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A region of extreme instability in the mitochondrial genome of yeast

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About half of the spontaneous petite mutants produced by wild-type *Saccharomyces cerevisiae* strain B (as well as by several other strains) have the same defective mitochondrial genome. Its repeat unit is a segment, 2200 base pairs (bp) long, which derives from an excision between the origins of replication *ori* 2 and *ori* 7 of the wild-type genome, and contains a hybrid *ori* 2 – *ori* 7 sequence. The spontaneous petites carrying this defective *ori*^{h} genome are supersuppressive, i.e., they very rapidly compete out the wild-type genome in crosses. The main reasons for the exceptional frequency of *ori*^{h} petites are an extremely high excision frequency, due to the extended homology between the two tandemly oriented *ori* sequences 265 bp long and the short distance separating them. Such an excision frequency is very strongly increased in petite genomes encompassing the *ori* 2 – *ori* 7 region, because of their higher concentration in these *ori* sequences.
Key words: mitochondrial DNA/petite mutation/yeast

Introduction

The molecular mechanism of the spontaneous petite mutations in yeast has been clarified by a series of investigations (Bernardi *et al.*, 1976, 1978, 1980; Faugeron-Fonty *et al.*, 1979; Gaillard *et al.*, 1980; Baldacci *et al.*, 1980; de Zamaroczy *et al.*, 1981, and in preparation) which have confirmed a model proposed in 1969 (see Bernardi, 1979, for a brief review). This mechanism can be summarized as follows. One of the 25 (or 50) units of mitochondrial genome of a haploid (or diploid) wild-type *Saccharomyces cerevisiae* cell undergoes an excision. The excised segment, carrying as a rule one of the seven origins of replication of the parental wild-type genome, then undergoes a tandem amplification to become the repeat unit of a defective genome unit, which replicates and segregates into the buds of the parental cell to form the mitochondrial genome of spontaneous petite mutants. The extremely high spontaneous mutation rate (~1%/generation in most strains) was thought to be correlated with the fact that excision events occur between pairs of the numerous sequence repeats (Bernardi and Bernardi, 1980; Bernardi, 1982) present in the very abundant non-coding sequences of the genome; these are formed by long AT spacers embedding a number of short GC clusters and represent >50% of the genome.

We have investigated an exceptionally frequent excision event in which two tandemly oriented origins of DNA replication, *ori* 2 and *ori* 7, only separated by 2200 base pairs (bp), act as excision sequences to produce a defective genome, with a hybrid *ori* 2 – *ori* 7 origin of replication. This genome segregates into a particularly frequent class of spontaneous, super-suppressive petite mutants, the *ori*^{h} petites.

Results

The initial observation that led to the present work was made during the course of investigations on the number of the *ori* sequences, the origins of DNA replication, and their localization on the mitochondrial genome of wild-type yeast cells (de Zamaroczy *et al.*, 1979, 1981; Goursot *et al.*, 1980; Bernardi *et al.*, 1980; Blanc and Dujon, 1980; see Figure 1). A probe used in this search was the mitochondrial DNA from petite a-1/1R/Z1 (Faugeron-Fonty *et al.*, 1979), whose repeat unit is 416 bp long, and contains the *ori* 1 sequence (265 bp long), plus 150 bp, essentially formed by A and T (Gaillard and Bernardi, 1979; Bernardi *et al.*, 1980; de Zamaroczy *et al.*, 1981). When this *ori* sequence probe was hybridized on the *Hae*III fragments from the mitochondrial DNA of wild-type strain B, those containing an *ori* sequence were visible in the autoradiogram (Figure 2), as expected from previous work (de Zamaroczy *et al.*, 1981; and in preparation). It should be noted that the mitochondrial genome of strain B lacks *ori* 4 and contains an *ori* 8 sequence, which is partially deleted and located between the 15S and the *oxi* 3 genes (G.Faugeron-Fonty, personal communication). This experiment revealed, in addition, that a sub-stoichiometric fragment, already observed in ethidium-stained gels and called b7 by Prunell *et al.* (1977), also hybridized with the *ori* probe. The yield of this fragment relative to that containing *ori* 2 (which is only 10% larger) was estimated to be 10–20%, on

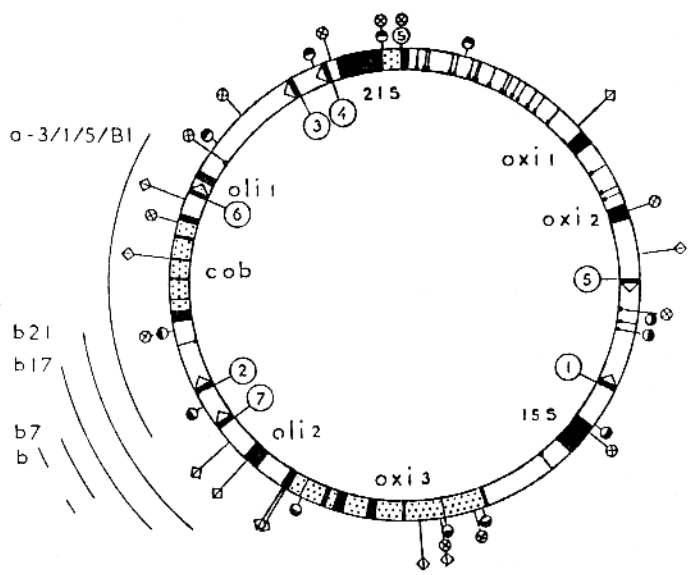

Fig. 1. Physical map of a mitochondrial genome unit of wild-type yeast strain A. The localization and orientation of *ori* sequences is shown (circled figures): triangles have their bases corresponding to cluster C (*Hph*I site of Figure 4) and point towards cluster A (*Ava*II site of Figure 4). This map is very similar to those of strains KL14/4A (Sanders *et al.*, 1977) and B (the latter, however, lacks *ori* 4). Thin lines indicate the petite genomes used in this work. The map shows the localization of mitochondrial genes (dotted areas correspond to intervening sequences; thin radial lines to tRNA genes). Restriction sites: ⊗ *Hinc*II; ◆ *Eco*RI; ◇ *Hinf*I; ● *Hha*I; S Sal I. (Modified from de Zamaroczy *et al.*, 1981).

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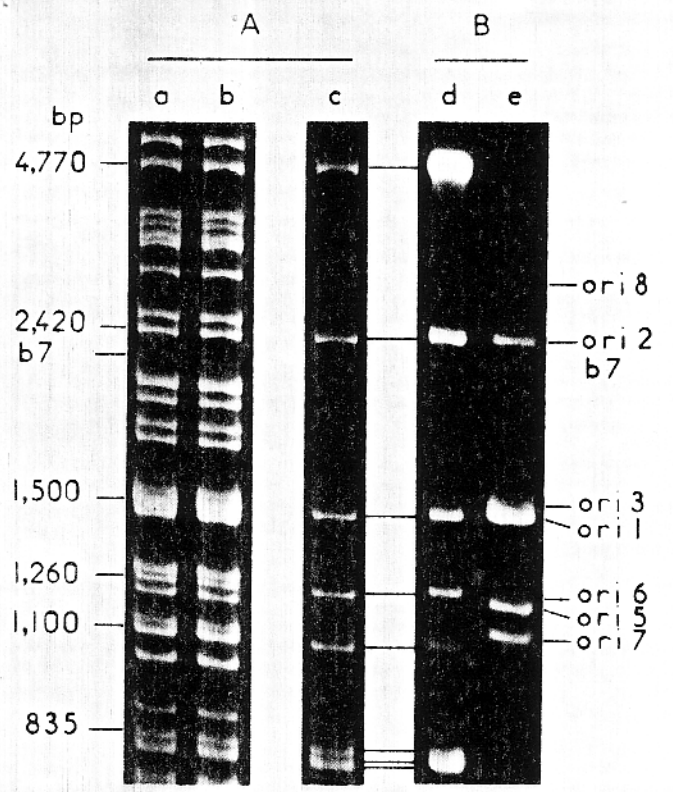

Fig. 2. (A) *Hae*III patterns on 2% agarose of mitochondrial DNAs from wild-type strain B **(lanes a** and **b)** and from petite a-3/1/5/B1 **(lane c**; this petite was obtained from a cross of petite a-3/1/5 with wild-type strain B, but its mitochondrial genome was the result of an excision in the genome of the latter; de Zamaroczy *et al.*, 1981). Note that *ori* sequences do not contain *Hae*III sites; therefore they are always contained in *Hae*III fragments. **(B)** Autoradiograms of DNA fragments from **lanes a** and **b** after transfer and hybridization with ^{32}P-labelled mitochondrial DNAs from petites a-3/1/5/B1 **(lane d)** and a-1/1R/Z1 **(lane e)**. The first probe revealed the *Hae*III fragments from DNA of wild-type strain B, which form the repeat unit of a-3/1/5/B1 and which include the fragments containing *ori* 2 and *ori* 6, (six additional smaller fragments do not appear on the 2% gel). The second probe revealed the wild-type DNA fragments containing previously identified *ori* sequences (de Zamaroczy *et al.*, 1981, and in preparation; note the absence of *ori* 4 in the genome of wild-type strain B). In addition, both probes hybridize on sub-stoichiometric fragment b7.

the basis of microdensitometry of the photographs of ethidium-stained gels as well as of autoradiograms. These findings raised the problem of the origin of the sub-stoichiometric fragment b7, which seems to contain an extra *ori* sequence.

As a step towards the solution of this problem, the presence of fragment b7 was looked for in seven additional wild-type strains and in a large number of spontaneous petites having mitochondrial genomes arising from different regions of the wild-type genome and covering most of it. Fragment b7 was found in six of the seven wild-type strains tested, *S. carlsbergensis*, KL-14/4A, A and AP3, (Figure 3), SM1313-1C and 777-3A (not shown), and in two petites, b17 and b21 (Figure 3), which were the only ones, among those examined, to carry on their genomes the two origins of replication *ori* 2 and *ori* 7 (Figure 4). All other petites tested, including a-3/1/5/B1 (whose repeat unit carried *ori* 2 and *ori* 6, but not *ori* 7) did not harbor fragment b7 (Figure 3). The stoichiometry of fragment b7 in the wild-type strains relative to the *ori* 2 fragment was found to be essentially the same as in strain B. These results pointed to a very wide-spread, but not general, occurrence of fragment b7 in wild-type strains. Furthermore, they indicated a possible relationship between the occurrence of fragment b7 and the presence of the segment encompassing *ori* 2 and *ori* 7 on the mitochondrial genome of both wild-type cells and petite mutants. The meaning of this relationship was clarified by the following observations.

First, a precise determination of the length of fragment b7 by fragment comigration experiments (not shown) revealed that it was identical to the distance, 2200 bp, between equivalent points of *ori* 2 and *ori* 7, two largely homologous sequences exhibiting the same orientation (Figures 1 and 4). Second, fragment b7 could be produced not only by *Hae*III, but also by *Hha*I; these are the only two enzymes, among those tested, with a single site between *ori* 2 and *ori* 7 (Figure 4). Enzymes with no site in that region, like *Eco*RI, *Bam*HI, *Hin*cII, and *Mbo*I, or having more than one site, like *Hpa*II, *Mbo*II, *Hph*I, and *Ava*II, did not produce fragment b7 (not shown). The DNA of petite a-3/1/5/B1, which lacks *ori* 7 (Figure 4) and does not produce fragment b7 upon *Hae*III digestion (Figure 3g), hybridizes on the latter (Figure 2d), since the right end of its repeat unit overlaps the left half of the *ori* 2 – *ori* 7 region (Figure 4). Third, linear or circular fragments corresponding to b7 were absent from undigested mitochondrial DNA preparations of strain B, where hybridization of the *ori* probe only took place on high mol. wt. DNA (Figure 5).

The above results indicate that fragment b7 is formed by breakage of an oligomeric DNA segment corresponding to the *ori* 2 – *ori* 7 region. They argue against the suggestion (Prunell *et al.*, 1977) that the low yield of fragment b7 is the result of a preferential degradation by a mitochondrial nuclease of the wild-type genome segment from which fragment b7 arose. Two other possibilities were therefore considered. The first was that of a tandem amplification of the *ori* 2 – *ori* 7 region on a minority of parental genome units (these would represent 2 – 4% of all genome units if the b7 oligomer was made up of five repeat units, and less if the oligomer was longer). If such were the case, one would expect to see no b7 fragment in DNAs from the vast majority of sub-clones from wild-type B cells, whereas every sub-clone showed the same sub-stoichiometric amount of b7 (Figure 6). This explanation should, therefore, also be ruled out. The second possibility was that fragment b7 simply corresponds to the repeat unit of the mitochondrial genome of a petite mutant present in the wild-type population. Assuming that such a petite has the same amount of mitochondrial DNA as wild-type cells, this would mean that the parental wild-type cell population contains 0.3 – 0.6% of a particular petite mutant harboring a mitochondrial genome made up of repeat units corresponding to b7. In other words, about half of the 1% spontaneous petites produced by strain B should contain the same defective genome. This expectation was fulfilled by the experimental finding that 16 out of 36 spontaneous petites originating from strain B did indeed harbor a mitochondrial genome formed by the tandem amplification of a DNA segment corresponding to fragment b7. A similar situation must exist in most of the other wild-type strains examined, as judged from the yield of fragment b7 (Figure 3). Furthermore, it was found that petites b21 and b17 produced even larger percentages of secondary petites belonging to the same category. In the case of b21, of 94 sub-clones 87 had the parental genome, two the b7 genome, and five other

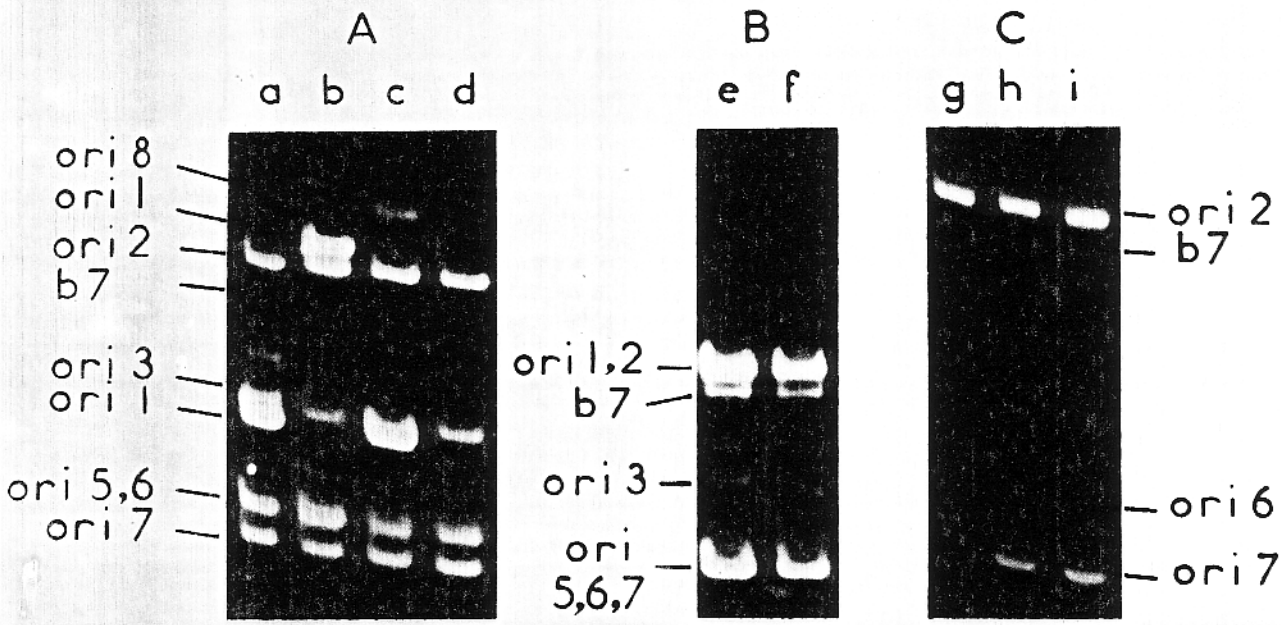

Fig. 3. Autoradiograms of *Hae*III fragments of mitochondrial DNAs from wild-type strains. *S. carlsbergensis* **(lane a)**, KL-14/4A **(lane b)**, B **(lane c)**, A **(lane d)**, AP 3 **(lane e)**, B **(lane f)**, and from petite mutants a-3/1/5/B1 **(lane g)**, b17 **(lane h)**, b21 **(lane i)** after transfer and hybridization with ^{32}P-labelled mitochondrial DNAs from petites a-1/1R/Z1 **(A;C)** and b **(B)**. The strain-dependent differences in the *Hae*III fragments carrying *ori* sequences will be reported in detail elsewhere.

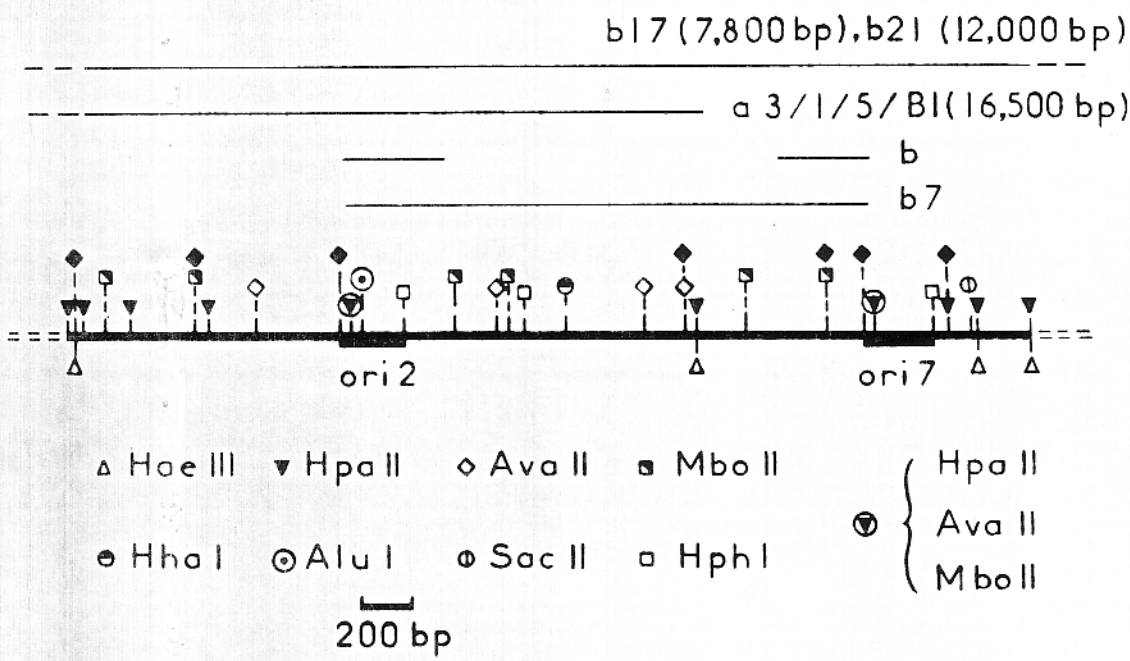

Fig. 4. Restriction map of several petite genomes derived from wild-type strain B in the *ori* 2 – *ori* 7 region. Top lines indicate the mapped regions of repeat units of the petite genomes.

genomes. In the case of b17, of 24 sub-clones, 19 harbored the parental genome, three the b7 genome, and two other genomes. As shown in Figure 5, the undigested mitochondrial DNA of these petites (which are identical with petite b6 of de Zamaroczy *et al.*, 1981) has a high mol. wt. with only a trace of monomeric circles. While complete degradation by *Hae*III or *Hha*I produces, as expected, monomeric linear units (Figure 5), partial degradation produces (M.Mangin, personal communication) the oligomeric ladder diagnostic of the tandem repetitions characteristic of spontaneous petites. Also, as expected, the restriction map of the repeat unit of these petites is identical to that of the *ori* 2 – *ori* 7 region (Figure 4). Obviously, these findings provide unambiguous evidence for the conclusions that fragment b7 corresponds to the repeat unit of defective, amplified mitochondrial genome units present in a class of very frequent petites. A small percentage of such genome units is probably present in the parental wild-type cells, as shown by the presence of fragment b7 in DNA digests from wild-type cells repeatedly sub-cloned in glycerol-containing culture media, in which petites cannot grow.

As far as the actual excision of the defective mitochondrial

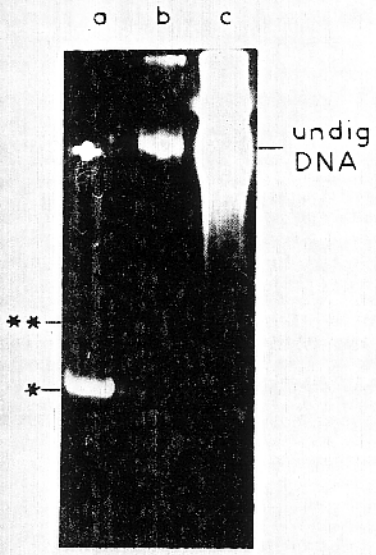

Fig. 5. Autoradiograms of mitochondrial DNAs from petite mutant b7 (digested with *Hae*III, **lane a**; undigested, **lane b**) and from wild-type strain B (undigested, **lane c**) after transfer and hybridization with ^{32}P-labelled mitochondrial DNA from petite a-1/1R/Z1. The linear repeat unit of petite b7 genome is indicated by one asterisk, that of its circular open form by two asterisks. Other oligomers, if present, would be found together with undigested material under the conditions used.

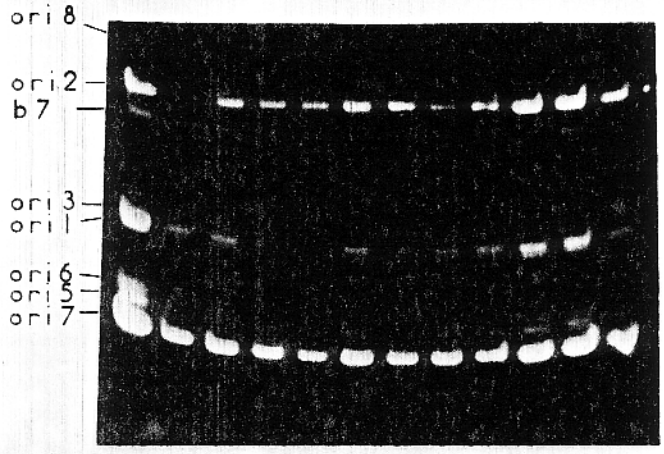

Fig. 6. Autoradiograms of *Hae*III fragments of mitochondrial DNAs from wild-type B (leftmost lane) and clones freshly prepared from it (other lanes) after transfer and hybridization with ^{32}P-labelled mitochondrial DNA from petite b whose repeat unit contains *ori* 2 (de Zamaroczy *et al.*, 1981). The use of this probe explains why the intensities of fragments containing *ori* sequences are slightly different compared with those obtained with DNA from a-1/1R/Z1, which contains *ori* 1 (see Figure 2e). Fifty additional clones were tested and gave the same results (not shown). In all cases, microdensitometric estimates showed the same relative amount of fragment b7. Note the presence of an extra hybridization band, possibly corresponding to another petite genome, in the third lane from the right.

genome is concerned, sequence work has shown that the crossing-over is located between GC clusters A and B (namely between the *Ava*II site and the *Hpa*II, *Ava*II, *Mbo*II site cluster; see Figure 4) of *ori* 2 and *ori* 7 (de Zamaroczy *et al.*, in preparation). The *ori* sequence present on the excised segment is, therefore, a hybrid *ori* 2 – *ori* 7 sequence, derived for the most part from *ori* 2 and containing the *Alu*I site of the latter sequence (Figures 4 and 7). We will call the petite genomes with such hybrid *ori* sequences *ori*^{h}; these appear to be as functional as normal genomes, as shown by the fact that

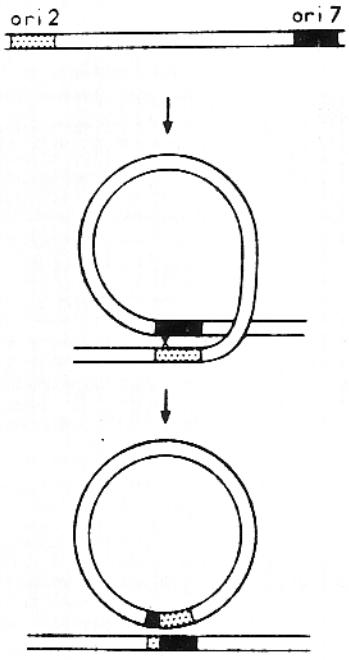

Fig. 7. Scheme of the excision process leading to the formation of the repeat units of petites b7 and b. Excision sequences are located within *ori* 2 and *ori* 7; the result of the excision is a defective unit carrying a hybrid *ori* 2 – *ori* 7 sequence and a large unit lacking the *ori* 2 – *ori* 7 region and carrying a hybrid *ori* 2 – *ori* 7 sequence complementary to that of the defective unit.

ori^{h} petites are super-suppressive (Goursot *et al.*, 1980), i.e., their genome very rapidly competes out the wild-type genome in crosses. Finally, petite b also appears to have been excised between *ori* 2 and *ori* 7; this primary event was, however, followed by a secondary excision involving two subsequent repeat units (see Figure 4).

Discussion

The fact that the mitochondrial genome of ~50% of the spontaneous petite mutants produced by wild-type strain B (and, apparently, by many others) is an *ori*^{h} genome appears to be due to three factors: (a) its very high replicative competitivity, witnessed by the super-suppressive character of the petites harboring it, is associated with the short length of the repeat units of the *ori*^{h} genome and with the presence in them of *ori* sequences that are functionally "normal" in spite of their "hybrid" nature; (b) its stability, which is also associated with the short length of its repeat unit; and (c) the very high excision rate associated with the *ori* 2 and *ori* 7 sequences, which is due to reasons discussed below. Obviously, the first two factors are necessary, but not sufficient, to generate the situation under discussion since other super-suppressive, very stable petites studied in our laboratory (de Zamaroczy *et al.*, 1981) harbor very different genomes. The third factor is clearly the major one and appears to be the rate-limiting factor in the production of spontaneous petite genomes. This is witnessed, in a most striking way, by the effect of increasing the concentration of excision sequences. Petite genomes b21 and b17 have repeat units of 12 000 bp and 7800 bp, respectively; their concentrations of *ori* 2 and *ori* 7 sequences are, therefore, ~six and 10 times higher than in the mitochondrial genome of isonuclear parental strain B (whose unit is 78 000 bp long). Accordingly, *ori* 2 – *ori* 7 petite production is increased from 0.5% in strain B, to 2% in

petite b21, to 12% in strain b17. The latter most probably is the highest spontaneous mutation rate associated with one and the same event ever found in any genetic system.

In turn, the high frequency of excision between *ori* 2 and *ori* 7, which is probably caused by the unequal, internal recombination process depicted in Figure 7, is due to two factors. The first is the extensive homology of *ori* 2 and *ori* 7 (two sequences that fulfil the prerequisite of having the same orientation) compared with other excision sequences; in this case, perfect homology covers 65 bp whereas homology with a few mismatches covers another 203 bp (de Zamaroczy *et al.*, in preparation). Experiments on model systems have indicated that excision frequencies between direct repeats increase with the square of the repeat length (Michel *et al.*, 1981). The second factor is the short distance between the two excision sequences, minimizing further excisions (which can, however, take place as shown in the case of petite b).

It is of interest to note that defective genomes might also be expected to arise by excisions between *ori* 3 and *ori* 4 in genomes containing these two tandem origins of replication, which are separated by the same distance as *ori* 2 and *ori* 7. These were not found, however, possibly owing to the presence of a large GC cluster, β, in *ori* 4, but not in *ori* 3 (de Zamaroczy *et al.*, 1981), interfering with sequence pairing. Other pairs of *ori* sequences are either not tandemly oriented or too distant to generate defective genomes endowed with enough stability and replicative competitivity. These facts justify calling the petite genomes specifically excised between *ori* 2 and *ori* 7 *ori*^{h}. Finally, while the uniform stoichiometry of the defective genome in the wild-type strains discussed so far indicates a comparable nuclear background, some yeast strains differ in this respect. For instance, one of the seven strains tested, strain D, showed no b7 fragment (Prunell *et al.*, 1977). However, clones from two of the four spores of this diploid strain produced normal amounts of fragment b7, whereas clones from the other two spores show no detectable b7 fragment (R.Goursot, unpublished data). This Mendelian segregation is indicative of a nuclear gene involved in the excision process, but further work is needed to clarify this point.

It is obvious that the simultaneous presence of *ori* 2 and *ori* 7 in the mitochondrial genome of wild-type cells is the source of a tremendous instability. The question may, therefore, be asked: what selective advantage do these two origins provide? [see also Bernardi (1982) for a discussion on this point]. Nothing is known at present about the physiological use of different *ori* sequences in the mitochondrial genome of wild-type cells, and the possibility exists that *ori* 2 and *ori* 7 are more important than other *ori* sequences for the replication of the wild-type genome. At least two additional reasons may, however, also contribute to a selective advantage associated with *ori* 2 and *ori* 7. The first is the possible use of these *ori* sequences as promotors or entry sites for the mitochondrial RNA polymerase (Baldacci and Bernardi, in preparation). The second is the presence of a mitochondrial locus indispensable for spore germination in the *ori* 2 – *ori* 7 region (M.Breitenbach, person communication).

As a final comment, it should be stressed that the excision and amplification of defective genome units from wild-type mitochondrial genomes are general phenomena. In the case of obligatory aerobes, namely almost all eukaryotes, such genome units co-exist with wild-type genomes; clearly, this is the only possible situation, since a certain number of wild-type genome units are required in order to ensure respiration, and since segregation of the defective genomes, if it takes place, is conducive to non-viable progeny. The best known examples are those of senescent cultures of *Podospora anserina* (Belcour *et al.*, 1981; Cummings *et al.*, 1980; Kück *et al.*, 1981), "ragged" mutants of *Aspergillus amstelodami* (Lazarus and Küntzel, 1981), *poky* and stopper mutants of *Neurospora crassa* (Manella *et al.*, 1979; Bertrand *et al.*, 1979; de Vries *et al.*, 1981), male-sterile mutants in maize (Weissinger *et al.*, 1982). Excision and amplification do not concern, however, only mitochondrial genomes; suffice it to mention here just a few examples concerning other genomes. Bleached mutants of *Euglena gracilis* have been found containing defective chloroplast genomes in which the ribosomal gene region and the origin of replication are preferentially retained; since the chloroplast genome is as dispensable in *Euglena* as the mitochondrial genome in yeast, these mutants only contain the defective genome (Heizmann *et al.*, 1981). *Drosophila* and mammalian cells are known to contain extrachromosomal circular DNA derived from nuclear DNA by an excision mechanism (Flavell and Ish-Horowicz, 1981; Calabretta *et al.*, 1982) and double minute chromosomes are known to be formed in methotrexate-resistant mouse cells (see Tyler-Smith and Alderson, 1981).

Materials and methods

Yeast strains

The wild-type *S. cerevisiae* strains used in this work were: D-243-2B-R_1, and C982-19d, (Faugeron-Fonty *et al.*, 1979); *S. carlsbergensis* NCY 74S, (Prunell *et al.*, 1977), DM, (Bernardi *et al.*, 1968; we indicate the preceding four strains as A, B, C, and D, respectively), KL 14-4A (Wolf *et al.*, 1973), AP 3 (Hartig *et al.*, 1982), SM 1313-1C (a, ura, his, trp), and 777-3A (α, ade, opl; these last two strains were obtained from W.Bandlow). All spontaneous petite mutants used in this work derived from strain B, with the exception of a-1/1R/Z1, derived from strain A.

Growth media

Cells were normally grown in YEPD (1% yeast extract, 2% bactopeptone, 2% glucose). YEPP (1% yeast extract, 2% bactopeptone, 0.1% glucose, 3% glycerol) was used to discriminate between wild-type and petite colonies. YEPG (1% yeast extract, 2% bactopeptone, 3% glycerol) is a medium on which petite mutants cannot grow. The tetrazolium overlay technique (Ogur *et al.*, 1957) was used for screening petites.

Mitochondrial DNA

Cells were grown in YEPD and harvested in late exponential phase. They were washed with 1 M sorbitol, 50 mM NaP pH 7.5, 0.1% β-mercaptoethanol, and resuspended in the same buffer (1 ml/g of cells); zymolyase (Kirin Brewery, Takasaki, Japan) was added to a final concentration of 0.5 mg/ml and the solution incubated for 30 – 60 min at 37°C. Spheroplasts were collected by centrifugation, resuspended in 0.6 M sorbitol, 10 mM EDTA, 50 mM Tris pH 7.5 (3 – 4 ml/g of starting cells), and vigorously shaken for 2 – 4 min. The lysate was centrifuged twice for 5 min at 3000 r.p.m. to eliminate nuclear and cell debris; the supernatant was centrifuged for 20 min at 15 000 r.p.m. to obtain a mitochondrial pellet, which was resuspended in the same buffer as above and the centrifugation steps repeated twice. Lysis of mitochondria was carried out in 10 mM EDTA, 1% sarkosyl, 50 mM Tris pH 7.5 at room temperature, followed by a phenol extraction and an ethanol precipitation, before running one or two CsCl gradients in the presence of bisbenzimide (Hoechst 33258, Riedel-de Häen AG, Seelze-Hannover; Hudspeth *et al.*, 1980).

A micro-scale method for rapid mitochondrial DNA isolation was also widely used. This was essentially as described by Dujon and Blanc (1980) with minor modifications.

Restriction enzyme digestions, gel electrophoresis, nick-translation of DNA probes, transfer of DNA to nitrocellulose filters, and filter hybridization were performed essentially as described (Faugeron-Fonty *et al.*, 1979).

Determination of stoichiometry

Determination of stoichiometry of fragment b7 relative to other wild-type DNA fragments was done by microdensitometry of photographic negatives taken from ethidium bromide-stained gels, as described (Prunell *et al.*, 1977). In cases where this fragment could be detected only by hybridization (e.g., in

micro-scale DNA preparations), autoradiograms were scanned and the intensity of the sub-stoichiometric fragment was compared with that of the fragment carrying *ori* 2, taking advantage of the observation that their efficiency of hybridization with the probe was the same. It should be stressed that the very close sizes of fragment b7 and of the reference fragment rule out differences in fragment yields due to differences in mol. wt. (Prunell *et al.*, 1977), and that the identical stoichiometries found for the ethidium-stained bands and for autoradiographic bands rule out differences in hybridization yields, such as those seen for fragments carrying different *ori* sequences (see Figure 2).

Acknowledgements

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