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Lessons from a small, dispensable genome: The mitochondrial genome of yeast

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Abstract

This article reviews the investigations on the mitochondrial genomes of yeast carried out in the author's laboratory during a quarter of a century (to be precise between 1966 and 1992). Our studies dealt with the structural basis for the cytoplasmic petite mutation, the replication, the transcription and the recombination of the mitochondrial genome, a genome which is dispensable and which comprises abundant non-coding sequences. This work led to some general conclusions on the nuclear genome of eukaryotes. Some recent results in apparent contradiction with our conclusions on *ori* sequences will also be briefly discussed.

Keywords: *Saccharomyces cerevisiae*; Cytoplasmic petite mutation; Replication origins; Non-coding sequences

1. Introduction

When I was asked to present a paper on the mitochondrial genome of yeast at the Meeting "Cross-talk between nucleus and organelles" (which was held on 15–18 September 2004 at the Stazione Zoologica of Naples), understandably I had some hesitations. Indeed, to prepare a short review of investigations that were finished more than 10 years before raised a number of obvious questions. Eventually, I decided to accept the invitation, not only because revisiting our results was a challenge and because it was interesting to see how our conclusions had stood the test of time, but also for a more general reason, namely that results on mitochondrial and/or chloroplast genomes are not widely known in comparative genomics. And yet what we learned from investigations on the mitochondrial genome of yeast (to mention the most thoroughly studied genome) is very relevant as far as structural, functional and evolutionary genomics is concerned. In this review, I will outline not only the major results that we obtained, but also the conclusions of general interest that came out from our investigations. A more detailed account, from which the present one is derived, was published in a recent book (Bernardi, 2004).

2. The mitochondrial genome of yeast and the petite mutation

2.1. The "petite colonie" mutation

The mitochondrial genome of yeast is of special interest for two major reasons: (i) because, in contrast to its very compact counterpart in animal cells, it comprises abundant non-coding sequences (Bernardi et al., 1970); this situation is not unique to *Saccharomyces cerevisiae*, since it is shared by other fungi and by *Euglena gracilis* (Fonty et al., 1975), nor to the mitochondrial genome since the chloroplast genome also shows it (Schmitt et al., 1981); in the case of *S. cerevisiae*, mitochondrial genome units are about five times larger than those of animal mitochondrial genomes; (ii) because it is dispensable, since *S. cerevisiae* can survive on fermentable carbon sources; mutants, showing a non-Mendelian inheritance, the cytoplasmic "petite colonie"

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mutants of Ephrussi (1949, 1953), having undergone massive deletions in their mitochondrial genome, or having lost it altogether, can therefore survive (dispensability also applies to the chloroplast genome of *E. gracilis*; see Heizmann et al., 1981).

In other words, although very small, the mitochondrial genome of yeast presents features that are common to those of the nuclear genome of eukaryotes. One can therefore study, for instance, the organization, the evolutionary origin and the function of non-coding sequences. Hence, our interest. Moreover, since the genome is dispensable, one can investigate genome changes which are normally incompatible with cell life.

2.2. The AT spacers and the deletion hypothesis

The mitochondrial DNA from wild-type yeast cells was found (Bernardi et al., 1970) to be extremely heterogeneous in base composition, about half of it melting at a very low temperature and being almost exclusively formed by long stretches of short alternating AT:AT and non-alternating A:T sequences (the AT spacers), and the rest melting over an extremely broad temperature range. In contrast, DNAs from spontaneous suppressive petite mutants (see Section 3.2) were shown to have lower GC levels (Bernardi et al., 1968; Mehrotra and Mahler, 1968), to lack a number of DNA stretches that melt at high temperature, and to renature very rapidly (Bernardi et al., 1970).

It should be stressed that we did not use the classical genetic approach to study the petite mutation, and confined ourselves to spontaneous, versus ethidium–bromide-induced mutants. These were wise decisions because in eukaryotic genetic systems, where so much of the DNA is non-coding, there is a real need for a molecular approach; indeed, approaches based on classical genetics, or on the study of gene products, suffer from serious intrinsic limitations and are unable to provide an overall picture of the genome. Using spontaneous mutants also was a good strategic choice since ethidium bromide causes a massive degradation of mitochondrial DNA as well as sequence rearrangements.

In 1969, I interpreted our results as indicating that the petite mutants had defective mitochondrial genomes, in which large segments of the parental wild-type genomes were deleted (see Faugeron-Fonty et al., 1979). I also suggested that such deletions arose by a mechanism (Campbell, 1969), involving illegitimate, unequal recombination events in the "AT spacers", which I supposed to contain sequence repetitions because of their extreme base composition. It was obvious that the loss of any known mitochondrial gene products (ribosomal RNAs, tRNAs and the sub-units of enzymes involved in respiration and oxidative phosphorylation) would have a pleiotropic effect and lead to a loss of respiratory functions. Incidentally, this could also happen as a consequence of mutations in nuclear genes that encode some sub-units of mitochondrial enzymes. These "nuclear petite mutants", that are characterized by a Mendelian inheritance, will, however, not be dealt with here.

Further work (Bernardi et al., 1972; Prunell and Bernardi, 1977) showed that the AT spacers formed about 50% of the wild-type mitochondrial genome, had a GC content lower than 5%, were likely to be repetitive in nucleotide sequences and to be endowed with sequence homology over stretches long enough to allow illegitimate site-specific recombination. Incidentally, such sequence homology was unambiguously shown in one of the first papers in the new field of computational genomics (Bernardi and Bernardi, 1980). In 1974, direct evidence was provided for both a deletion mechanism (Bernardi et al., 1975) and an accompanying amplification of the excised genome segment (Locker et al., 1974; Bernardi et al., 1975). Indeed, only a small number of the restriction fragments of wild-type mitochondrial DNA were present in petite genomes (in multiple copies per genome unit). These findings disposed of a number of strange ad hoc hypotheses put forward to explain the petite mutation and led to the scheme shown in Fig. 1 in which the excised segment from the wild-type genome (depicted as circular) becomes the repeat unit of the petite genome. This may, in turn, undergo further deletions leading to secondary petite genomes, characterized by shorter repeat units. Incidentally, the analysis of restriction patterns of mitochondrial DNA from several strains of wild-type cells provided the first unequivocal estimate of the size of the mitochondrial genome unit, about 50×10^6 Da or about 75 kb (Bernardi et al., 1975), an almost five-fold larger size than that of the mitochondrial

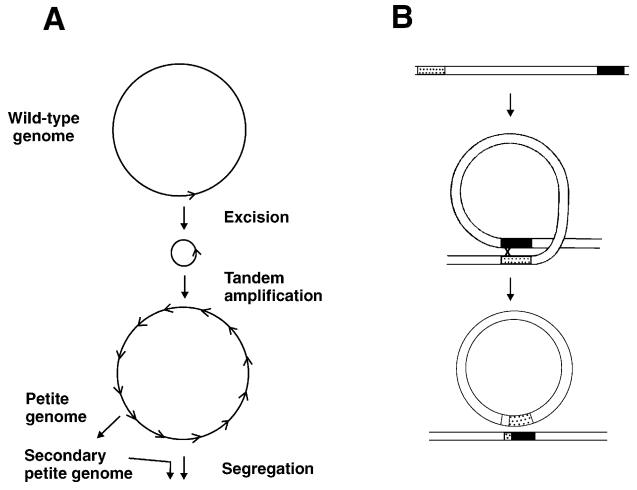

Fig. 1. (A) A scheme depicting the excision-amplification process leading to the formation of the genome of a spontaneous petite mutant. A segment of a wild-type mitochondrial genome unit is excised and tandemly amplified into a defective genome unit. This then replicates and segregates into the buds to form the genome of a petite mutant; the petite genome can undergo further excisions leading to the formation of secondary petite genomes. (B) A scheme of the excision process leading to the formation of a petite genome unit. Dotted and black areas represent two direct sequence repeats. Excision is followed by a tandem amplification (see panel A) (from Bernardi, 1983).

genome units of animals, about 17 kb. The strain-related size variation of restriction fragments provided in fact the first example of what was later called “restriction fragment length polymorphism or RFLP” (Botstein et al., 1980).

2.3. The GC clusters and the excision sites

Another important advance in our knowledge of the organization of the mitochondrial genome was the discovery of short segments of mitochondrial DNA extremely rich in GC, the “GC clusters” (Bernardi et al., 1976; Prunell et al., 1977; Prunell and Bernardi, 1977). Sequence analyses carried out in several laboratories indicated that the GC clusters were located in the middle of AT spacers and not at the ends of them, as first indicated as a working hypothesis (Prunell and Bernardi, 1977). This location suggests that they may fulfil a stabilizing role for the AT spacers (see also Section 3.5).

The basic idea of the deletion model was that the instability of the mitochondrial genome of yeast was due to the existence in each genome unit of a number of nucleotide sequences having enough homology to allow illegitimate, unequal recombination to take place. In this respect, clearly the GC clusters were at least as good candidates as the AT spacers. Indeed, investigations on a number of spontaneous petite mutants showed that most frequently the ends of the repeat units were formed by GC clusters, whereas they appeared to correspond to AT spacer sequences less frequently (Faugeron-Fonty et al., 1979; Gaillard et al., 1980). Furthermore, it was shown that repeat units were organized in a perfect tandem (head-to-tail) fashion. Interestingly, secondary excision of simpler repeat units from the petite genomes originally derived from the parental wild-type genome appeared to take place at the same sort of sites used in the primary process, the end product being even simpler and more stable petite genomes. The spontaneous, cytoplasmic petite mutation should, therefore, be visualized as a cascade of excisions, which is slowed down, or stopped, only because sequences appropriate for excision are used up in the process, or because the DNA looping needed for recombination becomes impossible due to the short distance between potential recombination sites.

2.4. Excision and recombination

The sequences used in the excision process were investigated in a number of spontaneous petite genomes (Marotta et al., 1982; de Zamaroczy et al., 1983). In all cases, excision sequences were perfect direct repeats located in the AT spacers or in the GC clusters, as predicted (see Bernardi, 1979). These results also indicated that the excision mechanism involved unequal crossing-over events within a genome unit and that this process was just a special case of the very active recombination processes taking place in the mitochondrial genomes of wild-type yeast cells (Fonty et al., 1978).

Indeed, when *S. cerevisiae* wild-type strains were crossed, recombinant mitochondrial genomes were present in 75% of the diploid clones. Such recombinant genomes had unit sizes different from, yet within 5% of, the parental ones and showed restriction fragment patterns of parental types, two strong indications that both the gene complement and the gene order were very largely preserved in the progeny. Indeed, such fragment patterns were characterized by (i) fragments originating from the DNAs of both parents; and (ii) new fragments, i.e. fragments absent in either parent. The new fragments appeared to arise from unequal crossing-over events occurring in the corresponding spacers of parental genetic units. These results provided the first evidence for physical recombination of mitochondrial DNA in crosses of wild-type yeast cells, indicated that recombination was very frequent in crosses and shed some light on mitochondrial segregation. They also provided examples of

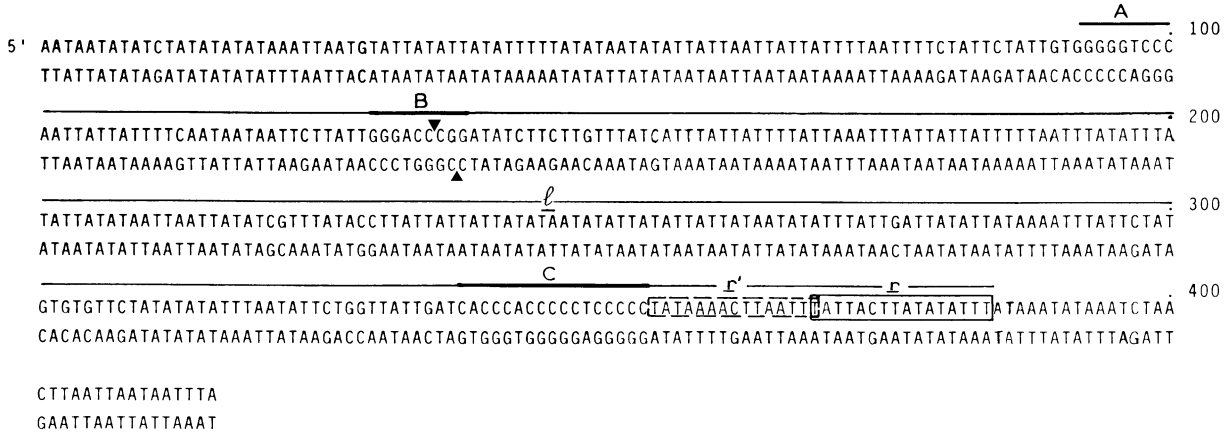

Fig. 2. Primary structures of the repeat unit of the mitochondrial genome from petite a-1/1R/Z1 (Gaillard and Bernardi, 1979). The *ori* sequence is indicated by a continuous line; A, B and C are the three GC clusters. The *r* sequence (box) includes the transcription starting point (Baldacci and Bernardi, 1982); *r*’ (box) indicates an AT stretch, located between cluster C and sequence *r*, only present in *ori* 1 and *ori* 2; *l* is the AT stretch separating GC clusters B and C. Black triangles indicate a *Hpa*II site (from Baldacci et al., 1984).

recombination phenomena in an interspersed system of unique and repetitive nucleotide sequences.

2.5. Genomes without genes

The first determination of the nucleotide sequence of the repeat unit of the mitochondrial genome of the spontaneous petite mutant $a_{1/1R/Z1}$ (see Fig. 2; Gaillard and Bernardi, 1979) and preceding work (Cosson and Tzagoloff, 1979; Macino and Tzagoloff, 1979) provided confirmations of several previous conclusions, namely that AT spacers are made up of short alternating and non-alternating AT sequences and contain direct and inverted repeated sequences and palindromes; that GC clusters are to some extent endowed with homology. These conclusions were further confirmed by one of the earliest investigations in compositional genomics (Bernardi and Bernardi, 1980; Bernardi, 1982a).

The genome of $a_{1/1R/Z1}$ was of interest in two other respects: (i) it did not contain any gene, and was therefore a clear example of the lethality of the petite mutation as far as mitochondrial functions are concerned; incidentally, the surprising finding of genomes without genes can be taken as the best evidence that genetics and genomics are not the same thing; (ii) it replicates; in fact, this is the only function left; since this genome was shown to be made up of a perfect tandem repetition of the basic unit (Faugeron-Fonty et al., 1979), the latter must contain a signal permitting the initiation of replication.

3. The origins of replication

3.1. The canonical and the surrogate origins of replication of petite genomes

The mitochondrial genomes of the vast majority of spontaneous petites are exclusively derived from the tandem amplification of a DNA segment excised from many regions of the parental wild-type genome (Faugeron-Fonty et al., 1979). Therefore, either the wild-type genome contains several origins of replication and at least one of them is present on the excised segment (Prunell and Bernardi, 1977), or sequences other than the origins of replication of the wild-type genome are used as surrogate origins of replication. In fact, both situations have been found to occur, although with very different frequencies.

Considering that the first explanation was the more likely one, when we first sequenced (Gaillard and Bernardi, 1979) the repeat units of two petite genomes excised from the same region of the wild-type genome, we looked for a putative origin of replication in the segment shared by them and found a region characterized by two short GC clusters, A and B, flanking a palindromic AT sequence, *p*, and a short AT segment, *s*; and one long GC cluster, C, separated from B by a long AT segment, *l* (see Fig. 2). We also noticed (de Zamaroczy et al., 1981) that the potential secondary structure of the A–B region, the primary structure of cluster C and the general arrangement of the whole region were remarkably similar to those found in animal mitochondrial origins of replication (Crews et al., 1979; Gillum and Clayton, 1979).

An *ori* sequence like the one just described was found in almost all the mitochondrial genomes of spontaneous petite mutants. Restriction mapping and hybridization of petite genomes on restriction fragments of wild-type genomes (de Zamaroczy et al., 1981; Bernardi, 1982b) provided evidence for the existence of eight *ori* sequences in the mitochondrial genome of wild-type cells. The primary structure of the *ori* sequences showed that they were extremely similar in sequence, particularly in their GC clusters. All these canonical *ori* sequences have been localized and oriented on the physical map of the wild-type genome (Fig. 3).

It should be noted: (i) that some *ori* sequences display one orientation and some the opposite one; (ii) that *ori* 2 and 7 as well as *ori* 3 and 4 are close to each other and tandemly oriented; (iii) that *ori* 4 is absent in a wild-type strain; (iv) that *ori* sequences containing the GC cluster γ were found only once (*ori* 4), or not at all (*ori* 6, *ori* 7) in extensive screenings of spontaneous petite genomes (see Table 1).

Ori^{o} petites, lacking a canonical ori sequence, were also found, although very rarely. An investigation of the mitochondrial genomes of such *ori*^{o} petites (Goursot et al., 1982) has revealed that their repeat units contain, instead of canonical *ori* sequences, one or more *ori*^{s} or *ori*

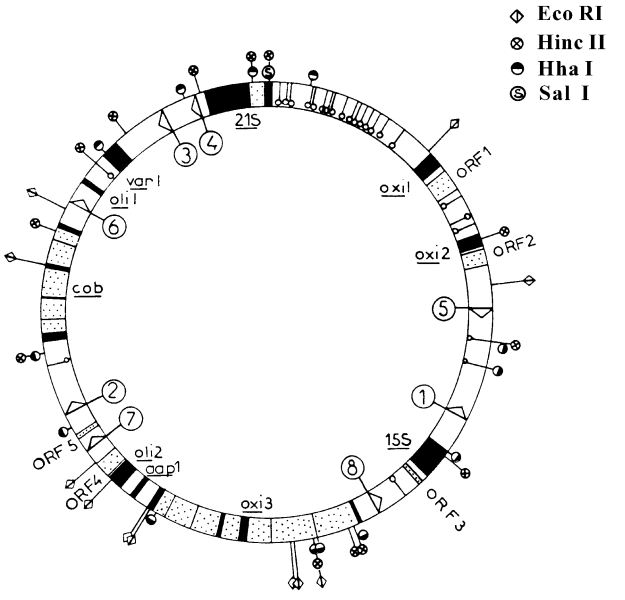

Fig. 3. Physical and genetical map of the mitochondrial genome unit of wild-type yeast (strain A). Some restriction sites are indicated. Circled numbers indicate the location of *ori* sequences 1–8 (arrowheads point in the direction cluster C to cluster A). Black and dotted areas correspond to exons and introns of mitochondrial genes, respectively. Thin radial lines ending in small circles indicate tRNA genes. White areas correspond to long AT spacers embedding short GC clusters (modified from de Zamaroczy et al., 1979).

Table 1
Replication and transcription of petite genomes[a]

Petites	Suppressivity[b]	Transcription	Petites	Suppressivity	Transcription
A Ori^+ petites			B Ori^- petites		
ori 1	>95%	+	*ori* 1 A^-	80%	+
ori 2	>95%	+	*ori* 1 C^-	n.d.	–
ori 3	85%	+	*ori* 3 C^-	<5%	–
ori 4[c]		–	C Ori^o petites		
ori 5	90%	+	a-15/4/1/10/3	~1%	–
ori 6[c]			a-3/1/B4[d]		–
ori 7[c]			D Ori^r petites	<5%	n.d.

[a] Modified from Baldacci and Bernardi (1982).
[b] Values found for petite genomes having repeat units ~900 (*ori* 1, 2) or 1800 (*ori* 3, 5) base pairs long.
[c] *Ori* 4 was only found once, *ori* 6 and 7 were never found alone in the extensive screenings of spontaneous petite genome.
[d] Diploid.

"surrogate" sequences. These ori^s sequences are reminiscent of GC clusters A and B from canonical *ori* sequences.

3.2. The replication of petite genomes and the phenomenon of suppressivity

A functional evidence that *ori* sequences are indeed involved in the replication of the mitochondrial genome came from crosses of spontaneous petites, characterized in their mitochondrial genome and their suppressivity (or suppressiveness), with wild-type cells (de Zamaroczy et al., 1979, 1981; Goursot et al., 1980). In the suppressivity test, petite mutants are crossed with wild-type cells, and the degree of suppressivity, namely the percentage of diploid petites in the progeny (which carry in every case the parental petite genome; Goursot et al., 1980; de Zamaroczy et al., 1981; Rayko et al., 1988) is determined by the replicative efficiency of the petite genome relative to the mitochondrial genome of the wild-type cells used in the cross (Bernardi et al., 1980a,b; Blanc and Dujon, 1980; de Zamaroczy et al., 1981). Crosses of spontaneous, highly suppressive petites having mitochondrial genomes formed by very short repeat units (400–900 base pairs) with wild-type cells produced diploids which harbored only the unaltered mitochondrial genomes of the parental petite. Such petites, first reported by Bernardi et al. (1978), were called supersuppressive petites (de Zamaroczy et al., 1979; Goursot et al., 1980). When petites with different degrees of suppressivity were used in the crosses, the genomes of diploid petite progeny had restriction maps identical to those of the parental haploid petites. Very few exceptions were found and these corresponded to new excision processes affecting one of the parental genomes.

There are two clear correlations between the *ori* sequence of the petite used in the cross and its degree of suppressivity. First, all other properties being comparable (namely, identical genetic background, the intact state of the *ori* sequence and the total amount of mitochondrial DNA per cell), the lower the overall density of *ori* sequences on the genome units, the lower the suppressivity. This can be called the repeat-unit size rule. Second (see Table 1), partial or total deletion of the *ori* sequences, or their rearrangement, affects suppressivity: (i) Ori^- petites, in which the *ori* sequence is partially deleted, show a decreased suppressivity relative to ori^+ petites carrying intact *ori* sequences; the loss of cluster C with its flanking sequence has a much more dramatic effect than the loss of cluster A; (ii) ori^r petites, which show an inverted orientation of two *ori* sequences within the same repeat units (the latter having, in turn, an alternate inverted and tandem orientation) have a very low degree of suppressivity; (iii) ori^0 petites, which lack the *ori* sequence altogether but contain ori^s sequences instead, have low to minimal degrees of suppressivity. These results provide a molecular basis for a replicative advantage being the explanation for suppressivity.

The first direct demonstration that the active *ori* sequences are indeed origins of DNA replication, as previously postulated on the basis of compelling but indirect evidence, was provided by Baldacci et al. (1984). These authors showed that in both *ori* 1 and *ori* 5, nascent DNA chains using as template the strand (the "*r* strand") containing sequence *r* (see Fig. 2) start at the *r* end of cluster C, are elongated towards sequence *l*, and follow an RNA primer starting at sequence *r*. Nascent DNA chains copied on the "non-*r* strand" start within cluster C, are elongated towards sequence *r*, and follow an RNA primer starting in sequence *l* just before cluster C. *Ori* 1 and 5 are, therefore, used as sites for RNA-primed bidirectional replication of mitochondrial DNA. That *ori* sequences also act as transcription initiation sites is indicated by results on petite genomes (Baldacci and Bernardi, 1982; see Table 1).

3.3. The effect of flanking sequences on the efficiency of replication of petite genomes

The mitochondrial genomes of progenies from crosses between cytoplasmic, spontaneous, suppressive, ori^+ petite mutants of *S. cerevisiae* showed that only parental genomes (or, occasionally, genomes derived from them by secondary excisions) were found in the progenies of the diploids investigated; no evidence for intermolecular unequal mitochondrial recombination was detected. One of the parental genomes was always found to predominate over the other

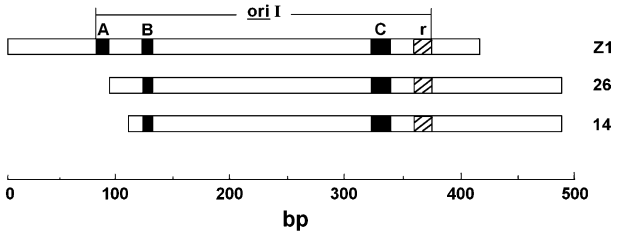

Fig. 4. The repeat units of the mitochondrial genomes of petites Z1, 26 and 14. The *ori* 1 sequence, with its main elements, GC clusters A, B, C (black boxes) and sequence *r* (hatched box), is also represented (from Goursot et al., 1988).

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 (i.e. with a shorter repeat unit size of the latter).

Exceptions to this 'repeat-unit size rule' (see preceding Section 3.2) were found, however, even when the parental mitochondrial genomes carried the same *ori* sequence. This indicates that non-coding, intergenic sequences flanking *ori* sequences also play a role in the modulation of 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 (Rayko et al., 1988).

3.4. The ori⁻ petites 14 and 26

The effect of temperature on the replicative ability of the mitochondrial genomes was investigated on three isonuclear, spontaneous, cytoplasmic petite mutants of *S. cerevisiae*, al/1R/14, al/1R/1/26 and al/1R/Z1, which here will be referred to as petites 14, 26 and Zl, respectively (Goursot et al., 1988). The primary structure of the mitochondrial genomes of these petites had been previously determined (Gaillard et al., 1980; de Zamaroczy et al., 1981, 1983, 1984). Each one of these genomes arose from the excision of a DNA segment from the mitochondrial genome of yet another petite, a1/1R/1. While the repeat unit of the parental petite genome is 884 bp long, the repeat units of petites Z1, 14 and 26 are 416, 392 and 398 bp long, respectively. Moreover, while each repeat unit of the mitochondrial genomes of petites al/1R/1 and Z1 (see Fig. 2) contains a complete *ori* sequence, those of petites 26 and 14 contain *ori* sequences that had lost 11 and 27 bp, respectively, in the excision process. In the case of petite 26, the deletion comprises GC cluster A; in the case of petite 14, it comprises also part of the neighbouring sequence *p* (see Fig. 4). In the complete *ori* sequences of *ori*⁺ petites al/1R/1 and Z1, a secondary structure, the 'A–B fold', is possible (see Fig. 5). This is a stem-and-loop structure, comprising 11 AT bp and 6 GC bp, which is formed by GC clusters A and B and the sequence in between. In contrast, the formation of this structure is impossible in the *ori*⁻ genomes of petites 14 and 26, which can form, however, 'replacement folds' made of pure AT, and endowed, therefore, with a lower thermodynamical stability (especially in the case of petite 14) compared to the A–B fold (Fig. 5).

The *ori*⁻ mitochondrial genome of petites 14 and 26, which lack a normal feature of the *ori* sequence, the A–B fold (a feature which is perfectly conserved in all *ori* sequences; de Zamaroczy et al., 1981, 1984), does exhibit lower replicative abilities compared to the *ori*⁺ mitochondrial genomes of petites al/1R/1 and Z1 (as well as of all *ori*⁺ petite genomes having repeat units shorter than about 1000 bp; Rayko et al., 1988). This can be judged by the suppressivity test (Ephrussi et al., 1955). Indeed, when crossed with wild-type cells, *ori*⁻ petites 26 and 14 yield progenies comprising only 85% and 79% diploid petites, respectively, instead of 99% produced by crosses involving

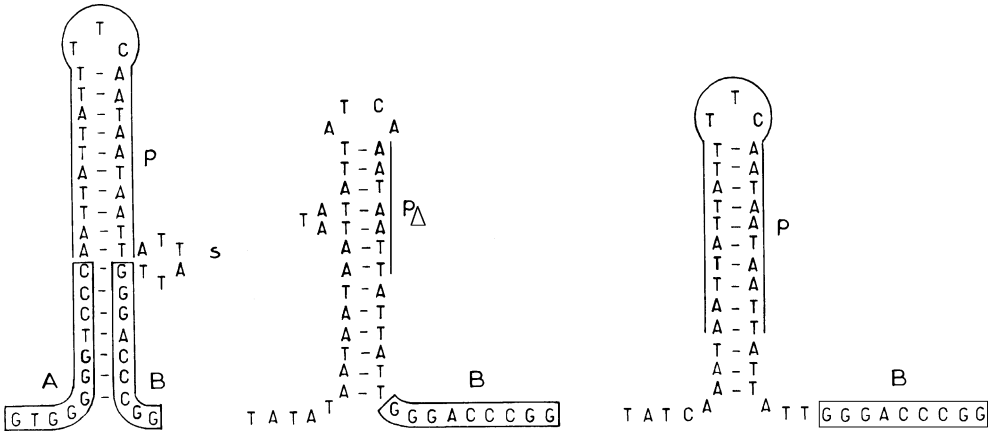

Fig. 5. Potential secondary structure of the A–B fold of the *ori* sequence present in the repeat units of mitochondrial genomes from petites a1/1R/1 and Z1 (*ori* 1) and of the replacement folds that can be formed in the *ori* 1 sequence present in the mitochondrial genomes of petites 14 and 26. In the case of petite 14, the residual nucleotides from the partially deleted *p* stretch (*p*Δ) can generate a hairpin structure with nucleotides from the preceding repeat unit, but the stem, only formed by 13 A/T nucleotides, carries different terminal and side loops compared to the A–B fold. In the case of petite 26, the upper part of the stem and terminal loop are identical to those of the A–B fold, but the lower part is replaced by three A:T pairs (three nucleotides are derived from the preceding repeat unit). The sequence involved in the structure shown (GC clusters A and B, sequences *p* and *s*) is that indicated in Fig. 4 (from Goursot et al., 1988).

ori^{+} petites al/1R/1 and Z1 (Table 2). Incidentally, much more dramatic decreases in suppressivity are associated with petite genomes deleted in the crucial C cluster region (de Zamaroczy et al., 1981; see Table 1), or lacking *ori* sequences altogether; as already mentioned, these latter *ori*^{0} petite genomes could replicate by making use of surrogate origins of replication, namely of *ori*^{s} sequences.

3.5. Temperature and the replicative ability of ori^{-} petites 14 and 26

In view of the presumed lower thermodynamical stability of replacement folds compared to the A–B fold, crosses of petites 14 and 26 with wild-type cells were also performed at both a higher (33 °C) and a lower (23 °C) temperature than the standard one, 28 °C. The results of Table 2 indicate that such temperatures decreased and increased, respectively, the replicative ability of the two mutants, whereas that of the control *ori*$^{+}$ petite Z1 did not show any significant change between these temperatures. The decrease of the replicative ability was stronger for petite 14 than for petite 26, as expected from the presumably lower thermodynamical stability of the replacement fold of the former. In the first case, the proportion of wild-type colonies obtained at 23 °C and 33 °C, respectively, was 10% and 55%, in the second 11% and 42%. The changes in replicative ability just described are immediate and reversible.

The results presented indicate that an environmental factor, temperature, can reversibly affect the replicative ability of a genome by altering its secondary (and possibly, its tertiary) structure. Indeed, (i) these changes cannot be ascribed to enzymes involved in DNA replication, as in temperature-sensitive mutants, since the petites discussed here are isonuclear, and lack mitochondrial protein synthesis, like all petites; (ii) the different effects of temperature on the replicative ability of petites Z1, 14 and 26 show an excellent correlation with those expected from the secondary structures of the postulated A–B fold and replacement folds (de Zamaroczy et al., 1981, 1984), an effect on tertiary structures being also possible.

Table 2
Suppressivity of petites Z1, 26 and 14 at different temperatures[a]

Temperature[b] (°C)	Suppressivity (%)[c]		
Petites:	Z1	26	14
23°	99	89	90
28°	99	85	79
33°	97	58	45

[a] From Goursot et al. (1988).
[b] Temperatures indicated were applied to precultures and to cultures used in crossings and in the incubation of diploids. Precultures in stationary phase were diluted 10 times upon setting up the cultures used in crossings.
[c] Suppressivity was tested by crossing petite strains (*MATa*, *ade1*) against the wild-type strain B (MATα, *trp1*, *his1*). The percentage of petite/total diploid colonies was determined by counting about 1000 colonies on minimal agar.

It should be pointed out that the repeat unit of petite Z1 extends 80 bp to the left of cluster A and only 40 bp to the right of sequence r, whereas those of petites 26 and 14 extend 115 bp to the right of sequence r (Fig. 4); in all cases, however, these extensions to the left of *ori* sequences are just made of AT spacer. Effects of flanking regions of *ori* sequences on the replicative ability of the latter are known (Rayko et al., 1988), but they are small compared with the different suppressivities exhibited by petites 14 and 26 relative to petite Z1. There is, therefore, no doubt that these differences are due to the deletions in the *ori* sequences of petites 14 and 26.

Interestingly, the conclusion that DNA secondary structure is required for *ori* activity in vivo was also reached for bacteriophage G4, where a strong temperature-dependent impairment of replication was found after introducing by site-directed mutagenesis point mutations which destabilize intra-strand base-pairing in the *ori* sequence (Lambert et al., 1987).

From a general viewpoint, our results indicate the existence of a novel type of environment–genome interaction, in which reversible changes in higher-order DNA structures are induced with profound consequences on a basic genome function, such as replication. These changes consist in genome transconformations which, although non-inheritable as such, can be maintained for many generations in the presence of the appropriate environmental condition. Genome transconformations can provide, therefore, strong selective advantages or disadvantages and play an important role in evolution, in addition to classical mutations, which involve changes in the primary structure of DNA. Moreover, similar phenomena might (i) be induced by other environmental factors; (ii) affect other genome functions (e.g. transcription) and also (iii) be operative in other organisms.

4. The organization and evolution of the mitochondrial genome of yeast

4.1. The organization of the mitochondrial genome of yeast

The organization of the mitochondrial genome of yeast, as it has emerged from our work, is similar to that of the nuclear genome of eukaryotes in that (i) coding sequences are interspersed with non-coding sequences, and (ii) the non-coding sequences are similar to the interspersed repeated sequences and the fold-back sequences, identical or similar sequences being present in many copies in the genome; moreover, (iii) the genome contains two genes, *cox1* (cytochrome oxidase sub-unit 1) and *cytb* (apocytochrome b), that comprise several introns (in some strains), some of which are translated into maturases, reverse transcriptases or site-specific endonucleases; the 21S RNA gene also may comprise an intron (see Foury et al., 1998, for a review). In conclusion, the mitochondrial genome is a useful, simple model for the nuclear genome. As already

mentioned, these genome features are not unique to the mitochondrial genome of yeast.

These results stress an important point, that mitochondrial genomes (to limit our discussion here to only one of the two organelle genomes) may range from the extremely compact structures that characterize not only most animal mitochondrial genomes, but also the mitochondrial genomes of some unicellular organisms (e.g. *Schizosaccharomyces pombe*), to structures in which non-coding sequences form the majority of the genome, the best studied case being that of the mitochondrial genome of yeast.

The monophyletic origin of the mitochondrial genome from a prokaryotic ancestor then raises two problems concerning the mechanism of formation and the biological role of non-coding sequences in the large mitochondrial genome of yeast.

4.2. The evolutionary origin of ori sequences, GC clusters, AT spacers and the *var 1* gene

The extremely high homology of the eight canonical *ori* sequences indicates that they arose as the result of duplication and translocation events. More precisely, we proposed that the canonical *ori* sequences derive from a primitive *ori* sequence (probably made of only a monomeric cluster C and its flanking sequences) through (i) a series of duplications and inversions generating clusters A and B; and (ii) an expansion process producing the AT stretches of *ori* sequences. It is possible that *ori* sequences are folded in a tertiary structure.

We studied the primary and secondary structures, the location and the orientation of the 196 GC clusters present in the 90% of the mitochondrial genome of *S. cerevisiae* which had been sequenced by 1986 (see de Zamaroczy and Bernardi, 1986b; the later completion of the sequence by Foury et al., 1998, did not change anything substantial in what follows). We found that (i) the vast majority of GC clusters is located in intergenic sequences (including *ori* sequences, intergenic open reading frames and the *var 1* gene; see next section) and in intronic closed reading frames (CRF's); (ii) most of them can be folded into stem-and-loop structures; (iii) both orientations are equally frequent. The primary structures of GC clusters permit to group them into eight families, seven of which are clearly related to the family formed by clusters A, B and C of the *ori* sequences. Most GC clusters apparently originated from primary clusters also derived from the primitive *ori* sequence in the course of its evolution towards the present *ori* sequences (de Zamaroczy and Bernardi, 1986a).

Intergenic sequences represent 63% of the mitochondrial 'long' (85 kb) genome of *S. cerevisiae*. They comprise 170 to 200 AT spacers that correspond to 47% of the genome and are separated from each other by GC clusters, ORFs, *ori* sequences, as well as by protein-coding genes. Intergenic AT spacers have an average size of 190 bp, and a GC level of 5%; they are formed by short (20–30 nt on the average) A/T stretches separated by mono-to tri-C/G. An analysis of the primary structures of intergenic AT spacers has shown that they are characterized by an extremely high level of short sequence repetitiousness and by a characteristic sequence pattern; the frequencies of A/T isostichs (oligonucleotides having the same size) conspicuously deviate from statistical expectations, and exponentially decrease when their (AT+TA)/(AA+TT) ratio, R, decreases. A situation essentially identical was found in the AT spacers of the small mitochondrial genome (19 kb) of *T. glabrata*. The sequence features of the AT spacers indicate that they were built in evolution by an expansion process mainly involving rounds of duplication, inversion and translocation events which affected an initial oligodeoxynucleotide (characterized by a particular R ratio) and the sequences derived from it. In turn, the initial oligodeoxynucleotide appears to have arisen from an ancestral promoter–replicator sequence which was at the origin of the nonanucleotide promoters present in the mitochondrial genomes of several yeasts (de Zamaroczy and Bernardi, 1987).

Common sequence patterns indicate that the AT spacers so formed gave rise to the *var 1* gene (by linking and phasing of short ORFs), coding for the only mitochondrially encoded protein of the large ribosome sub-unit, to the DNA stretches corresponding to the untranslated mRNA sequences and to the central stretches of *ori* sequences. The case of *var 1* gene (Hudspeth et al., 1982) is of special interest in connection with the idea of the expansion of *ori* sequences. This gene is 10% GC and contains a 46 base pair GC cluster accounting for 38% of total GC. Its similarity with spacer-cluster sequences is so striking that it suggests that this gene arose from an intergenic sequence only recently. Incidentally, *var 1* provided the first example of the generation of coding from non-coding sequences (de Zamaroczy and Bernardi, 1987), a subject of great current interest (see Jordan et al., 2003).

4.3. The non-coding sequences: evolutionary origin and biological role

To sum up the preceding sections, it is conceivable that the intergenic sequences were derived from *ori* sequences by the expansion process proposed by Bernardi (1982a) on the basis of sequence comparisons (Bernardi and Bernardi, 1980). This might have taken place through three different mechanisms, all of which are likely to have played a role. First, a slippage of the replicase could occur at the *ori* sequences; this is a well-known phenomenon first studied in the reiterative replication of poly (dAT:dAT) by DNA polymerase I of *E. coli* (Kornberg et al., 1964). A second mechanism is unequal crossing-over; evidence for the high frequency of such a phenomenon in mitochondrial recombination is available (Fonty et al., 1978). A third mechanism is insertion. Almost all GC clusters are inserted in AT spacers; some rare ones are inserted in AT-rich regions of

rRNA genes (Sor and Fukuhara, 1982) and even in a protein-coding gene, *var 1* (see above). Interestingly, these insertions are not only transcribed but, in the case of *var 1*, also translated.

Another interesting result (Bernardi, 1982a; de Zamaroczy and Bernardi, 1986a) was that the closed reading frames (CRF) of the intervening sequences of *oxi* 3 and *cob* genes share all the features of intergenic non-coding sequences. Indeed, when the relative amounts of di-to hexa-nucleotides were compared with those from random sequences having the same sizes and compositions, they were found to exhibit the same deviations as the intergenic non-coding sequences of the mitochondrial genome. In contrast, intronic open reading frames (ORFs) showed oligonucleotide patterns which were generally quite distinct from those of CRFs, although some similarities could be detected in some cases (especially for aI5α). The mitochondrial introns of yeast, therefore, are endowed with a mosaic structure, in which CRFs derive from mitochondrial intergenic sequences, whereas ORFs have a different origin (indicated as exogenous by other evidences).

It is evident that regulatory sequences acting as promotors, operators, sites for the initiation of replication and sites involved in the processing of transcripts are present in the non-coding sequences of yeast mitochondrial DNA. Another function of the non-coding sequences is also well documented and has to do with illegitimate, unequal recombination. The excision of the spontaneous petite genomes just described is an example of these extragenic recombinational events. The same basic mechanism appears, however, to be more general and to account for: (i) the divergence of the mitochondrial genomes of wild-type yeast cells; it has been shown (Bernardi et al., 1975; Prunell et al., 1977) that different strains have mitochondrial genomes differing in the length of AT spacers, apparently the result of unequal crossing-overs in the sequences of spacers; (ii) similar changes in the mitochondrial genomes of the progeny arising from crosses of different wild-type strains (Fonty et al., 1978).

In conclusion, the evidence available at the present time appears to support the idea that the complex sequence organization of the mitochondrial genome of yeast corresponds to the needs of very active and finely regulated replication, transcription and recombination processes. This seems to be achieved at the price of an exceptional genomic instability.

I discussed this genome instability at a Symposium held in Brno in 1982 in honour of Gregor Mendel (Bernardi, 1983). The following is a verbatim quotation. The non-coding sequences of the mitochondrial genome of yeast are the source of three disadvantages for the genome. The main one is that the abundant direct repeats that they contain are potential excision sequences; as such, they are responsible for the extreme instability of the genome. The other two are that they increase replication time and energy expenditure. These disadvantages would quickly change wild-type yeast cells into suppressive and neutral petite mutants, if this intracellular selection was not counterbalanced by an intercellular selection in which the faster growing respiratory-competent wild-type cells compete out the respiratory-deficient petite mutants (incidentally, this accounts for the fact that in nature only wild-type yeast cells are found). Even if the disadvantages associated with the non-coding sequences do not lead, therefore, to the elimination of the mitochondrial genome, they should at least lead to the elimination of the non-coding sequences themselves. We know, however, that, although *S. cerevisiae* strains exist which lack a number of intervening sequences (introns) and also *ori* 4, in general, non-coding sequences tend to be largely conserved. This indicates that the removal of non-coding sequences is selectively disadvantageous or, in other words, that non-coding sequences provide selective advantages which compensate for the disadvantages associated with them. This obviously raises the question of the nature of these advantages, namely of the physiological roles played by non-coding sequences.

It is clear from the genome map of the mitochondrial genome of yeast that the deletion of intergenic sequences, where most excision sequences used in the spontaneous mutation are located (de Zamaroczy et al., 1983), will frequently remove canonical *ori* sequences from the wild-type genome. Even if a wild-type genome lacking *ori* 4 has been found, it is evident that in general such elimination will affect replication and also transcription. There is therefore a selective advantage in keeping *ori* sequences in the wild-type genome. As far as non-coding sequences outside *ori* sequences are concerned, it should first of all be stressed that the expansion process does not propagate non-sense sequences, but propagates instead sequences which have been highly selected and conserved in evolution and whose primary role is to interact specifically with enzymes involved in DNA replication and transcription. Thus, the expansion of *ori* sequences leads to the propagation of potential regulatory signals, which may be used in the regulation of gene expression, in repression (anaerobiosis or glucose can shut off transcription) and derepression, in the processing of primary transcripts and in the regulation of nucleo-mitochondrial interactions. Another physiological role of non-coding sequences concerns recombination, since evidence exists (Fonty et al., 1978) that repeated and palindromic non-coding sequences are involved in mitochondrial site-specific recombination. Finally, it should be recalled that some non-coding sequences appear to be inserted into transcribed genes or even to be transformed into genes. In summary, a number of physiological roles apparently provide selective advantages compensating for the disadvantages inherent in the very existence of non-coding sequences. What we know about the non-coding sequences of the mitochondrial genome of yeast suggests that their conservation in the genome is due to selective advantages associated with their physiological roles; these sequences, or at least their majority, cannot, therefore, be

considered "selfish DNA sequences" (sensu Doolittle and Sapienza, 1980; Orgel and Crick, 1980).

A final point on which the mitochondrial genome of yeast is relevant to the "selfish DNA" issue is the occurrence of functionless genomes in suppressive petites. Many of these genomes contain no gene, and yet replication, transcription and even transcript processing may still go on. In nature, as already pointed out, these genomes rapidly disappear since petites are competed out by the faster growing wild-type cells. Many of these genomes have such a replicative advantage over wild-type genomes that they could spread out through crosses with wild-type cells, if haploid. This does not occur in nature because parental wild-type cells and the derived petites have the same mating type. When isolated from competition with wild-type cells in the laboratory, however, petites not only survive, but also frequently end up with very stable genomes which are the result of a selection on the basis of replication efficiency. These "genomes without genes" are practically made up of repeat units containing barely more than an *ori* sequence; replication is most efficient, the corresponding petites being supersuppressive, and transcription is only preserved because of the role played in replication. In other words, functionless genomes like the mitochondrial genomes of suppressive petites not only can exist and be quite stable, but they also undergo a selection favouring those which are closest to the ultimate situation of being just a set of replication origins; this in vivo selection is very much the same found in the Qβ replicase in vitro system by Mills et al. (1967). These "selfish genomes", exemplifying primordial self-replicating systems, will however be lost in the long run, when mitochondrial or nuclear mutations will inactivate the initiation of replication.

5. Discussion

This discussion will be limited to three points concerning *ori* sequences. The first one concerns nomenclature. *Ori* sequences were also called *rep* sequences (Blanc and Dujon, 1980). The former terminology should, however, be preferred for two reasons, priority (see de Zamaroczy et al., 1979; and Bernardi, 1979) and correctness. Indeed, while *ori* is the term established in the literature to indicate the sequences corresponding to the origin of replication, *rep* indicates genes encoding sub-units of the replication machinery. Needless to say that the nomenclature *ori/rep* used by several authors can be only taken as a diplomatic not as a scientific wording. Likewise, "supersuppressive petites" should be preferred to "hypersuppressive petites" (Blanc and Dujon, 1980) because of priority (de Zamaroczy et al., 1979; Goursot et al., 1980), and because a latin–latin combination is to be preferred to a greek–latin one in neologisms.

The second point is that petite genomes only consisting of AT sequences do replicate and may even show reduced suppressivity (Fangman and Dujon, 1984), a finding, that might put in doubt that *ori* sequences are origins of replication. Now, the observation concerns two petites isolated (probably after ethidium bromide treatment) from a supersuppressive mutant (itself the result of ethidium bromide mutagenesis; Blanc and Dujon, 1980). The two mutants, one neutral and one moderately suppressive, retained only 70 and 89 A/Ts, respectively, from the wild-type genome. This situation is in fact similar to that of the neutral petite RD1A (also the result of a drastic ethidium bromide treatment) which had a mitochondrial genome mostly formed by short repeat units of 68 nucleotides (Van Kreijl and Bos, 1977). In all these cases, either the petite genomes do not carry an origin of replication per repeat unit, but carry instead (as suggested by de Zamaroczy et al., 1979) one (or a few) origin(s) of replication per genome unit (which makes them weak replicators), or, more likely, they carry a sequence which is an extremely weak surrogate origin of replication, but which is, however, repeated so many times as to be able to have a weak, or even moderate, replication activity. In any case, they do not disprove *ori* sequences being the canonical origins of replication.

The third point concerns the possibility, suggested by Fangman and Dujon (1984), that *ori* sequences may have a role in mitochondrial genome segregation. This line of investigation has been explored by Butow and coworkers (see Kaufman et al., 2003), who raised "the interesting proposition that, rather than functioning primarily in DNA replication, yeast mitochondrial DNA *ori* sequences together with Hsp60 (a yeast mitochondrial chaperonin) may function in nucleoid division, which is likely dependent on membrane association of mitochondrial DNA (Albring et al., 1977)". Solid data needed to support the proposition of Kaufman et al. (2003) and outweigh the overwhelming evidence in favour of *ori* sequences being the origins of mitochondrial DNA replication are, however, still lacking.

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