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# Regions flanking *ori* sequences affect the replication efficiency of the mitochondrial genome of *ori*$^+$ petite mutants from yeast

(*Saccharomyces cerevisiae*; cytoplasmic petite colonie mutation; suppressivity; chromatin; genetic crosses; modulation; recombination)

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SUMMARY

The mitochondrial genomes of progenies from 26 crosses between 17 cytoplasmic, spontaneous, suppressive, *ori*$^+$ petite mutants of *Saccharomyces cerevisiae* have been studied by electrophoresis of restriction fragments. Only parental genomes (or occasionally, genomes derived from them by secondary excisions) were found in the progenies of the almost 500 diploids investigated; no evidence for illegitimate, site-specific mitochondrial recombination was detected. One of the parental genomes was always found to predominate over the other one, although to different extents in different crosses. This predominance appears to be due to a higher replication efficiency, which is correlated with a greater density of *ori* sequences on the mitochondrial genome (and with a shorter repeat unit size of the latter). Exceptions to the 'repeat-unit-size rule' were found, however, even when the parental mitochondrial genomes carried the same *ori* sequence. This indicates that noncoding, intergenic sequences outside *ori* sequences also play a role in modulating replication efficiency. Since in different petites such sequences differ in primary structure, size, and position relative to *ori* sequences, this modulation is likely to take place through an indirect effect on DNA and nucleoid structure.

## INTRODUCTION

When respiratory-competent (wt, grande colonie, rho$^+$) *S. cerevisiae* cells are crossed, the parental mitochondrial genomes undergo recombination. The original observation of this phenomenon (Thomas and Wilkie, 1968) concerned intragenic recombination of mitochondrial genetic markers, due to 'legitimate' events taking place in regions of extended nucleotide homology between the two parental genomes.

Site-specific, 'illegitimate' recombination, only re-

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Abbreviations: bp, base pair(s); EtdBr, ethidium bromide; kb, 1000 bp; *ori*, origin of DNA replication; TBE, 0.05 M Tris-(hydroxymethyl) aminomethane, 0.05 M boric acid, 0.001 M EDTA; TTC, 2,3,5-triphenyltetrazolium chloride; wt, wild type.

quiring local homology, also does take place in crosses of wild-type cells (Fonty et al., 1978). Indeed, the vast majority, or the totality, of the progenies from such crosses exhibit mitochondrial DNA restriction fragments that derive from both parental genomes; 'new' (junction) fragments, absent in either parent, and arising from non-reciprocal recombination events involving intergenic sequences are also present. Needless to say, intergenic recombination events, as observed genetically in the progeny of crosses of wt cells, can be the result of both 'legitimate' and 'illegitimate' recombination phenomena, the latter being much more frequent than the former.

Illegitimate recombination events can also lead to the formation of the defective mitochondrial genomes of spontaneous cytoplasmic petite mutants (respiratory-deficient, rho$^-$; see Bernardi, 1979, for a brief review). Indeed, detailed investigations (Faugeron-Fonty et al., 1979; Gaillard et al., 1980; Marotta et al., 1982; de Zamaroczy et al., 1983) showed that the mitochondrial genomes of such mutants are formed by recombination at direct repeats predominantly located in the abundant, intergenic sequences of the wt genomes. Excision events are followed by the amplification of the excised DNA segments, which become the repeat units of the petite mitochondrial genome and are generally arranged in a tandem (head-to-tail) fashion.

Genetic recombination has also been shown in wt diploids after crossing wt and petite cells (see Dujon, 1981, for a review), but no information at the molecular level is available in this case. The percentage of petite diploids issued from such crosses (namely, suppressivity; Ephrussi et al., 1955) was shown to be determined by the replicative competition between the two parental genomes (de Zamaroczy et al., 1979; 1981; Goursot et al., 1980; Bernardi et al., 1980; Blanc and Dujon, 1980) and not by the destructive recombination of the petite genome with the wt genome (Coen et al., 1970; Lazowska and Slonimski, 1977). Indeed, the structure (intact, rearranged, partially or completely deleted) and the density of *ori* sequences on the mitochondrial genomes of the parental petites are the main factors determining the result of the replicative competition with the mitochondrial genome from wt cells (de Zamaroczy et al., 1981).

In the case of petite × petite crosses, the situation is much less clear. Illegitimate recombination has been claimed to occur (Michaelis et al., 1973; 1976a,b; Lazowska and Slonimski, 1977), but conclusive evidence is still lacking. Moreover, no information is available on the replicative competition between parental mitochondrial genomes in such crosses.

In the present work we have studied the outcome of crosses of spontaneous, suppressive, *ori*$^+$ petites (containing active *ori* sequences in their mitochondrial genomes) with the aim of (*i*) investigating the possibility of recombination in such crosses; and (*ii*) studying the factors that determine the replicative competitiveness of petite genomes.

## MATERIALS AND METHODS

### (a) Yeast strains and growth media

All strains used in this work were respiratory-deficient, spontaneous, cytoplasmic, suppressive, *ori*$^+$ petite colony mutants, derived from laboratory strains A (*MAT*α *ade1*), and B (*MAT*α *his1 trp1*; see Bernardi et al., 1970 for the origin of these strains). The only exception was strain JF11-2A/B17/Hp5 (*MAT***a**, *met2*; called henceforth hp5), kindly provided by Dr. F. Foury.

Three growth media were mainly used. YPG consists of 1% yeast extract, 2% bactopeptone and 2% glucose. M contains 0.67% yeast nitrogen base, without amino acids, and 2% glucose. SDG is M with 0.1% (instead of 2%) glucose and 3% glycerol.

### (b) Petite × petite crosses

Overnight cultures (0.5 ml) of parental strains in YPG were mixed and diluted in 5 ml of YPG, and incubated with agitation at 28°C for 4 h. Samples were plated on M agar, which only allows the diploids to grow. Zygotic clones were isolated after four days at 28°C, and subcloned in several cases.

### (c) Petite × grande crosses

Crosses of petite × wt cells were performed as described above to determine suppressivity; diploids were then plated on SDG agar, which easily permits

to distinguish the grande (oxidizing glycerol) from the petite (fermenting the limiting glucose) diploids. In some cases, colonies were stained by the TTC overlay technique (Ogur et al., 1957).

### (d) Mitochondrial DNA

Micro-scale DNA preparations from all 485 diploids investigated were obtained, essentially as described by Dujon and Blanc (1980), about 40

TABLE I

Properties of spontaneous cytoplasmic petite mutants used in the present work

| Petite mutant | Wild-type parental strain[a] | *ori* sequence[b] | Repeat unit(bp)[c] | Suppressivity[d] (%) | References[e] |
|---|---|---|---|---|---|
| a1/1R/Z1 | A | *ori*1 | 416 s | >95 | 1, 2 |
| a1/1R/34 | A | *ori*1 | 515 m | >95 | 3 |
| a1/1R/40 | A | *ori*1 | 606 m | >95 | 2 |
| a1/1R/1 | A | *ori*1 | 884 s | >95 | 4, 5 |
| hp5 | ....[f] | *ori*2 | 700 m | >95 | 6 |
| b11 | B | *ori*2 | 1855 s | 85 | 7 |
| b20 | B | *ori*2 | 4500 m | 80 | 8 |
| b | B | *ori*$^{h}$2-7[g] | 852 s | >95 | 9 |
| b7 or k7[h] | B | *ori*$^{h}$2-7[g] | 2187 s | >95 | 9, 10 |
| b17 | B | *ori*2 + *ori*7[i] | 7800 m | 80 | 5, 8 |
| b21 | B | *ori*2 + *ori*7[i] | 13200 m | 55 | 8 |
| a*1/7/8 | A | *ori*3 | 1760 m | 85 | 5 |
| a*1/7/20 | A | *ori*3 | 2125 m | >95 | 3 |
| a*1/7/12 | A | *ori*3 | 4500 m | 65–75 | 2, 11 |
| a3/1 | A | *ori*3 + *ori*4[i] | 4500 m | 65–75 | 5 |
| a-23/3 | A | *ori*5 | 750 s | >95 | 12 |
| b13/1 | B | *ori*5 | 1085 m | >95 | 13 |
| b103 | B | *ori*5 | 1200 m | >95 | 3 |
| b13 | B | *ori*5 | 1400 m | >95 | 3 |
| b106 | B | *ori*5 | 1750 m | >95 | 3 |
| a15/3/2 | A | *ori*5 | 4300 m | 50–60 | 12 |

[a] A is strain D243-2B-R1; B, strain C982-19D (see MATERIALS AND METHODS, section a).

[b] For the primary structures and properties of *ori* sequences, see de Zamaroczy et al. (1984).

[c] s indicates that the repeat unit (see INTRODUCTION) has been entirely sequenced, m that it has been mapped and located on the wt genome sequence compiled by de Zamaroczy and Bernardi (1986).

[d] The suppressivity of a petite strain is $\frac{X - Y}{100 - Y} \times 100$ where $X$ is the fraction of petite colonies produced by the zygote(s) and $Y$ is the fraction of petite cells in the rho$^+$ haploid tester strain.

[e] Numbers provide the references where information on the petites and on sequencing and mapping data can be found: (1) Gaillard and Bernardi (1979); (2) de Zamaroczy et al. (1981); (3) R.G. (unpublished); (4) Gaillard et al. (1980); (5) de Zamaroczy et al. (1984); (6) F. Foury (pers. commun.); (7) R.G. and B. Aissani (unpublished); Aissani (1985); (8) B.C.-Z. (in preparation); (9) Colin et al. (1985); (10) Marotta et al. (1982); (11) de Zamaroczy et al. (1983); (12) Mangin et al. (1983); (13) E.R. (unpublished).

[f] For the origin of this petite, see MATERIALS AND METHODS, section a.

[g] The hybrid *ori*$^{h}$2-7 sequence contains GC clusters A and B derived from *ori*7 and the rest of the sequence derived from *ori*2 (see Fig. 4). The *ori*$^{h}$2-7 sequence is strictly identical to *ori*2 (Colin et al., 1985).

[h] k7 is a cytoductant of b7 into strain JC8/AA1, (*MAT*a *kar1*, *leu1* rho°), constructed by M. Breitenbach, Vienna (Austria).

[i] *ori*4 and *ori*7 have been shown to be inactive *ori* sequences (de Zamaroczy et al., 1984).

generations after zygote formation; this was required to produce enough cells for restriction fragment analysis of mitochondrial DNA. Large-scale preparations of purified DNA were also used, in particular for sequencing (Maxam and Gilbert, 1980).

## RESULTS

### (a) The petite mutants used

Twenty-two spontaneous, cytoplasmic, suppressive, *ori*$^+$ petite mutants were used in the present work. They were characterized in their suppressivity and in their mitochondrial genomes. These were either completely sequenced or mapped with restriction enzymes and localized on the wt genome sequence compiled by de Zamaroczy and Bernardi (1986b). All petite genomes were, therefore, known in their primary structure, with the only exception of petite hp5, which was not from our collection. All petite genomes were made up of tandem repeat units, as is the rule for spontaneous petites (Faugeron-Fonty et al., 1979; 1983). The petite mutants used differed from each other in one or more of the following properties: (*i*) suppressivity; (*ii*) size of the repeat units of mitochondrial genomes; (*iii*) *ori* sequence; (*iv*) sequences flanking the *ori* sequences; (*v*) amount of mitochondrial DNA (Table I). Restriction maps and primary structures of the mitochondrial

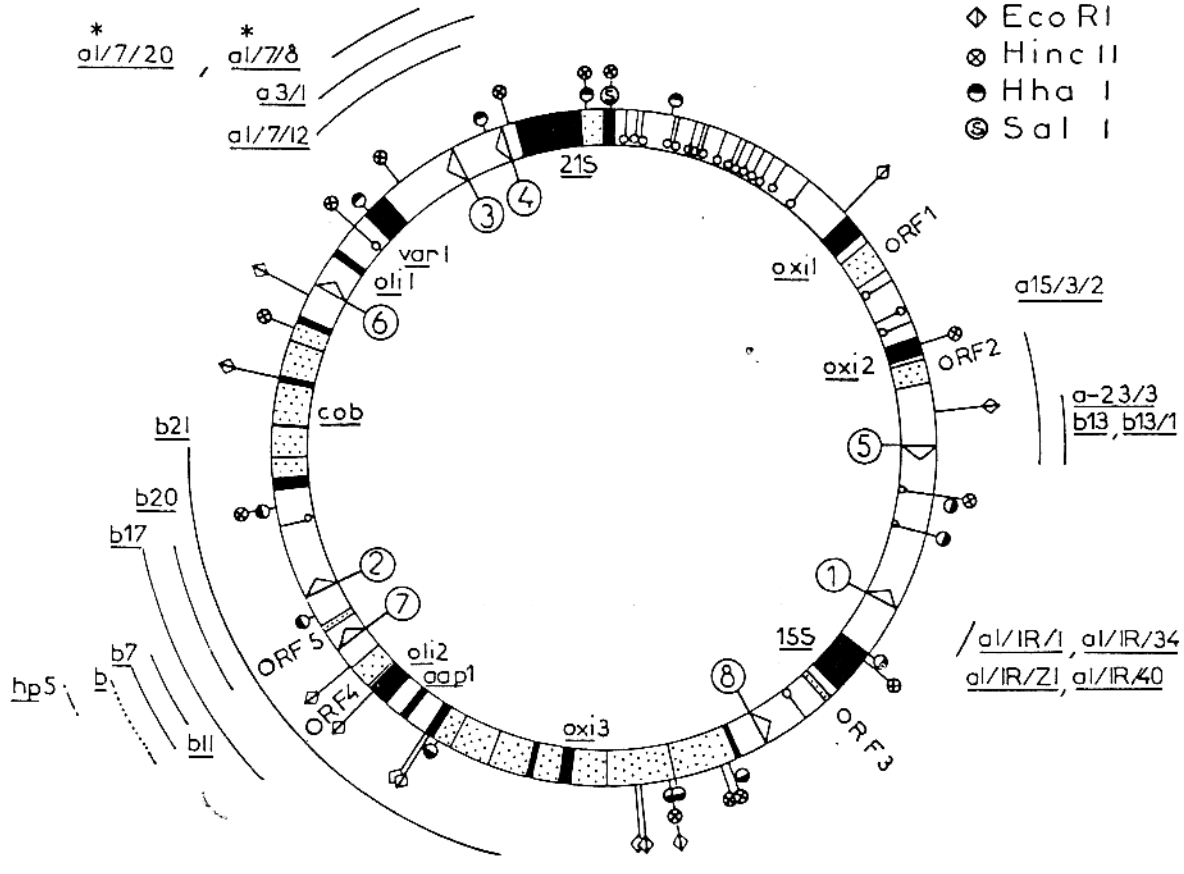


Fig. 1. Localization of the repeat units from the mitochondrial genomes of petite mutants used in the present work on the physical map of the mitochondrial genome of wt cells. When several petite genomes correspond to the same localization, they are of about equal sizes. In the case of petite b, the dotted line corresponds to a central deletion; in that of petite hp5, excision sequences were not determined. Some restriction sites are indicated. Circled numbers indicate the location of *ori* sequences 1-8 (arrowheads point in the direction from cluster C to cluster A). Blackened and stippled areas correspond to exons and introns of mitochondrial genes, respectively. Thin radial lines indicate tRNA genes. White areas correspond to long AT spacers with embedded short GC clusters.

genomes from most petites used here are published and the appropriate references are given in Table I. Fig. 1 presents the location of these petite genomes on the physical and genetical map of the mitochondrial genome from wt cells. The petite genomes studied derive from the three major intergenic regions of the latter; these comprise all the four active *ori* sequences (*ori*1, *ori*2, *ori*3 and *ori*5; de Zamaroczy et al., 1984).

The data of Table I show that supersuppressivity (i.e., suppressivity equal to, or higher than, 95%) was found (with two exceptions, petites b11 and a*1/7/8) to be associated with repeat unit sizes up to about 2 kb (in agreement with previous results; de Zamaroczy et al., 1981; Blanc and Dujon, 1982). Petites carrying mitochondrial genomes made up of longer repeat units exhibited lower suppressivities. Suppressivity seemed, however, also to depend upon the particular *ori* sequence (and/or its flanking sequences) carried by the mitochondrial genome. Indeed, petites b20 (*ori*2), a*1/7/12 (*ori*3) and a15/3/2 (*ori*5) which harbor mitochondrial genomes with repeat units of the same size (about 4500 bp), exhibit different suppressivities (80%, 65–75% and 50–60%, respectively). Likewise, petites b11 (*ori*2), a*1/7/8 (*ori*3) and b106 (*ori*5) with repeat units about 1800 bp in size, show suppressivities of 85%, 85% and >95%, respectively.

### (b) Uniparental inheritance of the mitochondrial genome

When petites exhibiting large differences in suppressivity levels were crossed, a uniparental inheritance of the mitochondrial genome was found about 40 generations after zygote formation. In this case,

TABLE II

Uniparental inheritance of the mitochondrial genome in the progeny from crosses of suppressive petites[a]

| | Cross | | | | Number of diploids analyzed | Mitochondrial genome in all diploids from |
|---|---|---|---|---|---|---|
| | Parent 1 | × | Parent 2 | | | |
| (A) | | | | | | |
| M2 | b (>95%) 852 bp | × | a15/3/2 4500 bp | (60%) | 12 | parent 1 |
| R1 | b | × | a3/1 4500 bp | (70%) | 15 | parent 1 |
| R2 | b | × | a*1/7/12 4500 bp | (70%) | 11 | parent 1 |
| R3 | b | × | a*1/7/8 1760 bp | (85%) | 30 | parent 1 |
| E22 | hp5(>95%) 700 bp | × | b11 1855 bp | (85%) | 7 | parent 1 |
| (B) | | | | | | |
| E23 | hp5(*ori*2) 700 bp | × | b13 1400 bp | (*ori*5) | 11 | parent 1 |
| E5 | b (*ori*$^h$2-7) 852 bp | × | k7 2187 bp | (*ori*$^h$2-7) | 10 | parent 1 |
| R5 | b | × | a1/1R/34 515 bp | (*ori*1) | 12 | parent 2 |
| R4 | b | × | a1/1R/Z1 416 bp | (*ori*1) | 8 | parent 2 |
| EM9 | b | × | a-23/3 750 bp | (*ori*5) | 25 | parent 1 |

[a] Part **A** of this Table presents the outcome of crosses involving petites exhibiting different suppressivity levels (indicated by values in parentheses); part **B** concerns crosses of supersuppressive petites.

the diploid progeny only carried the mitochondrial genome from the petite endowed with the higher suppressivity level. The results of these experiments are shown in Table IIA. In all cases, the petites showing lower suppressivity levels were endowed with mitochondrial genomes made up of much longer repeat units (1760–4600 bp) than those (700 or 852 bp) of the petites having a higher suppressivity.

A uniparental inheritance was also found in many crosses of supersuppressive petites in which no difference in suppressivity could be measured (Table IIB), since very small differences at such high suppressivity levels are not significant. The predominance of one genome was, however, clearly correlated with a shorter repeat unit size, irrespective of the *ori* sequence present.

TABLE III

Biparental inheritance of the mitochondrial genome in the progeny from crosses of suppressive petites[a]

| Cross | | | Mitochondrial genomes in diploids from[b] | | | Total colonies analyzed |
|---|---|---|---|---|---|---|
| Parent 1 | × | Parent 2 | Parent 1 | Parent 2 | Parents 1 + 2 | |
| (A) | | | | | | |
| E26 a*1/7/8 1760 bp | × | b20 4500 bp | 11 | 0 | 1 | 12 |
| E11 a1/1R/1 884 bp | × | b13 1400 bp | 9 | 0 | 1 | 10 |
| E12 a1/1R/1 884 bp | × | b13/1 1085 bp | 9 | 0 | 1 | 10 |
| E21 hp5 700 bp | × | b7 2187 bp | 7 | 0 | 4 | 11 |
| E29 a-23/3 750 bp | × | b13 1400 bp | 5 | 0 | 7 | 12 |
| E20 b 852 bp | × | hp5 700 bp | 8 | 2 | 0 | 10 |
| E27 k7 2187 bp | × | b20 4500 bp | 9 | 1(1) | 1 | 12 |
| (B) | | | | | | |
| R8 a1/1R/1 884 bp | × | b 852 bp | 3(4) | 0 | 12(1) | 20 |
| E2 a*1/7/8 1760 bp | × | b11 1855 bp | 3 | 1 | 2 | 6 |
| E30 b13/1 1085 bp | × | a-23/3 750 bp | 8 | 2 | 16 | 26 |
| E31 b7 2187 bp | × | a*1/7/20 2125 bp | 4 | 1 | 29 | 34 |
| E24 b13/1 1085 bp | × | a*1/7/8 1760 bp | 2 | 0 | 10 | 12 |
| E15 k7 2187 bp | × | b11 1855 bp | 3 | 2 | 11 | 16 |
| E25 k7 2187 bp | × | b13 1400 bp | 1 | 0 | 9(2) | 12 |
| E1 a*1/7/8 1760 bp | × | b7 2187 bp | 0 | 0 | 6 | 6 |
| E3 a*1/7/8 1760 bp | × | b13 1400 bp | 0 | 0 | 9(1) | 10 |

[a] For the two parts, **A** and **B**, see RESULTS, section **c**.

[b] Values in parentheses indicate additional new genomes derived by secondary excisions from one of the parental genomes.

The only exception to the predominance of the petite genome made up of shorter repeat units was cross EM9 (b × a-23/3). In this case, the cross yielded a progeny only carrying the b genome, which is characterized by a repeat unit size of 852 bp, larger than that of the other parent (750 bp).

### (c) Biparental inheritance of the mitochondrial genome

In all crosses other than those of Table II, both parental genomes were present in the progeny after a standard number of 40 generations from zygote formation. In most cases (Table III), both heteroplasmic and homoplasmic clones were produced; much more rarely, the progeny consisted only of homoplasmic clones containing either parental genome (cross E20), or only of heteroplasmic clones (crosses E1 and E3). It should be noted that the latter may not only contain heteroplasmic cells, but also mixtures of homoplasmic segregants (as shown by subcloning experiments; Table IV).

Table III divides the crosses with biparental inheritance according to whether one parental genome was clearly predominant in the progeny or not. In the first case (Table IIIA), one parental genome either was the only one appearing in homoplasmic clones or was clearly predominant in those clones. In the second case (Table IIIB), the predominance of one genome was not obvious in homoplasmic clones, or heteroplasmic clones were predominant.

The second case was further examined by assessing the relative amounts of the two parental genomes in the heteroplasmic clones and/or by subcloning. Indeed, the relative abundance of parental genomes provided a criterion for evaluating the predominance of one of the two parental genomes in heteroplasmic clones (see Fig. 2,A and B). Subcloning experiments established the predominance of one parental genome, even when weak (Table IV).

Like the crosses with uniparental inheritance (Table II), those with biparental inheritance (Tables III and IV) also showed the predominance of one parental genome, which was generally corre-

TABLE IV

Subcloning of heteroplasmic diploid petites

| Clones[a] | | Mitochondrial genomes in diploids from[b] | | | Total subclones analyzed |
|---|---|---|---|---|---|
| | | Parent 1 | Parent 2 | Parents 1 + 2 | |
| R8/1 | (a1/1R/1 × b) | 11(1) | 2 | 4 | 18 |
| E2/2 | (a*1/7/8 × b11) | 5 | 0 | 1 | 6 |
| E24/1 | (b13/1 × a*1/7/8) | 2 | 0 | 10 | 12 |
| E24/1′ | | 7 | 0 | 2(1) | 10 |
| E24/1′′ | | 4 | 0 | 5 | 9 |
| E25/7 | (k7 × b13) | 0 | 0(1) | 10 | 11 |
| E25/11 | | 0 | 0(5) | 0(6) | 11 |
| E1/2 | (a*1/7/8 × b7) | 0 | 0 | 6 | 6 |
| E1/2′ | | 2 | 0 | 10 | 12 |
| E1/2′ | (5 months later) | 5 | 0 | 3 | 8 |
| E3/7 | (a*1/7/8 × b13) | 5 | 2(1) | 2(1) | 11 |
| E3/10 | (2 years later) | 5 | 0 | 1 | 6 |
| E3/1 | (2 years later) | 3 | 2 | 0 | 5 |

[a] These clones are derived from crosses of Table III. The number after the bar indicates the heteroplasmic zygotic clone analyzed. Symbol ′ (prime) corresponds to a heteroplasmic primary subclone of a heteroplasmic zygotic clone. Symbol ″ (double prime) corresponds to a heteroplasmic secondary subclone of a heteroplasmic primary subclone.

[b] See the corresponding footnote b of Table III.

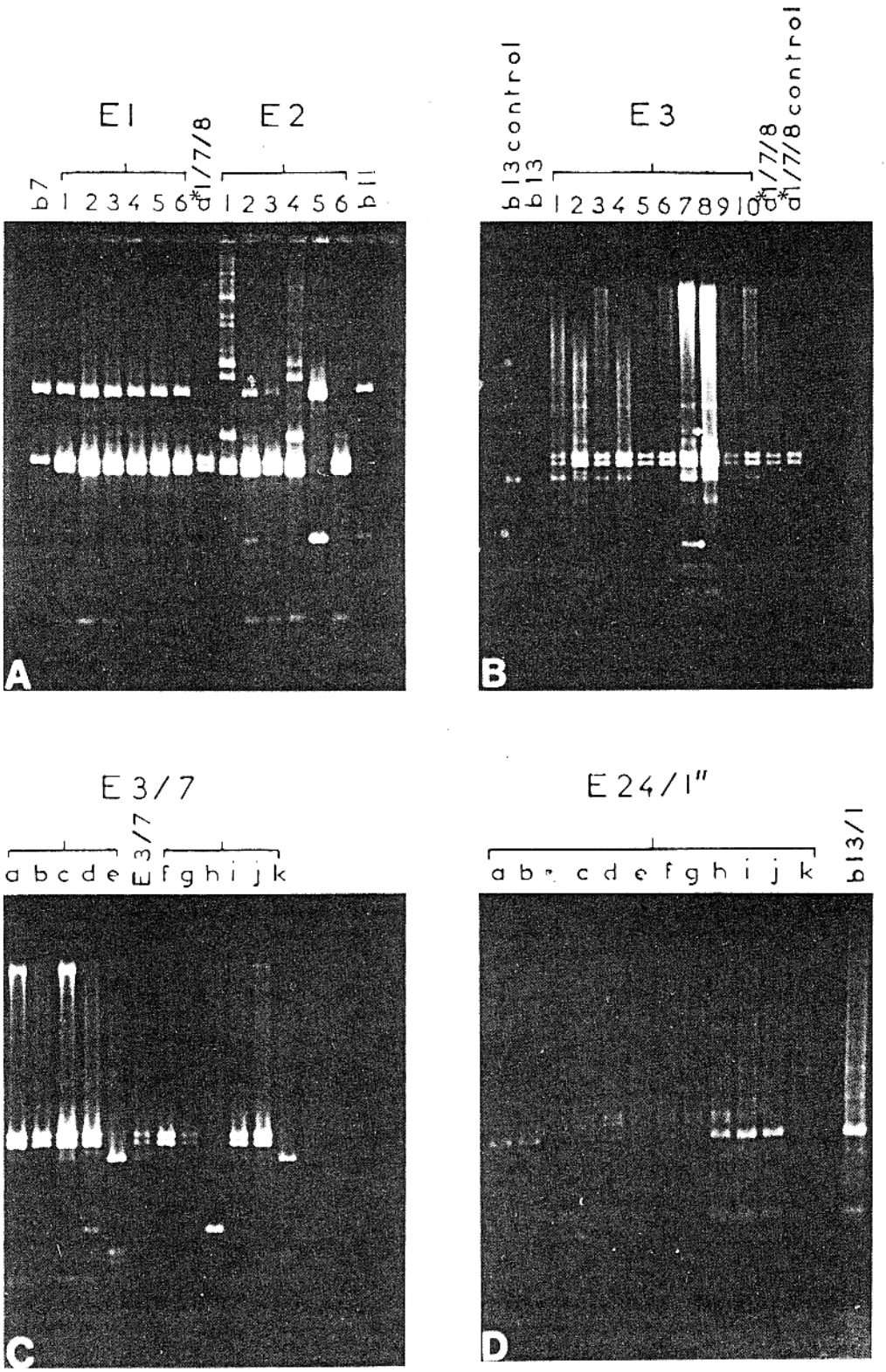


Fig. 2. *Hpa*II digests of the mitochondrial DNAs from haploid parental strains and their diploid progeny, visualized on 1.5% agarose gels, run in TBE-buffer overnight (40 V) and stained by EtdBr. (**A**) Cross E1 (a*1/7/8 × b7) shows only heteroplasmic diploids, with the a*1/7/8 genome being very slightly predominant over that of the b7 genome. Cross E2 (a*1/7/8 × b11) shows homoplasmic diploid

lated with a shorter repeat unit size. This rule showed, however, some exceptions which concerned crosses E20, E30, E31, E3, E15, R8 and E25 (Table III). These results were confirmed by subcloning experiments in the case of three of these crosses, E3, R8 and E25 (Table IV).

Two additional remarks, concerning the crosses of Tables III and IV, are (*i*) that heteroplasmy persisted over more than 200 generations in the progenies from at least three crosses (E1, E2, R8; see also Fig. 2D); (*ii*) that secondary excisions were found in the mitochondrial genome from the progeny of some crosses (see the following section).

### (d) Secondary excisions

In some cases, new restriction fragments, not detectable in the genome of either parent, were found in the progeny of crosses with biparental inheritance (Fig. 2,B and C). These fragments could vary in relative amounts in different clones, and they could also segregate into homoplasmic clones. These findings indicated that these fragments did not correspond to junction fragments of recombined parental genomes, but that they belonged to new molecular species arisen in diploids.

Restriction analysis showed that all these new genomes contained in their repeat units a complete, active *ori* sequence with its flanking regions, which was derived from one or the other parental genome. For instance, all 'new' genomes derived from cross R8 contained *ori*1 (from the a1/1R/1 parent) and its flanking regions; those derived from crosses E3, E24, and E25 contained *ori*5 (from the b13 or b13/1 parents) and its flanking regions. In the case of the diploid E3/7h (Fig. 2,C and D), the new repeat unit was too short to be identified by restriction analysis alone. Primary sequence determination and comparison with the a-23/5 genome (Baldacci et al., 1984) containing *ori*5 showed, however, that this genome contained exclusively the *ori*5 sequence and its flanking regions (results not shown).

All these results demonstrate that the new fragments appearing in diploids were secondary petite genomes formed, like all spontaneous petite genomes, by site-specific recombination events. In some cases, the haploid parental strains already contained these genomes as minority genomes. Indeed, extensive subcloning of haploid petites a1/1R/1 and b13 finally gave rise to the same *ori*$^+$ genomes as those appearing in the progeny from their crossings (results not shown).

## DISCUSSION

### (a) Recombination in petite × petite crosses

Out of almost 500 diploid clones examined here in their mitochondrial genomes by restriction fragment analysis, not a single one exhibited physical evidence for illegitimate recombination. In some cases, 'new' restriction fragments were found, which could be interpreted as due to 'recombinant' restriction fragments. Subcloning and restriction mapping (followed in one case by primary structure determination), showed, however, that these fragments originated from mitochondrial genomes arisen by secondary excisions from one of the parental genomes.

This absence of site-specific, illegitimate recombination in petite × petite crosses is in full contrast

clones containing parental DNAs of either a1*/7/8 (E2/1, E2/4, E2/6) or of b11 (E2/5). In the case of E2/1 and E2/4 digestion was incomplete. In the two heteroplasmic clones E2/2 and E2/3, the a*1/7/8 genome is in large excess relative to the b11 genome. (**B**) Cross E3 (a*1/7/8 × b13). In all cases, except in E3/1, the a*1/7/8 genome is more abundant than the b13 genome. In the zygotic clone E3/7, new bands, indicated by lozenges, are present in addition to those from the parental genomes. (This clone is further analysed in panel C.) Several other faint bands correspond to nuclear DNA, as indicated by their absence in pure mitochondrial DNA preparations (lane labeled 'control'). (**C**) The heteroplasmic zygotic clone, E3/7 (see also panel B) and its subclones (E3/7a-k). Note the segregation of the parental genomes a*1/7/8 (E3/7a,b,f,g,i,j) and b13 (E3/7e and E3/7k) and also of the new genome E3/7h. Two diploid subclones are still heteroplasmic; one, (E3/7c), contains the two parental genomes, a*1/7/8 being largely predominant; the other, (E3/7d), contains the a*1/7/8 and the E3/7h genomes. Comparing these two heteroplasmic strains, note the greater abundance of the E3/7h genome relative to the longer b13 genome, both genomes containing *ori*5. (**D**) Parental strain b13/1 and tertiary subclones issued from the cross E24 (b13/1 × a*1/7/8). Only 4 out of 11 subclones contain a 'purified' b13/1 genome (E24/1'' a,b and i,j), (five subclones are still heteroplasmic (E24/1'' c,d and f-h); two subclones are *rho*° (E24/1'' e and k).

with the results of Michaelis et al. (1973), who reported that about 80% of the progeny from crosses of chloramphenicol-resistant by erythromycin-resistant petites carried recombinant genomes harboring both resistance markers. These genomes were visualized by electron microscopy of partially denatured DNA molecules as made up of repeat units from both parental genomes illegitimately recombined end-to-end (Lazowska and Slonimski, 1977). These results never were, however, substantiated by restriction mapping and/or sequencing of recombinant mitochondrial genomes and, in any case, they only concerned EtdBr-induced petite mutants containing unstable, heterogeneous mitochondrial genomes which were deprived of *ori* sequences, made of palindromic repeat units, endowed with very poor replicative ability, and derived from the same omega locus region (Lazowska, 1979; Michel, 1982). The replication of these genomes will be discussed in detail together with the outcome of crosses of *ori*°, *ori*$^r$ and *ori*$^-$ petites (R.M., R.G., B.C.-Z., E.R. and G.B., paper in preparation).

**(b) Factors responsible for the replicative competition of petite genomes**

The results obtained in the present work show that the outcome of crosses of *ori*$^+$ petites is mainly determined by the replicative competition of the parental mitochondrial genomes. In turn, this is mainly dependent upon the density of active *ori* sequences that such genomes carry, namely upon the same factor which mainly determines the suppressivity of *ori*$^+$ petites (Table I). Incidentally, this conclusion does not suggest that segregation (a phenomenon concerning mitochondria and only indirectly mitochondrial DNA) plays a role in the phenomena under discussion.

Out of 26 crosses studied, 18 showed a predominance of the parental genome made up of a shorter repeat unit, and eight, EM9 (Table IIB), E20, E30, E31, E15, E25, R8 and E3 (Tables III and IV) showed results against the 'repeat-unit-size rule'. Out of these eight cases, two (EM9 and E20) showed a clear-cut predominance, whereas in the other cases

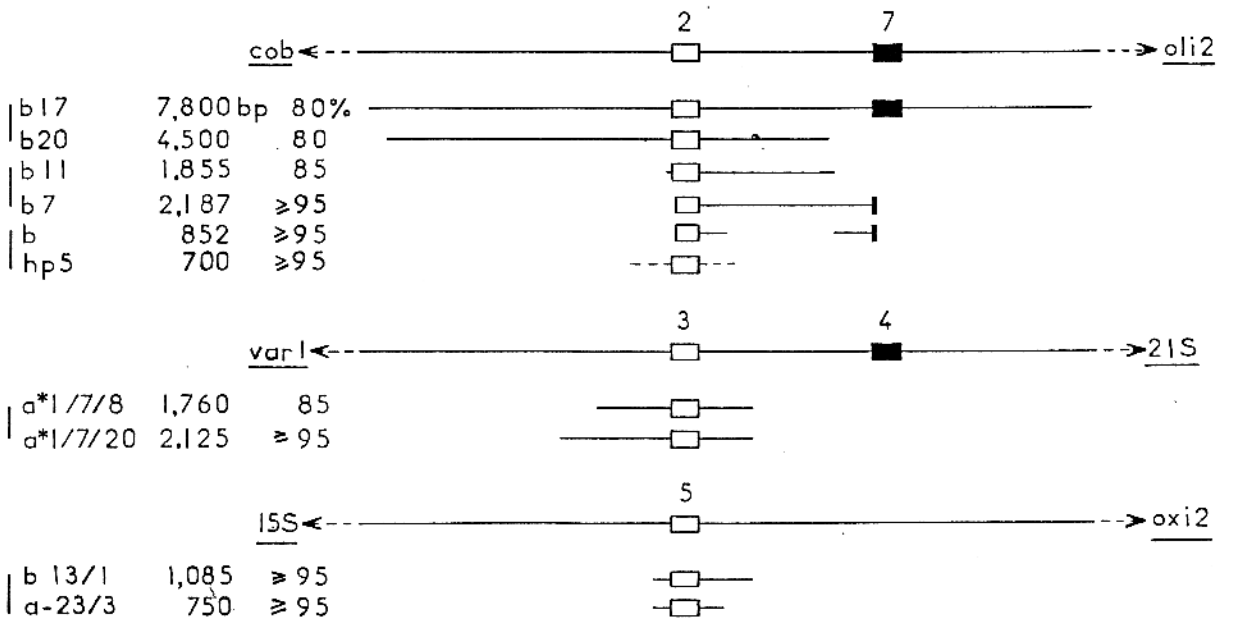


Fig. 3. Repeat units of petites harboring mitochondrial genomes carrying identical *ori* sequences and exhibiting suppressivities and/or competitive abilities in petite × petite crosses which do not follow the repeat unit size rule (see section b of DISCUSSION). Repeat unit sizes and suppressivities are indicated. Open boxes indicate active *ori* sequences 2, 3 and 5, blackened boxes indicate inactive *ori* sequences 4 and 7 (de Zamaroczy et al., 1984). The *ori* sequences are all oriented with cluster C to the right of cluster A. Flanking genetic markers are indicated (see also Fig. 1). Vertical bars on the left indicate the pairs of petite repeat units which are compared and discussed (DISCUSSION, section b).

the predominance of the genome with longer repeat units was weak.

The exceptions to the 'repeat-unit-size rule', as well as those cases where suppressivity was not correlated with the repeat unit size, point to the existence of other factors which play a role in determining the replicative efficiency of *ori*$^+$ petite genomes. (The case of *ori*$^\circ$ petites, which was investigated by Goursot et al., 1982, and by Fangman and Dujon, 1984, will be dealt with elsewhere.) Conceivably, two of these factors could be (*i*) the input of parental mitochondrial DNA into the zygote; and (*ii*) different specific activities of different *ori* sequences.

As far as the mitochondrial DNA input is concerned, results obtained in this work indicate that this cannot be an important factor. Indeed, a petite widely used in our crosses, petite b, is characterized (Bernardi et al., 1970) by a particularly low level of mitochondrial DNA (2% of total DNA), whereas other petites are much closer to the wt cell level (about 14% of total DNA; Bernardi et al., 1970). Now, the mitochondrial genome of petite b behaved in crosses essentially as expected on the basis of its repeat unit size; only in cross R8 (Tables IIIB and IV), this genome appeared to be slightly less competitive than that of the other parent, a1/1R/1, which had a barely larger repeat unit (884 bp vs. 852 bp). Obviously, this slight difference might also be due to a better efficiency of *ori*1 (present in a1/1R/1) compared to *ori*$^h$ 2-7 (present in b). The importance of differences in specific activity of different *ori* sequences in determining the replicative competitivity of petite genomes cannot, however, be judged at present because of the existence of another factor, which definitely plays a role.

This factor is the influence of sequences located outside the *ori* sequence, and is best illustrated by the exceptions to the 'repeat-unit-size-rule' which involve mitochondrial genomes carrying the same *ori* sequence (Fig. 3).

Among petites carrying *ori*2 (or the identical *ori*$^h$ 2-7), (*i*) petite b (852 bp) predominated over petite hp5 (700 bp; Table IIIA); and (*ii*) petite b7 (2187 bp) had a higher suppressivity (> 95%) than petite b11 (1855 bp; 85% suppressivity); this difference in suppressivity was confirmed by petite × petite crosses; indeed, in cross E15, the b7 genome (strain k7) competes out that of petite b11 (Table IIIB) and, not surprisingly, the mitochondrial genome of petite b7 is less well competed out by that of a*1/7/8 than that of b11 (compare crosses E2 and E1 in Tables IIIB and IV).

Among petites carrying *ori*3, petite a*1/7/20 (2125 bp) has a higher suppressivity (> 95%) than a*1/7/8 (1760 bp; 85% suppressivity; Table I). Here again, the higher replicative efficiency of a*1/7/20 is confirmed by petite × petite crosses, since the mitochondrial genome of a*1/7/8 is less good than that of a*1/7/20 in competing out that of b7 (compare crosses E1 and E31 in Tables IIIB and IV).

Finally, among petites carrying *ori*5, the mitochondrial genome of petite b13/1 (1085 bp) can compete out that of another supersuppressive petite, a-23/3 (750 bp, cross E30).

In all four cases presented above, genomes made up of longer repeat units show a better replicative ability than genomes made up of shorter repeat units. This different replicative ability has been proven by significant differences in suppressivity levels and/or in the outcome of petite × petite crosses. Since the *ori* sequences carried by each pair of genomes compared in Fig. 3 are the same, the better replicative ability of some petite genomes made up of longer repeat units leads to the conclusion that the extra sequences carried by such longer repeat units somehow favor replication.

This conclusion is of interest in three different respects. First of all, it may indeed apply to other situations met in the present work, such as that of petites exhibiting the same suppressivity in spite of carrying the same active *ori* sequence on repeat units of different sizes; this is the case of petites b17 (7800 bp) and b20 (4500 bp) which both carry *ori*2 and have a suppressivity of 80% (Fig. 3; it should be recalled that the second *ori* sequence present on the b17 genome, *ori*7, is an inactive *ori* sequence; see de Zamaroczy et al., 1984).

Second, it suggests that the exceptions to the 'repeat-unit-size rule' for petites carrying different *ori* sequences may be not (or not only) due to different specific efficiencies of such *ori* sequences (which might exist, however), but rather to the effects of flanking sequences. This applies to (*i*) petites showing different suppressivities and carrying different *ori* sequences, but having mitochondrial genomes with repeat units very close in size (like petites b20, a*1/7/12 and a15/3/2, or petites b11, a*1/7/8 and

b106; Table I); and (*ii*) to petites carrying different *ori* sequences and exhibiting better replication efficiencies in spite of longer repeat units, as in crosses EM9 (Table IIB), E20, E30, E31, E15 (Table III), R8, E3 and E25 (Tables III and IV).

Third, the conclusion appears to be of general interest, since it does not seem to be limited to mitochondrial *ori* sequences of yeast. Indeed, the deletions in a 500-bp region of plasmid pT181 (a 4.4-kb multicopy plasmid of *Staphylococcus aureus*) external to the minimal replicon decreased the ability of the plasmid to compete with a coexisting incompatible plasmid, whereas in the homoplasmic state the deletions affected neither copy number nor plasmid stability (Gennaro and Novick, 1986). These deletions appeared to affect the interaction of RepC, a *trans*-acting initiator protein which is rate-limiting for replication, and the plasmid origin of replication.

Concerning the mechanism by which the extra sequences favor replication, it should be pointed out that in different cases, the extra sequences are different in primary structure, size and position relative to the *ori* sequences (Fig. 3). This makes it unlikely that replication is favored by specific 'replication enhancer' sequences, and rather suggests that the effect is due to differences in the DNA or nucleoid (see for instance Rickwood et al., 1981) structure in the different petite genomes under consideration. Such differences can be visualised as differences in DNA bending, DNA superhelicity and/or DNA–protein interactions which may modify the secondary and tertiary structure of the *ori* sequences themselves (see de Zamaroczy et al., 1984) and, consequently, the initiation of DNA replication; obviously effects on the elongation rate of newly synthesized DNA strands are also conceivable.

Since the flanking sequences of *ori* sequences in the petite genomes under consideration are made up of AT spacers and GC clusters, our results provide evidence that intergenic noncoding sequences play a role in the modulation of an essential genome function, such as replication, and substantiate the general idea, supported by other lines of evidence (Bernardi, 1982; 1983; de Zamaroczy and Bernardi, 1986a; 1987; Bernardi and Bernardi, 1986), that noncoding sequences do play a physiological role in genome function.

As a final remark, it should be noted that effects similar to those described here might be responsible for cases in which recombinant plasmids carrying particular inserts, fail to replicate.

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REFERENCES

Aissani, B.: Contribution à l'Etude du Génome Mitochondrial d'un Mutant 'petite' de la Levure *Saccharomyces cerevisiae*. Diplôme d'Etudes Approfondies, Université Paris VI, 1985.

Baldacci, G., Chérif-Zahar, B. and Bernardi, G.: The initiation of DNA replication in the mitochondrial genome of yeast. EMBO J. 3 (1984) 2115–2120.

Bernardi, G.: The petite mutation in yeast. Trends Biochem. Sci. 4 (1979) 1–5.

Bernardi, G.: Evolutionary origin and the biological function of non-coding sequences in the mitochondrial genome of yeast. In Slonimski, P.P., Bort, P. and Attardi, G. (Eds.), Mitochondrial Genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, pp. 269–278.

Bernardi, G.: Genome instability and the selfish DNA issue. Folia Biol. 29 (1983) 82–92.

Bernardi, G. and Bernardi, G.: Compositional constraints and genome evolution. J. Mol. Biol. 24 (1986) 1–11.

Bernardi, G., Faurès, M., Piperno, G. and Slonimski, P.P.: Mitochondrial DNA's from respiratory-sufficient and cytoplasmic respiratory-deficient mutant yeast. J. Mol. Biol. 48 (1970) 23–42.

Bernardi, G., Baldacci, G., Bernardi, G., Faugeron-Fonty, G., Gaillard C., Goursot, R., Huyard, A., Mangin, M., Marotta, R. and de Zamaroczy, M.: The petite mutation: excision sequences, replication origins and suppressivity. In Kroon, A.M. and Saccone, C. (Eds.), The Organization and Expression of the Mitochondrial Genome. Elsevier, Amsterdam, 1980, pp. 21–31.

Blanc, H. and Dujon, B.: Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. Proc. Natl. Acad. Sci. (USA) 77 (1980) 3942–3946.

Blanc, H. and Dujon, B.: Replicator regions of the yeast mitochondrial DNA active in vivo and in yeast transformants, in: Slonimski, P.P., Borst, P. and Attardi, G. (Eds.) Mitochondrial Genes, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, pp. 279–294.

Coen, D., Deutsch, J., Netter, P., Petrochilo E. and Slonimski, P.P.: Mitochondrial genetics, I. Methodology and phenome-

nology. Symposia of the Society for Experimental Biology, Symposium 24, Control of Organelle Development. Cambridge University Press, London, 1970, pp. 449–496.

Colin, Y., Baldacci, G. and Bernardi, G.: A new putative gene in the mitochondrial genome of *Saccharomyces cerevisiae*. Gene 36 (1985) 1–13.

de Zamaroczy, M. and Bernardi, G.: The GC clusters of the mitochondrial genome of yeast and their evolutionary origin. Gene 41 (1986a) 1–22.

de Zamaroczy, M. and Bernardi, G.: The primary structure of the mitochondrial genome of *Saccharomyces cerevisiae* — a review. Gene 47 (1986b) 155–177.

de Zamaroczy, M., Baldacci, G. and Bernardi, G.: Putative origins of replication in the mitochondrial genome of yeast. FEBS Lett. 108 (1979) 429–432.

de Zamaroczy, M., Marotta, R., Faugeron-Fonty, G., Goursot, R., Mangin, M., Baldacci, G. and Bernardi, G.: The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity. Nature 292 (1981) 75–78.

de Zamaroczy, M., Faugeron-Fonty, G. and Bernardi, G.: Excision sequences in the mitochondrial genome of yeast. Gene 21 (1983) 193–202.

de Zamaroczy, M., Faugeron-Fonty, G., Baldacci, G., Goursot, R. and Bernardi, G.: The *ori* sequences of the mitochondrial genome of a wild-type yeast strain: number, location, orientation and structure. Gene 32 (1984) 439–457.

de Zamaroczy, M. and Bernardi, G.: The AT spacers and the *var1* genes from the mitochondrial genomes of *Saccharomyces cerevisiae* and *Torulopsis glabrata*: evolutionary origin and mechanism of formation. Gene 54 (1987) 1–22.

Dujon, B.: Mitochondrial genetics and functions. In Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance. Cold Spring Harbor Laboratory, NY, 1981, pp. 505–635.

Dujon, B. and Blanc, H.: Yeast mitochondria minilysates and their use to screen a collection of hypersuppressive rho$^-$ mutants. In Kroon, A.M. and Saccone, C. (Eds.). The Organization and Expression of the Mitochondrial Genome. Elsevier, Amsterdam, 1980, pp. 33–36.

Ephrussi, B., de Margerie-Hottinguer, H. and Roman, H.: Suppressiveness: a new factor in the genetic determinism of the synthesis of respiratory enzymes in yeast. Proc. Natl. Acad. Sci. USA 41 (1955) 1065–1071.

Fangman, W.L. and Dujon, B.: Yeast mitochondrial genomes consisting of only A-T base replicate and exhibit suppressiveness. Proc. Natl. Acad. Sci USA 81 (1984) 7156–7160.

Faugeron-Fonty, G., Culard, F., Baldacci, G., Goursot, R., Prunell, A. and Bernardi, G.: The mitochondrial genome of wild-type yeast cells, VIII. The spontaneous cytoplasmic 'petite' mutation. J. Mol. Biol. 134 (1979) 493–537.

Faugeron-Fonty, G., Mangin, M., Huyard, A. and Bernardi, G.: The mitochondrial genome of spontaneous *ori*$^r$ petite mutants of yeast have rearranged repeat units organized as inverted tandem dimers. Gene 24 (1983) 61–71.

Fonty, G., Goursot, R., Wilkie, D. and Bernardi, G.: The mitochondrial genome of wild-type yeast cells, VII. Recombination in crosses. J. Mol. Biol. 119 (1978) 213–235.

Gaillard, C. and Bernardi, G.: The nucleotide sequence of the mitochondrial genome of a spontaneous 'petite' mutant of yeast. Mol. Gen. Genet. 174 (1979) 335–337.

Gaillard, C., Strauss, F. and Bernardi, G.: Excision sequences in the mitochondrial genome of yeast. Nature 283 (1980) 218–220.

Gennaro, M.L. and Novick, R.R.: Cmp, a *cis*-acting plasmid locus that increases interaction between replication origin and initiator protein. J. Bacteriol. 168 (1986) 160–166.

Goursot, R., de Zamaroczy, M., Baldacci, G. and Bernardi, G.: Supersuppressive 'petite' mutants of yeast. Curr. Genet. 1 (1980) 173–176.

Lazowska, J.: Electron microscopic analysis of the yeast mitochondrial DNA segment conferring chloramphenicol resistance. Mol. Gen. Genet. 172 (1979) 81–92.

Lazowska, J. and Slonimski, P.P.: Site-specific recombination in 'petite colony' mutants of *Saccharomyces cerevisiae*, I. Electron microscopic analysis of the organization of recombinant DNA resulting from end to end joining of two mitochondrial segments. Mol. Gen. Genet. 156 (1977) 163–175.

Mangin, M., Faugeron-Fonty, G. and Bernardi, G.: The *ori*$^r$ to *ori*$^+$ mutation in spontaneous yeast petites is accompanied by a drastic change in mitochondrial genome replication. Gene 24 (1983) 73–81.

Marotta, R., Colin, Y., Goursot, R. and Bernardi, G.: A region of extreme instability in the mitochondrial genome of yeast. EMBO J. 1 (1982) 529–534.

Maxam, A.M. and Gilbert, W.: Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. 65 (1980) 499–599.

Michaelis, G., Petrochilo, E. and Slonimski, P.P.: Mitochondrial genetics, III. Recombined molecules of mitochondrial DNA obtained from crosses between cytoplasmic petite mutants of *Saccharomyces cerevisiae*. Physical and genetic characterization. Molec. Gen. Genet. 123 (1973) 51–65.

Michaelis, G., Michel, F., Lazowska, J. and Slonimski, P.P.: Recombined molecules of mitochondrial DNA obtained from crosses between cytoplasmic petite mutants of *Saccharomyces cerevisiae*: the stoichiometry of parental DNA repeats within the recombined molecule. Mol. Gen. Genet. 149 (1976a) 125–130.

Michaelis, G., Pratje, E., Dujon, B. and Weill, L.: Recombination of yeast mitochondrial DNA segments conferring resistance either to paromomycin or to chloramphenicol. In Bandlow, W., Schweyen, R.J., Thomas, D.Y., Wolf, K. and Kaudewitz, F. (Eds.) Genetics, Biogenesis and Bioenergetics of Mitochondria. De Gruyter, Berlin, 1976b, pp. 49–56.

Michel, F.: Etudes Expérimentales et Théoriques des Transitions Thermiques de l'ADN et leur Application à l'Etude Génétique de l'ADN Mitochondrial de Levure. Thèse de Doctorat d'Etat ès-Sciences Naturelles, Université Paris VI, 1982.

Ogur, M., St. John, R., Nagai, S.: Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 125 (1957) 928–929.

Rickwood, D., Chambers, J.A.A. and Barat, M.: Isolation and preliminary characterization of DNA-Protein complexes from the mitochondria of *Saccharomyces cerevisiae*. Exp. Cell. Res. 133 (1981) 1–13.

Thomas, D.Y., Wilkie, D.: Recombination of mitochondrial drug-resistance factors in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 30 (1968) 368–372.

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