J. Mol. Biol. (1978) **119**, 213–235

The Mitochondrial Genome of Wild-type Yeast Cells

VII. †Recombination in crosses

GODELEINE FONTY, REGINA GOURSOT, DAVID WILKIE[1] AND GIORGIO BERNARDI‡

Laboratoire de Génétique Moléculaire, Institut de Biologie Moléculaire Paris 5°, France and [1]University College, Department of Botany and Microbiology, London, England

(*Received 14 June 1977, and in revised form, 6 October 1977*)

Two *Saccharomyces cerevisiae* wild-type strains were crossed, and 26 diploid clones were obtained from (1) mass mating; (2) individual buds in zygote lineages; (3) individual zygotes. The mitochondrial DNAs from these diploids were investigated in their recombination and segregation by analyzing their restriction fragment patterns.

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 *Eco*RI and *Hin*dII + III fragment patterns of parental types, two strong indications that both the gene complement and the gene order were very largely preserved in the progeny.

Fragment patterns produced by *Hpa*II and *Hae*III were characterized by (1) fragments originating from the DNAs of both parents; and (2) new fragments, namely fragments absent in either parent. The new fragments appear to arise from unequal crossing-over events occurring in the spacers of allelic parental genetic units and usually have preferential localizations in the genome.

These results provide the first evidence for physical recombinations of mitochondrial DNA in crosses of wild-type yeast cells, indicate that recombination is very frequent in crosses, and shed some light on mitochondrial segregation. They also have interesting implications for recombination phenomena in interspersed systems of unique and repetitive nucleotide sequences.

1. Introduction

The mitochondrial genome of wild-type yeast cells is made up of approximately equal amounts of interspersed G+C-rich (G+C = 32%) and A+T-rich (G+C < 5%) stretches having an average length of 500 to 600 base-pairs. It has been suggested that the A+T-rich stretches, internally repetitive in sequence (Ehrlich *et al.*, 1972), are spacers separating the G+C-rich stretches, which correspond to the mitochondrial genes with their regulatory elements (Prunell & Bernardi, 1974).

Recent investigations have shown that, in general, G+C-rich stretches start with a cluster (having an average length of 35 base-pairs) of C-C-G-G and G-G-C-C sequences and a contiguous larger cluster of G+C-rich sequences not containing those tetranucleotides; it has been suggested that the (C-C-G-G, G-G-C-C) clusters and the G+C-

† Paper VI in this series is Prunell & Bernardi (1977).
‡ To whom all correspondence should be addressed.

rich clusters are regulatory sequences, and that the mitochondrial genome of yeast is made up of about 60 (the number of (C-C-G-G, G-G-C-C) clusters) genetic units formed by a regulatory sequence, a gene and a spacer (Prunell & Bernardi, 1977). Restriction enzymes, like *Hpa*II and *Hae*III†, splitting C-C-G-G or G-G-C-C, respectively, essentially break down the mitochondrial genome of yeast into its constituent genetic units.

Other experiments have demonstrated that large differences in the *Hae* and *Hpa* electrophoretic patterns exist among mitochondrial DNAs from different *Saccharomyces cerevisiae* strains. Such differences appear to originate from additions and deletions in the spacers and are accompanied by a large preservation of gene order. Unequal crossing-over events at allelic spacers (namely spacers contiguous to allelic genes) belonging to different genome units seem to be the source of deletions and additions and to underlie the divergence of the mitochondrial genome of yeast (Prunell *et al.*, 1977*a*).

The results just summarized suggested to us that an experimental approach based on the use of restriction fragment patterns could provide an interesting insight into the molecular phenomena underlying recombination of mitochondrial genomes in crosses of yeast cells.

In the present work, we report data on the restriction patterns of mitochondrial DNAs from diploid clones issued from crosses of two haploid strains previously studied. The results obtained (1) provide physical evidence for mitochondrial DNA recombination in wild-type yeast cells, a phenomenon for which genetical evidence was first provided by Thomas & Wilkie (1968); (2) indicate that recombination occurs very frequently in crosses; (3) show that unequal crossing-over events in the allelic spacers of the parental genomes always occur in the recombinant genomes; (4) support the mechanism of genome divergence proposed by Prunell *et al.* (1977*a*); (5) shed some light on mitochondrial segregation; and (6) have some general implications for recombination phenomena in interspersed systems of unique and repetitive nucleotide sequences.

Preliminary reports on the present work were published elsewhere (Bernardi, 1975, 1976).

2. Materials and Methods

(a) *Yeast strains*

The 2 haploid strains used in the crosses, A and B, have been described by Bernardi *et al.* (1970). Strain A, of mating type a, was ω^+; strain B, of mating type α, was ω^- (B. Dujon, personal communication).

(b) *Crossing procedure*

(1) Heavy inocula of the parent strains were placed on YED medium (1% yeast extract, 2% glucose, 2% agar) and incubated overnight at 30°C. Cells were then mixed and re-incubated for 1·5 to 2 h, and the resulting zygotes, (usually comprising about 20% of the mating mixture), were isolated by micromanipulation. Ten isolated zygotes were incubated for about 48 h (corresponding to about 20 cell generations) and the resulting zygotic clones, all capable of sporulation, were sampled, mixed, and plated on a minimal medium. Nine individual diploid colonies, representing subcloned random diploids from the mixture of zygotic clones, were isolated after about 48 h incubation, and grown for

† Restriction endonucleases are indicated here according to Smith & Nathans (1973). *Hpa*II and *Hae*III will be indicated henceforth as *Hpa* and *Hae*, respectively.

48 h. These further subclones (1·1 to 9·1), essentially corresponding to random diploids from a mass mating, were used. The cell cultures used for DNA preparation were about 100 generations apart from the zygotes.

(2) To obtain zygote cell lineages, daughter cells of individual zygotes were isolated by micromanipulation, as they formed on YED medium. Daughter cells and residual zygotes, namely the zygotes from which the early buds had been removed by micromanipulation, were incubated for about 48 h, and resulting colonies isolated, again representing about 20 cell generations. All progeny from zygotes, when subsequently tested, grew on minimal medium and sporulated.

(3) Diploid clones were also obtained from individual zygotes isolated by micromanipulation. In this case, as well as in the previous one, cell cultures used for DNA preparation were 40 generations apart from the zygotes. For further details of these methods, see Wilkie (1973). Figure 1 presents a scheme of the procedures used to obtain the diploid clones.

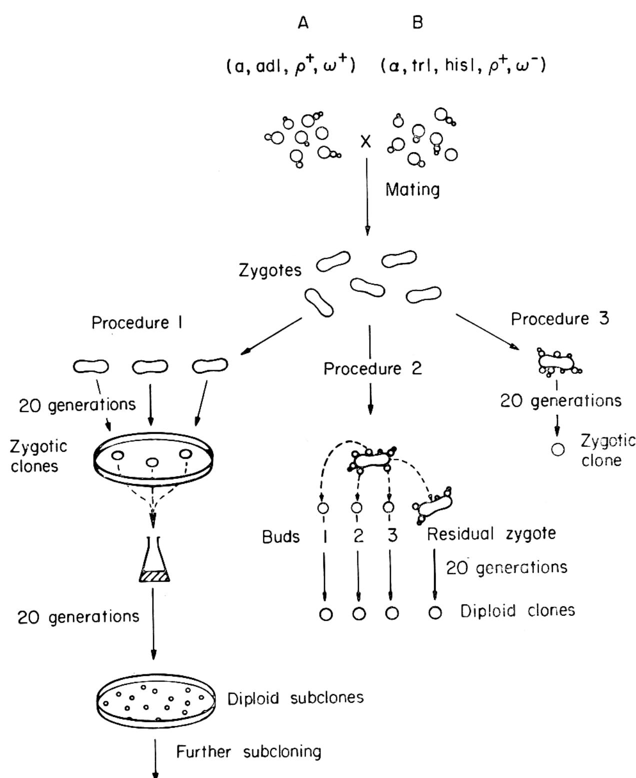

FIG. 1. Scheme of the procedures used to obtain diploid clones from the parent haploid strains (see Materials and Methods). The number of generations separating the cells used for DNA preparation from the zygotes was about 100 in procedure 1, and about 40 in procedures 2 and 3. Furthermore, a sub-cloning process took place in procedure 1.

(c) *Mitochondrial DNA preparation*

The diploid yeast cells obtained as described above were grown in 1% yeast extract, 3% glycerol, 0·5% glucose, 0·01% $(NH_4)_2SO_4$ to mid- or late-log phase, harvested, washed twice with distilled water and once with 0·15 M-NaCl, 0·1 M-EDTA, 4% sodium dodecyl sulphate, pH 9·5. Wet-packed cells (100 g) were then suspended in 2 vol. of this solvent, frozen, thawed (Smith & Halvorson, 1967; Bicknell & Douglas, 1970), and incubated for 10 min at 65°C and 1 h at 37°C with 100 μg/ml of proteinase K (Merck, Darmstadt)

preincubated at 37°C for 2 h in 0·01 M-Tris, pH 7·6. The very viscous lysate so obtained was diluted 3 times with a NaCl solution (final concn 1 M), cooled to 0°C, and centrifuged. The supernatant was precipitated with 1 vol. ethanol. The precipitate was dissolved in 0·05 M-NaCl, 0·28 M-Na phosphate (pH 6·8) and adsorbed batchwise on hydroxylapatite equilibrated with 0·28 M-Na phosphate (pH 6·8). Mitochondrial DNA was eluted with 0·4 M-Na phosphate (pH 6·8). The yield was about 10 A_{260} units/preparation. Sedimentation coefficients of mitochondrial DNA were close to 28 S, corresponding to a molecular weight of about 15×10^6 (Studier, 1965). Compared to procedures previously used in our laboratory (Bernardi *et al.*, 1970, 1972; Prunell *et al.*, 1977*a*), the present one had the advantage of rapidity and higher molecular weight of DNA, and the disadvantage of lower yields, because not all cells were broken down, and of some nuclear DNA contamination; the latter, however, did not interfere with the analysis of restriction patterns, since it only contributed to slightly increasing the background smear. This smear was mainly due (Prunell *et al.*, 1977*a*) to the fact that the original DNA preparations had a molecular weight much lower than the mitochondrial genome size, $\sim 50 \times 10^6$.

All other materials and methods were as described elsewhere (Prunell *et al.*, 1977*a*), except that: (1) gel electrophoresis was performed at room temperature; this led to slight differences in the *Hpa* and *Hae* restriction patterns of the DNAs from parent strains A and B, compared to those previously obtained at 4°C; the origin of such differences has already been discussed (Prunell *et al.*, 1977*a*), and will be further commented upon elsewhere; (2) 3 gels only (0·5% agarose/2% polyacrylamide, 0·5% agarose/3% polyacrylamide, and 6% polyacrylamide), instead of 6 (Prunell *et al.*, 1977*a*), were used to resolve the *Hpa* restriction fragments; (3) *Hin*dII + III patterns were studied on 1% agarose, instead of 0·8% agarose (Prunell *et al.*, 1977*a*). (4) A 0·5% agarose/3% polyacrylamide gel was not used to detect the 2 lowest molecular weight bands ($0{\cdot}37 \times 10^6$ and $0{\cdot}27 \times 10^6$) in the *Hin*dII + III digest of strain A and of the diploid clones showing the A pattern.

The great similarity of the *Hpa* or *Hae* restriction patterns of mitochondrial DNAs from different diploids (Fig. 2) made easy the detection on the large scans (2 m per 40 cm gel) used (Prunell *et al.*, 1977*a*) of even small differences in band mobilities; furthermore, differences were checked in many cases by experiments performed on mixtures of mitochondrial DNAs (Fig. 3). As in previous work, the photographic method of Prunell *et al.* (1977*b*) was used.

3. Results

(a) *Diploid sub-clones from zygotic clones*

The *Eco*RI and *Hin*dII + III restriction patterns of the mitochondrial DNAs obtained from the diploid sub-clones derived from the mixture of the zygotic clones (see Materials and Methods, section (b) (1) and Procedure 1 of Fig. 1) were identical to those exhibited by the B parent, with two exceptions: (a) sub-clone 4.1 showed the *Eco*RI and *Hin*dII + III patterns of parent A; (b) sub-clone 2.1 showed the *Eco*RI pattern of parent B and the *Hin*dII + III pattern of parent A. Clone 9.1 was not examined. Figure 5 shows the *Eco*RI and *Hin*dII + III patterns of parental DNAs.

Figures 2, 6 and 8 show the *Hpa* restriction patterns on agarose/polyacrylamide gels exhibited by the mitochondrial DNAs mentioned above. The main features of the patterns (Figs 6 to 8 and Table 1) are the following. (i) Three out of nine sub-clones (3.1, 6.1, 7.1) showed restriction patterns identical to that of one parent, B; the corresponding patterns are not shown. (ii) All other sub-clones exhibited: (a) bands from both parents and (b) bands differing in mobility or multiplicity from parental bands; these bands (indicated in red in Figs 6 to 8), belong in three classes: (1) single bands differing in mobility from parental bands; these will be called here new bands; (2) double bands corresponding to single parental bands; in this case a new band is

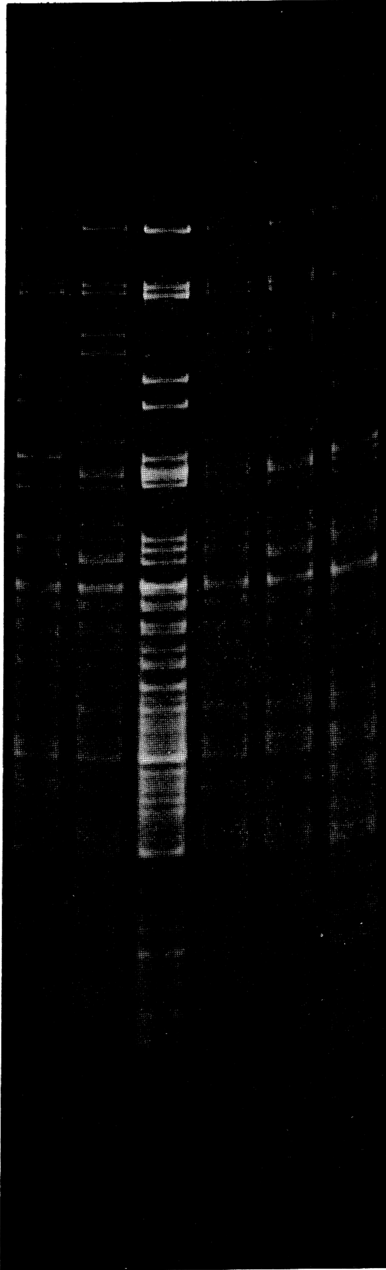

FIG. 2. Electrophoretic pattern on 0·5% agarose/2% polyacrylamide gel of *Hpa* digests of mitochondrial DNAs from 6 diploid clones obtained by procedure 1 of Fig. 1 (see Fig. 6).

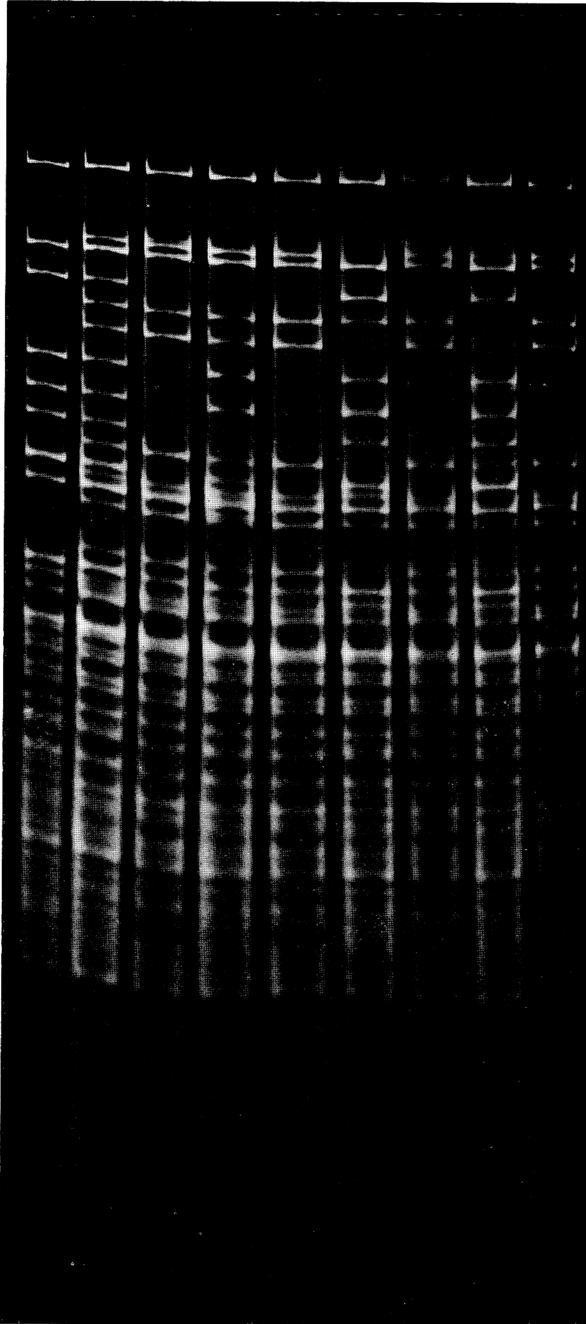

FIG. 3. Electrophoretic pattern on 0·5% agarose/2% polyacrylamide gel of *Hpa* digests of mitochondrial DNAs from the 2 parental strains (A and B) and of mixtures of DNAs from parental strains and from diploid clones (indicated as in Figs 6 and 10) obtained from them.

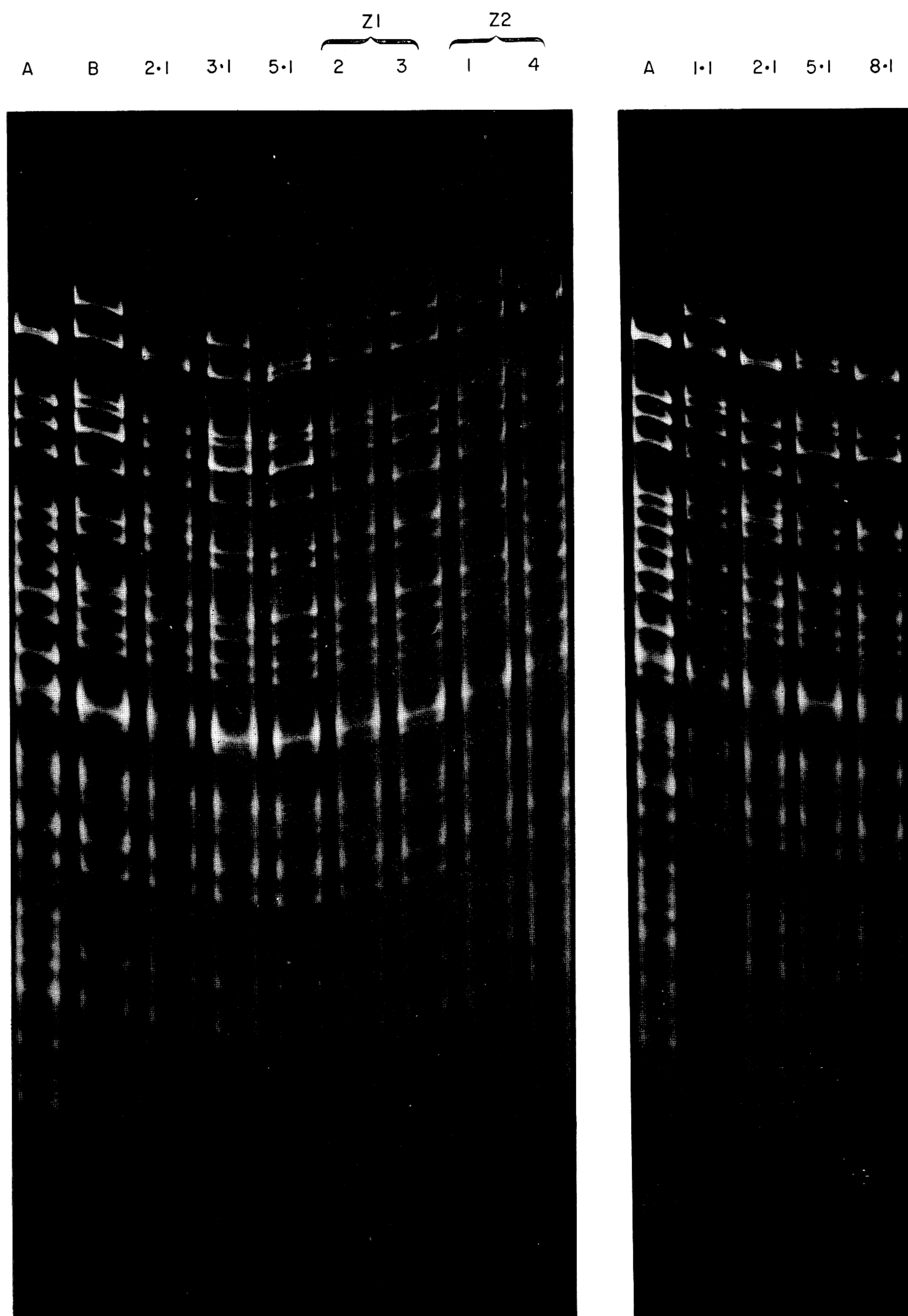

FIG. 4. Electrophoretic patterns on 0·5% agarose/2% polyacrylamide gel of *Hae* digests of mitochondrial DNAs from the 2 parental strains (A and B) and some diploid clones issued from them (see Fig. 13; see also Figs 6 and 10 for the indications of the strains).

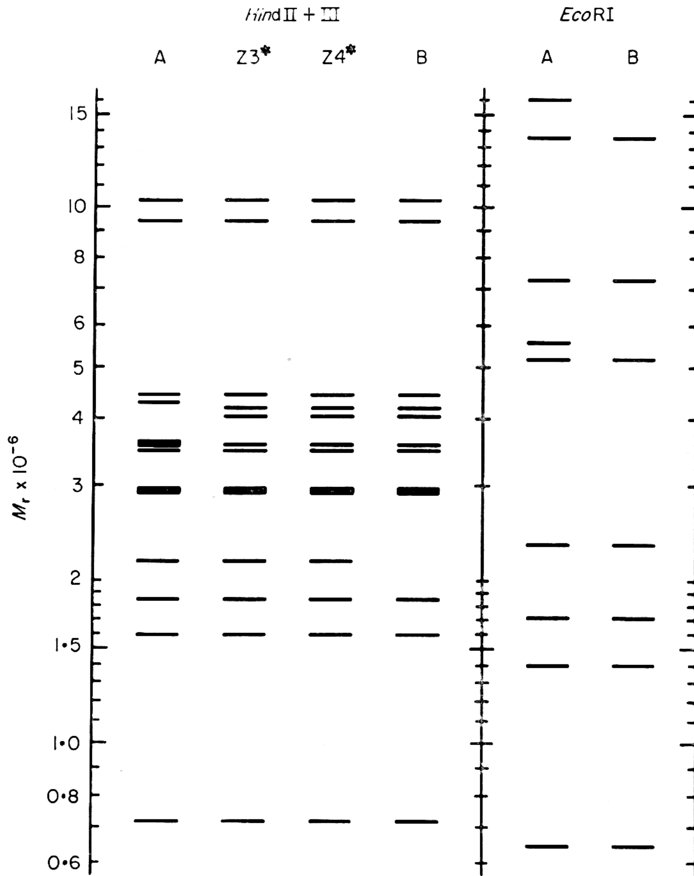

FIG. 5. *Hind*II + III and *Eco*RI band patterns of mitochondrial DNAs from parental strains and zygotic clones Z3* and Z4*. The top *Eco*RI band of mitochondrial DNA from strain B was not seen because of its very poor yield. The 2 lowest molecular weight ($0{\cdot}37 \times 10^6$ and $0{\cdot}27 \times 10^6$) *Hind*II + III bands of DNA from strain A do not appear on this gel.

not resolved from a parental band; (3) single bands corresponding to double parental bands; these usually indicate the disappearance of one parental band. Figure 9 presents a scheme of these different situations and stresses the fact that not all of the red bands of Figures 6 to 8 correspond to new bands. Three important features of new bands are that : (a) they show the same yield as neighboring parental bands; (b) they seem to replace parental bands of rather similar mobility; (c) they often have the same mobility in *Hpa* digests of mitochondrial DNAs arising from different subclones. (iii) Bands common to both parents were always found to maintain the same mobility in the progeny with a single exception (clone 2.1; fragments of molecular weight close to 4×10^5; Fig. 7); here, a parental doublet was changed into a progeny doublet with slightly different mobilities; interestingly, the sum of the molecular weights of the two bands was the same in the parents ($3{\cdot}97 \times 10^5 + 3{\cdot}93 \times 10^5 = 7{\cdot}90 \times 10^5$) and in clone 2.1 ($4{\cdot}02 \times 10^5 + 3{\cdot}88 \times 10^5 = 7{\cdot}90 \times 10^5$). (iv) Two subclones (5.1, 5.2) from the same diploid, 5, showed identical banding patterns; the pattern of clone 5.2 is not shown. (v) Faint bands, namely bands showing a lower yield (10 to 50%) compared to those of the neighboring bands, were absent. (vi) The

TABLE 1

Hpa *digests*†

Strains	No. of bands A	B	AB	New‡	Total	No. of fragments A	B	AB	New‡	Total	Weight of fragments (%) A	B	AB	New	Faint bands (×10⁻⁶)	Genome unit size (×10⁻⁶)§	Difference in genome size (×10⁻⁶)¶
A	49		28		77	72		37		109	60		40		—	54·5	
B		44	28		72		58	37		95		58	42		—	52·3	
1·1	2	39	28	1 + (1)	71	2	51	37	1 + (1)	92	3	49	42	6	—	52·4	−1
2·1	34	6	26	8 + (3)	77	50	7	35	9 + (3)	104	37	11	40	12	—	53·5	+0·1
4·1	33	6	28	6 + (8)	81	49	9	37	6 + (8)	109	34	12	39	15	—	56·3	+2·9
5·1	3	42	28	1	74	3	54	37	1	95	4	52	43	2	—	50·9	−2·5
8·1	16	15	28	13 + (6)	78	20	20	37	18 + (6)	101	18	20	42	20	—	51·7	−1·7
9·1	36	4	28	5 + (3)	76	54	5	37	6 + (3)	105	42	5	40	13	—	54·8	+1·4
1	5	39	28	1 + (1)	74	7	52	37	1 + (1)	98	8	43	42	6	3·7	51·6	−1·8
2	5	39	28	2	74	7	52	37	2	98	8	40	40	7	3·0	52·4	−1·0
3	5	39	28	2	74	7	52	37	2	98	8	43	42	7	3·4	52·0	−1·4
4	17	30	27	1 + (4)	79	22	41	36	1 + (4)	104	21	35	42	3	4·7	52·5	−0·9
5	15	32	27	1 + (6)	81	20	44	36	1 + (6)	107	19	36	40	5	6·0	54·0	+0·6
6	17	28	27	1 + (3)	76	24	39	36	1 + (3)	103	23	33	41	3	4·5	53·5	+0·1
7	19	25	27	1 + (3)	75	27	35	36	1 + (3)	102	26	30	41	3	2·5	53·0	−0·4
Z1	19	27	27	1 + (4)	78	27	37	36	1 + (4)	105	26	30	41	3	2·5	53·9	+0·5
1	5	40	28	4 + (2)	79	6	53	37	4 + (2)	102	6	42	39	12	2·0	55·7	+2·3
2	1	42	28	1	72	1	55	37	1	94	2	53	44	2	3·4	50·2	−3·2
3	2	41	28	2	73	3	54	27	2	96	3	50	45	3	3·7	49·1	−4·3
Z2	45	2	28	3	78	67	2	37	3	109	48	3	39	9	5·8	55·9	+2·5
Z3*	22	28	27	1 + (4)	82	33	37	36	1 + (4)	111	23	35	39	3	9·6	55·7	+2·3
Z4*	20	29	27	1 + (4)	81	29	39	36	1 + (4)	109	20	37	40	3	9·6	54·7	+1·3

† Clones 3.1, 6.1 and 7.1 from the mass mating experiment and clones 4, 5 and 6 from the second zygote lineage (Z2) experiments are not included in the Table because they gave *Hpa* patterns identical to those of parents B and A, respectively (see the text). Clone 5.2 is also not included, since it showed a *Hpa* pattern identical to that of clone 5.1.

‡ The number of new bands (or fragments) is given; the number of bands (or fragments) differing in multiplicity from the parental bands is given in parentheses.

§ Not including the weight of faint bands.

¶ Differences from the average of the parental genome unit sizes.

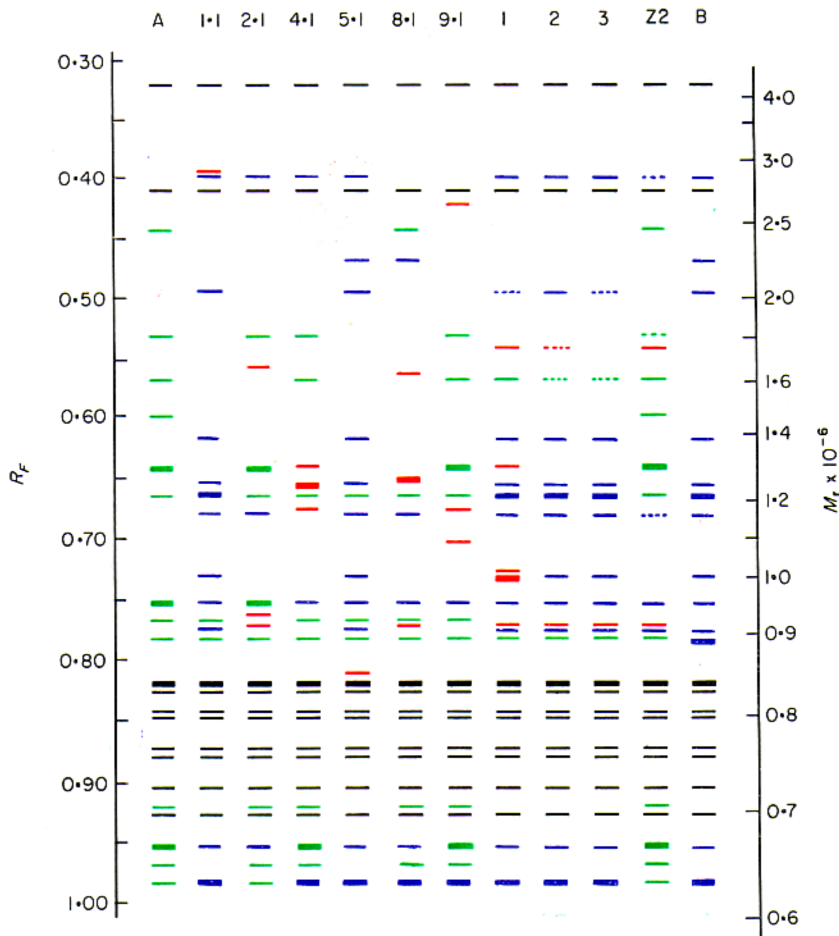

FIG. 6. Scheme of the *Hpa* band patterns on 0·5% agarose/2% polyacrylamide gel shown by the DNAs from parent strains A and B, the diploid clones issued from the mass-mating experiment (1.1, 2.1, 4.1, 5.1, 8.1, 9.1), the diploid clones issued from individual buds (1 to 3) and from the residual zygote of the second zygote lineage (Z2). The relative mobilities and molecular weights of the fragments are indicated. Bands containing 1 or 2 (or more) fragments are indicated by a different thickness. This scheme only depicts the bands present in the best-resolved region of the gel and does not show the overlap region between different gels. Black bands are bands common to both parental strains; green and blue bands are bands only present in 1 of the 2 parents; red bands are bands differing in mobility or multiplicity from parental bands (see Fig. 9). Faint bands are indicated by broken lines (the faintest bands being indicated by 4 dashes, the others by 3). The parental patterns of clones 3.1, 6.1, 7.1, of clones 4, 5 and 6 from the second zygote lineage and the pattern of clone 5.2 are not shown (see the text).

genome sizes of recombinant diploid clones (Table 1), calculated by summing the molecular weights of all fragments, including those present in new bands, always were different from the parental values ($52·3 \times 10^6$ and $54·5 \times 10^6$); they never were, however, very far from them (Table 1).

The *Hae* pattern of some DNAs on the 2% gel (Figs. 4 and 13) was basically similar to that just described for *Hpa*; the most interesting features were: (a) the larger number of new bands in the *Hae* digests compared to the *Hpa* digests; (b) the fact that *Hae* new

FIG. 8. Scheme of the *Hpa* band patterns on 6% polyacrylamide gel. All other indications as in Fig. 6.

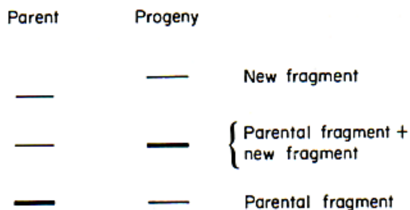

FIG. 9. Scheme of progeny *Hpa* bands differing in mobility or multiplicity from the parental bands.

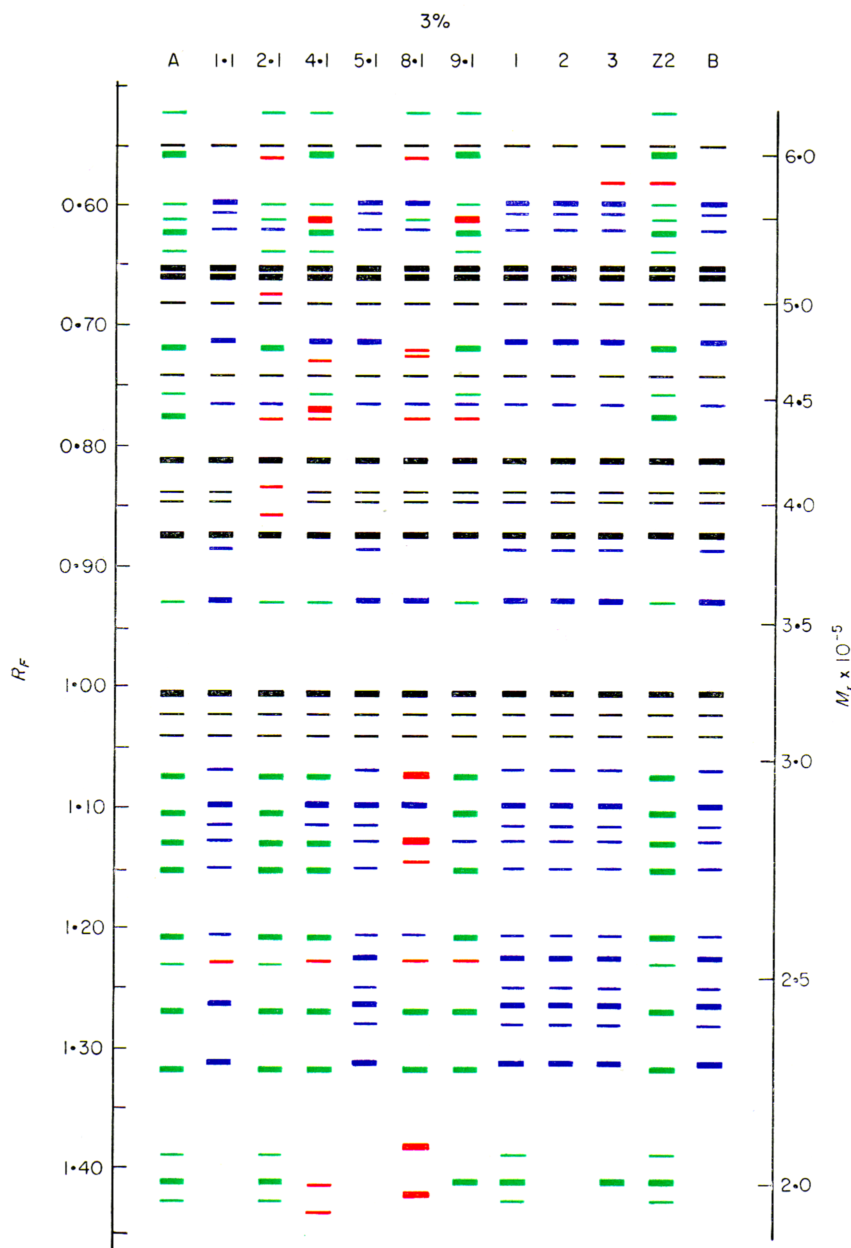

FIG. 7. Scheme of the *Hpa* band patterns on 0·5% agarose/3% polyacrylamide gel. All other indications as in Fig. 6.

bands were regularly similar in size to *Hpa* new bands and regularly larger in size than the latter (Table 2 and Fig. 13). A comparison of the molecular weights of *Hae* and *Hpa* bands common to both parents and progeny also showed that *Hae* bands were systematically larger in size than the corresponding *Hpa* bands (Fig. 13). A detailed quantitative comparison of *Hae* and *Hpa* bands is in progress. No new band was found in the *Hae* digest from clone 3.1, which also did not show new *Hpa* bands (Figs. 4 and 13).

6%

A 1·1 2·1 4·1 5·1 8·1 9·1 1 2 3 Z2 B

R_F: 0·50 0·60 0·70 0·80 0·90 1·00 1·10 1·20 1·30 1·40

$M_r \times 10^{-5}$: 2·0 1·8 1·6 1·4 1·2 1·0 0·8 0·6 0·4

FIG. 8. Scheme of the *Hpa* band patterns on 6% polyacrylamide gel. All other indications as in Fig. 6.

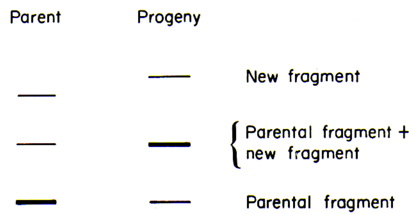

FIG. 9. Scheme of progeny *Hpa* bands differing in mobility or multiplicity from the parental bands.

(b) *Diploid clones from individual buds*

In both zygote lineages, the first three clones showed *Eco*RI and *Hin*dII + III patterns identical to those of parent B, whereas the following ones and the residual zygotes exhibited patterns identical to those of A; clone 7 from Z1 and the residual zygote Z2 showed an *Eco*RI pattern of A type and a *Hin*dII + III pattern of B type.

Figures 6 to 8 and 10 to 12 show the *Hpa* restriction patterns exhibited by diploid clones obtained from individual buds sequentially formed by zygotes Z2 and Z1 and from the residual zygotes, namely the zygotes from which the early buds had been removed by micromanipulation. Quantitative results are shown in Table 1. (i) In the case of Z1 (Figs 10 to 12), the clones derived from the first three buds (1 to 3) originated restriction patterns almost identical with each other and very similar to that of the parent strain B; bands from the parent strain A, new bands and faint bands were, however, also present (Table 1). The clones from the four subsequent buds (4 to 7) and from the residual zygote (Z1) exhibited restriction patterns in which bands from parent A were more represented; the residual zygote showed a pattern very similar to that of the later buds; new bands and faint bands were present in all cases. (ii) In the case of Z2 (Figs 6 to 8), the clones from the first three buds (1 to 3) originated restriction patterns very similar to that of parent B; a few new bands could be seen, as well as some faint bands. In contrast, the clones from the subsequent buds (4 to 6, not shown) originated restriction patterns identical to that of the parent strain A. The residual zygote (Z2) showed a restriction pattern quite similar, yet not identical with that of A. (iii) In all cases, new bands showed mobilities close to those of parental bands missing in the patterns. Again, new bands often showed the same mobility in different clones. (iv) In all cases, faint bands often were new and exhibited the same mobility in different clones. Faint bands were only estimated on the 2% gel, the more diffuse aspect of bands on the 3% and 6% gels preventing any assessment of faint bands on them. (v) Bands common to both parents were always present in the progeny pattern. (vi) In all cases, the genome sizes of recombinant diploid clones, calculated as indicated in the preceding section, were comprised between $49{\cdot}1 \times 10^6$ and $55{\cdot}7 \times 10^6$ and were always different from the parental values. Faint bands were not taken into account in this calculation; if they were, genome sizes as high as 60×10^6 would be obtained (Table 1).

The *Hae* patterns of some DNAs are shown in Figures 4 and 13; they exhibit the same characteristics described above for random diploids; in addition they show faint bands. Clone 4 (Z2), which did not show new *Hpa* bands, also did not show *Hae* new bands on the same gel (Figs. 4 and 13).

(c) *Diploid clones from zygotes*

The mitochondrial DNAs from both zygotic clones showed an *Eco*RI pattern identical to that of DNA from strain A; the *Hin*dII + III patterns were identical to that of DNA from strain B, with however one additional band ($2{\cdot}2 \times 10^6$) originating from strain A (Fig. 5).

Zygotic clones Z3* and Z4* (Figs 10 to 12) showed *Hpa* restriction patterns very similar to each other; practically all bands had the same mobility as bands from either parent; the only new band and the only new faint band were common to both clones.

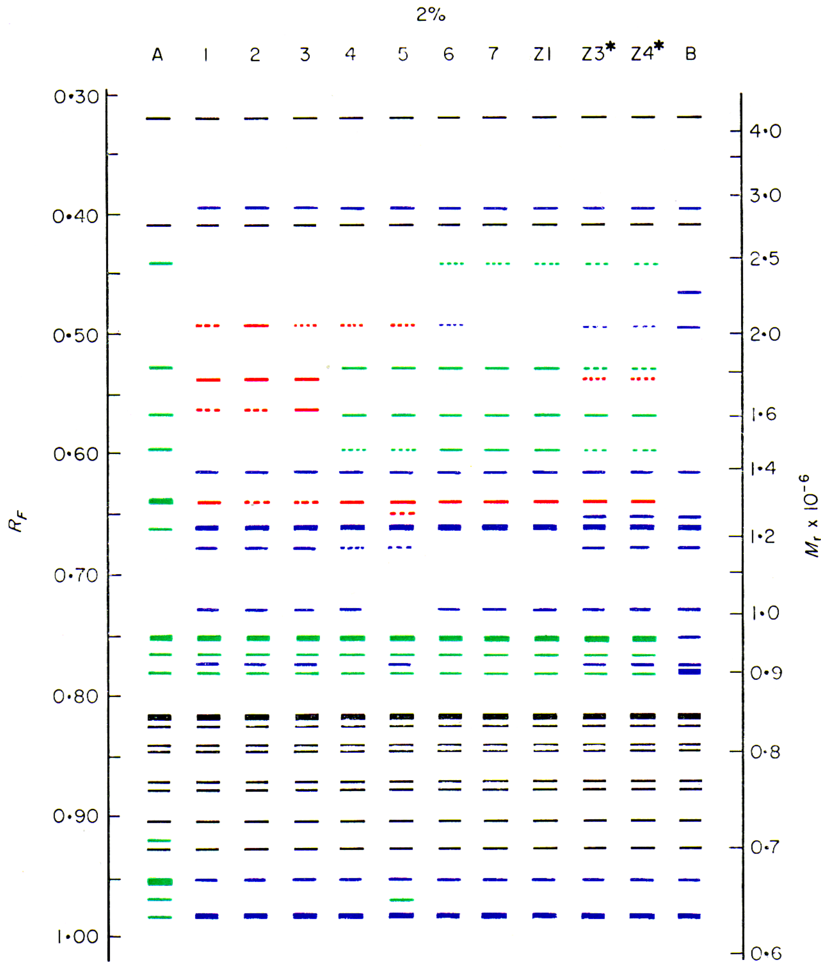

FIG. 10. Scheme of the *Hpa* band patterns on the 0·5% agarose/2% polyacrylamide gel shown by the DNAs from parent strains A and B, the diploid clones issued from individual buds (1 to 7) and from the residual zygote of the first zygote lineage (Z1) and the zygotic clones Z3* and Z4*. All other indications as in Fig. 6.

The genome sizes, neglecting the faint bands, were equal to 55·7 × 10^6 and 54·7 × 10^6, respectively. If the contribution of faint bands was taken into consideration, genome sizes in excess of 64 × 10^6 would be calculated (Table 1).

4. Discussion

(a) *Genome homogeneity*

A major difference between the *Hpa* and *Hae* restriction patterns of the mitochondrial DNAs from the random diploid clones (Figs 6, 7, 8 and 13) and those from the other diploids (Figs 6 to 8, 10 to 13) is the presence in the latter of faint bands, namely bands appearing in lower yields compared to neighboring bands.

Such faint bands are not due to a specific DNA degradation, as was the case for two bands from low molecular weight preparations of the parental DNAs (Prunell *et al.*,

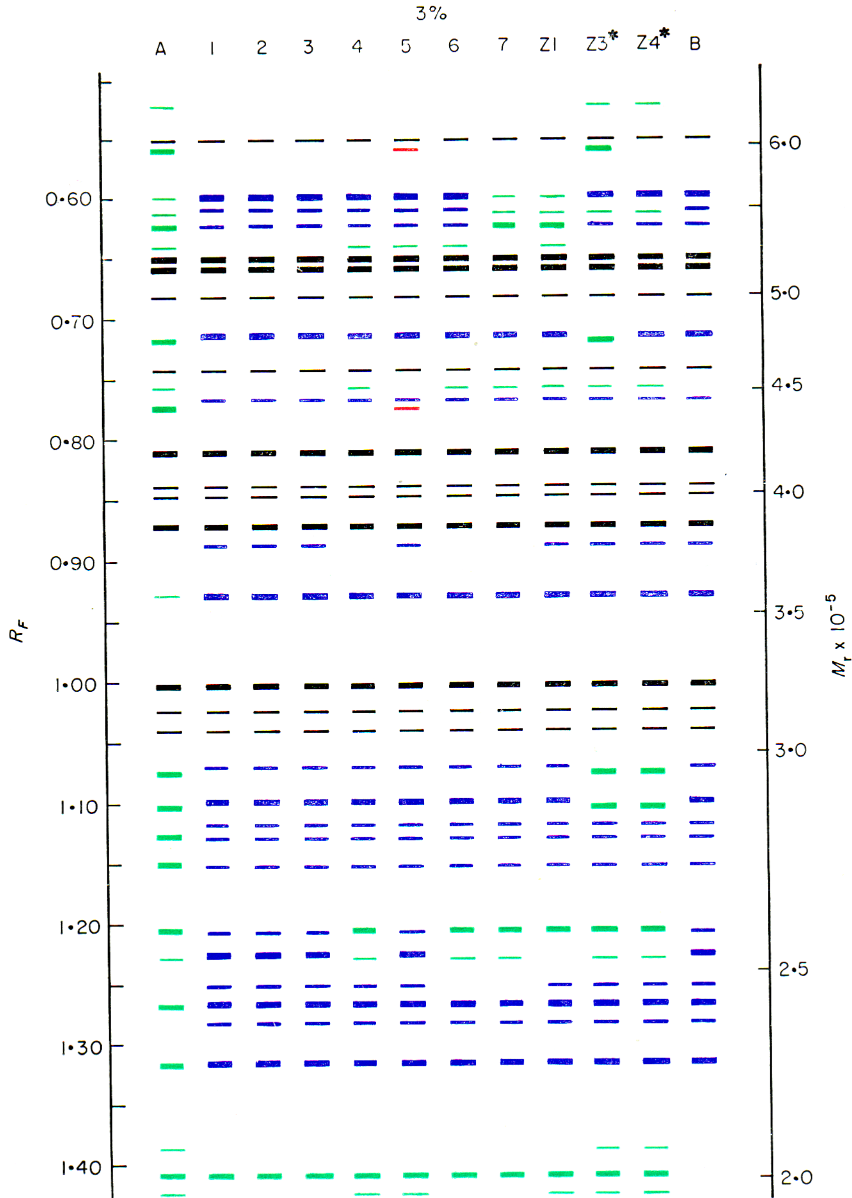

FIG. 11. Scheme of the *Hpa* band patterns on the 0·5% agarose/3% polyacrylamide gel. All other indications as in Fig. 10.

1977*a*), because (i) they do not correspond to those bands; (ii) they were found in high molecular weight preparations, where a specific degradation was not previously found; (iii) they correspond to additional bands, since when their molecular weights are added to those of the other fragments, genome sizes in the 55×10^6 to 65×10^6 range are obtained (Table 1).

It should be concluded, therefore, that the faint bands are due to the presence, in the DNA preparations under consideration, of a certain percentage of genomes

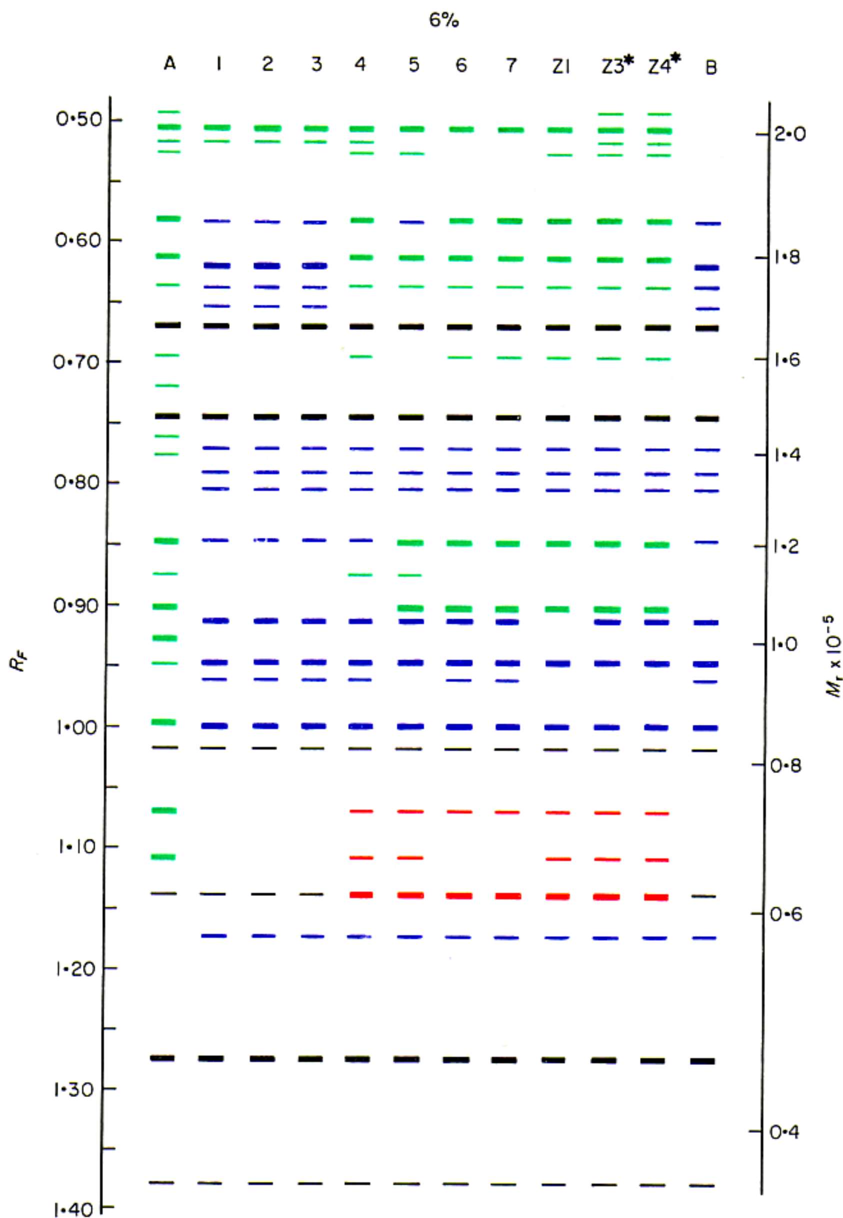

FIG. 12. Scheme of the *Hpa* band patterns on the 6% polyacrylamide gel. All other indications as in Fig. 10.

different from the majority population. Such genome heterogeneity seems to be associated with (i) the short number of generations, about 40, separating the cell population used for DNA preparation from the starting zygotes; (ii) the fact that diploids were not subcloned. In the case of the random diploids, where no faint bands were seen, the number of generations was about 100 and sub-cloning was done. It is conceivable that both genome segregation at sub-cloning and genome selection taking place over a larger number of cell generations are responsible for the apparent genome

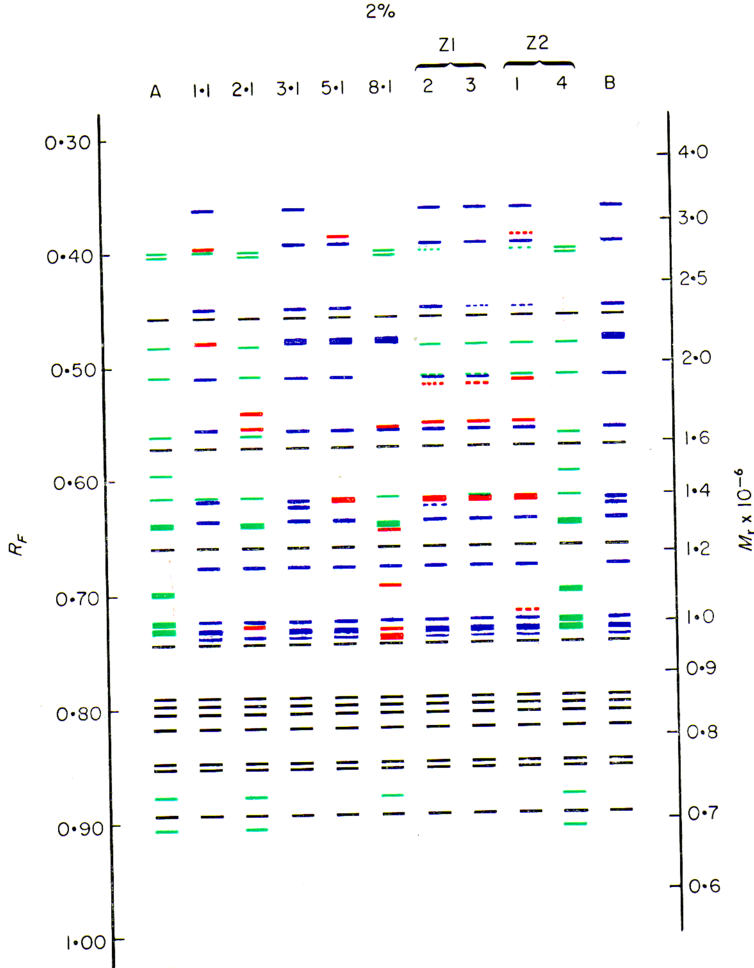

FIG. 13. Scheme of the *Hae* band patterns on 0·5% agarose/2% polyacrylamide gel shown by the DNAs from parent strains A and B, and the diploid clones issued from the mass-mating experiment or from individual buds. For all other indications see the legends to Figs 6 and 10.

homogeneity of random diploids; recombination phenomena may also have played a role.

Interestingly enough, the further sub-cloning of the random diploids of procedure 1 showed that the mitochondrial genomes of the latter were already homogeneous; in fact, subclones 5.1 and 5.2 were identical with each other.

(b) *Genome segregation*

(i) The finding of purely parental restriction patterns in some of the diploids investigated here may have two not mutually exclusive explanations. (1) Segregation of parental genomes into the buds occurred prior to recombinational events; (2) recombination between the parent genomes took place in regions of fragment homology only (see below).

(ii) Both A and B parental patterns were found in the progeny. 25% (6 out of 24) of the diploid clones investigated (zygotic clones are not taken into consideration here)

Table 2

A comparison of the molecular weights of new bands in Hae *and* Hpa *digests*†

Diploids:	1.1		2.1		5.1		8.1		2 (Z1)		3 (Z1)		1 (Z2)	
	Hae	*Hpa*	*Hae*	*Hpa*	*Hae*	*Hpa*	*Hae*	*Hpa*	*Hae*	*Hpa*	*Hae*	*Hpa*	*Hae*	*Hpa*
	2·85	2·93			2·92								2·92	
	2·15									2·07		2·07‡		
			1·76	1·70					1·83	1.80	1·89‡	1·80	1·91	1·79
			1·69				1·69	1·66	1·70	1·66‡	1·70	1·66	1·70	
									1·40	1·30‡	1·40	1·30§	1·40	1·30§
							1·29	1·25						
			1·00	0·92			0·99						1·03‡	1·02
														0·97
				0·89										0·90
						0·85								

† Data concern new fragments as seen on the 2% gel; values are $M_r \times 10^{-6}$.
‡ Faint bands.
§ Single band corresponding to a double band of strain A.

were of parental type, three of each one of them (see Table 1, footnote† and Fig. 6). In this connection, it is relevant to mention that the amount of mitochondrial DNA was found, in the present work, to be the same in both parents, namely 12% of total DNA. While the statistical significance of the values quoted above is weak, because of the small number of clones investigated and of the non-independent origin of many of them, it is quite clear that recombination is very frequent in crosses.

(iii) In our second cell lineage (Figs 6 to 8), the segregation of recombinant genomes into the buds corresponded to a central location of the buds, and that of parental genomes to a polar location, in agreement with findings by Strausberg &Perlman (1974). This might, however, be coincidental, or specific for the particular cross investigated here, since, in experiments by Waxman *et al.* (1973), bud position did not seem to be a factor in determining inheritance pattern.

(c) *Genome size*

The genome sizes of all recombinant diploid clones investigated here are significantly different from those of the parental genomes, yet they are all within ± 5% of the average of the parental genomes. This conclusion is in apparent conflict with the finding that all *Eco*RI and *Hin*dII + III restriction patterns of recombinant genomes are of parental type. In fact, this discrepancy is in all likelihood due to the fact that differences in size of the very large fragments which account for the majority of mitochondrial genomes in *Eco*RI and *Hin*dII + III digests may go unnoticed. Taking into account that the number of *Hpa* fragments, which largely reflects that of the genetic units (Prunell & Bernardi, 1977), falls within the parental range for all recombinant genomes (Table 1), it is conceivable that all or most of the differences in genome sizes are due to variations in the total amount of DNA in the spacers. If this conclusion is correct, it implies (1) that all or most mitochondrial genes in *Saccharomyces* are required for respiratory function (see also section (d), below); (2) that even the variation in the amount of spacer sequences is kept within narrow limits: if no changes affect the genes, the variation in spacer amount is ± 10%.

(d) *Gene order*

In spite of the differences in the *Hpa* restriction patterns of recombinant diploids (see below), all of them show *Eco*RI and *Hin*dII + III patterns of parental type; in other words, over 20 restriction sites maintain the same (or, rather, a very close; see section (c), above) location on the recombinant genomes as they had on the parental ones. An implication of these data is that the overall gene order is very largely preserved in the recombinants. Such a conclusion had already been reached for different *Saccharomyces* strains (Bernardi *et al.*, 1976; Prunell *et al.*, 1977*a* and manuscript in preparation; Sanders *et al.*, 1975,1976) on the basis of the similarity of *Eco*RI and *Hin*dII + III patterns of their mitochondrial DNAs. Here, this point is much stronger, since *Eco*RI and *Hin*dII + III patterns of recombinants showing different *Hpa* patterns are indistinguishable from each other, instead of being just similar. Obviously, these findings strengthen the arguments given in section (c) above, on the preservation of mitochondrial genes in different diploids.

(e) *Genome recombination*

(i) The *Hpa* and *Hae* restriction patterns of most diploids investigated here are characterized by fragments originating from each parent and by new fragments. The

first finding provides by itself unequivocal evidence for physical recombination of the parental genomes. The second finding provides a very important clue concerning the mechanism underlying the recombination process.

(ii) It may be interesting at this point to consider the expected *Hpa* or *Hae* restriction patterns of recombinants and to compare these expectations with the experimental findings. Keeping in mind what is known about the organization of the mitochondrial genome of yeast (Prunell & Bernardi, 1977; see also Introduction), recombination events can affect the *Hpa* or *Hae* pattern in the following ways. (1) A pair of recombination events (the minimum needed to lead to DNA exchanges between circular molecules) takes place in two regions homologous in nucleotide sequence, like the allelic genes from the two parent strains. Then, the recombinant genomes will exhibit, after segregation and replication, just an exchange of *Hpa* fragments (Fig. 14). A

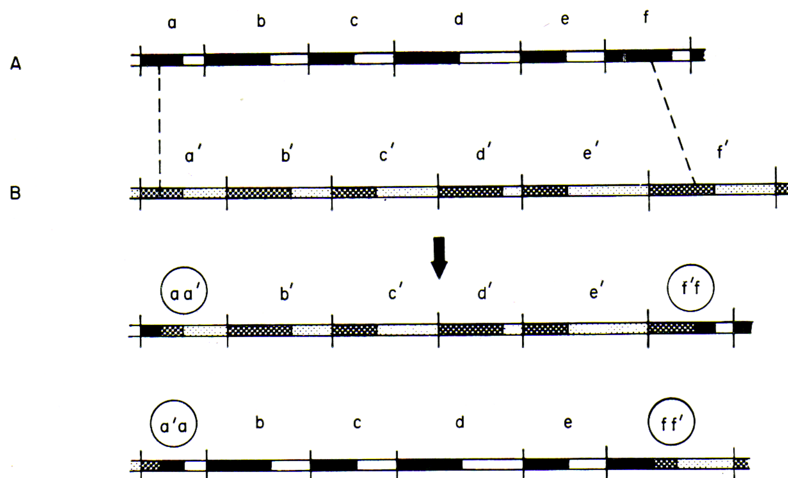

FIG. 14. Scheme of crossing-over events leading to exchanges of DNA segments between the parental genomes A and B. Segments a to f and a′ to f′ indicate genetic units as split by *Hpa*; in each segment, dark stretches correspond to genes, clear stretches to spacers. In this scheme, crossing-overs take place between allelic genes.

special case is that in which the pair of recombination events encompasses genome regions, where all *Hpa* or *Hae* fragments have identical lengths in the two strains: obviously here no sign of recombination will appear in the fragment patterns of recombinant genomes. Similarly, recombination events in the (C-C-G-G, G-G-C-C) clusters of allelic genetic units, if they take place at all, will be missed. (2) At least one of the two recombinational events is unequal. Then the fragments where the unequal crossing-over occurred will have a different length in the recombinant compared to the parent molecules and the fragments comprised between the two crossing-overs will be exchanged. The overall result will then be as in (1) above with, however, the important additional feature of the presence of two new fragments replacing two parental fragments (Fig. 15) in the pattern. The most likely localization of unequal crossing-overs is at the spacers of allelic genetic units (Fig. 15), where internal sequence repetition provides several opportunities for unequal crossing-over events at homologous or quasi-homologous nucleotide sequences. This second mechanism has certainly been operative in all the recombinant genomes investigated, as

shown by the general presence of new *Hae* and *Hpa* bands in them. Its frequency relative to the first one cannot be assessed at the present time, but is being investigated. (3) The alternative possibility of crossing-over events at non-allelic genetic units should be ruled out as a source of recombination in wild-type cells, since it would lead to large deletions and additions, which are not found; such unequal crossing-over seems to be responsible for the production of the defective genomes of spontaneous petite mutants (paper in preparation).

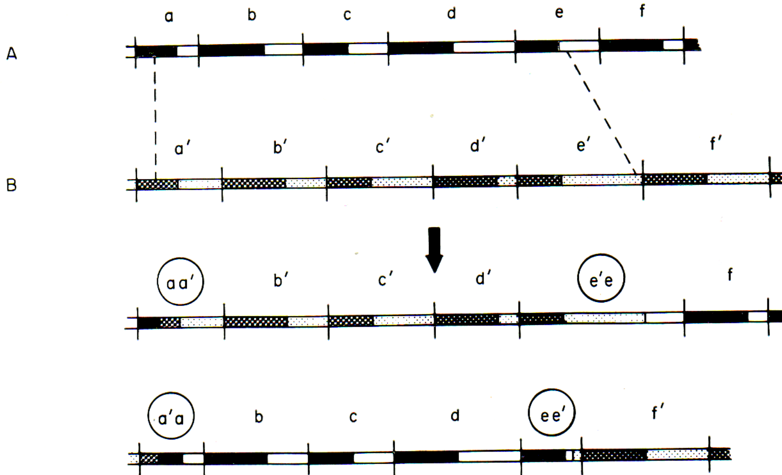

FIG. 15. Scheme of crossing-over events leading to exchanges of DNA segments between the parental genomes A and B (see Fig. 14). In this scheme, one of the crossing-overs takes place between allelic spacers and is unequal.

The unequal crossing-over model just presented is essentially identical to that proposed by Prunell *et al.* (1977*a*) to account for the different *Hpa* patterns found in different yeast strains. It should be stressed that the underlying unequal recombination events are likely to be much more frequent in zygotes and early diploids than in vegetative cells. In the first case, mitochondrial genomes having allelic genetic units with spacers of different length, and therefore of largely different nucleotide sequences, are in the presence of each other, and recombination events occurring in these spacers can very frequently be of the unequal type. In the second case, mitochondrial genomes are identical with each other and unequal crossing-overs require a more rare event like a mismatch at the heteroduplex formed between two allelic spacers. It should also be stressed that the unequal crossing-over mechanisms underlying recombination events in crosses, in genome divergence and in the production of petite mutants, are essentially identical at the molecular level. Moreover, the ideas just presented apply to any system in which unique nucleotide sequences are interspersed with internally repetitive ones, like the nuclear genome of eukaryotes. They also stress the possible importance of inaccuracy in recombinational events at spacer sequences in genome evolution.

(iii) Several predictions of the model discussed above appear to be fulfilled by our experimental results. Two points are of special interest. (1) It is known that allelic genetic units are of similar size in the mitochondrial genomes of different yeast

strains (Prunell *et al.*, 1977*a*); it is expected, therefore, that the new bands formed by unequal crossing-over have a size similar to that of the parental band from which they were derived; our results appear to fit this expectation. (2) Since both *Hae* and *Hpa* fragments largely correspond to the genetic units of the mitochondrial genome (Prunell *et al.*, 1977*a*), it is expected, and found experimentally (Table 2), that the new bands formed by these enzymes are generally similar in size. The slightly larger size of new and common *Hae* bands compared to the corresponding *Hpa* bands (Table 2) is in agreement with the conclusion (Prunell & Bernardi, 1977) that *Hpa* sites are external to *Hae* sites in (C-C-G-G, G-G-C-C) clusters. The larger number of *Hae* new bands compared to *Hpa* new bands on the 2% gel may be due to the fact that some of them are split by *Hpa*, an enzyme known to insert a rather large number of isolated breaks. The smaller fragments so produced may therefore appear only on the 3% and 6% gels, which have not been used to analyze *Hae* digests. The absence of *both Hae* and *Hpa* new bands in progeny DNAs showing a restriction fragment pattern of parental type also is an expected finding.

Other predictions of the model depicted in Figure 15 only apply to the primary events. These predictions are that: (1) two new bands are formed for every unequal crossing-over; (2) two parental bands disappear at the same time; (3) the sum of the molecular weights of new bands is equal to that of the corresponding parental bands. It is obvious that genome segregation and additional crossing-over events in the same spacers can modify the original situation.

(iv) The experimental results of this work provide some additional information: (1) the number of new bands allows an estimate of the number of unequal crossing-over events; however, this is a minimum estimate, since exchanges leading to very short deletions and/or additions can be missed; the data of Table 1 indicate that such a minimum number can be as high as 13. (2) Most of the new bands have exactly the same molecular weight in different clones. Out of a total of 64 new bands (including new faint bands) seen in the *Hpa* restriction patterns of the mitochondrial DNAs from the different clones examined, 39 have the same molecular weight in 2 up to 8 different DNAs, and only 25 are unique to a given DNA. This finding points to the existence of strongly preferred locations for unequal crossing-over events.

(v) The number of new bands appears to be smaller in clones from individual buds and, even more so, in the two zygotic clones compared to the random diploids. The reason for this finding is in all likelihood associated with the heterogeneity of the former clones compared to the latter. Since new bands are largely different in different recombinants, they will tend to be lost in the background; in agreement with this explanation, a number of new bands are found among faint bands.

(vi) The results obtained here are also interesting in that they rule out the unlikely possibility that the different *Hpa* and *Hae* restriction patterns of strains A and B are due to a different specific methylation at *Hpa* or *Hae* sites. If such were the case (and whatever the origin, nuclear or mitochondrial, of the hypothetical methylase) one would not expect that (a) the vast majority of DNA bands from recombinant diploids are identical in size to bands from either parent, while (b) purely parental diploids also exist. The first phenomenon would imply that *Hae* and *Hpa* restriction sites of recombinant genomes are methylated in the diploids in part according to the methylation pattern of one parent and in part according to that of the other parent. The second phenomenon, in contrast, would imply that a parental genome in the diploid is methylated according to the pattern it had in its haploid parent.

(vii) No feature in the restriction pattern has been detected which could be associated to the polar recombination phenomena occurring in a small region of the genome in crosses of $\omega^+ \times \omega^-$ strains (Bolotin *et al.*, 1971).

We thank our colleague Helena Kopecka for her help with initial restriction enzyme degradations; A. Meier and H. Chalumeau for their help in growing yeast cultures; and Dr B. Dujon for useful discussions.

REFERENCES

Bernardi, G. (1975). In *Fed. Eur. Biochem. Soc. Proc. Tenth Meeting, Paris*, July, 1975 (Bernardi, G. & Gros, F., eds), vol. 38, pp. 41–56, North-Holland, Amsterdam.

Bernardi, G. (1976). In *The Genetics and the Biogenesis of Chloroplasts and Mitochondria* (Bücher, T. *et al.*, eds), pp. 503–510, Elsevier-North-Holland, Amsterdam.

Bernardi, G., Faurès, M., Piperno, G. & Slonimski, P. P. (1970). *J. Mol. Biol.* **48**, 23–42.

Bernardi, G., Piperno, G. & Fonty, G. (1972). *J. Mol. Biol.* **65**, 173–189.

Bernardi, G., Prunell, A., Fonty, G., Kopecka, H. & Strauss, F. (1976). In *The Genetic Function of Mitochondrial DNA* (Saccone, C. & Kroon, A. M., eds), pp. 185–198, North-Holland, Amsterdam.

Bicknell, J. N. & Douglas, H. C. (1970). *J. Bacteriol.* **101**, 505–512.

Bolotin, M., Coen, D., Deutsch, J., Dujon, B., Netter, P., Petrochilo, E. & Slonimski, P. P. (1971). *Bull. Inst. Pasteur (Paris)*, **69**, 215–239.

Ehrlich, S. D., Thiery, J. P. & Bernardi, G. (1972). *J. Mol. Biol.* **65**, 207–212.

Prunell, A. & Bernardi, G. (1974). *J. Mol. Biol.* **86**, 825–841.

Prunell, A. & Bernardi, G. (1977). *J. Mol. Biol.* **110**, 53–74.

Prunell, A., Kopecka, H., Strauss, F. & Bernardi, G. (1977*a*). *J. Mol. Biol.* **110**, 17–52.

Prunell, A., Strauss, F. & Leblanc, B. (1977*b*). *Anal. Biochem.* **78**, 57–65.

Sanders, J. P. M., Borst, P. & Weijers, P. J. (1975). *Mol. Gen. Genet.* **143**, 53–54.

Sanders, J. P. M., Heyting, C., Di Franco, A., Borst, P. & Slonimski, P. P. (1976). In *The Genetic Function of Mitochondrial DNA* (Saccone, C. & Kroon, A., eds), pp. 259–272, North-Holland, Amsterdam.

Smith, D. & Halvorson, H. O. (1967). In *Methods in Enzymology* (Grossman, L. & Moldave, K., eds), vol. 12, part A, pp. 538–541, Academic Press, New York.

Smith, H. & Nathans, D. (1973). *J. Mol. Biol.* **81**, 419–423.

Strausberg, R. L. & Perlman, P. S. (1974). *Genetics*, **77**, 62–63.

Studier, F. W. (1965). *J. Mol. Biol.* **11**, 373–390.

Thomas, D. Y. & Wilkie, D. (1968). *Biochem. Biophys. Res. Commun.* **30**, 368–372.

Waxman, M. F., Eaton, N. & Wilkie, D. (1973). *Mol. Gen. Genet.* **127**, 277–284.

Wilkie, D. (1973). *Brit. Med. Bull.* **29**, 263–268.