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THE MITOCHONDRIAL GENOME OF YEAST : ORGANIZATION, EVOLUTION AND THE PETITE MUTATION

Giorgio Bernardi, Ariel Prunell
Godeleine Fonty, Helena Kopecka and François Strauss
Laboratoire de Génétique Moléculaire,
Institut de Biologie Moléculaire, Paris, France.

Introduction. Previous investigations from our laboratory[1-6] have led to the recognition of two distinct sequence elements in the mitochondrial genome of wild-type S.cerevisiae cells : A+T-rich stretches (G+C < 5 %), formed by short, repeated dAdT : dAdT and dA : dT sequences, and G+C-rich stretches (average G+C = 32 %), exhibiting a very strong compositional heterogeneity ; we called the first ones spacers, and we thought that the latter ones corresponded to genes with their regulatory regions. Both sequence elements have the same average size, higher than $1.6.10^5$, are equally represented in the mitochondrial genome; and are interspersed with each other. We will briefly report here a study of the fragments obtained by degrading yeast mitochondrial DNA with two restriction enzymes, Hae III and Hpa II. These investigations have led not only to the discovery of two new sequence elements, which we believe to play a regulatory role, but also to new ideas on the organization and evolution of the mitochondrial genome of yeast, and on the changes it undergoes in cytoplasmic petite mutants. Preliminary reports on parts of this work have been published [7-9].

THE ORGANIZATION OF THE MITOCHONDRIAL GENOME IN WILD-TYPE YEAST CELLS.

The Hae and Hpa restriction fragments. Degradation of mitochondrial DNAs from four different yeast strains with Hae or Hpa (as we will indicate hencefrom Hae III and Hpa II) yield 71 to 113 fragments according to the enzyme and the yeast strain used. These range in molecular weight from 4.10^6 to 10^4 and can be resolved by electrophoresis on gels of different polyacrylamide concentration. Genome unit sizes, calculated by adding up the molecular weights of all fragments produced by Hae or Hpa were different in different strains, yet always rather close to 50.10^6 (ref. 8).

The number of Hae or Hpa restriction sites, (GGCC and CCGG, respectively), as judged from the number of fragments seen on the gels, is much larger than that expected, 5, for a statistical DNA having the size of the mitochondrial genome unit, about 50.10^6, and its base composition, 18 % G+C. Since the number of the restriction sites under consideration increases with the fourth power of the G+C content, a possible explanation for the discrepancy between the experi-

mental findings and the statistical expectations may be the strong compositional heterogeneity of yeast mitochondrial DNA, and the consequent very high concentration of Hae or Hpa sites in its sequences highest in G+C. The fact that the restriction enzyme Hha I, (whose site GCGC has the same statistical frequency as those of Hae or Hpa), only produces about 10 fragments suggests, however, that a high degree of non-randomness exists in such sequences. Under these circumstances the distribution of Hae or Hpa sites on the mitochondrial genome becomes an interesting problem.

The Hae + Hpa double digest. A clustering of Hae and Hpa sites, already suggested by the similar size distributions of Hae and Hpa fragments [8], was demonstrated by an analysis of the Hae + Hpa double digests. In the case of the DNA from S.carlsbergensis, for instance, the double digest exhibited 107 fragments on the gel, whereas the total number of Hae + Hpa sites is at least 178, (71 Hae sites + 107 Hpa sites). When Hae digests were degraded by Hpa, a number of additional bands appeared, mostly in the lowest molecular weight range, and almost all Hae bands were shifted down to positions generally corresponding to only slightly smaller molecular weights (Fig. 1). In contrast, Hae

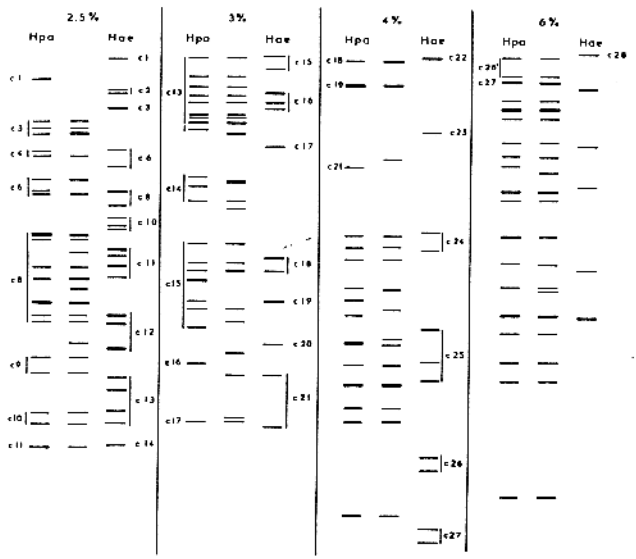

Fig. 1

Scheme of the band patterns obtained on agarose (0.5 %)-polyacrylamide (2.5 % - 6 %) gels with S.carlsbergensis DNA degraded by Hpa and Hae (simple digests) and Hpa + Hae (double digests). Overlaps from gel to gel were not shown. 10 very low molecular weight bands having the same mobility in Hpa and Hpa + Hae digests, as well as 2 Hae bands, are not shown on the 6 % gel. Double and triple bands are indicated with thicker bars.

digestion of the Hpa hydrolysate led to a band pattern extremely similar to that of the Hpa digest (Fig. 1) ; the number of bands did not change, but some fragments were shifted down (Fig. 1). Whatever fragments were formed by Hae, these were lost, suggesting that all Hae sites are clustered with Hpa sites. The number of (Hae, Hpa) site clusters should, therefore, be equal to the number of Hae fragments, 71. In fact, since a few non-clustered Hae breaks do occur, this number is slightly lower than 70. Obviously, Hpa sites not clustered with Hae sites also occur. In S.carlsbergensis DNA about 40 such isolated sites occur.

Hydroxyapatite chromatography of Hae and Hpa digests. These findings prompted an analysis of Hae and Hpa digests by chromatography on hydroxyapatite, the purpose of the experiment being to reveal the presence of single-stranded fragments arising from the melting of DNA segments in which Hae or Hpa sites were clustered. Chromatography on hydroxyapatite showed that Hpa digests of mitochondrial DNA were characterized by the presence of a small amount of material eluting at 0.05 M, 0.1 M and 0.15 M sodium phosphate and by a major peak eluting at 0.22-0.32 M phosphate (Fig. 2). The main peak material was formed by double-

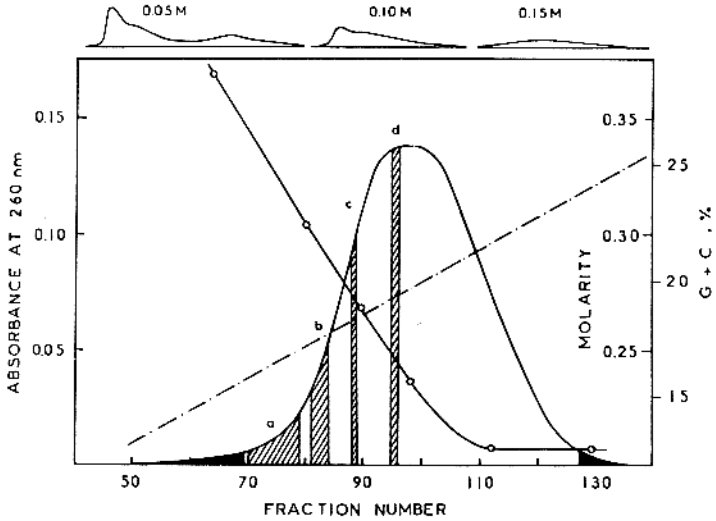

Fig. 2

Chromatogram of a Hpa digest of mitochondrial DNA from S.cerevisiae, strain B, on a hydroxyapatite column. After loading, the column was washed with the equilibration buffer, 0.001 M sodium phosphate, pH 6.8, until the 2-mercaptoethanol of the DNA solvent was completely eluted. The corresponding peak (not shown) was free from mono- and oligonucleotides. Steps of increasing molarities were then applied to the column. The three peaks above the frame are the enlarged transmission recordings, at 253.7 nm, of the material eluted at 0.05 M, 0.10 M and 0.15 M sodium phosphate, respectively. A linear gradient (0.15-0.40 M) of sodium phosphate, pH 6.8, was then applied. The dashed line indicates the phosphate molarities of the fractions. Open circles indicate the base composition, which was determined on pooled fractions in some cases (black areas).

stranded fragments, as indicated by its elution molarity[10] and by the fact that its lowest eluting 1.2 %,(left black area in Fig. 2), contained all the smallest fragments detected by gel electrophoresis. The low-eluting material, in contrast, was formed by single-stranded oligonucleotides, as indicated by its elution molarity range[10]. Basically similar results were obtained with Hae digests. The amounts and G+C contents of the oligonucleotides released by Hpa or Hae are shown in Table I. These results showed that Hae site clusters and Hpa site

TABLE I

Amount and base composition of oligonucleotides released by Hae and Hpa from the mitochondrial DNA of S.carlsbergensis.

First degradation	Second degradation	Amount		G + C
		%	millions per genome unit	%
Hpa	-	2.1	1.05	68
Hpa	Hae	0.5	0.25	37
Hae	-	1.7	0.85	33
Hae	Hpa	1.3	0.65	60
Hpa + Hae		2.6	1.3	62
Hae + Hpa		3.0	1.5	45

clusters exist in the (Hae,Hpa) clusters. Furthermore, the data concerning the amounts of oligonucleotides released upon a second degradation (Table I) suggest that part of the Hae clusters are contained in Hpa clusters ; in fact the amount of Hpa clusters remains large 1.3 % versus 2.1 % after removing the Hae clusters, whereas the amount of Hae clusters is more strongly reduced, 0.5 % versus 1.7 %, after removing the Hpa clusters. This points to the existence of two classes of (Hae, Hpa) clusters, the first of which, (Hpa-Hae-Hpa), has Hae sites contained in Hpa clusters, whereas the second, (Hpa-Hae), does not.

The restriction site clusters. i. The data of Table I indicate that 2.6-3 % of mitochondrial DNA, namely 1.3-1.5 millions per genome unit, is present in (Hae, Hpa) site clusters. Since the number of the latter is about 70, each site cluster is formed, on the average, by 30 base pairs. Because of their high G+C content, 45-62 %, it is unlikely that these melt unless they become shorter than about 10 base pairs, which implies that each site cluster contains on the average no less than 3-4 sites, corresponding to 12-16 base pairs. This indicates that the number of restriction sites for both Hpa and Hae is much larger than that of restriction fragments. A conservative estimate may be about 200 Hpa sites

and about 100 Hae sites per genome unit. Now, if one takes into account the fact that the sites have a G+C level of 100 %, and that the clusters have a level of 45-62 %, then most of the base pairs alternating with the restriction sites in the clusters must be dA:dT base pairs.

The clustering of Hae and Hpa sites in mitochondrial DNA suggested that such sites might be concentrated on the G+C-rich fragments which can be prepared by degradation with micrococcal nuclease[6]. Such fragments were, therefore, prepared again; their yield, 9 %, and their G+C content, 62 %, were in agreement with previous results[6]. The fragments were then degraded with Hpa and chromatographed on hydroxyapatite. 15 % of this material, therefore 1.4 % of the DNA, was eluted as oligonucleotides, the rest as double-stranded fragments having a G+C-content of 60 %. This experiment showed 1) that most of the Hpa clusters are found in the G+C-rich fragments prepared by micrococcal nuclease ; 2) that sequences resistant to Hpa, Hae, and Hha I, having a G+C content of 60 % also exist in such fragments ; we will call these sequences G+C-rich clusters.

The G+C-rich clusters. In spite of their very high G+C level (60 %), these sequences do not contain any of the quadruplets CCGG, GGCC, GCGC, which are the targets of Hpa, Hae, and Hha I, respectively. This implies that the G+C-rich clusters are highly non-statistical in sequence, like the restriction site clusters.

If most of the G+C-rich fragments released by micrococcal nuclease (which form about 9 % of the DNA) are formed by restriction site clusters (which represent about 3 % of the DNA), and by G+C-rich clusters, then the latter could represent around 5 % of the DNA.

The G+C-rich clusters, like the restriction site clusters are certainly not located within the genes, since each gene would then contain such peculiar sequences. It is also certain that they are not located within the spacers, since in such a case they would be completely released by micrococcal nuclease at 50 % degradation, a possibility ruled out by a previous experiment[6]. The only possible localization of the two sorts of clusters, therefore, is at the border of subsequent gene-spacer units, where they can only be contiguous to each other. It is quite possible that each restriction site cluster is contiguous to a G+C-rich cluster.

The restriction site clusters are characterized by 1) a high concentration in symmetrical nucleotide sequences and 2) the alternation of short A:T and G:C sequences. The first feature suggests by itself a binding role for proteins having a dyad axis of symmetry; such a symmetry relationship[11,12] has been found in all specific protein-DNA interactions[13]. Both features found in the (Hae, Hpa) site clusters are also present in promotor and operator sites of phage and bacterial DNAs[14]: this strongly suggests that the site clusters may correspond to regulatory sequences of the mitochondrial genome.

The number of (Hpa, Hae) restriction site clusters in the S.carlsbergensis mitochondrial DNA, is about twice the number of yeast mitochondrial genes known so far. These include 24-tRNA genes[15,16] 2-rRNA genes[17] and, very probably, 7 genes coding for polypeptide chains[18]. 70 mitochondrial genes are, however, compatible with the coding capacity of the mitochondrial genome of yeast, since it would mean an average gene size of about 350,000, which is not too low a figure for a genome in which the small t-RNA genes represent such a large percentage (over 30 %) of the total.

The sequence properties of the G+C-rich clusters and their contiguity with the restriction site clusters suggest a regulatory role. An attractive possibility is that one of the two sets of clusters might correspond to operator sequences and the other one to promotor sequences.

The genetic units of the mitochondrial genome. If the stoichiometry and the topology of the genome elements are those suggested in the preceding section, then the mitochondrial genome of yeast is organized in a number of genetic units, each one of which contains the four sequence elements depicted in Fig. 3. The size distribution of genetic units covers a relatively large range; the average size is about 8.10^5.

The presence of very small fragments in the Hpa digest is not in conflict with the genetic units just discussed. These fragments almost exclusively arise from isolated Hpa breaks (Fig. 1). They are the first ones eluted from hydroxyapatite and have a G+C content close to 29 % (Fig. 2), two results suggesting that they do not contain spacers ; moreover they are largely homologous even in digests from S.cerevisiae and S.carlsbergensis. These findings suggest that the isolated Hpa sites are located in mitochondrial genes, an attractive possibility being that they are present in the tRNA genes and correspond to self-complementary sequences in the stems of these RNAs.

The few small Hae fragments are also likely to arise from isolated Hae breaks.

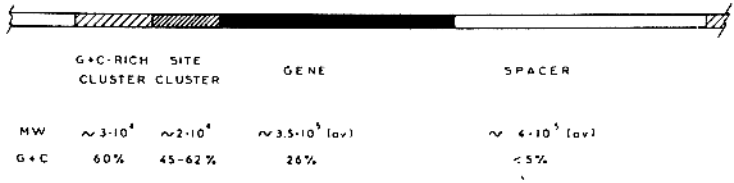

Fig. 3

Hypothetical scheme of the organization of genetic units from the mitochondrial genome of yeast. The relative order of G+C-rich clusters and restriction site clusters in unknown.

If the model of Fig. 3 is essentially correct, as we believe, it implies that : 1) transcription of the mitochondrial genome of yeast yields monocistronic messages ; in agreement with this idea, there is no evidence for spacer transcripts in wild-type yeast mitochondria ; 2) coordinate expression of genes apart from each other, (an example being the genes for the large and small ribosomal RNAs[19]), as in the aerobic induction, may take place through the interaction of regulatory proteins with operator sites having identical nucleotide sequences ; 3) transcription occurs very differently in yeast and animal cell mitochondria ; in the latter case both mitochondrial DNA strands are fully transcribed into RNAs[20] which are then processed to yield rRNAs, tRNAs, and mRNAs: 4) the mitochondrial genome of yeast shows a eukaryotic and not a prokaryotic type of organization in being an interspersed system of repetitive sequences and genes transcribed monocistronically; 5) recombination phenomena taking advantage of the high level of sequence homology in the spacers and in the regulatory segments can be expected.

THE EVOLUTION OF THE MITOCHONDRIAL GENOME IN WILD-TYPE YEAST CELLS.

Hae and Hpa fragment patterns of mitochondrial DNAs from different strains. The comparison of the electrophoretic restriction patterns obtained by degrading the mitochondrial DNAs from one S.carlsbergensis and three genetically unrelated S.cerevisiae wild-type strains revealed large differences[7,8]. The interstrain percent fragment homologies by fragment weight (w) or fragment number (n), are presented in Table II. This shows, in particular, that fragment homology between S.carlsbergensis (strain C) DNA and S.cerevisiae (strains A, B and D) DNAs is extremely small.

TABLE II

Interstrain fragment homologies[(a)]

Strains	Hpa		Hae	
	w	n	w	n
A-B	40	43	32	35
A-D	28	20	37	29
B-D	29	16	23	22
A-C	0	7	0	10
B-C	10	19	6	14
D-C	0	6	0	11

In apparent full contradiction with these results, a number of findings indicate a high level of sequence homology in the mitochondrial genomes investigated : 1) All DNAs released approximately the same number of Hae and Hpa fragments ; these had similar size distributions. 2) Fragments of similar size from strains B and C showed similar base compositions[7] and similar chromatographic behavior on hydroxyapatite. 3) The DNAs from S.carlsbergensis and S.cerevisiae showed 100 % homology, by DNA-DNA hybridization, with no mismatch in the hybrids[21]. All these results are not surprising in view of the large similarity of all physical and chemical properties shared by the DNAs under consideration : buoyant density, G+C content, melting curve, pyrimidine isostichs, A+T-rich spacers, restriction site clusters, G+C-rich clusters. An explanation of this apparent paradox is given in the following two sections.

Origin of differences in the fragment patterns. Two, not mutually exclusive, possibilities can explain the differences : 1) Point mutations (and/or base modifications) at the restriction sites ; 2) Deletions and/or additions between restriction sites.

The first possibility can be ruled out as a mechanism accounting for more than a minor part of the changes. 1) Since practically all fragments of S.carlsbergensis DNA have electrophoretic mobilities different from those of the S.cerevisiae DNAs, at least half of the restriction sites are different in the two DNAs. Assuming a random point mutation mechanism and a single base pair change per two sites, at least one nucleotide out of eight (the number of nucleotides present in two sites) has mutated in the two DNAs. Such a situation should give rise to a measurable mismatch in the DNA-DNA hybrids ($\Delta T_m \simeq 10°$), whereas none was observed[21]. 2) Since the number and the size distribution of the restriction fragments is very similar in DNAs from different strains, the disappearance of "old" restriction sites should be accompanied by the formation (caused by the same point mutation mechanism) of an approximately equal number of "new" restriction sites having a very similar distribution on the genome. 3) Since the number of Hae or Hpa sites in mitochondrial DNAs is more than 20 and 40 times, respectively, the number expected for a statistical DNA having the same base composition, a random mutation process cannot preserve such a number since it will randomize the nucleotide sequences ; 4) Since restriction sites are clustered in mitochondrial DNA, several point mutations at neighboring sites are needed to change the restriction fragment patterns.

Base modifications at the sites can also be ruled as an explanation on the basis of results which we will not present here.

We conclude, therefore, that the predominant mechanism underlying the changes in restriction site distributions is one involving deletions and additions between such sites.

Localization of deletions and additions in the restriction fragments. It should be recalled here that practically all Hae fragments and most Hpa fragments essentially correspond to the genetic units of the mitochondrial genome, as defined above, and, therefore, they contain a gene and a spacer element flanked by G+C-rich segments containing Hae and Hpa site clusters. Under these circumstances, the important question concerns which one of these elements is the seat of deletions and additions. It is obvious that such phenomena cannot affect the genes themselves since in the case of the DNAs from S.carlsbergensis and S.cerevisiae all allelic gene pairs should be different in length. Deletions and additions must, therefore, affect either the A+T-rich spacers or the G+C-rich segments (i.e., the (Hae, Hpa) site clusters and the G+C-rich clusters). In both cases, one could expect absence of mismatch in the DNA-DNA hybrids because of precise pairing of the gene sequences present in the fragments and of foldback pairing in the extra lengths of A+T-rich spacers or G+C-rich segments, both of which are known to contain hyphenated palindromic sequences.

It is very likely that the A+T-rich spacers are the seat of changes in length of restriction fragments for the following reasons : 1) The A+T-rich spacers represent 50 % of the genome, the G+C-rich segments less than 10 % ; if the number of these two elements is the same, as it seems to be the case, the G+C-rich segments are just too short to allow for the changes in length observed; 2) If the hypothesis that the G+C-rich segments correspond to regulatory sequences is correct, it is obvious that these sequences cannot be altered without functional losses ; in contrast, the A+T-rich spacers are not likely to play the same role nor to be under the same evolutionary contraints.

Mechanisms underlying deletions and additions in the restriction fragments. Several mechanisms may conceivably be responsible for the deletion-addition events occurring in the A+T-rich spacers of the mitochondrial genome of yeast. The basic mechanism which we favor is one involving unequal crossing-over events in the A+T-rich spacers. Three points are worth stressing : 1) gene order is very largely preserved in the diverged genomes under consideration, as shown by the overall similarity of the band patterns exhibited by Hind II + III and Eco RI digests (Fig.4); this is an important conclusion, since Hae and Hpa patterns are compatible with very extensive translocations, since these enzymes unlink genetic units; 2) recombination events are likely to affect different genome units, in view of the well-proven intergenomic recombination in diploids issued from AxB crosses (ref.8; fig. 6); this process occurs at a very high rate compared to point mutations[8]; 3) recombination events may lead to petite mutants if they cause deletions of genes indispensable for the respiratory function.

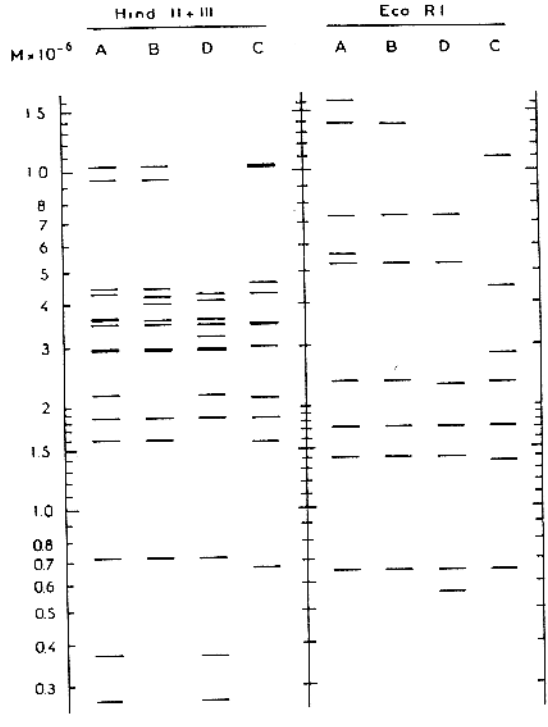

Fig. 4

Scheme of the band patterns obtained with Hind II + III and Eco RI digests of mitochondrial DNAs on 0.8 % agarose and 0.5 % agarose-3 % polyacrylamide. The top bands of Hind II + III digest of DNA from strain D and of Eco RI digests of DNAs from strains B,D and C are missing.

General implications. The conclusion that, in an interspersed system of genes and internally repetitive spacers, evolution goes about essentially by recombination, this process being several orders of magnitude faster than point mutation, is very interesting because it may apply to the nuclear genome of eukaryotes which is also made up of genes and interspersed repetitive sequences. It is conceivable that recombination processes taking place at a high rate at such sequences have played a very important role in the evolution of eukaryotes. A key feature of evolution by recombination at interspersed repetitive sequences is that it can generally affect the genome essentially by translocations, inversions, duplications and deletions of genes whereas it is very conservative as far as gene nucleotide sequences are concerned ; only the much slower process of point mutation is operative on these. A major difference between prokaryotes and eukaryotes might precisely be that eukaryotes developed an interspersed system of repetitive sequences and this allowed them to evolve much more rapidly than prokaryotes.

THE ORGANIZATION OF THE MITOCHONDRIAL GENOME IN CYTOPLASMIC PETITE MUTANTS.

Introduction. Several years ago, it was demonstrated[22,23] that the mitochondrial DNAs from two independent, acriflavine-induced petite mutants had a greatly altered base composition (G+C = 4 %) compared to the DNAs from their parent wild-type cells (G+C = 18 %). Subsequent investigations[1,2] showed that the DNAs from three spontaneous petite mutants 1) had G+C contents in the 16 %-13 % range ; 2) had multimodal melting profile exhibiting the main low-melting component corresponding to dAT:dAT and dA:dT sequences, but lacking, to different degrees, high-melting components ; 3) renatured very rapidly.

On the basis of these data and of what was known on the structure of mitochondrial DNA from wild-type cells, it was proposed [24,4,6] that petite mutants had defective genomes in which mitochondrial genes had been deleted as a result of internal recombinations in the A+T-rich stretches. The high frequency of the spontaneous petite mutation was considered to be the result of high recombination rates, the latter being due to the high level of sequence homology in the A+T-rich stretches.

More recently, the organization of the mitochondrial genomes from several spontaneous petite mutants has been investigated again using the same approaches applied to the study of the mitochondrial genome from wild-type cells. Physical and chemical analyses and the use of enzymatic degradations by spleen acid DNase and by micrococcal nuclease has demonstrated that the basic organization of the petite genome is characterized, like that of the wild-type genome, by an interspersion of A+T-rich stretches and G+C-rich stretches.
The most interesting results came, however, from the use of restriction enzyme degradations. We will briefly describe, here, the results concerning one particular case because of its general interest.

The mitochondrial genome of strain a_1^*. The Hae and Hpa fragment patterns of the mitochondrial DNA from spontaneous petite strain a_1^* (ref. 7) showed the following features (Plate I) : 1) practically all the bands had mobilities identical to those of the DNA from the parent strain A ; only 2 Hpa and 4 Hae bands had different mobilities ; 2) about one third of the bands (34 Hpa and 32 Hae bands) of the DNA from strain A were missing ; 3) a certain number of bands in the petite DNA had higher intensities than the corresponding bands in the wild-type DNA.

These findings not only provide a direct evidence for the deletion and amplification phenomena characterizing the petite mutation, but lead in a straightforward way to an important new conclusion, namely that these phenomena concern segments of wild-type DNA having Hae or Hpa sites at both their ends.

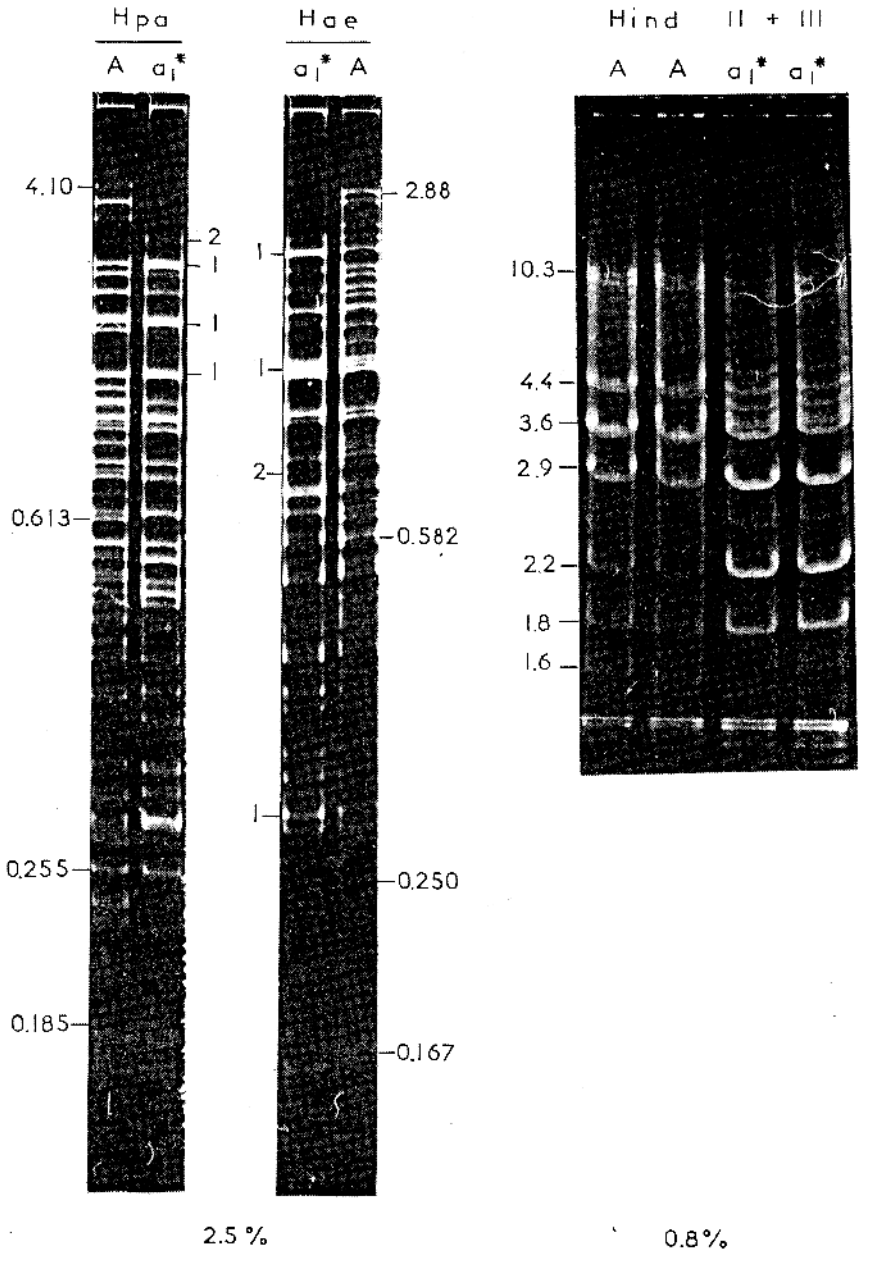

Plate I

Electrophoretic patterns of Hae, Hpa, and Hind II + III digests of mitochondrial DNAs from strains A and a_1^*. The molecular weights, in millions, corresponding to some bands are indicated ; 1 and 2 indicate reinforced and new bands.

Recalling that these are clustered at the border of subsequent genetic units, an inescapable conclusion is that most deletions and amplifications concern individual genetic units or series of subsequent genetic units. Unequal crossing-over events at (Hae, Hpa) site clusters are likely to be responsible for these phenomena.

The fact that only 2 Hpa bands out of 61 and 4 Hae bands out of 53 are "new bands" in the petite DNA, namely bands having a mobility different from those of wild-type DNA, suggests that only rarely the crossing-over phenomena occur elsewhere than in the (Hae, Hpa) site clusters, a likely localization in this case being the spacers (see above). In sharp contrast, 8 "new bands" out of 17, are seen in the Hind II + III pattern of petite DNA (Plate I). Since the sum of the molecular weights of all Hind II + III bands (regardless of their multiplicity) is 70 millions, more than twice that of all Hae or Hpa bands, 30 millions, this result suggests that all or most of Hind II + III "new bands" contain tandem repeats of sequences present in the wild-type DNA ; such tandem repeats obviously lack Hind II + III sites, yet contain Hae and Hpa sites and lead, upon digestion with the latter enzymes, to the production of reinforced bands.

It should be noted that petite strain a_1^* is heterogeneous in terms of mitochondrial genome, as indicated by the different suppressiveness of ten clones issued from it. This means that all deletion and amplification phenomena are seen at a population level, but does not modify the general conclusions drawn above.

Recombination phenomena taking advantage of the sequence homology existing in the mitochondrial genome of yeast, seem to underlie both the divergence of wild-type genome and the petite mutation. Crossing over events appear to occur essentially in the spacers in the first case and preferentially in the G+C-rich segments in the second one.

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