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# Molecular genetics of yeast mitochondria

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I would like to report at this Symposium recent results on a number of problems concerning the mitochondrial genome of yeast, which have been studied using as major tools two restriction enzymes. A detailed presentation of these data will be given elsewhere (Prunell et al.,1975 ; Prunell and Bernardi,1975 ; Fonty et al.,1975).

As an introduction to the problems to be discussed here, it is appropriate to mention our earlier results on the physical organization of wild-type S.cerevisiae m-DNA. The investigations we did along this line (Bernardi et al.,1970 ; Bernardi and Timasheff,1970) immediately revealed that yeast mitochondrial DNA is characterized by two particular features.

The first one is that a number of properties, like buoyant density, $T_m$, $[\alpha]_{290}$, elution molarity from hydroxyapatite, silver binding, terminal nucleotides released by DNases are "anomalous" compared to what could be expected for a bacterial DNA having the same base composition. The origin of such "anomalies" does not reside in chemically modified nucleotides, which we had shown not to exist in yeast mitochondrial DNA (at least to a level high enough to account for the "anomalies"), but in the deviation of nucleotide sequences from randomness. In fact, all the properties under consideration are sequence-dependent. In bacterial DNAs,where short nucleotide sequences are essentially random (Josse et al., 1961), the sequence-dependence is not apparent because of the averaging out of the contributions from a very large number of random sequences ; for this reason the properties we are examining now have been usually considered as composition-dependent. In DNAs containing short repetitive sequences (both synthetic and natural), in contrast, the sequence-dependence is quite evident, as we have shown for mammalian satellite DNAs ( Corneo et al.,1968). In other words the "anomalies" of yeast mitochondrial DNA point to the presence of short repetitive sequences in it.

The second characteristic feature is a striking compositional heterogeneity. In sharp contrast with repetitive DNAs which melt in an extremely cooperative way because of their high compositional homogeneity, yeast mitochondrial DNA exhibits a most complex melting pattern. The differential melting curve (fig. 1)

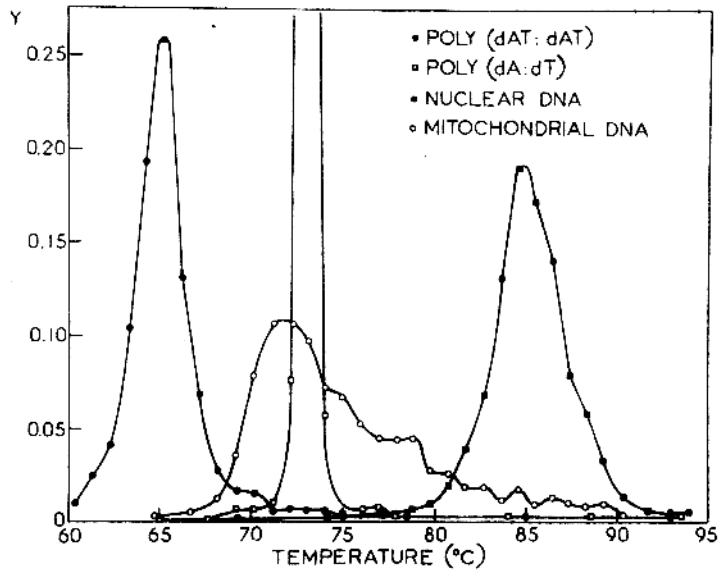



Fig. 1 Differential melting curves obtained with poly(dAT:dAT) ( ● ),poly(dA:dT) ( □ ),mitochondrial DNA from strain B ( o ),and nuclear DNA from strain B ( ■ ).The ordinate indicates the increment in relative absorbance per degree : $Y = \frac{A_{t_1} - A_{t_2}}{A_{100} - A_{25}} / (t_1 - t_2)$, where $A_{t_1}, A_{t_2}, A_{100}, A_{25}$ are absorbance measured at temperature $t_1$, $t_2$, 100°C and 25°C, respectively.The abscissa values are equal to $t_1 + t_2 / 2$. $Y_{max}$ of poly(dA:dT) had a value of 0.71 (Bernardi et al., 1970).

is characterized by : 1) a gaussian component which represents about half of the DNA ; this must be extremely rich in A+T,because of its very low $T_m$, and must contain both alternating and non-alternating AT sequences, because of its circular dichroism spectrum, and 2) by a number of discrete melting components covering an extremely wide range of G+C levels. These two sorts of compo-

nents are intimately interspersed with each other, as shown by the fact that mitochondrial DNA having a molecular weight as low as $1.2.10^6$ (versus the $50.10^6$ size of the intact genome, see below) still show a unimodal, symmetrical band in CsCl density gradients (Bernardi et al.,1970).

At this stage of the game, it is inescapable to think of the mitochondrial genome of yeast in terms of a system in which very A+T-rich stretches are interspersed with stretches having higher levels of G+C (fig. 2). Subsequent investigations in our laboratory

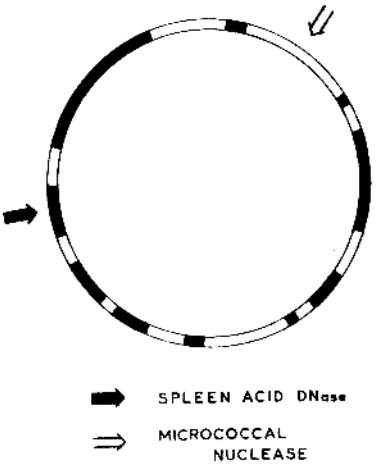



Fig. 2 A scheme of our working model for the organization of yeast mitochondrial DNA and of the experimental approaches used to test it.Black stretches correspond to the G+C-rich regions, white stretches to the A+T-rich region.Spleen acid DNase splits mitochondrial DNA with a slight preference for the G+C-rich regions ; micrococcal nuclease splits mitochondrial DNA with a very high specificity for the A+T-rich regions (Prunell and Bernardi, 1974).

aimed at checking the validity of the interspersion model and at better defining the organization of the mitochondrial genome of yeast. Three approaches were used : 1) degradation of mitochondrial DNA with spleen acid DNase, an enzyme which preferentially breaks G+C-rich sequences ; 2) degradation with micrococcal nuclease, an enzyme which breaks A+T-rich sequences with a high selectivity, and 3) pyrimidine tract analysis.

Spleen acid DNase degradation combined with chromatography of the fragments on hydroxyapatite led to the preparation of short fragments as rich as 27 % in G+C (versus the 18 % of the total DNA) and particularly of fragments having a molecular weight of $0.24.10^6$ and a G+C content of only 10 % ; these were recovered in a yield of 12 % and shown to melt in a very narrow temperature range and to be responsible for the "anomalous" properties. These results (Bernardi et al.,1972 ; Piperno et al.,1972) provided the first direct evidence for the interspersion model (Table I).

| Spleen acid DNase | Yield | $M_w$ | G+C |
|---|---|---|---|
| A+T-rich fragments isolated on HAP | 12 % | $2.4.10^5$ | 10.4 % |
| Micrococcal nuclease | | | |
| Material derived from : | | | |
| A+T-rich segments | 50 % | oligonucleotides | <5 % |
| G+C-rich segments | 50 % | $1.6.10^5$ | 32 % |
| | (10 % | $0.4.10^5$ | 65 %) |

Table I Results of enzymatic degradation of yeast mitochondrial DNA.

Micrococcal nuclease digestion at 6° or at the melting temperature combined with separation by gel filtration of the large G+C-rich fragments from the short oligonucleotides derived from the spacers revealed that spacers on one hand and genes with their regulatory elements on the other were present in equal amounts in mitochondrial DNA ; the G+C-level of the former was lower than 5 %, whereas the average G+C level of the latter was about 32 %. Very interestingly, fragments of 40,000 daltons as high as 65 % in G+C could be prepared in a yield of 10 % by this method. These data (Prunell and Bernardi,1974) gave a quantitative estimation of the relative amounts of genes and spacers in the mitochondrial genome and of their G+C levels (Table I ).

The pyrimidine isostich analysis (Ehrlich et al.,1972) confirmed our previous conclusion (Bernardi and Timasheff,1970) that the A+T-rich spacers are basically formed by intermingled short alternating and non-alternating AT sequences. Some isostich components, $T_1$, $T_2$ and $T_3$ are very frequent, since they appear in every 3, 8 and 25 base pairs, respectively. This suggests that short runs of 10 to 30 nucleotides having the same or a very similar base sequence may often exist in the A+T-rich spacers.

To summarize our results on the organization of mitochondrial genome of yeast the major conclusions arrived at were 1) the demonstration that this genome is an interspersed system of genes, with their regulatory elements, and spacers ; 2) the estimation of the amount (50 % each), the average length (over $1.5.10^5$) and the average G+C level (32 % for the genes, <5 % for the spacers) of these two elements ; 3) the indication that the A+T-rich spacers are formed by short repetitive sequences.

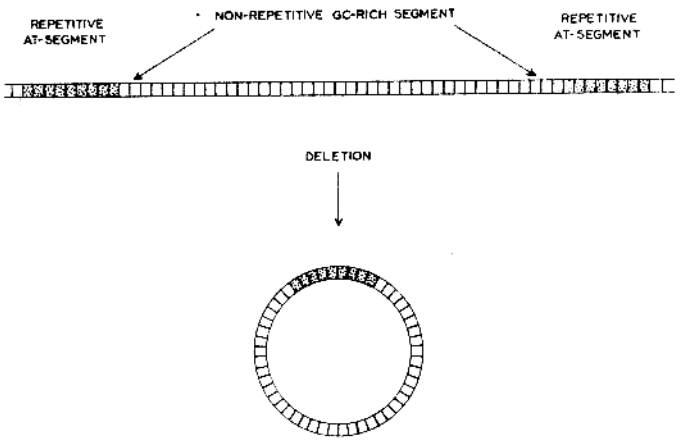



Fig. 3 The deletion model.

The interest of this interspersed gene and spacer model, is that it not only accounted for all the data available at that time, but also suggested a mechanism for the "petite" mutation[(a)] (fig. 3). In fact, if the spacers are internally repetitive and contain short identical or quasi-identical sequences, this means that the yeast mitochondrial genome has an extremely high level of sequence homology and that deletions can easily take place by internal crossing-overs which eliminate genes essential for the

(a) This model was first presented in a lecture at the Karolinska Institute, Stockholm, in October 1969. It was subsequently presented at a number of Meetings and mentioned by Piperno et al. (1972) and by Prunell and Bernardi (1974). A very similar model has been developed by Clark-Walker and Miklos (1974).

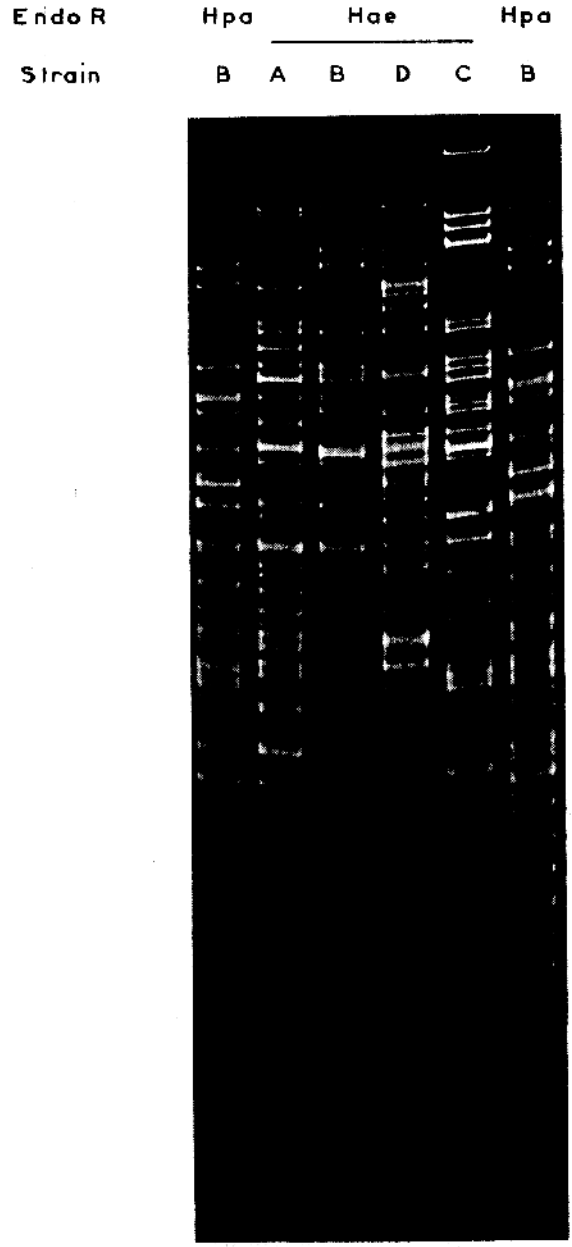
synthesis of mitochondrial respiratory enzymes. This simple model accounts for the very high frequency of spontaneous "petite" mutation in terms of the very high level of sequence homology in mitochondrial DNA and for the fact that mitochondrial DNAs from most "petites" have either the same or a lower G+C content than the parent wild-type strain. The deletion model was another addition to an already rather long series of hypothesis put forward to explain the "petite" mutation, but, unlike its predecessors, it was there to stay. The only modification brought in by further work was that deletions are accompanied by amplifications, a phenomenon indicating that the recombinational events underlying the "mutation" may involve the spacers of different genome units (see below).

Our more recent investigations dealt with a number of problems such as the unit size, the homogeneity and the evolution of the mitochondrial genome of wild-type yeast. All these problems were essentially investigated by studying in detail the fragment patterns obtained with Hae III and Hpa II, two restriction enzymes, from the mitochondrial DNAs of one S.carlsbergensis (C) and three S.cerevisiae (A,B,D) strains. The sequences split by these enzymes are GG↓CC and C↓CGG, respectively.

a) Unit size and homogeneity of the genome

The number of fragments varied from 71 to 116 according to the DNA and the enzyme used. The molecular weight of fragments ranged from $4.10^6$ to $10.10^3$. A satisfactory separation of the fragments could only be achieved on a series of long slabs of 2 to 6 % polyacrylamide plus 0.5 % agarose. Plate I is given as an example of the fragment patterns obtained. The scheme corresponding to the 2 % gel is shown in fig. 4. Since five gels of different porosity were used to resolve most of the fragments, the first problem was to identify corresponding bands, as observed on different gels, in order to establish the necessary overlaps. This could be easily done on the basis of the molecular weights of the fragments and with the help of characteristic features in the band patterns, such as fragment clusters and multiple bands.

Plate I Electrophoresis patterns obtained on a 2 % polyacrylamide - 0.5 % agarose gel. The enzyme used and the DNA source are indicated.




2%

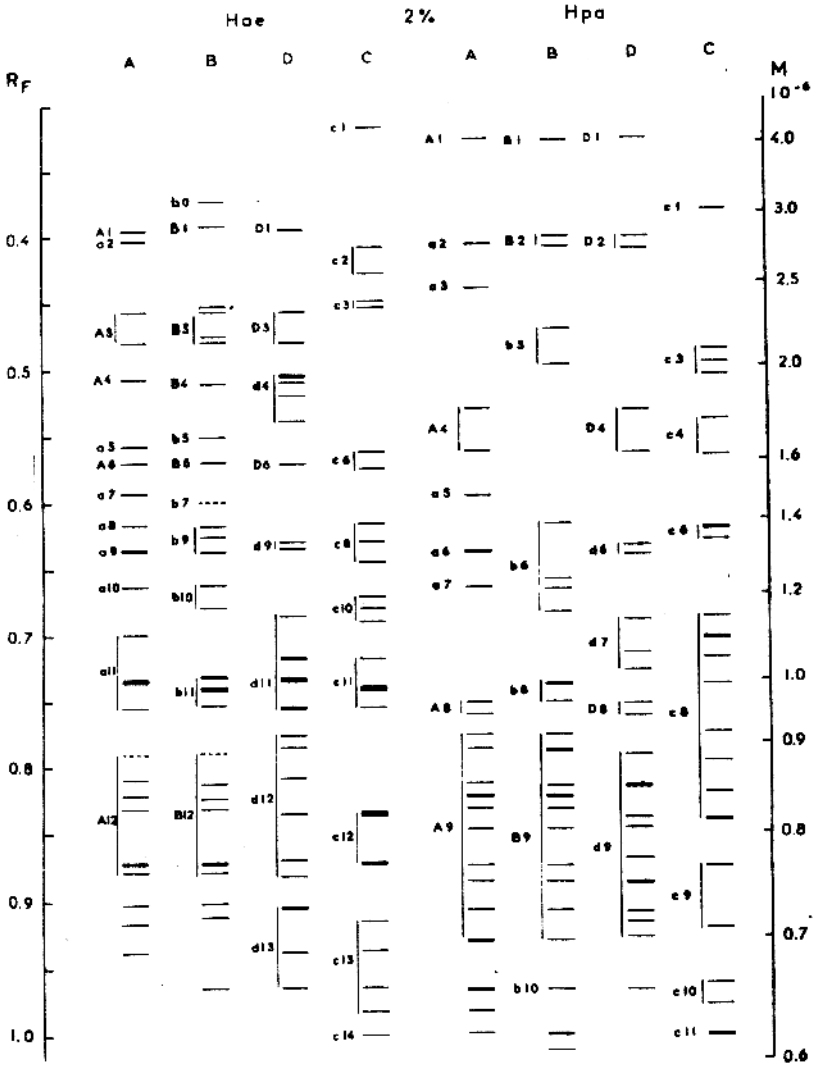



Fig. 4 Scheme of the band patterns obtained on the 2% gel. The relative mobilities and molecular weights of the fragments are indicated. Bands containing one fragment, two fragments, and more than two (3 or 4) fragments are indicated by different thicknesses; faint bands are indicated by dashed lines. Corresponding bands, or band clusters, as seen on different gels, are indicated with the same letter-number combination. Capital letters indicate bands exhibiting interstrain homology, low-case letters all other bands. The homology between $B2_2$, $D2_2$ and $a_2$ of the Hpa pattern and between $A_4$, $B_4$ and $d4_2$ of the Hae pattern were not indicated.

An inspection of Plate I reveals a number of bands showing higher intensities as well as a few bands showing lower intensities than the neighboring ones. Multiple bands could be shown to derive in the majority of the cases from lack of resolution. Faint bands were shown to result from a very specific degradation of the starting DNAs by an enzyme localized in all likeliness in yeast mitochondria.

Genome unit sizes could be calculated by adding up the molecular weights of all fragments (Table II). These ranged from 52 to $55.10^6$ for the three S.cerevisiae strains, whereas a value of $50.10^6$ was found for the S.carlsbergensis strain. The quoted values refer to results derived from Hae digests ;these are considered to be more reliable than those derived from Hpa digests because of the smaller number of bands, the smaller contribution of band multiplicity and the lack of formation of small fragments not observable on gels. These values are in general agreement with the physical size of circular twisted yeast mitochondrial DNA, as observed by Hollenberg et al. (1970)and indicate that the mitochondrial DNA of a given yeast strain is highly homogeneous in terms of nucleotide sequences.

| | | Strains | | | |
|---|---|---|---|---|---|
| | Enzyme | A | B | D | C |
| Number of fragments | Hae | 84 | 81 | 83 | 71 |
| | Hpa | 116 | 107 | 113 | 107 |
| Genome unit size($\Sigma M_i.10^{-6}$) | Hae | 52 | 55 | 52 | 50 |
| | Hpa | 55 | 52 | 52 | 49 |

Table II Restriction fragments and unit size of four yeast mitochondrial genomes.

b) Homology and divergence of yeast mitochondrial genomes

i. Sequence homology. Qualitative indications of sequence homology in the mitochondrial DNAs from all strains investigated come from the following findings concerning the fragments released by the two restriction enzymes used : 1) The approximately equal number of fragments and therefore of restriction sites (Table II);

2) the similar size distribution of the restriction fragments (fig. 5) ; 3) the similar base composition of fragments in DNAs

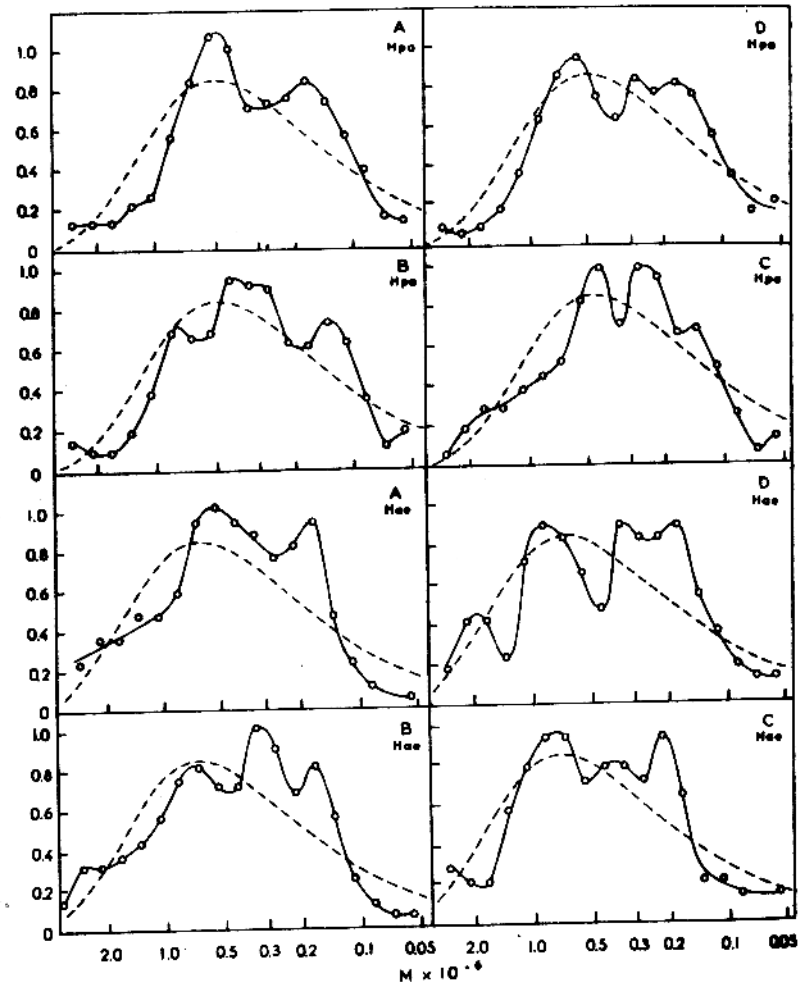



Fig. 5 Semilogarithmic plot of n versus M for the restriction enzyme fragments obtained from mitochondrial DNAs.
$n = \frac{1}{N_o} \cdot \frac{\Delta N}{\Delta \log M}$, value $N_o$ is the total number of fragments, $\Delta N$ the number of fragments between log M and logM + $\Delta$ log M. Intervals overlapping each other by 50 % were taken in order to increase the number of points. $\Delta$ logM values equal to 0.2 were used; 0.1 and 0.4 values gave the same results. The dashed line correspond to random distributions for the experimental number of fragments of each digest (from Prunell et al., 1975).

from strains A and C (Prunell and Bernardi, 1975) ; 4) the same bias in the number of Hpa versus Hae sites, the former being largely predominant (Table II ; see further comments in Prunell and Bernardi, 1975). It should be noted that homology among the mitochondrial DNAs from S.cerevisiae strains is larger than between them and S.carlsbergensis as judged by all possible criteria : number of sites, fragment homology, $M_w/M_n$ ratio of fragments.

All these findings point to a remarkable conservation of the genes and their regulatory elements in all the strains under consideration, if account is taken of the distribution and clustering of restriction sites (Prunell and Bernardi, 1975).

ii. Origin of differences in fragment patterns in the mitochondrial DNAs from different strains. Two, not mutually exclusive, possibilities exist : 1) mutations (point mutations, insertions or deletions) and/or modifications (methylation) at the sequences split by the restriction enzymes ; and/or 2) deletions and/or additions between such sites. It is of obvious interest to judge the relative importance of these mechanisms.

Since the number and the size distribution of fragments is very similar in different strains, the disappearance of "old" restriction sites should be accompanied by the formation (by point mutations, deletions, insertions, demethylation) of an approximately equal number of "new" restriction sites ; in addition, these should have a very similar distribution on the genome. This explanation clearly is so unlikely that it can definitely be ruled out as a mechanism counting for more than a very small percentage of the changes observed. Two additional problems with a point mutation mechanism arise from : 1) the clustering of restriction sites (Prunell and Bernardi, 1975), which requires point mutations at several neighboring sites to cause changes in the patterns ; and 2) the inacceptably high divergence time (mutation rate of $10^{-7}$ per generation) between strains like S.carlsbergensis and S.cerevisiae, which show 100 % homology in their nuclear and mitochondrial genomes by DNA-DNA hybridization (Groot et al.,1975).

It is evident, therefore, that the predominant mechanism underlying the changes in restriction site distributions is one involving deletions and/or additions between such sites. This allows the number of sites to be conserved and the distribution of the restriction fragments to be affected much less than if

the site number greatly changed. The deletion/addition mechanism is supported by two findings : 1) the differences in the mitochondrial genome unit sizes observed among S.cerevisiae strains and, more so, between S.cerevisiae and S.carlsbergensis (Table II); such differences are quite remarkable in view of the internal compensations between additions and deletions ; 2) deletions and additions very clearly underlie the cytoplasmic "petite" mutation (Bernardi et al.,1975 ; and paper in preparation); this suggests that the same mechanism is responsible for the "petite" mutation and for mutations in which the wild-type phenotype is preserved because gene functions essential for respiratory competence are not affected (Plate II).

iii. Localization of deletions and additions. Deletions and additions certainly affect essentially the A+T-rich spacers, which make up to 50 % of mitochondrial DNA (Prunell and Bernardi,1974) and do not contain any restriction sites (Prunell and Bernardi, 1975). This explanation is the only one resolving the apparent conflict between : 1) the 100 % homology found by DNA-DNA hybridization of mitochondrial DNAs from S.cerevisiae and S.carlsbergensis, which is associated with an absence of mismatch in the hybrids as judged by their melting curves (Groot et al.,1975) and 2) the almost complete absence of homologous restriction fragments found in the present work. In fact, base pairing in the DNA-DNA hybrids can be expected to take place first in the genes and their regulatory elements, because of their higher G+C level ; differences in length of the spacers due to deletions and/or additions are not very important since longer spacers could undergo looping out; moreover some extent of base pairing by foldback could take place in the loops, if palindromic sequences exist in the spacers. An additional observation pointing in the same direction is that fragment homology between S.cerevisiae and S.carlsbergensis was seen in the smallest fragments, which do not contain spacers (Prunell and Bernardi,1975). Finally, it is interesting to note that the mitochondrial DNAs of four yeasts which in all likeliness

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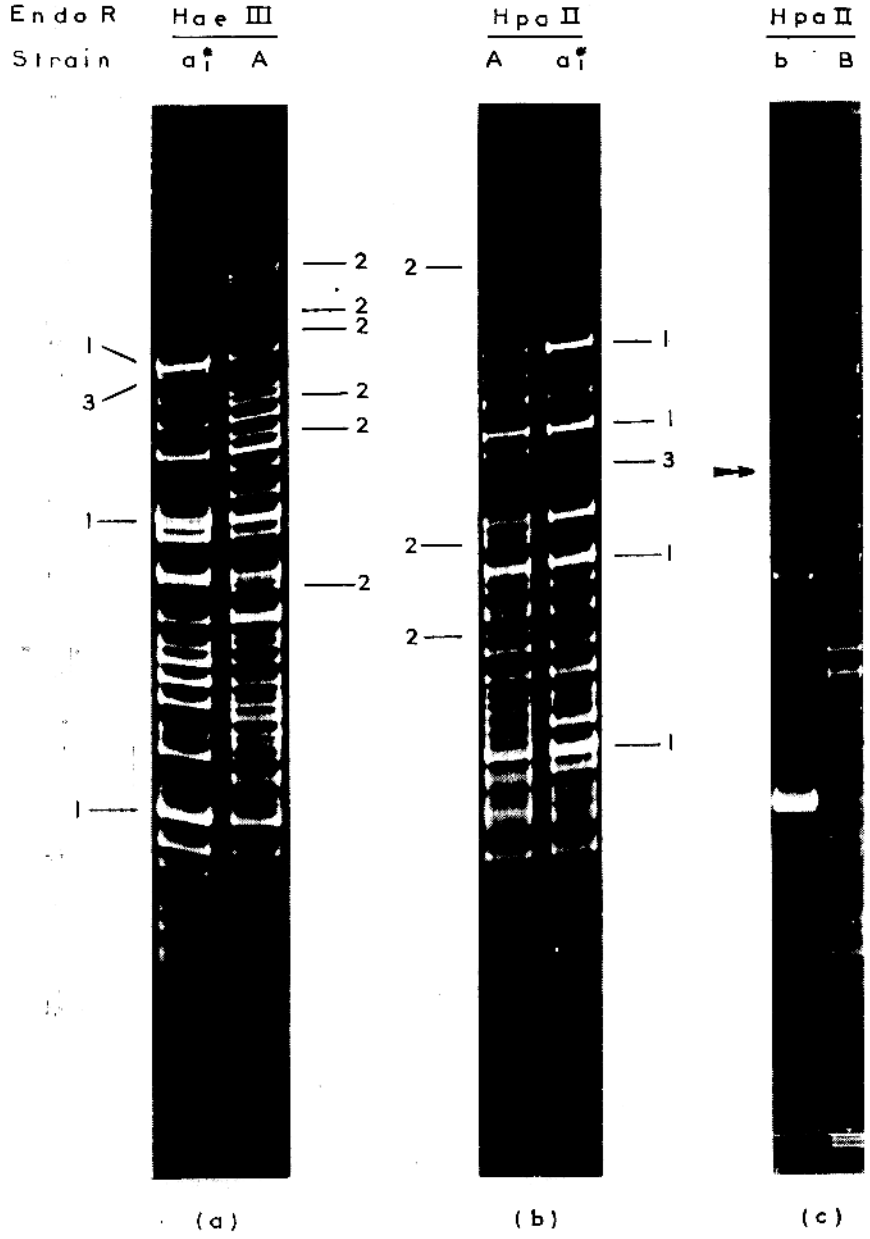
Plate II Electrophoretic patterns obtained on a 2 % polyacrylamide - 0.5 % agarose gel. The enzyme used and the DNA source are indicated.
(a) and (b) : 1,2,3 indicate bands which are reinforced, missing or novel, respectively, compared to the parent wild-type.
(c) : the arrow indicates a very faint band.


EndoR
Strain
Hae III
af A
Hpa II
A af
Hpa II
b B
1
3
1
1
2
2
2
2
2
2
2
2
1
1
3
1
1
(a)
(b)
(c)

do not contain A+T-rich spacers, since their physical genome size is comprised between 1/2 and 1/4 of that of Saccharomyces and their G+C content is in the 32-42 % versus the 17-18 % of Saccharomyces, are "petite negative" (O'Connor et al.,1975).

iv. Mechanism underlying deletions and additions. The most likely basis for the deletion/addition mechanism is given by unequal crossing-overs events taking place in the spacers of different genome units. Such events can obviously lead to unequal exchanges of genome segments and therefore to changes in the location of restriction sites on the genome units which will eventually segregate. It is clear that such mechanism can lead to the formation of defective genomes and give rise to "petite" mutants as well. In other terms, we consider here recombination as the basic phenomenon underlying both mitochondrial genome evolution and the "petite" mutation, which is a frequent accident in this process. This proposal is simply an extension of the deletion model by internal recombination at the spacers previously proposed. It should be mentioned that a very strong evidence in favor of this mechanism is given by the changes in restriction fragment patterns which we have demonstrated in diploids issued from zygotes of strains A and B (Fonty et al., 1975). The Hpa band patterns (fig.6) of 6 such diploids (1.1, 2.1, 4.1, 5.1, 8.1,9.1) were characterized by bands identical to those of either parents and by a number of new bands, and by genome unit sizes differing by only a few percent from those of the parents. An important feature of the recombination events so observed for the first time is their very high rate, since the pattern changes were observed after as few as 20 generations. Very interestingly, three (3.1, 6.1, 7.1) diploids showed banding patterns identical to that of one and the same parental strain, B. This may mean either that recombinational events were not detectable because involving homologous genome fragments or, more likely, that mitochondrial DNA segregation in the buds took place before recombination. In one particular case, DNA from two subclones of clone 5 were also examined and found to be identical in banding pattern (5.1,5.2).

v. General implications. In summary, what has been shown in the present work is 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. This conclusion is very

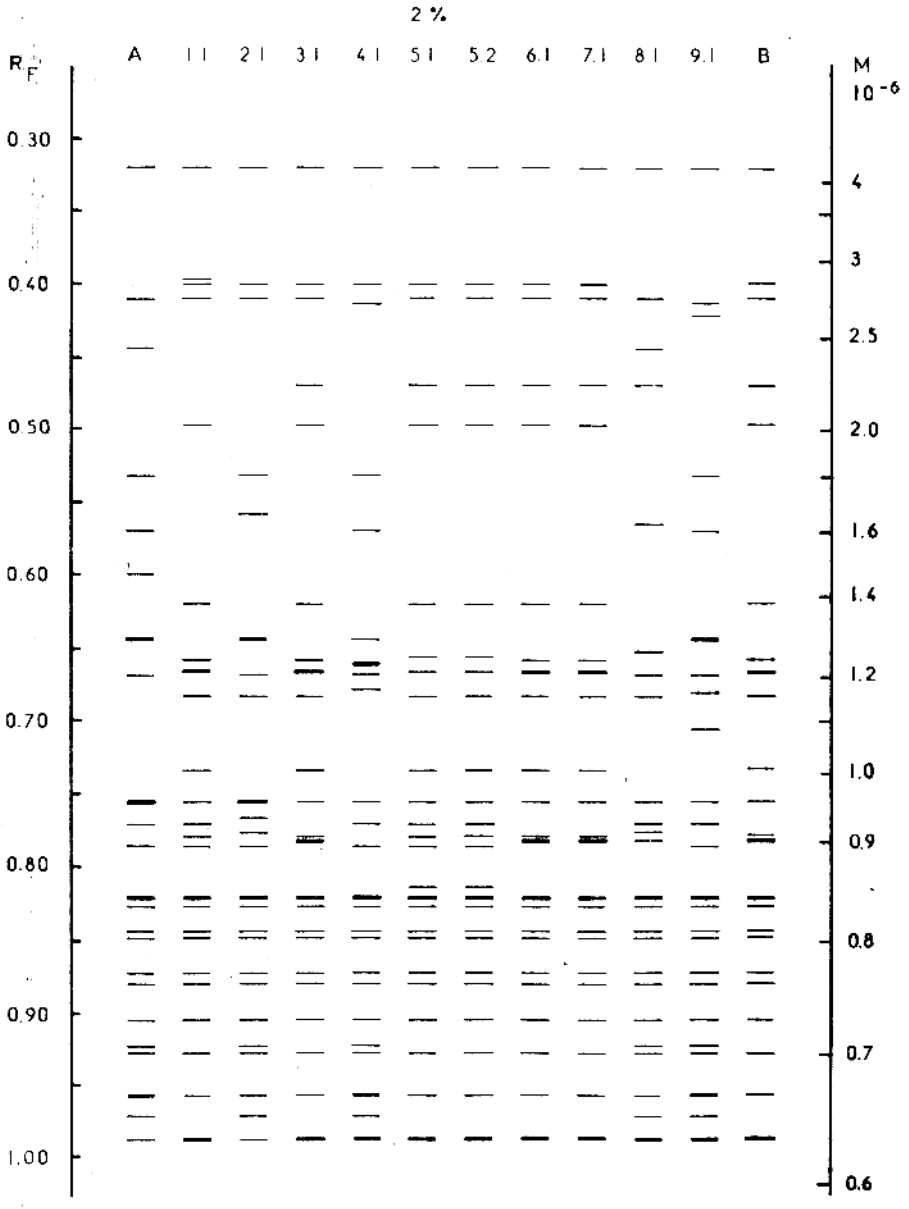



Fig. 6 Scheme of the band patterns obtained with mitochondrial DNAs from strains A, B and a number of diploids from zygotes issued from crosses of these strains.

interesting because it may apply to the 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.

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