EXCISION AND REPLICATION SEQUENCES IN THE MITOCHONDRIAL GENOME OF YEAST

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I. INTRODUCTION

It is now well established (Bernardi, 1979; Faugeron-Fonty *et al.*, 1979) that the mitochondrial genomes of spontaneous cytoplasmic petite mutants of *Saccharomyces cerevisiae* originate by a mechanism involving: a) the excision of a segment from one of the 50-100 mitochondrial genome units of the parental wild-type cells; b) the amplification of the excised segment into tandem repeat units to form a defective mitochondrial genome unit (Fig. 1). In general, the latter will: a) segregate into one of the buds originating from the parental wild-type cell; b) give rise, in a few generations time, to the mitochondrial genome of petite mutant cells. The genome units of the petite mutant may in turn undergo further excision-amplification processes leading to secondary petite genomes having shorter tandem repeats.

We report here recent results on the nucleotide sequences involved in the excision process giving rise to the mitochondrial genomes of petite mutants. In addition, we present data on sequences which appear to correspond to the origins of replication of the mitochondrial genome.

In order to put the present work in a proper perspective, it will be useful to mention two points: a) previous work from our laboratory had shown that the mitochondrial genome units

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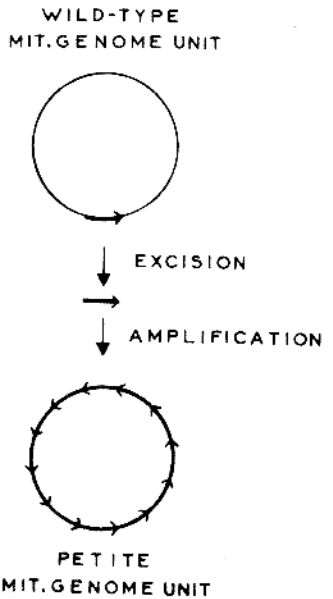

Fig. 1. Scheme of the process leading to the formation of spontaneous petite genomes (see Text).

of wild-type yeast cells are characterized by the presence of long AT spacers (which form at least half of each 50.10^6 genome unit and are formed by short alternating and non-alternating A:T sequences) and of many (a hundred or so) short GC clusters which are embedded in the AT spacers and consist, in most cases, of CCGG sequences clustered with GGCC or other GC-rich sequences; b) ten years ago, we proposed a deletion model as the basic mechanism underlying the petite mutation and suggested that excisions were due to illegitimate, site-specific recombination events taking advantage of homologous sequences which we supposed to occur frequently in the spacers of the mitochondrial genome units and to account for the extreme instability of the latter, the spontaneous petite mutation having a rate of 1-5% per generation (Bernardi, 1979).

II. RESULTS

A. Excision Sequences Belong in Two Classes

Restriction mapping of the repeat units of the mitochondrial genomes from six spontaneous petites (Fig. 2) and hybridization of these genomes on restriction fragments from the parental wild-type genomes have shown that these petite genomes and their excision sequences belong into two classes (Faugeron-Fonty *et al.*, 1979; Bernardi *et al.*, 1980). The repeat units of petite genomes of the first class appear to have been excised at (CCGG, GGCC) clusters, since: a) they are formed by Hpa II and Hae III fragments having the same sizes as Hpa II and Hae III fragments from the parental wild-type

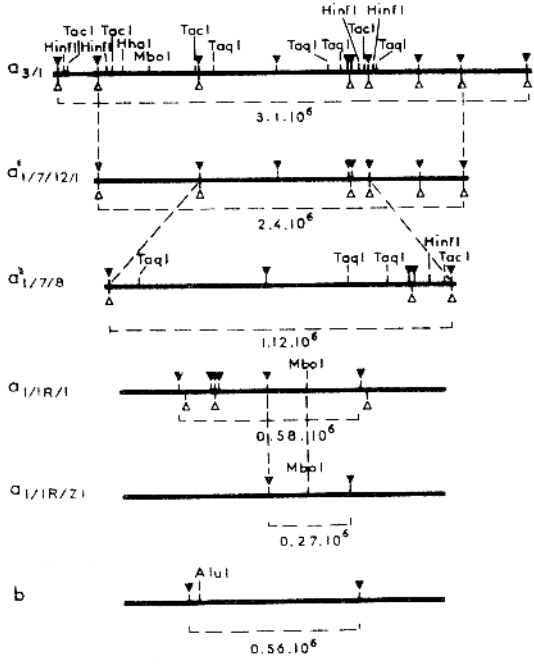

Fig. 2. Restriction enzyme maps of the repeating units of the mitochondrial genomes of six spontaneous petite mutants. The molecular weights of the repeat units are indicated, along with the positions of Hae III (Δ), Hpa II (Δ) and other restriction sites.

genomes (Hpa II and Hae III are restriction fragments splitting the sequences CCGG and GGCC, respectively); b) they hybridize on such parental fragments. Petite genomes of this class are exemplified by a*-1/7/8 and a-1/1R/1 (Fig. 2; for typographical reasons subscripts in the original code numbers of petites follow a dash here). In contrast, the repeat units of petite genomes of the second class were not excised at (CCGG, GGCC) clusters. Examples of these genomes are those of a-1/1R/Z1 and b (Fig. 2). The first of these genomes when degraded by Hpa II released a fragment corresponding in size to a Hpa II parental fragment; the petite genome did not, however, hybridize on this fragment, but did so on the two parental fragments corresponding to the two largest Hpa II fragments and to the larger Hae III fragment of a-1/1R/1 (Fig. 2). The second of these genomes, that of b, when degraded by Hpa II also released a fragment corresponding in size to a Hpa II fragment; its hybridization took place on two parental Hpa II fragments, one of which had about the same size as the petite Hpa II fragment; very recent sequences data (see below) indicate, however, that the latter finding is the result of a coincidence and that the repeat unit of b was not excised at (CCGG, GGCC) clusters nor at GC clusters containing CCGG sequence.

B. Excision Sequences are Repeated Sequences

So far, we have obtained definitive information on the excision sequences of two petites of the second class and preliminary results on those of one petite of the first class.

Several results strongly suggested that the repeat unit of a-1/1R/1 contained the repeat unit of a-1/1R/Z1 plus its flanking sequences (Faugeron-Fonty *et al.*, 1979): a) both petite genomes were originally present in the same spontaneous heterogeneous petite a-1/1R, from which they could be isolated by sub-cloning; b) they derived from the same region (map positions 27-46 of Sanders *et al.*, 1977) of the parental wild-type genome; c) they shared a Hpa II-Mbo I fragment (Fig. 2); d) they showed overlapping hybridization patterns on Hpa II and Hae III parental fragments (see above). All these data suggested that the genome of a-1/1R/Z1 could be a secondary excision product of a-1/1R/1; alternatively, the two petite genomes could derive from two overlapping segments of the parental wild-type genome. As expected, the 884 nucleotide sequence of the repeat unit of a-1/1R/1 (Fig. 3) was found (Gaillard *et al.*, 1980) to contain a 416 nucleotide sequence which was identical to that, previously sequenced by Gaillard and Bernardi (1979), of a-1/1R/Z1, except for two base pair changes. Very interestingly, the initial 13-nucleotide sequence of a-1/1R/Z1 was found to be repeated (with one A:T to T:A change) just after the end of the repeat unit.

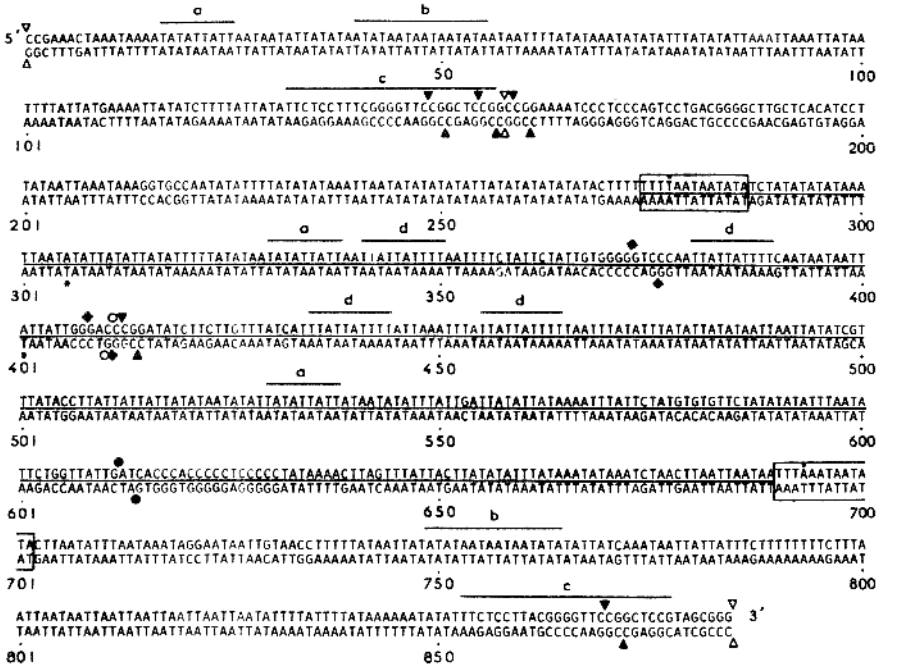

Fig. 3. Nucleotide sequence of the repeat unit of the mitochondrial genome of spontaneous petite mutant a-1/1R/1. Cutting sites of Hae III (▼), Hpa II (▼), Mbo I (●), Mbo II (○) and Ava II (◆) are indicated. The sequence marked by the continous line is identical with the repeat unit of a-1/1R/Z1 (Gaillard and Bernardi, 1979), except for the asterisked base pairs. Boxed sequences are the direct repeats flanking the sequence of a-1/1R/Z1 (asterisks mark a base pair difference). Lines indicate some of the repeated sequences.

The sequence of the repeat unit of another petite, provisionally called a-1/1R/14, also related to a-1/1R/1 revealed that again an initial 13-nucleotide sequence is repeated just after the end of the repeat unit. The sequence under consideration is AATAATTATTATT at positions 394-406 and 773-785 on the a-1/1R/1 repeat (Fig. 3).

Finally, the partial primary structure of the excision sequences of a*-1/7/8 (Fig. 2) has shown that they correspond to two direct repeats of a-3/1, each one of which is characterized by the clustering of a Tac-I, a Hae III and a Hpa II site (Fig. 2).

In conclusion, the three excision sequences known so far correspond to three sets of direct repeats, two of which are in AT spacers and one in GC clusters.

C. *Genomes without Genes*

An inspection of the sequence of a-1/1R/1 confirms the sequence features predicted by our previous work for both AT spacers and GC clusters, but fails to reveal the presence of any gene or gene fragment, a conclusion supported by the current knowledge of codons used for the synthesis of mitochondrially encoded proteins (Macino and Tzagoloff, 1979) and of tRNA gene sequences (Martin *et al.*, 1979). In all likelihood, the only function left in this petite genome is replication. Since the excision mechanism leading to the formation of spontaneous petite genomes is extremely conservative as far as the excised sequence is concerned (in contrast to that induced by ethidium, where sequence rearrangements of different kinds are frequent; Lewin *et al.*, 1978), each repeat unit of a-1/1R/1 should contain an origin of replication. A good candidate for this role is the 80-nucleotide sequence centered around position 412, since it is characterized by features already found in replication origins of other genomes. In its left half, this sequence contains a palindrome 23 nucleotides long (378-400) and a small symmetrical sequence TTAATT (402-406), which are flanked by two inverted repeats formed by G:C base pairs only; in its right half, the sequence contains a decanucleotide formed by A:T base pairs only, which is a direct repeat of another sequence present in the left half. The central feature of the sequence is a very characteristic cluster of a Ava II, Mbo II and Hpa II cutting sites (the recognition site of Mbo II being a pentanucleotide 8 nucleotides away from the cutting site, this cluster corresponds to 13 nucleotides in a sequence of 16; see Figs. 3 and 5).

D. *Origins of Replication of the Mitochondrial Genome*

To test the hypothesis that the 80-nucleotide sequence just discussed corresponds to the origin of replication of a-1/1R/1 we decided to look for its most salient features, the central restriction site cluster, in the repeat units of b and a*-1/7/8, two petites whose genomes arose from two other regions (Fig. 4) of the mitochondrial genome of the parental wild-type cells. This search having proved successful, we determined the two sequences flanking the cluster (de Zamaroczy *et al.*, 1979) and found them to be identical to that of a-1/1R/1, except for a few base pair changes (Fig. 5). Incidentally, the sequence of Fig. 5 proves that the repeat unit of

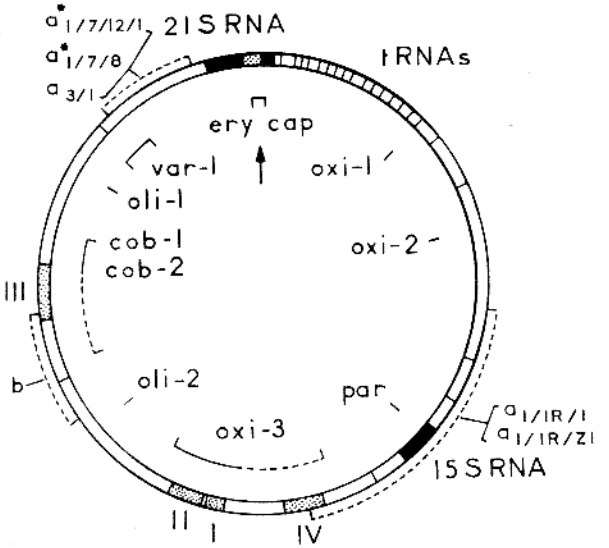

Fig. 4. Regions of excision of the petite genomes of Fig. 2 on a general map (adapted from Borst and Grivell, 1978) of the mitochondrial genome of wild-type yeast cells.

b was not excised at or around the Hpa II site, since the 80 nucleotides around it clearly were not interrupted by the excision process.

The finding of the 80-nucleotide sequence in the mitochondrial genome of a*-1/7/8 strongly suggests that this sequence is also present in the genomes of a*-1/7/12/1 and a-3/1, since their repeat units encompass that of a*-1/7/8. If such is the case, then all six spontaneous petite genomes of Fig. 2 contain the sequence under consideration, in spite of their origin from three distinct regions of the parental wildtype genome (Fig. 4). Interestingly, one of these petites, b, originated in fact from a different parental strain (strain B), compared to all the other ones, which originated from strain A; its mitochondrial DNA showed, however, the same hybridization patterns on restriction fragments from the mitochondiral DNAs of both strains A and B.

Finally, it should be mentioned that petite a-1/1R/14 lacks the 20 or so left-most nucleotides in the putative replication origin, showing that this stretch is not essential.

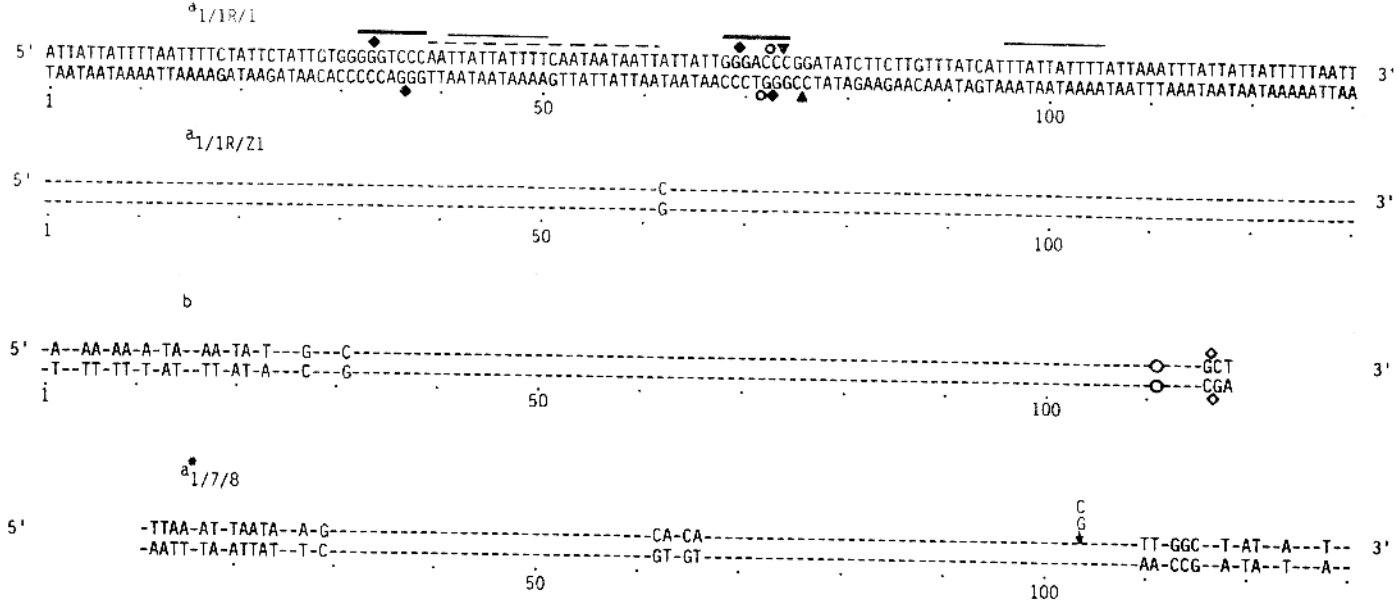

Fig. 5. Nucleotide sequence of the repeat unit of the mitochondrial genome of a-1/1R/1 (Fig. 3) between base pairs 340 and 470, of the corresponding segment of a-1/1R/Z1 and of the region around the Hpa II, Mbo II, Ava II cluster of the repeat units of b and a-1/7/8. Dashes indicate nucleotides identical to those found in a-1/1R/1, taken as a reference. One base pair deletion in b is indicated by a double empty circle, one base pair addition in a*-1/7/8 by an arrow. Restriction sites are indicated as in Fig. 3; Alu I by ◇. The 23-base pair palindrome is indicated by a broken line, the 7-base pair inverted repeats by heavy lines, the 10-base pair direct repeat by thin lines.*

E. *Supersuppressive Petites*

Even if the circumstantial evidence just presented is very strong, no direct evidence is available yet to prove that the 80-nucleotide sequences correspond to origins of replication of mitochondrial DNA. Two additional, independent lines of evidence support this view, however: a) the results presented in the previous section imply that the mitochondrial genome units of wild-type cells have at least three (actual or potential) origins of replications; in agreement with this idea is the finding that the regions from which the petite genomes of Fig. 2 arose, although in need of a better definition, overlap with the regions rom which spontaneous petites arise with the highest frequency (Mathews *et al.*, 1977); b) the four petite genomes of Fig. 5 are supersuppressive (Goursot *et al.*, 1980; the evidence for a*-1/7/8 being still preliminary), namely they are selectively transmitted to the progeny from crosses with wild-type cells without any apparent sequence change; the simplest explanation for this phenomenon is that multiple replication origins are present in these petite genomes, one per repeat unit, and this allows them to compete out the mitochondrial genome of wild-type cells in crosses, and this is spite of the fact that the wild-type cells issued from the cross have a selective growth advantage over the petite mutants.

III. DISCUSSION

A. *The Petite Mutation, Neutrality and Suppressivity*

The results just described are directly relevant for our understanding of the mechanism of the spontaneous petite mutation as well as of the phenomena of neutrality and suppresivity. As far as the first point is concerned, while it will be of interest to know about the primary structure of more excision sequences, it is already clear that the excision mechanism experimentally found completely fulfills the hypothesis put forward ten years ago. In contrast, nothing is known about the amplification mechanism. Concerning the neutrality-suppresivity issue (crosses of wild-type cells with netural petites yield wild-type cells only; those with suppressive petites yield both wild-type cells and petite mutants in ratios essentially dependent upon the petite used in the cross), this is clarified to some extent by the results on the replication origin. Two situations appear to be quite clear. Supersuppressive petites, when crossed with wild-type cells, yield almost exclusively petite mutants, the mitochondrial genome of which seems to be identical to that of the petite entering the cross.

As already mentioned, this situation appears to stem from the high replicative efficiency of supersuppressive genomes, which are characterized by very short repeat units comprising an origin of replication, and which can therefore compete out the wild-type genomes. It is obvious that a supersuppressive genome of very low complexity and very low concentration of excision sequences can only undergo mutations affecting the replicative function; when this is lost, cells without mitochondrial genomes are formed (Nagley and Linnane, 1970; Goldring *et al.*, 1970), which will behave as neutral in crosses. The other two situations require further investigations. Some neutral petites contain a mitochondrial genome, the best documented case being that of RD1A (Moustacchi, 1972). In this case, it is known (van Kreijl and Bos, 1977) that *most* of the mitochondrial DNA is present in an extremely short (68 nucleotides) repeat unit which has nothing in common with the origins of replication sequenced here. Since this petite is the result of a drastic ethidium treatment, which is known to cause a massive breakdown of mitochondrial DNA (Goldring *et al.*, 1970) followed by genome rearrangements (Lewin *et al.*, 1978), we suggest that the lack of replicative competitivity of this genome is due to the fact that it may only carry one origin of replication translocated on the amplified repeat unit. The final case, that of suppressive petite, may be due to a number of reasons. The study of the suppressivity of a-1/1R/14, which lacks part of the origin of replication, will be of great interest in this respect. For instance, the length of the repeat unit and changes in the origin of replication may affect the replicative competitivity of a petite. Fig. 6 presents a scheme of the different situations encountered so far in petites, along with suggested abbreviations.

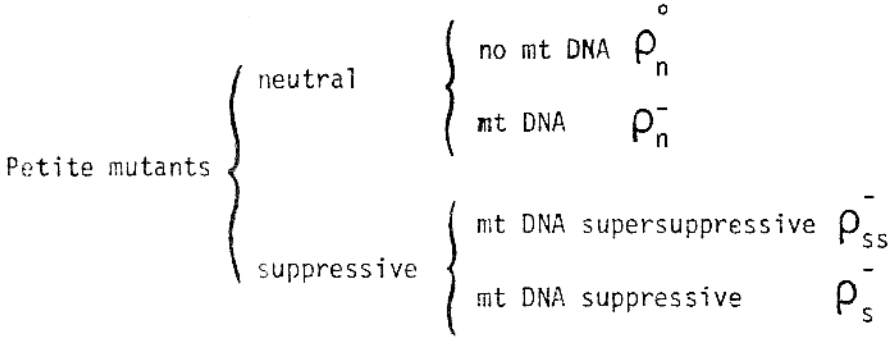

Fig. 6. Scheme of a classification of petite mutants (see text). Suggested abbreviations are indicated.

B. *The General Significance of Excision Sequences*

There is little doubt that the direct repeats investigated here are responsible, by a mechanism involving a site-specific, illegitimate recombination, for the excision of the repeat units of petite genomes, and therefore for the extreme instability of the mitochondrial genome of wild-type cells. It is also quite possible that mitochondrial genome segments bordered by such sequences can be transposed, by an excision-insertion mechanism, onto other mitochondrial genome units present in the same cell. This mechanism might account for three different findings: a) the different lengths of "allelic" spacers (namely of spacers contiguous to allelic genes) which were observed in the mitochondrial genomes of different yeast strains (Bernardi *et al.*, 1975; Prunell *et al.*, 1977); b) the insertions which are present in the genomes of some strains (Sanders *et al.*, 1977); c) the existence of multiple origins of replication (de Zamaroczy *et al.*, 1980).

One may wonder, however, about the "physiological" role of "excision" sequences in the mitochondrial genome of yeast. There are at least three sorts of answers to such question. Taking into account the diversity of excision sequences, these answers need not be mutually exclusive. Since excision sequences are so reminiscent of those found, for example, on both sides of insertion sequence IS1 and of transpoon Tn 9 in *E. coli* (Calos *et al.*, 1978; Grindley, 1978; Johnsrud *et al.*, 1978), the first answer may be that excision sequences play indeed the same role as those prokaryotic sequences; if this answer is correct, it implies that this type of sequence is conserved in organisms very far away from prokaryotes. The second sort of answer is that these sequences are normally used in site-specific recombination processes among genome units of the same vegetative cells or of different cells in crosses; evidences for the high frequency of both processes have been presented elsewhere (Bernardi *et al.*, 1975; Prunell *et al.*, 1977; Fonty *et al.*, 1978). The thid sort of answer has to do with regulatory functions of some kind; GC clusters, for instance, might play a primary role as sequences recognized by DNA replicase, RNA polymerase, enzymes of RNA processing (once they are transcribed), regulatory proteins (Prunell and Bernardi, 1977). Further work should clarify these issues.

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DISCUSSION

P. BERG: What is the state of the DNA in the yeast mitochondria in terms of the proteins with which it is associated? How does it compare to the DNA in the nucleus?

G. BERNARDI: According to a very recent paper by F. Caron, C. Jacq and J. Rouviere-Yanic (Proc. Natl. Acad. Sci. USA 76, 4265-4269) yeast mitochondria do not contain histones but have an abundance of 20,000 dalton DNA-binding protein which introduced superhelical turns in relaxed circular DNA in the presence of a nicking-closing enzyme like histones. This protein is slightly basic and heat-stable and might play the role of histones.

P. BERG: The reason for my question is whether the kind of recombinational excision you proposed is something that could occur in genomic DNA elements with which histones and other accessory proteins are associated; or, is this phenomenon restricted to "unprotected" types of DNA genomes?

G. BERNARDI: I agree with you that one may wonder about the inhibiting role on recombination by the complex structure of chromosomes. On the other hand, it is also true that the relative amount of interspersed repetitive sequences is much higher in the mitochondrial genome of yeast compared, for instance, to its nuclear counterpart.

C. HERSCHBERGER: If you introduce suppressive petite DNA into a rho^{o} strain can you get transformation or are you dependent strictly on a genetic cross? Can you displace the wild-type mitochondrial genome in a transformation type system with the petite DNA?

G. BERNARDI: You can transfer, without any problem, any mitochondrial genome into rho^{o} cells, namely cells deprived of mitochondrial genome. In fact, this is a nice way to see the effect of different nuclear backgrounds on the mitochondrial genome function.

C. HERSCHBERGER: Are these transferred by genetic crosses?

G. BERNARDI: Yes.

C. HERSCHBERGER: Can you do a similar experiment using yeast transformation system where you introduce the purified DNA into the rho^{o} or wild-type cells?

G. BERNARDI: I suppose so, but to my knowledge nobody has managed, so far, to do it. I think some attempts have been made, and probably somebody will succeed one day or another.

A. BOLLON: Are there any common sequences at all between the mitochondrial genome and the nuclear genome?

G. BERNARDI: Some old hybridization experiments done when nick translation was not a current technique, which are against the idea of common sequences, but those experiments should be repeated because now techniques are so much more sophisticated.