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Excision sequences in the mitochondrial genome of yeast

(Petite mutation; deletions; AT spacers; GC clusters; origins of DNA replication)

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SUMMARY

We report an analysis of the sequences used in the excision of the mitochondrial genomes of 22 spontaneous and ten ethidium bromide (EtBr)-induced *Saccharomyces cerevisiae* petite mutants. In all cases, excision sequences were found to be perfect direct repeats, often flanked on one or both sides by regions of patchy homology. Sequences used in the excision of the genomes of spontaneous petites were always located in the AT spacers and GC clusters of intergenic regions of the genome; the GC clusters corresponded to *ori* and *ori*^{s} sequences, namely to canonical and surrogate origins of DNA replication, respectively. In the case of the ethidium bromide-induced petites, excision sequences were found not only in intergenic sequences, but also in the introns and exons of mitochondrial genes.

INTRODUCTION

Previous investigations from our laboratory led to the discovery of long AT stretches in the mitochondrial genome of *S. cerevisiae* (Bernardi et al., 1968; 1970; Bernardi and Timasheff, 1970), and to the suggestion that recombination in these sequences, presumed to be internally repetitive, were responsible for the high frequency of the spontaneous excision of DNA segments from this genome (see Bernardi, 1979, for a brief review. These segments are then tandemly amplified to become the *repeat units* forming the *genome units* of petite mutants; Fig. 1A). Subsequent work (Bernardi et al., 1976; 1978; Faugeron-Fonty et al., 1979) indicated that excision sequences are localized in the noncoding regions that comprise > 50% of the genome, namely the long AT spacers and the short GC clusters embedded in them. Sequence analysis (Gaillard et al., 1980; Baldacci et al., 1980; Bernardi et al., 1980) provided the demonstration that, indeed, direct repeats located in AT spacers or in GC clusters were used in the excision of the mitochondrial genomes of three spontaneous petites.

Here we have extended these investigations by analysing the excision sequences of a total of 22 spontaneous petites. In all cases studied, perfect direct repeats located in the AT spacers or in the

Abbreviations: bp, base pairs; EtBr, ethidium bromide.

GC clusters were found to function as excision sequences. In many cases, these were flanked on one or both sides by sequences with a significant homology. For comparison, we have also examined the excision sequences of ten EtBr-induced petites studied in other laboratories (Bonitz et al., 1980; Nobrega and Tzagoloff, 1980; Thalenfeld and Tzagoloff, 1980; Blanc and Dujon, 1980; Tzagoloff et al., 1980; Coruzzi et al., 1981; Osinga and Tabak, 1982). Direct repeats were used as excision sequences in this case as well; however, they occurred not only in noncoding sequences, but also in introns and exons of mitochondrial genes.

MATERIALS AND METHODS

Yeast strains were described elsewhere (de Zamaroczy et al., 1981; Mangin et al., 1983). Culturing conditions and DNA preparation were also described elsewhere (Faugeron-Fonty et al., 1979). The primary structure of DNA was determined according to Maxam and Gilbert (1977).

RESULTS

The experimental approach used in the present work (see Fig. 1B) involved several steps. The first one was to establish a restriction map of the repeat unit of the petite genome under consideration. This defined the nature of the ends, namely GC clusters containing restriction sites or other sequences (usually AT spacers). The second step was to sequence the ends of the repeat unit. This was done by sequencing the DNA segment covering the junction of two subsequent repeat units, when the repeat unit ends had been identified by restriction mapping; alternatively, the region containing the ends, or the whole repeat unit were sequenced. The third step was to sequence the segments corresponding to the ends of the repeat unit on the longer repeat unit of a second petite genome derived from the same region of the wild-type genome and encompassing the first one. The last step was to compare the two sets of sequences.

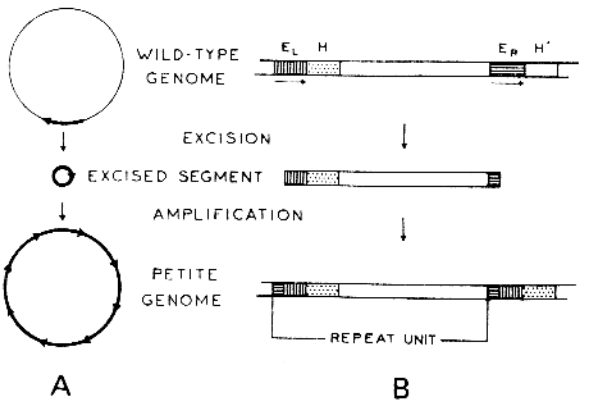

Fig. 1. (A) Scheme depicting the excision-amplification process leading to the formation of the genome of a spontaneous petite mutant. A segment of a unit of a wild-type mitochondrial genome is excised and tandemly amplified into the *repeat units* forming a defective *genome unit*. This then replicates and segregates into the buds to form the genome of a petite mutant; the petite genome can undergo further excisions leading to the formation of secondary petite genomes. (B) Scheme showing the perfect direct repeats forming the left and right (E_L, E_R) excision sequences. These are present on the parental wild-type genome region from which the repeat unit of the petite genome was excised. H, H' indicate sequences flanking the excision sequences and sharing a significant but imperfect homology.

When the approach just outlined was used, two perfect direct repeats, left and right, could be unambiguously defined on a linear representation of the (clockwise oriented) map (see Fig. 1B, boxes E_L and E_R). These are the excision sequences, namely the sequences within which the crossing-over event leading to the excision of the petite genome took place (see DISCUSSION). After crossing-over, one of the resulting hybrid excision sequences remains on the repeat unit of the petite genome; we have arbitrarily placed it at one end of the repeat unit in Fig. 1B, but obviously it can be placed at the other end or be split between the two ends since the precise crossing-over point cannot be determined. Our results also indicate in many cases a patchy homology in the flanking regions, H and H', which are placed at the right of the excision sequences in Fig. 1B, but can also be found at the left or on both sides of the excision sequences.

Two remarks should be made here. The first one is that the repeat units of all the petite genomes studied were tandemly arranged. While this

is the rule for spontaneous petite genomes (Faugeron-Fonty et al., 1979), it is not for EtBr-induced petite genomes, which very often display complex genome rearrangements (Lewin et al., 1978). The genomes of induced petites studied here showed, however, a tandem arrangement of their repeat units and did not differ, therefore, in this respect from spontaneous petite genomes. The second remark is that the overlapping repeat units of different petite genomes derived from the same region of the same parental wild-type genome are identical in sequence, with the exception of extremely rare single-base changes. This means that the approach used is equivalent to comparing the ends of the repeat units of petite genomes with the corresponding segments of the parental wild-type genome (Fig. 1B).

Fig. 2 presents a physical and genetic map of the mitochondrial genome unit of wild-type yeast cells, indicating the three regions, ⓐ, ⓑ and ⓒ, from which the repeat units of the spontaneous petites studied here were excised. These encompassed the canonical *ori* sequences, with the exception of *ori*6. Fig. 2 also shows the location of repeat units of EtBr-induced petites studied by Thalenfeld and Tzagoloff (1980) and by Bonitz et al. (1980); these petite genomes derived from the *oxi*2 and *oxi*3 gene regions, respectively.

Fig. 3 presents the *Hpa*II-*Hae*III restriction map of the ⓐ, ⓑ and ⓒ regions of the mitochondrial genome of wild-type yeast, as well as a simplified nomenclature of the petite strains studied. The repeat unit of one EtBr-induced petite (petite 18), falls in one of these regions; in this case we determined the primary structure of the sequences overlapping the ends of the repeat unit sequenced by Blanc and Dujon (1980).

Fig. 4 displays the left (top line) and right (bottom line) excision sequences, as well as the flanking sequences, of 22 petite genomes, (1–17 from the spontaneous petites and 18–22 from the induced petites), determined on the corresponding regions of overlapping, longer petite repeat units. In four cases, one of the two excision sequences was not established since the corresponding overlapping sequence of the petite genome was not available; in these cases, putative excision sequences were indicated (brackets). Moreover, in the case of petite 1, the junction fragment between

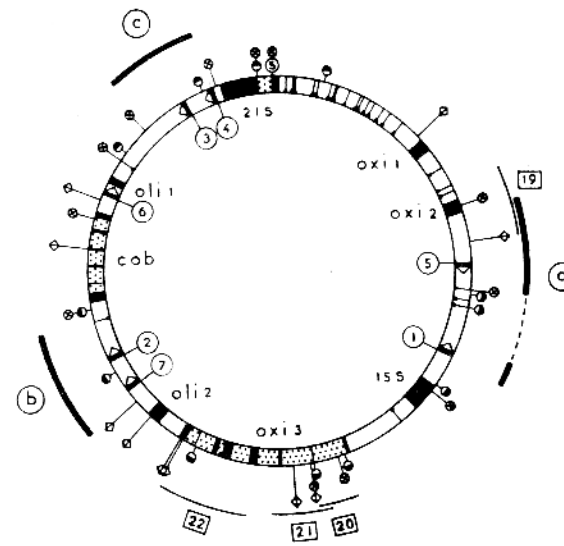

Fig. 2. Map of the mitochondrial genome unit of wild-type strain A. (Strain B, also used in this work, has an almost identical map lacking, however, *ori*4). Some restriction sites are indicated: *Hin*cII (⊗), *Hha*I (◓), *Eco*RI (◇), *Sal*I (ⓢ). Circled numbers indicate the location of *ori* sequences 1-7 (arrowheads point from cluster C to cluster A; see de Zamaroczy et al., 1981). Black and dotted areas correspond to exons and introns of mitochondrial genes, respectively. Thin radial lines indicate tRNA genes. ⓐ, ⓑ, ⓒ indicate the three genome regions mapped in Fig. 3; solid bars correspond to the segments studied and almost completely sequenced in our laboratory; boxed numbers, 19–22, indicate the repeat units of petites sequenced by Thalenfeld and Tzagoloff (1980) and Bonitz et al. (1980) in the *oxi*2 and *oxi*3 regions, respectively; petites 19, 20, 21 and 22 correspond to petite strains DS40, DS6/A400, DS6/A402 and DS6/A407, respectively.

subsequent repeat units was not sequenced. The locations of the regions of excision were established by restriction mapping and hybridization experiments, and sequences corresponding to these presumed excision sites were determined on the left side by Bonitz and Tzagoloff (1980), on the right side by us, and found to be almost identical; differences may be attributed to sequencing errors and/or genetic differences between the strains used.

Additional excision sequences from the genomes of five spontaneous and five induced petites are not listed in Fig. 4, either because sequences coincided with listed ones or because information was incomplete (see below).

In all 32 cases studied excision sequences are perfect direct repeats. With the exception of rare

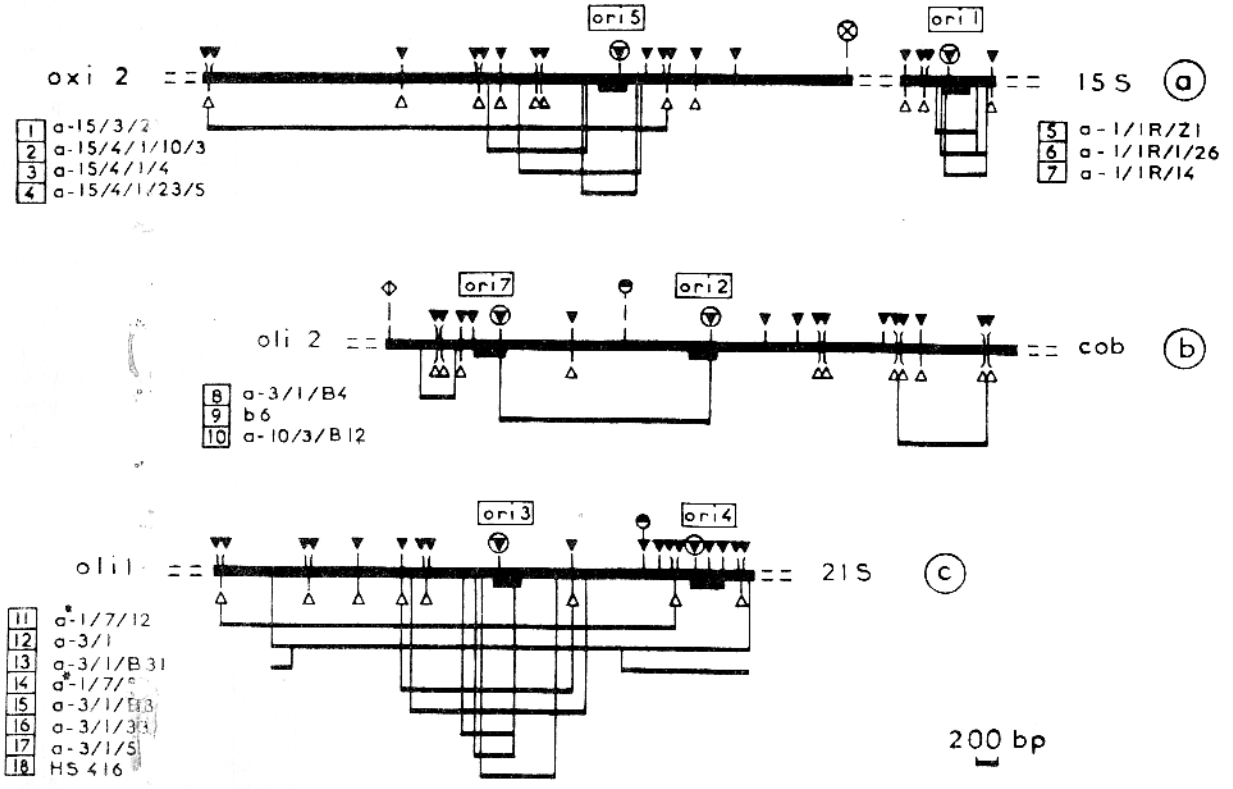

Fig. 3. *Hpa*II (▼) and *Hae*III (△) restriction maps of regions ⓐ, ⓑ, and ⓒ of the mitochondrial wild-type genome. These are the regions where excision of petite repeat units studied here took place; the left to right direction on these linear maps corresponds to the clockwise direction on the circular map of the genome unit. The *Hpa*II, *Ava*II, *Mbo*II site cluster (⏷) of GC cluster B of *ori* sequences are also indicated. More detailed maps can be found in de Zamaroczy et al. (1981). Regions ⓐ and ⓒ are from the genome of strain A, region ⓑ from strain B. Horizontal bars correspond to the repeat units of petites; a simplified nomenclature (1–18) of the petites is given. Petite 13 is a special case in which excision involved sequences present on subsequent repeat units of a parental petite genome: this can only occur in the excision of secondary petites genomes and was already found in another case, that of petite b (Marotta et al., 1982).

cases of induced petites, mentioned at the end of this section, excision sequences belonged in either AT spacers of GC clusters. In the first case, they were 5–26 nucleotides long and were almost always flanked by regions of patchy homology. This was the rule in the case of the shorter excision sequences. In the second case, they were 45–65 nucleotides long and corresponded to GC clusters A and B of *ori*2 and *ori*7 (de Zamaroczy et al.,1981), two canonical origins of replication (petite 9), to *ori*^{s} sequences (Goursot et al., 1982), the surrogate origins of replication (petites 1, 10, 11), or to sequences of the *ori*^{s} family (Goursot et al., 1982 and work in preparation), namely to sequences related to the *ori*^{s} sequences (petites 14 and 15).

The ten pairs of excision sequences not listed in Fig. 4 also corresponded to *ori*^{s} sequences, or to sequences of the *ori*^{s} family. Excisions in *ori*^{s} sequences occurred for seven of these petites. One of them, a-15/4/1/1, was a petite studied in detail elsewhere (Mangin et al., 1983), having a repeat unit 1560 nucleotides long and whose excision sequences have been determined. Its right excision sequence coincided with the right excision sequence of petite 1, which is an *ori*^{s} sequence (Fig. 4), but its left excision sequence corresponded to an *ori*^{s} sequence mapping at a different location from that of petite 1. The repeat units of two other petites encompassing *ori*1, b7/1 (1400 nucleotides long and almost completely sequenced; Le Van Kim C., personal communication), and a-1/1R/1 (Gaillard et al., 1980) were excised between two pairs of *ori*^{s} sequences; in

each case, the primary structure of one of the two *ori*^{s} sequences was determined whereas the other was just mapped with restriction enzymes. Excision sequences of two more spontaneous petites, b-28/1 and a-10/3/2/B11 (Goursot et al., 1982), coincided with complex restriction site clusters, diagnostic of *ori*^{s} sequences. Finally, we have observed that the same *ori*^{s} sequence acts as the right excision sequence of DS-400/M4 and the left excision sequence of DS-400/M11; the other two excision sequences of these induced petites (Nobrega and Tzagoloff, 1980) corresponded to other *ori*^{s} sequences, as shown by restriction mapping and partial sequencing. Excisions in sequences of the *ori*^{s} family occurred for three additional induced petites, DS-401 (Tzagoloff et al., 1980), DS-302 (Coruzzi et al., 1981), and LH26D7-10 (Osinga and Tabak, 1982; Martin et al., 1982), as shown by restriction mapping and partial sequencing.

While the excision sequences of all 22 spontaneous petite genomes studied here and of five induced petite genomes (petites 18, DS-401, DS-302, 19 and LH26D7-10) were located in AT spacers or GC clusters of intergenic sequences, those of the other induced petites were not. Two of them (DS-400/M4 and DS-400/M11) had their common excision sequence in the closed reading frame of *cob* intron 2 and the other ones in the intergenic regions flanking the *cob* gene. Petites 20 and 21 had excision sequences located in the open reading frames of *oxi*3 introns (1 and 2 in the first case, 1 and 3 in the second one). Finally, petite 22 had excision sequences located in exons 4 and 8 of *oxi*3.

DISCUSSION

These investigations provide a definitive confirmation of the hypothesis advanced many years ago (see Bernardi, 1979) that the excision process leading to the defective genome of spontaneous petites involves repeated sequences located in intergenic regions and that the abundance of such sequences in the mitochondrial genome of wild-type yeast cells (Bernardi and Bernardi, 1980; Bernardi, 1982) accounts for the extremely high frequency of the spontaneous petite mutation. They also show that the use of direct repeats located in AT spacers and GC clusters, first observed in three spontaneous petite genomes (Gaillard et al., 1980; Baldacci et al., 1980; Bernardi et al., 1980), is general in the excision process. It is pertinent to emphasize that the spontaneous petites studied here were not selected in any way and represent the totality of those whose excision sequences were analyzed. In addition, the present results provide novel information on the primary structure of excision sequences and lead to several new conclusions.

The first point that can be considered on the basis of the data of Fig. 4 concerns the extent of nucleotide homology in the excision sequences, and the stability of the heteroduplex formed by them during the excision process. In order to judge the minimal requirements to be fulfilled for excision to take place, it is useful to first examine the excision sequences formed by AT base pairs that are not flanked by homologous regions. As seen in Fig. 4, the shortest excision sequences are those of petites 6 (11 nucleotides) and 21 (12 nucleotides). Thus, sequences of 11–12 AT base pairs are sufficient to allow the formation of a stable heteroduplex leading to excision.

All shorter excision sequences (such as those of petites 4, 13, 5) are flanked by a considerable nucleotide homology, presumably contributing to the stabilization of the heteroduplex. If so, while excision sequences may lead by themselves to the formation of the heteroduplex, alternatively they may be part of a more complex pairing region involving flanking sequences. Under the latter circumstances, the excision sequences stand out as those exhibiting the highest stability within the pairing region. This can be seen by looking at the length of excision sequences vs. other direct repeats present in the flanking sequences, if they are all only made of AT base pairs, or the length and the base composition if the sequences also contain GC base pairs. For example, in petite 8 the excision sequences are 11 nucleotides long, whereas two other direct repeats in the flanking sequences are only nine nucleotides long; similarly, in petite 13 excision sequences are eight nucleotides long, whereas two other direct repeats in the flanking sequences are seven nucleotides long. On the other hand, in petite 15, excision sequences are 20

Fig. 4. Excision sequences of the mitochondrial genomes of petites. Left (top line) and right (bottom line) sequences used in the excision of petite genomes 1 to 22 (see Figs. 2 and 3) are separated by a horizontal line; sequences are written 5′ to 3′ on the nontranscribed strand. Flanking sequences are in capital letters whenever they share homology; they correspond to the H and H′ sequences of Fig. 1; vertical dashes indicate mismatches, horizontal dashes deletions. In four cases, one of the two excision sequences

nucleotides long and so are direct repeats in the flanking sequences, but excision sequences are 70% in GC, whereas the direct repeats in the flanking sequences are only 60% in GC. In another case, petite 9, excision sequences correspond to the regions comprising GC clusters A and B of *ori*2 and *ori*7, which are 32% in GC and 65 nucleotides long, and not to GC cluster C, which is 64% in GC, but only 22 nucleotides long; this result is in agreement with the predominant stabilizing effect of homology length, as discussed below.

If one considers the frequency of excision sequences in AT spacers and GC clusters, one finds that both types of sequences are equally represented in the petite genomes studied. This indicates that GC clusters are favored over AT spacers as excision sequences, since the former ones correspond to a much smaller number of potential excision sequences compared to the latter ones.

It is very likely that the most stable (longest and/or richest in GC) excision sequences are associated with the highest rates of excision; this is in agreement with experiments on model systems, indicating that excision rates between direct repeats increase with the square of the sequence length (Michel et al., 1981). In the case of petite 9, the excision sequences are located in *ori* sequences. Not only are these excision sequences the longest ones (65 nucleotides), but they are also flanked by two largely homologous AT regions, the first one preceding cluster A (68 nucleotides), and the second one following cluster B and encompassing cluster C (203 nucleotides). In this case, the excision rate is extremely high (Marotta et al., 1982) and the resultant petite 9 accounts for 50% of all spontaneous petites produced by the parental wild-type cells. In general, however, it is difficult to assess excision rates, since the production of petite mutants also depends upon two other parameters, namely the replication rate of the newly formed defective genome relative to the parental one and upon its stability, namely its resistance to secondary excisions (Marotta et al., 1982). These factors favor the production of petites formed by short repeat units containing canonical *ori* sequences. The repeat units mapped in Fig. 3 are short, ranging in size from 341 to 4450 bp (petites 17 and 12, respectively) and contain in most cases one canonical *ori* sequence.

The fact that all spontaneous petite genomes investigated here are excised in AT spacers and GC clusters of intergenic sequences can be attributed in part to the large extension of these regions in the mitochondrial genome. However, a selection at the level of replication is also obvious, since the vast majority of spontaneous petite genomes contain canonical *ori* sequences located in intergenic regions. The minority *ori*$^{\circ}$ petites (Goursot et al., 1982), on the other hand, contain *ori*^{s} sequences that are also located in intergenic sequences. It is conceivable therefore, that provided excision sequences of comparable stability are used, the process may take place at the same rate elsewhere, for instance in the closed reading frames of introns, which possess the same sequence features as the intergenic regions (Bernardi, 1982). Selection against the defective genomes least proficient in replication, however, eliminates these mutants and leads to the large excess of *ori*$^{+}$ petite genomes observed. Obviously, one can counteract this trend by genetically selecting petites with a poor replication efficiency and/or resulting from rare events, on the basis of the presence of a given gene or gene segment. Such is the case for several EtBr induced petites studied here, like those excised in introns and exons. The results presented here suggest, however, that the basic mechanisms for the formation of petite genomes may be the same in both spontaneous and induced petites. The main difference between spontaneous and induced mutation seems to be just the highly increased rate of excision, which leads to a disin-

was not determined since the corresponding overlapping sequence of the petite genome was not available; in these cases, putative excision sequences were indicated (in brackets); these were arbitrarily represented as 10 nucleotides long, except in the case of petite 11, where it was assumed, as based on the restriction map of Fig. 3, that the missing excision sequence was another *ori*^{s} sequence. In the case of petite 2, the putative left excision sequence is immediately followed by a sequence that is identical to it, except for one nucleotide. The excision sequences of petite 14 corrects some mistakes in previously published data (Baldacci et al.,1980). Partial data for the excision sequences of some petites were obtained by Marguerite Mangin (petite 4), René Goursot (petite 6), Yves Colin (petite 15), Renzo Marotta (petites 16, 17) or are based on published results (Blanc and Dujon, 1980) (petite 18). The excision sequences of petites 5, 7, 8 and 10 are from previously published results (Gaillard et al., 1980; Bernardi et al., 1980; Goursot et al., 1982).

tegration of the parental wild-type genome. Another difference (not found, however, in the induced petite genomes under consideration) is the frequent occurrence of inversions, in which the recombination process (see below) affects not direct, but inverted repeats. A case belonging to this category has been studied in detail and will be reported elsewhere (Mangin et al., 1983).

Two possibilities should be considered for the mechanism of excision: (*i*) unequal crossing-over events within a genome unit; and (*ii*) "slipped mispairing" during DNA synthesis (Farabaugh et al., 1978; Efstratiadis et al., 1980). The latter was originally proposed for the generation of small frameshift mutations (Streisinger et al., 1966).Two arguments, however, favor the former mechanism in the present case. First of all, recombination is a very active process in the mitochondrial genomes of wild-type yeast cells (Fonty et al.,1978; more recent unpublished data have shown that all the progeny of crosses of *S. cerevisiae* and *S. carlsbergensis* are of the recombinant type), and seems to preferentially involve the same sequences used in excision. The excision of petite genomes from wild-type genomes would thus be a special case of recombination, in which the event occurs within the same genome unit and leads to the deletion of a genome segment depicted in Fig. 5. Second, the repeat units of spontaneous petites are very often too large to allow single-stranded intermediates formed during DNA replication to undergo "slipped mispairing". Proof of an unequal crossing-over process could be obtained by demonstrating the formation of a second genome containing the sequences absent in the petite genome. Such genomes may be difficult to detect due to their being counterselected relative to intact genomes, or to disintegration through further excisions. Wild-type genomes, like that of strain B, lacking *ori*4 and its flanking sequences (Faugeron-Fonty, G., unpublished results), however, may be representative of this class of genomes.

It is well known that spontaneous deletions in prokaryotic genomes also take place between short direct repeats (Farabaugh et al., 1978; Brake et al., 1978; Studier et al., 1979; Ghosal and Saedler, 1979; Ross et al., 1979; Post et al., 1980; Wu et al., 1980; Pribnow et al., 1981; Albertini et al., 1982). The main differences between our results for the mitochondrial genome of yeast and those obtained in prokaryotic genomes are the longer lengths of the repeats and the much higher mutation rate. For example, the production of petite 9 from wild-type strain B is about 0.25% per generation, that of petite b7 (a secondary petite excised at the same loci as petite 9) from petite b17 is about 12% per generation (Marotta et al., 1982), whereas deletions in the *lacI* system of *Escherichia coli* never have frequencies higher than 10^{-5} per generation.

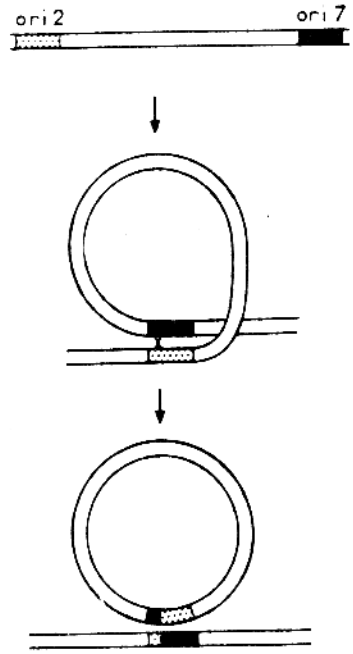

Fig. 5. Scheme of the excision process leading to the formation of the repeat unit of petite 9. Excision sequences are located within *ori*2 and *ori*7, two tandemly oriented *ori* sequences separated by 2200 bp. As a result of a crossing-over process, two defective units, a small one and a large one, carrying complementary hybrid *ori*2-*ori*7 sequenced are formed (Marotta et al., 1982).

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