recombination and high transmission of the *capl-r* marker, whereas crosses to L2200 (ω^+) tester strains showed all the properties of nonpolar crosses. In nonpolar crosses of both petites and parent *rho*$^+$ strains, the frequency of recombination was between 12 and 18%, whereas for polar crosses the variation was between 24 and 34%. Two additional petites retaining both the *eryl* and *capl* loci were isolated from strain 413-16 (ω^-) and crossed to tester strains (Table 7). One of these, T7, gave polarity results similar to those of the *rho*$^+$ parent, although extreme recombination frequencies of 81% for the polar and 4% for the nonpolar cross were observed. The second petite, T6, likewise displayed a high polarity of recombination when crossed to strain L410 (ω^+). However, a high but reciprocal polarity was observed in a cross to strain D253-3C (ω^-), a cross that, by comparison with the parental cross, would be expected to be nonpolar. Crosses to both L2200 and D253 tester strains showed about 30% recombination between *eryl* and *capl* and 30 and 60%, respectively, for co-transmission of the petite markers. The inverse

TABLE 7. *Analysis of rho⁻ by rho⁺ crosses*

Strain	Markers		Crossed with:	% Transmission		Recombination		Suppressiveness
	Retained	Lost		*eryl-r*	*capl-r*	%	Polarity[a]	
770-7B	*rho*$^+$		L2000	44	47	12	1.55	
			D253-3C	42	75	34	196/0	
U7	*eryl-r*, *capl-r*	*mik1-r*, *oli1-r*	L2000	68	71	11.5	1.6	12
			D253-3C	65	90	25	137/0	10
761-7A	*rho*$^+$		L2300	45	42	13	0.67	
			D253-3C	63	87	24	128/0	
P1	*eryl-r*, *capl-r*	*oli1-r*	L2300	25	26	18	1.2	12
			D253-3C	44	74	31	73	2
P2	*capl-r*	*oli1-r*, *eryl-r*	L2300		45			18
			D253-3C		9			2
413-16	*rho*$^+$		L410	36	9	27	0/114	
			D253-3C	45	45	8	1.1	
T7	*eryl-r*, *capl-r*	*oli1-r*	L410	94	13	81	0.004	3
			D253-3C	87	88	4.2	1.3	9
T6	*eryl-r*, *capl-r*	*oli1-r*	L410	65	31	33	0/239	4
			D253-3C	61	86	26	180/0	6.5

[a] Expressed as *eryl-s*, *capl-r*/*eryl-r*, *capl-s*.

nature of the polarity on crossing of this petite with strains of different polarity type suggests that both polarity types may act in a similar but reciprocal manner, rather than one having an active and the other a passive role in the generation of polarity.

A petite, P2, retaining only the *cap1* locus, isolated as a subclone of P1, was also crossed to the two tester strains (Table 7). This petite, in contrast to both the parent *rho*$^+$ and P1 strains, showed a decreased transmission of *cap1-r* when crossed to D253-3C (ω^-) relative to the cross with L2300 (ω^+). The highly polar nature of the D253 cross had thus been lost. This petite has previously been shown (Table 6) not to transmit the polarity determinant in a polar cross, whereas the parent clone P1 retained the polarity determinant. Thus, these two independent tests demonstrate the deletion of the polarity determinant ω^+ during the transition of *oli1-O, ery1-r, cap1-r* to *oli1-O, ery1-O, cap1-r*.

In conclusion, these crosses demonstrate that petites from strains of both polarity types are able to retain normal polarity of recombination and that neither an intact mitochondrial genome nor the presence of products of mitochondrial protein synthesis in the cells prior to crossing is required for the determination of polarity of recombination. The petite mutation can, however, have a marked influence on polarity of recombination, as exemplified by abnormal polarity results obtained with petite T6.

DISCUSSION

A primary objective of this investigation has been to determine whether gene retention in petites offers a potentially useful tool for the ordering of genes on mitochondrial DNA. The ability to determine the order of markers by deletion analysis requires that, in general, continuous segments of the genome are deleted or retained in petite mutants and that the retention of noncontiguous segments is either not observed or is of low frequency. The gene retention observed among spontaneous petites derived from strains carrying three or four mitochondrial markers is consistent with the proposal that continuous sequences are generally retained. Thus, the stable petite types purified during this study were: *cap1-r; ery1-r; oli1-r; mik1-r; par-r; ery1-r, cap1-r; oli1-r, ery1-r; mik1-r, oli1-r; oli1-r, ery1-r, cap1-r;* and *mik1-r, oli1-r, ery1-r, cap1-r*. Considering the *oli1, ery1,* and *cap1* loci, the order of which has been clearly demonstrated by recombination (1, 17), it was found that the only petite types able to be purified were those retaining consecutive markers, i.e., all combinations other than *oli1-r, cap1-r*. Similarly, for the *mik1, oli1,* and *ery1* loci the only stable petite types isolated retaining two of the three loci were *mik1-r, oli1-r* and *oli1-r, ery1-r*.

On the basis of frequency of co-retention of markers and the ability to obtain stable clones of particular genotypes, the loci *mik1, oli1, ery1,* and *cap1* behave as a linear sequence, in that order, with respect to deletion. However, analysis of strain 829-5B carrying the *par-r* marker suggests that the *par* locus is located outside the *oli1, ery1, cap1* region, and the ready isolation of petites of types *oli1-r, ery1-r, cap1-r* and *par-r, oli1-r,* and *par-r, ery1-r, cap1-r* is indicative of functional circularity with respect to petite formation. It is generally accepted, as a result of direct microscope observation, that yeast mitochondrial DNA can exist as closed circular molecules with a contour length of about 25 μ (3). These results provide the first evidence of a genetic nature in support of this conclusion.

The order of gene markers most consistent with the data on gene retention in petites is shown in Fig. 1. This order varies only in the location of the *mik* locus from that derived from the analysis of polarities of recombination and transmission in polar *rho*$^+$ crosses (14; N. Howell and P. Molloy, unpublished data). Polarity values suggest a gene order of *par, oli1, mik1, ery1, cap1*. However, polarities of recombination observed between *par, oli1,* and *mik1* loci are low, and deviations from a polarity of one may be due to other nuclear or mitochondrial influences. For example, Wolf et al. (30) were unable to assign a gene order for the *oli1* and *par* loci in a series of different polar crosses. Use of the petite mutation for ordering of genes by deletion may be most applicable when considering such loci, which show low polarities of recombination.

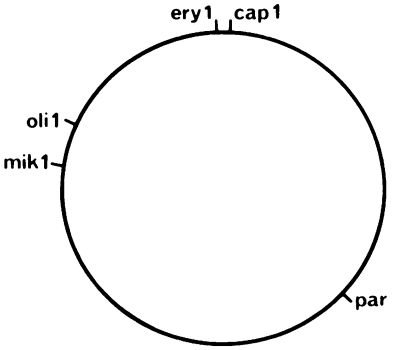

FIG. 1. *Mitochondrial map most consistent with gene retention analysis in petites.*

The nature of deletion events involved in petite formation is not known, but a mechanism that would lead to retention of continuous sequences in petites has recently been proposed by Prunell and Bernardi (26). These authors have shown that information-containing regions in mitochondrial DNA are interspersed with A,T-rich sequences and they suggest that petite mutants may arise by internal recombinations between A,T-rich segments to generate smaller defective molecules. Although this proposal is attractive in its simplicity, deletion alone is not sufficient to account for the structure of mitochondrial DNA shown to be present in a few petite isolates. Thus, Rabinowitz and co-workers (12, 19) have obtained evidence in three cases, indicating the presence of tandem inverted repeated sequences and, in one case, the presence of sequences in a petite not homologous to *rho*$^+$ mitochondrial DNA. It should therefore be stressed that if the petite mutation is not a simple deletion the apparent ordering of loci could be artifactual. The general consistency with recombination ordering, however, makes this unlikely.

A further marker on mitochondrial DNA, which differs in nature from antibiotic resistance genes, is the allele pair, ω^+/ω^-, involved in the determination of polarity and transmission of mitochondrial genes. The ability of petite strains to transmit the ω^+ determinant to recombinant diploids was shown to coincide with retention of the *ery1* and *cap1* loci. Thus, all 13 petites retaining the *ery1*, *cap1* region transmitted the ω^+ determinant, whereas all 11 petites studied that had lost the *ery1*, *cap1* region likewise had lost the ω^+ determinant. Loss of the determinant in two petites, one retaining *cap1* alone and the other retaining *ery1*, is suggestive of a location for ω between these two loci, in agreement with other evidence recently reported from this laboratory (13).

The relative distance between markers on mitochondrial DNA has thus far been estimated from transmission and recombination frequencies in highly polar crosses. It might be expected that the frequency of separation of markers by deletion in petite mutants may also relate to the distance between loci. However, the possible occurrence of preferred sites of deletion or of deletion end points would invalidate the extension of the frequency of marker separation to relative physical distances between markers. The frequencies of marker separation by petite mutation, taken from Tables 2 through 5, have been pooled in Table 8 for comparison with recombination frequencies. The most striking feature of the petite data is the close linkage observed between the *ery1* and *cap1* loci, in comparison with the ready separation of these markers by recombination. Similar tight linkage of *ery* and *cap* in ethidium bromide-induced petites has been reported by Suda and Uchida (28, 29) and by Deutsch et al. (7). Close linkage is also observed between *ery1* and either the *spi4* or *cap2* loci (Molloy, unpublished data). The region of the mitochondrial genome that encompasses the *ery1*, *cap1*, *cap2*, and *spi4* loci is of particular significance with regard to recombination due to its proximity to the polarity determinant, ω, and markers in this region show extreme polarity of recombination. The relatively high recombination seen between *ery1* and *cap1* (30%, Table 8) may be misleading, and the close linkage indicated by the petite analysis may be more truly representative of the physical separation of these markers.

The frequency of separation of the *oli1*, *ery1*, and *mik1* loci observed in petites is similar to that found by recombination, with the *oli1* and *mik1* showing greatest linkage. The *par* locus shows a high frequency of separation in petite mutants from all other loci, with slightly tighter linkage to *ery1* and *cap1* than *oli1*. In contrast,

TABLE 8. *Comparison of the frequency of gene separation by petite mutation with the frequency of gene recombination in rho*$^+$ *crosses*[a]

Gene pair	Petite mutation					Recombination		
	770-7B	761-7A	432-31	829-5B	Mean	770-7B	829-5B	432-31
ery1, *cap1*	0	0		0.3	0.1	34	32	
oli1, *ery1*	19.4	17	26	18	20	13	20	16.0
mik1, *ery1*	25		27		26	15		16.5
mik1, *oli1*	6.6		4.3		5.5	7.3		7.0
ery1, *par1*				49	49		27	
oli1, *par1*				55	55		12.5	

[a] The frequency of separation by petite mutation is the frequency (as percentages) with which either locus of a pair is retained in a spontaneous petite while the other is deleted. Recombination frequencies are those observed in polar crosses of parent strains to D253-3C. Recombination frequencies in nonpolar crosses are about 13% for the *ery1*, *cap1* and *mik1*, *oli1* pairs and 20 to 25% for all other marker pairs.

par shows a higher frequency of recombination with *ery1* than *oli1* in a polar cross (Table 8), although this may be related to their order on the genome. In summary, the lack of direct correlation for degree of marker separation determined by analysis of recombination or by marker retention studies in petites makes it difficult to interpret either procedure in terms of distances between loci. It is clear that an understanding of distances between markers on the mitochondrial genome will require some direct physical measurements, e.g., analysis of heteroduplexes formed between specifically marked petite and *rho*$^+$ genomes.

Considerable variation was seen in the proportions of different petite types obtained, although marker separation in spontaneous petites was similar among the several strains studied. This variation is reflected in the relative retention of the *oli1* and *ery1* loci alone. In the case of ethidium bromide-induced petite mutants, there were only minor differences from the pattern of petite types seen in spontaneous petites, suggesting that ethidium bromide acts to increase the frequency of spontaneous events yielding petite cells, rather than acting by an independent mechanism. This is not the case, however, for petites derived by UV induction, where significant changes are seen in proportions of different petite types. For strains 770-7B, 432-31, and 761-7A, all of L2200 background, the frequency of retention of the *mik1* and *oli1* loci in UV-induced petites is reduced relative to that of *ery1* and *cap1*. However, strain 829-5B, which is only partially of L2200 background, but which shows recombination properties typical of an L2200 strain, showed an opposite response to UV irradiation in which retention of *ery1* and *cap1* relative to *oli1* was decreased. In view of this result, the effects of UV on petite induction cannot be directly related to the polarity type of the strain.

This difference in response to UV irradiation and differences observed in relative marker retention among spontaneous petites isolated from different strains raises the possibility that there are considerable differences in the structure or replication of genomes of different *rho*$^+$ strains. These results are at variance with those of Deutsch et al. (7), in that the relative frequency of marker retention is both strain and mutagen dependent. Their conclusion that target size is an intrinsic property of the marker, independent of both the strain used and type of mutagenesis, is probably due to their use of only a limited number of related strains.

Petite mitochondrial genomes may interact normally with *rho*$^+$ mitochondrial DNA with regard to the expression of polarity of recombination. However, petites showing aberrant polarity behavior can be obtained, and two petites of this type isolated in this study are of interest. One petite retaining only the *cap1* locus was shown to have lost ω^+ by two criteria: first, in that it failed to display polarity of transmission in a cross with a ω^- strain and, further, that in such a cross the ω^+ determinant was not transmitted to recombinant diploids. The second petite showing aberrant behavior was derived from a ω^- strain and retained the *ery1* and *cap1* loci, and in preliminary studies with this petite high polarity of recombination was observed in crosses to both ω^+ and ω^- strains.

ACKNOWLEDGMENTS

The excellent technical assistance of Val Mack and assistance in the preparation of the manuscript by N. Howell, P. Nagley and G. Kellerman is much appreciated.

This work was supported by the Australian Research Grants Committee, Project D65/15930.

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