ELEVATED LEVELS OF PETITE FORMATION IN STRAINS OF *SACCHAROMYCES CEREVISIAE* RESTORED TO RESPIRATORY COMPETENCE. I. ASSOCIATION OF BOTH HIGH AND MODERATE FREQUENCIES OF PETITE MUTANT FORMATION WITH THE PRESENCE OF ABERRANT MITOCHONDRIAL DNA

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Manuscript received April 15, 1984
Revised copy accepted May 11, 1985

ABSTRACT

When recently arisen spontaneous petite mutants of *Saccharomyces cerevisiae* are crossed, respiratory competent diploids can be recovered. Such restored strains can be divided into two groups having sectored or unsectored colony morphology, the former being due to an elevated level of spontaneous petite mutation. On the basis of petite frequency, the sectored strains can be subdivided into those with a moderate frequency (5–16%) and those with a high frequency (>60%) of petite formation. Each of the three categories of restored strains can be found on crossing two petites, suggesting either that the parental mutants contain a heterogeneous population of deleted mtDNAs at the time of mating or that different interactions can occur between the defective molecules. Restriction endonuclease analysis of mtDNA from restored strains that have a wild-type petite frequency showed that they had recovered a wild-type mtDNA fragmentation pattern. Conversely, all examined cultures from both categories of sectored strains contained aberrant mitochondrial genomes that were perpetuated without change over at least 200 generations. In addition, sectored colony siblings can have different aberrant mtDNAs. The finding that two sectored, restored strains from different crosses have identical but aberrant mtDNAs provides evidence for preferred deletion sites from the mitochondrial genome. Although it appears that mtDNAs from sectored strains invariably contain duplications, there is no apparent correlation between the size of the duplication and spontaneous petite frequency.

THE yeast *Saccharomyces cerevisiae* spontaneously produces respiratory deficient mutants (petites) at the high rate of approximately 1% per generation (EPHRUSSI 1953; NAGAI, YANAGISHIMA and NAGAI 1961). This mutation is irreversible, cytoplasmically inherited (EPHRUSSI 1953; WRIGHT and LEDERBERG 1957) and associated with an element termed ρ (SHERMAN 1963). The ρ

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factor has since been identified as mitochondrial DNA (MOUNOLOU, JAKOB and SLONIMSKI 1966; MEHROTA and MAHLER 1968; GOLDRING *et al.* 1970; NAGLEY and LINNANE 1970), and petite mutants have large deletions from the circular 70–80 kilobase pairs (kbp) mitochondrial genome (MOUNOLOU, JAKOB and SLONIMSKI 1966; SANDERS *et al.* 1973; FAYE *et al.* 1973; CASEY, GORDON and RABINOWITZ 1974). Investigators have subsequently been interested in the deletion mechanism.

Physical studies have centered on characterizing the mtDNA retained in petite mutants and in identifying the sequences involved in the deletion process. In terms of genome organization, two broad classes of petites have been identified. In one mutant type the retained sequence is reiterated through a series of tandem duplications (FAYE *et al.* 1973; LOCKER, RABINOWITZ and GETZ 1974a; HEYTING *et al.* 1979), deletions apparently arising by recombination at short, tandemly orientated sequences that are, in general, between 10–20 nucleotides long (DEZAMAROCZY, FAUGERON-FONTY and BERNARDI 1983). In the other class the remaining mtDNA is organized as inverted repeats (LOCKER, RABINOWITZ and GETZ 1974b; LAZOWSKA and SLONIMSKI 1976; HEYTING *et al.* 1979) that appear to result from unequal excision between pairs of oppositely oriented, short, repeated sequences flanking the deleted segment (SOR and FUKUHARA 1983).

Genetic investigations undertaken in parallel with the physical studies showed that petite mutants can retain markers from different regions of the mitochondrial genome (LINNANE *et al.* 1968; GINGOLD *et al.* 1969). In these studies, although recombination could be demonstrated between the defective mtDNA molecules on crossing established petites, restoration of respiratory competence was not observed (MICHAELIS, PETROCHILO and SLONIMSKI 1973). However, crosses performed in our laboratory at this time showed that respiratory competence could be recovered provided that spontaneous petites of recent origin were used (CLARK-WALKER and MIKLOS 1975). This finding demonstrated that deletions do not always involve a common sequence. During our investigations it was observed that a proportion of the respiratory competent $\rho+$ restored colonies were sectored to varying extents. Colony sectoring on selective medium has subsequently been shown to be due to a raised level of spontaneous petite mutant formation that reaches over 80% per generation in some strains (OAKLEY and CLARK-WALKER 1978). Genetic analysis revealed that the high frequency of spontaneous mutation is cytoplasmically inherited (OAKLEY and CLARK-WALKER 1978), and further experiments involving crosses between haploid petites derived from an individual high-frequency petite-forming diploid, showed that these petites, unlike those from the original parental strains, fell into a small number of complementation groups (CLARK-WALKER *et al.* 1976). These studies suggested that abnormal mitochondrial genomes sometimes are formed on crossing recently arisen petites and that the loss of respiratory competence at high frequency is associated with a nonrandom breakdown of the restored mitochondrial genome.

In the investigation described below, and in the accompanying paper (EVANS and CLARK-WALKER 1985), we provide evidence from restriction endonuclease

analysis of mtDNA for the existence of abnormal mitochondrial genomes in restored strains having a higher than normal frequency of spontaneous petite formation.

MATERIALS AND METHODS

Yeast strains: *S. cerevisiae* strains used in this study are different from those employed in previous work, due to a decrease in the mating efficiency of one of the original strains. However, as in the earlier reports, we have constructed isomitochondrial haploid parents by sporulation and ascus dissection of a diploid arising from mating D13.1A (*α, ρ+, his3-532, trp1*) (STRUHL *et al.* 1979) with 500e*ρ*− (*a, ρ°, ade1, arg4-16*); this latter strain has had its mtDNA eliminated (CLARK-WALKER 1972) and was obtained as an ascospore from a diploid produced by mating strains F and T, which have been previously described (CLARK-WALKER and MIKLOS 1975). After screening ascospores from the D13.1A × 500e*ρ*− diploid, we obtained a suitable culture, T3/3 (*a, ρ+, ade1, arg4-16*), which was used in conjunction with D13.1A as a parental strain for the production of petite mutants.

Complementation tests: Isolation of recently arisen petite mutants, crossing of mutants and detection of respiratory competent restored forms have been described in detail in previous publications (CLARK-WALKER and MIKLOS 1975; OAKLEY and CLARK-WALKER 1978).

Nomenclature of strains restored to respiratory competence: Restored strains are named according to their complementation experiment of origin, their D13.1A *ρ*− parent, their T3/3 *ρ*− parent and their colony morphology (s = sectored, e = entire), in that order. They also may have a strain number if more than one colony of a particular morphology was picked from a single complementation-yielding matrix position. For example, the strain [1]9.6s3 was obtained from the first complementation experiment involving petite #9 derived from D13.1A and petite #6 derived from T3/3; it is the third picked strain of sectored colony morphology.

Determination of culture petite frequencies: Strains were grown and spread on GGYP plates, as described for the selection of petites for complementation experiments; however, in this case, colonies were allowed to develop for 4–5 days at 30° to allow unequivocal identification of petite and grande colonies. Classification was principally on the basis of color, as some of the mutant strains produced respiratory competent colonies that varied in their size and shape. Mixed colonies were scored as respiratory competent, as there is a greater probability that the mutation to respiratory deficiency has occurred after, rather than before, plating (OGUR *et al.* 1959). Depending on the proportion of petites in the culture, as judged by preliminary spreads, between 20 and 100 GGYP plates were scored per strain. Culture petite frequencies are expressed as the number of petite colonies divided by the total number of colonies multiplied by 100%. Under conditions that do not support proliferation of petites, this value has been shown to approximate to the petite frequency per generation of the strain (OGUR *et al.* 1959).

Other procedures: Media and culture conditions have been described previously (OAKLEY and CLARK-WALKER 1978). GlySV is a glycerol minimal medium supplemented with synthetic vitamins; GGYP contains yeast extract and bactopeptone and has glycerol as the major carbon source and glucose as a minor component. GlyYP is as GGYP, except that glycerol is the sole carbon source. For details of these media see OAKLEY and CLARK-WALKER (1978). Preparation of mtDNA was by dye-buoyant density centrifugation using bisbenzimide H 33258, as described previously (CLARK-WALKER, MCARTHUR and SRIPRAKASH 1981). Restriction endonuclease digestion of mtDNA and electrophoretic separation of fragments in 0.8% agarose gels was as previously described (CLARK-WALKER *et al.* 1980), except that the enzyme-specific digestion buffers were replaced in these studies by TA buffer (O'FARRELL, KUTTER and NAKANISHI 1980). Restriction endonuclease *Hha*I was produced locally according to the method of GREENE *et al.* (1978), and *Cla*I and *Pvu*II were supplied by Boehringer Mannheim, West Germany. The sizes of the restriction fragments on gels were estimated according to their migration relative to fragments of known size. The marker fragments were derived from plasmid pAN124 (CHANG and COHEN 1978; MACINO and TZAGOLOFF 1979; STRUHL *et al.* 1979) and were the products of digestion with *Xho*I (14.0 kbp), *Eco*RI (8.0 and 6.0 kbp) and *Bam*HI/*Eco*RI (4.65, 3.35, 2.35, 1.98 and 1.65 kbp).

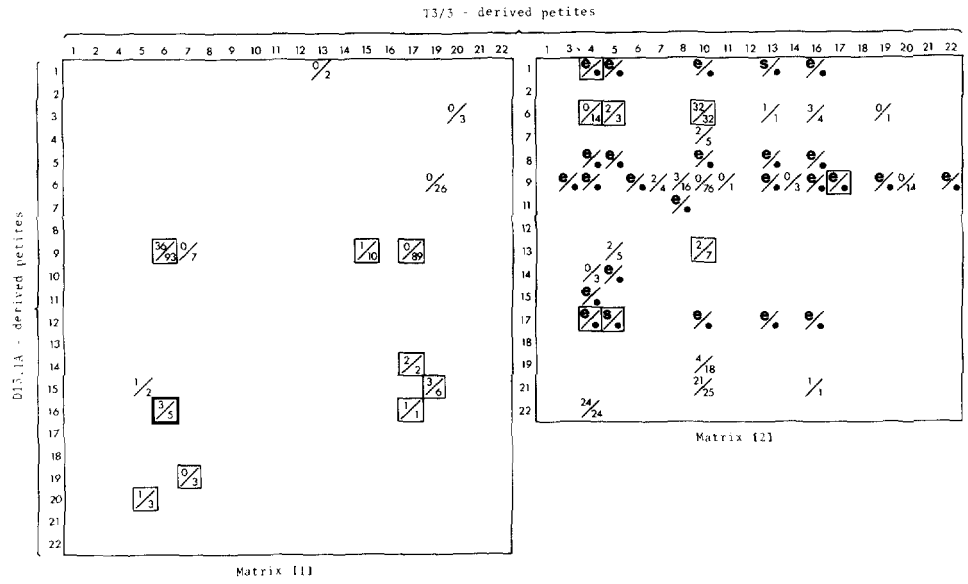

FIGURE 1.—Restoration to respiratory competence (complementation) observed in two experiments using petites obtained 64 hr (matrix [1]) and 42 hr (matrix [2]) after plating. Individual petites arising from D13.1A were crossed in all pairwise combinations with petites arising from T3/3. A mating resulting in the appearance of colonies restored to respiratory competence is indicated by a diagonal bar. The number below the bar is the number of restored colonies observed, and that above the bar is the number of restored colonies that showed a sectored colony morphology. In matrix [1], 14 of the 440 matings (3.2%) gave complementation; in matrix [2], 46 of the 304 matings (15.1%) gave complementation. Complementations having >100 colonies are indicated by a black circle below the diagonal bar. In these cases, the letter above the bar indicates the morphology of the colonies (s = sectored, e = entire) seen when loops of cells from each successful matrix position were restreaked onto five GlySV plates. A total of approximately 300 colonies was scored in each case, and the colonies were found to be either all sectored or all entire. Where colonies were picked from the matrices for further study, the complementation event is enclosed.

RESULTS

Isolation of sectored and unsectored colonies from complementation experiments: Crossing recently arisen spontaneous petite mutants of *S. cerevisiae* can yield respiratory competent progeny (Figure 1). As observed in previous experiments, some petites are prolific in producing restored forms, whereas others fail to complement. Proficiency is influenced by the age of the parental petites, as those obtained 64 hr after plating produced only 3.2% complementation (matrix [1]), compared with 15.1% for those picked after 42 hr (matrix [2]).

A characteristic of restored forms is their variable colony morphology. Some crosses produced colonies that were either all unsectored or all sectored, whereas other crosses produced mixtures of sectored and unsectored colonies. In addition, the degree of sectoring is variable and can range from mild (just detectable) to severe (see OAKLEY and CLARK-WALKER 1978). Colonies representative of each of the above characteristics were sampled, as well as colonies

TABLE 1

Culture petite frequencies (CPF) of restored strains

Strain	CPF (%)[a]	Strain	CPF (%)
[2]22.4s5	99	[1]9.6s7	11
[2]22.4s1	93	[1]9.6s5	9
[2]6.5s	90	[1]9.6s4	6
[1]14.17s	86	[1]16.6s	5
[1]15.19s	83		[b]
[2]17.5s	80	[1]9.6e1	<1
[1]9.6s1	79	[1]9.6e2	<1
[1]9.6s2	78	[1]9.6e3	<1
[2]13.10s	78	[1]9.15e	<1
[1]9.6s3	76	[1]9.17e	<1
[1]9.15s	75	[1]19.7e	<1
[2]22.4s4	74	[2]1.4e	<1
[2]22.4s3	72	[2]6.4e	<1
[2]22.4s2	64	[2]9.17e	<1
[2]22.4s6	60	[2]17.4e	<1
[2]6.10s	38	Dip1	0.6
[1]16.17s	16	Dip2	0.6
[1]9.6s6	14	Dip3	0.5

[a] ±3% at 75% to ±1% at 5%.

[b] The strains above the bar have a sectored colony morphology (s), while those below the bar are entire (e) (unsectored).

sharing one or both parental petites. The positions in the matrices from which these strains were picked are indicated in Figure 1.

Frequency of respiratory deficient cells in cultures of sectored and unsectored colonies: The proportion of petite cells in exponentially growing cultures was determined for each of the selected colonies (Table 1). Strains that have an entire (unsectored) colony morphology invariably have a culture petite frequency comparable with wild-type diploids, whereas sectored morphology is associated with an elevated culture petite frequency.

Results presented in Table 1 also show that the culture petite frequencies from sectored restored strains fall into two classes. One group have frequencies of 5–16% and are subsequently referred to as moderate-frequency petite-forming (mfp) strains. Members of the other group, referred to as high-frequency petite-forming (hfp) strains, show frequencies of 60–99%. The degree of colony sectoring is less marked in mfp strains than in hfp strains. Only one exception was found to these groups, and this strain proved to be unstable. The initial culture petite frequency of [2]6.10s (38%) dropped on subculture to a stable 6%. This instability has not been detected in other strains (see below).

Heterogeneity of culture petite frequency among restored colonies from within a cross is apparent not only between sectored and unsectored colonies but also between separate sectored colonies. For example, colonies picked from the cross [2]22.4 all showed a highly sectored morphology, but the culture petite frequencies of these strains ranged from 60–99% (Table 1).

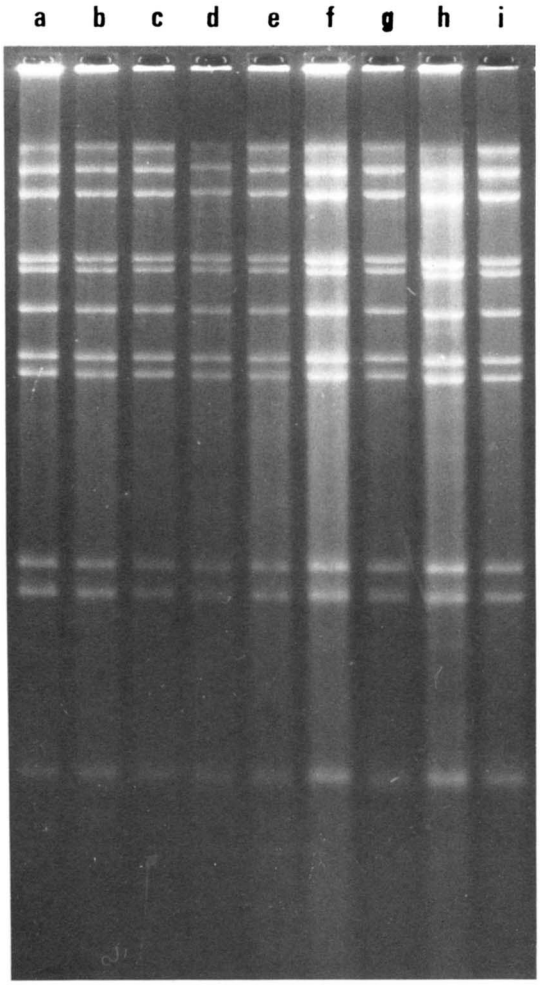

FIGURE 2.—*Hha*I digestion patterns of mtDNA from haploid and diploid respiratory competent yeast strains and from three unsectored strains arising in complementation experiments. Separation of fragments is by electrophoresis in a 0.8% agarose gel. Key to strains: a, D13.1A; b, Dip 1; c, Dip 2; d, Dip 3; e, T3/3; f, [1]9.6e1; g, [1]9.15e; h, [2]6.4e; i, D13.1A.

Mitochondrial DNA in wild-type and unsectored restored strains: Correlation of higher than normal petite frequency with aberrant mtDNA structure is contingent on the demonstration that the mitochondrial genome remains unperturbed upon crossing wild types and in restored forms with unsectored morphology. To establish this basis for analysis, mtDNA was prepared from the parental haploid strains, as well as from three diploids produced by crossing these cultures. MtDNA was also isolated from ten restored forms having a normal level of petite formation. Patterns produced on *Hha*I cleavage of these mtDNAs are shown in Figure 2. These results demonstrate (1) that the parental strains D13.1A and T3/3 are isomitochondrial by this analysis, (2) that diploids produced from crossing the respiratory competent parents have iden-

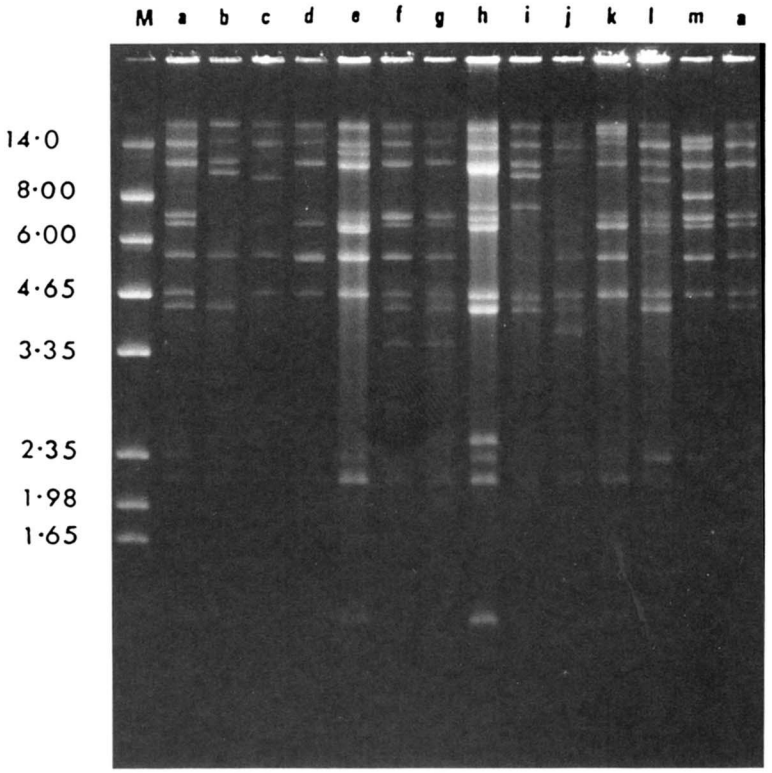

FIGURE 3.—Fragmentation patterns of mtDNA from 12 sectored strains and a wild-type diploid following digestion by *Hha*I and electrophoresis in a 0.8% agarose gel. Standard marker fragments are included (channel M) and their sizes, in kilobase pairs, are given to the left of the photograph. Key to strains (culture petite frequency is given in brackets): a, Dip 2 (0.6%); b, [2]6.5s (90%); c, [1]14.17s (86%); d, [1]15.19s (83%); e, [2]17.5s (80%); f, [1]9.6s3 (76%); g, [1]9.15s (75%); h, [2]13.10s (78%); i, [2]6.10s (38%); j, [1]16.17s (16%); k, [1]9.6s5 (9%); l, [1]9.6s4 (6%); m, [1] 16.6s (5%).

tical cleavage patterns and (3) that unsectored restored forms arising from crossing petites contain no detectable change in their mtDNAs (only three of the ten strains are illustrated). Likewise, no differences were detected upon digestion of the mtDNAs with *Taq*I, which cleaves at more sites than *Hha*I and, hence, should reveal smaller changes not necessarily detected by *Hha*I (data not shown).

Mitochondrial DNA in sectored strains: In contrast to the above results, mtDNA from 12 sectored strains (both hfp and mfp) showed abnormalities on digestion with *Hha*I (Figure 3) and *Pvu*II plus *Cla*I (Figure 4). In each case, mtDNAs from the sectored strains are flanked by similarly digested mtDNA from a wild-type diploid. Strains are arranged in both figures in order of decreasing culture petite frequency from left to right. The mtDNAs from all hfp and mfp strains have fragmentation patterns differing from the wild type in two respects: in the size and number of fragments present and in the relative fluorescence intensity of some bands.

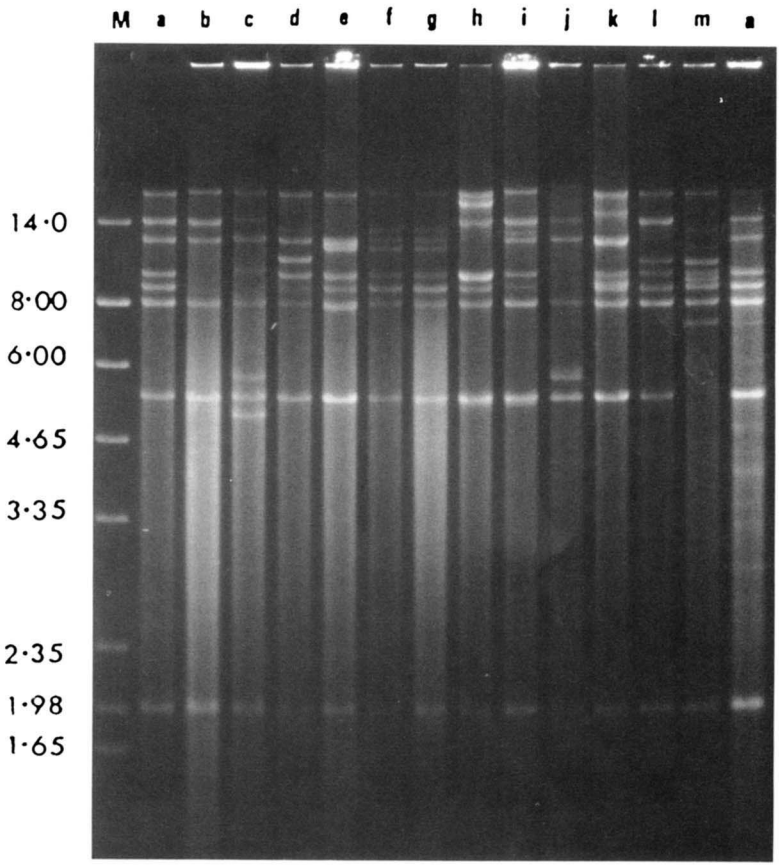

FIGURE 4.—Fragmentation patterns of mtDNA from 12 sectored strains and a wild-type diploid following digestion by *Pvu*II and *Cla*I and electrophoresis in a 0.8% agarose gel. Standard marker fragments are included (channel M) and their sizes, in kilobase pairs, are given to the left of the photograph. See Figure 3 legend for identification of the mtDNAs.

Stability of mitochondrial genomes in sectored strains: Because of the high rate of petite formation in sectored strains, it is important to know, when considering likely mechanisms of restoration, whether the aberrant mitochondrial genomes undergo change with time. Changes would be reflected both in an alteration to the petite frequency of a strain and in the restriction endonuclease pattern of the digested mtDNA. To investigate stability, the petite frequencies of six strains were followed over a period of 3 wk, and mtDNA preparations were made at weekly intervals.

During the course of this experiment, no significant differences were detected in culture petite frequencies (Table 2). With the possible exception of strain [1]9.15s, this result correlates with the observation that the restriction pattern of mtDNA from each strain remains constant (Figure 5). For strain [1]9.15s, it has not been determined whether the variations in band intensity between the 0- and 21-day samples is due to a change in the mtDNA or is an artifact arising from overloading of the latter sample. However, even with this

TABLE 2

Culture petite frequencies (%) on GlyYP subculture of sectored strains

Strain	Days of subculture[a]			
	0	7	14	21
[1]14.17s	86	88	85	85
[1]15.19s	81	80	84	80
[1]9.6s3	76	74	76	73
[1]9.15s	74	72	72	76
[1]16.17s	17	17	10	15
[1]16.6s	5	5	7	6

[a] The measured doubling time for wild-type diploid Dip2 is 155 min in GlyYP (data not shown). Assuming that the generation time of the $\rho+$ cells in the sectored strains is approximately equivalent to this value, the strains used in this experiment would have undergone 190–200 generations in 21 days.

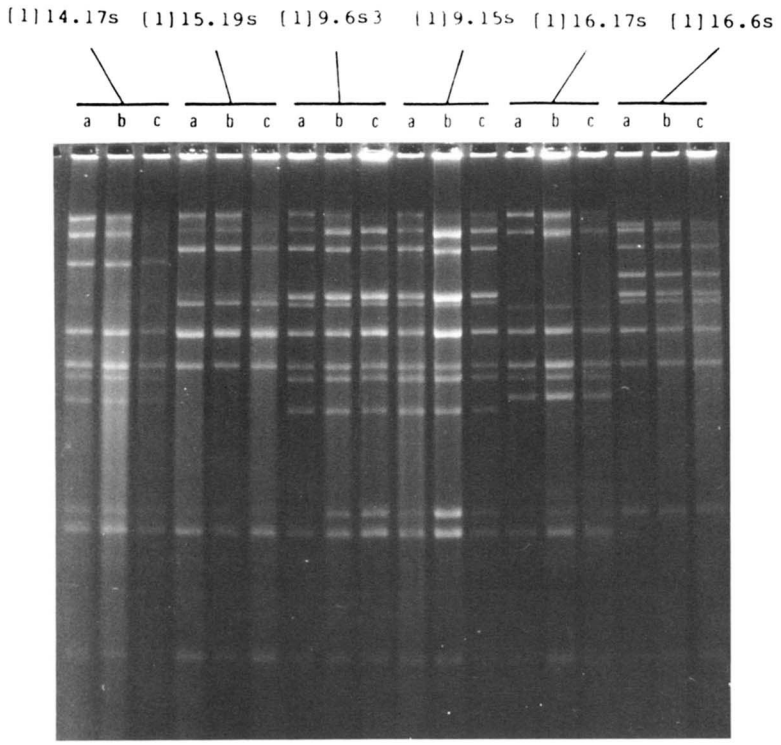

FIGURE 5.—Fragmentation patterns of mtDNA from six sectored strains digested with *Hha*I and separated in 0.8% agarose. MtDNA was prepared as follows: a, at the beginning of the experimental time course; b, after 21-days' growth in nonfermentable medium (glycerol); c, after growth for 24 hr in a fermentable medium (glucose).

strain it is apparent that no new bands have been gained or old ones lost. Also included in Figure 5 are mtDNAs isolated from glucose-grown cultures of each strain. It can be seen that growth in the fermentable medium does not bring

TABLE 3

Culture petite frequency and size of mtDNA in sectored strains

Strain	CPF (%)	mtDNA size (kbp)[a]	Duplication size (kbp)[b]
[1]9.6s3	76	100	19
[1]9.15s	75	100	19
[1]9.6s5	9	130	49
[1]9.6s4	6	113	32

[a] Estimates for mitochondrial genome sizes were determined by digestion with *Bam*HI, *Cla*I, *Hha*I, *Hpa*I, *Pvu*II and *Sph*I, both singly and in combination.

[b] The amount of duplicated sequence is estimated by subtracting the wild-type mtDNA size (81 kbp, see EVANS and CLARK-WALKER 1985) from that of the sectored strain.

about changes in the number of fragments; however, it is apparent that there is a reduced relative yield of the larger molecular weight fragments in mtDNA from glucose-grown cells.

Evaluation of the effect of putative duplications on culture petite frequency: After observing that fragmentation of mtDNAs from mfp and hfp strains showed both novel bands and ones that were judged by eye to differ in relative fluorescence intensity, we adopted as a working hypothesis that these changes may be produced by internal duplicated regions. We were interested, then, to learn whether a correlation existed between culture petite frequency and the extent of duplications.

The size of the putative duplication within each aberrant mtDNA can, in principle, be obtained by subtracting the size of the wild-type mtDNA from the total size of all the fragments plus the size of the bands that were adjudged to be duplicated. Estimates of these two paramenters would vary with different restriction enzyme digestion, as values are dependent on the number and position of cleavage sites within an aberrant molecule carrying a duplication. Enzymes that do not cleave twice or more within a duplicated region would not produce duplicated bands; nevertheless, in such cases larger novel fragments would appear.

Attempts at estimating the total mitochondrial genome size and duplication size for each of the 12 sectored strains were made, using several restriction enzyme digest combinations (for details see EVANS 1983). In only four cases (three unique genomes) could a consensus value for mtDNA size be established using separate enzyme digestions (Table 3). Even with these mtDNAs it is apparent that there is no correlation between the size of the duplication and the culture petite frequency. Indeed, the two strains that display culture petite frequencies of below 10% have the largest duplications.

DISCUSSION

In an earlier publication we reported that sectored colony morphology of respiratory competent restored forms is correlated with higher than normal levels of spontaneous petite formation and that this trait is cytoplasmically

inherited (OAKLEY and CLARK-WALKER 1978). In the present report we provide evidence that elevated levels of petite production are associated with the presence of aberrant mtDNA. In addition, the present studies have shown that two types of sectored colony forms can be distinguished on the basis of culture petite frequency. Forms that have high rates of spontaneous petite formation (hfp) (>60%) can be distinguished from those that show a moderate frequency of petite formation (mfp) (5–16%). These two types, together with restored unsectored strains having wild-type mtDNA, can be found among siblings from a cross between two petite parents. Thus, siblings [1]9.6s3 (76% petite formation), [1]9.6s5 (9%) and [1]9.6s4 (6%) differ not only in their rate of petite formation but also in the fragmentation pattern of their mtDNAs. It is unclear whether this result arises from heterogeneity of defective mtDNA molecules within the parents or is due to different recombination events between identical defective molecules. However, circumstantial evidence for heterogeneity among defective molecules in the parents comes from the observation that the level of complementation in matrices is dependent on petite age. This result suggests that further deletions do occur to the primary defective mtDNAs, leading, ultimately, to molecules that fail to complement when brought together by crossing.

Another indication of the complexities involved in interpreting the processes of deletion and restoration in the mitochondrial genome comes from the observation that two sectored strains [1]9.6s3 and [1]9.15s from different crosses have identical but aberrant mtDNAs. The simplest interpretation of this result is that the two different petite cultures, #6 and #15 from the T3/3 parent, contained the same defective mtDNA that underwent recombination with like molecules in the shared petite parent #9 from D13.1A. If this is the correct view, then the conclusion to be drawn is that the wild-type mtDNA could have preferred sites for deletions. However, this statement needs to be qualified by the possibility that some constraints on the restoration process could select the types of defective molecules that can participate (see accompanying paper, EVANS and CLARK-WALKER 1985).

Before the detailed mapping studies of the aberrant mtDNAs in mfp and hfp forms, we were curious to know from summation of fragment sizes whether a correlation existed between size and culture petite frequency. Notwithstanding the difficulties encountered in this analysis (see EVANS and CLARK-WALKER 1985), it was apparent that a simple relationship did not exist and that genomes with larger duplications were present in the mfp forms, while a smaller duplication existed in the hfp strain. An explanation for this observation has come from analysis of aberrant mtDNA structure. These studies, presented in the accompanying report, have enabled us to conclude that a fundamental difference exists between the mitochondrial genome organization in mfp and hfp strains. On the one hand, hfp strains have genomes that contain duplications in tandem that are stabilized by a deletion in a nonessential region; on the other hand, mfp strains contain inverted duplications. Furthermore, either of these structural characteristics of aberrant mtDNAs can explain the persistence

of these genomes during prolonged culture of hfp and mfp strains as opportunities for internal rearrangements are precluded.

R. J. EVANS and K. M. OAKLEY acknowledge the support of an Australian National University Ph.D. Scholarship and a Commonwealth Postgraduate Scholarship, respectively.

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Communicating editor: I. HERSKOWITZ