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Ariel Prunell and Giorgio Bernardi

Laboratoire de Génétique Moléculaire
Institut de Biologie Moléculaire
Faculté des Sciences, Paris 5e, France

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The organization of the mitochondrial genome of wild-type *Saccharomyces cerevisiae* cells has been investigated further, by degrading mitochondrial DNA with micrococcal nuclease. Under the conditions used, this enzyme very strongly degrades the A + T-rich stretches (spacers) whereas it only inflicts a limited number of breaks into the G + C-rich stretches (genes). The macromolecular fragments derived from the "genes" have been separated from the oligonucleotides originating from the "spacers" by gel filtration, and both sorts of products have been investigated. It has been shown (a) that the spacers are very homogeneous in base composition and have a G + C content lower than 5% (mitochondrial DNA has a G + C content of 18%); (b) that the genes are very heterogeneous in base composition, the G + C content ranging from about 25% to 50%, when the average size of the fragments is $1{\cdot}2\times10^5$; smaller fragments, molecular weight 4×10^4, having a G + C level as high as 65%, have been isolated in a yield of 10%; the average G + C content of genes is about 32%; (c) that genes and spacers are present in about equal amounts in the mitochondrial genome and that they have comparable average sizes.

1. Introduction

A series of physical and chemical investigations on yeast mitochondrial DNA (Bernardi *et al.*, 1968,1970; Bernardi & Timasheff, 1970) led us to think that the mitochondrial genome of wild-type *Saccharomyces cerevisiae* cells is organized as depicted in Figure 1. The basic feature of this model, which has been our working hypothesis for the past four years and which has been presented elsewhere (Bernardi *et al.*, 1972; Piperno *et al.*, 1972), is an interspersion of A + T-rich and G + C-rich stretches, corresponding, in our view, to spacers and genes (with their regulatory elements and, possibly, transcribed spacers), respectively. One of the implications of this model is that the extremely high spontaneous and induced rate of the cytoplasmic petite mutation can be explained by internal crossing-overs, leading to large deletions and essentially involving homologous nucleotide sequences of the spacers.

Recent investigations (Bernardi *et al.*, 1972; Piperno *et al.*, 1972; Ehrlich *et al.*, 1972) provided the first direct evidence in favour of the model. Yeast mitochondrial DNA (which has a G + C content of 18% and shows unimodal, symmetrical bands in CsCl at molecular weights as low as 2×10^6) was shown to exhibit a striking "inter-

† Paper III in this series is Ehrlich *et al.*, 1972.

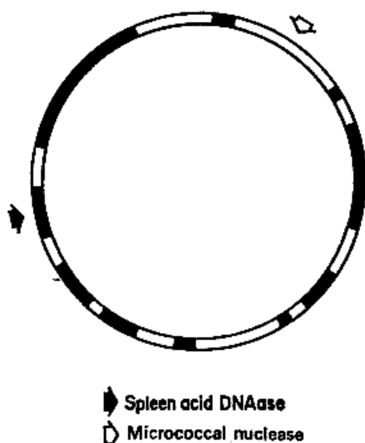

FIG. 1. A scheme of our working model for the organization of yeast mitochondrial DNA and of the experimental approach used to test it. Black stretches correspond to the G + C-rich regions, white stretches to the A + T-rich regions. Spleen acid DNAase splits mitochondrial DNA with a slight preference for the G + C-rich regions; micrococcal nuclease splits mitochondrial DNA with a very high specificity for the A + T-rich regions.

molecular" compositional heterogeneity when degraded by spleen acid DNAase, an enzyme preferring G + C-rich sequences; at an average molecular weight of $4{\cdot}7\times10^5$, fragments having a G + C content of 26% and a molecular weight of 1 to 2×10^5 were obtained, as well as fragments having a G + C content of 14% and a molecular weight of 8×10^5. By recurrent degradation of this latter material, fragments having a G + C content of 10·4% and a molecular weight of $2{\cdot}5\times10^5$ could be isolated in an overall 12% yield. On the other hand, an analysis of the pyrimidine tracts showed that these were compatible with the presence of short, repetitive sequences in the A + T-rich stretches.

In the present work we have looked at the compositional heterogeneity of yeast mitochondrial DNA by degrading it with an enzyme having a different specificity, micrococcal (staphylococcal) nuclease. Degradation has been performed (a) at the melting temperature (69°C), where the A + T-rich stretches are melted and the enzyme (which is endowed with a high-thermal stability) preferentially degrades at random single-stranded regions, and (b) at low temperatures (6 or 25°C), where the enzyme preferentially degrades A + T-rich sequences (von Hippel & Felsenfeld, 1964). The results obtained with micrococcal nuclease not only are complementary to those previously reported with spleen acid DNAase, but also lead to a much more precise definition of the organization of the mitochondrial genome.

In fact, it has been possible to show that the A + T-rich stretches are very homogeneous in base composition, have a G + C content lower than 5%, and represent about 50% of the mitochondrial genome. The G + C-rich stretches, which have a G + C content of about 32%, are very heterogeneous in base composition, ranging from about 25% to 50% G + C, when their average size is about $1{\cdot}2\times10^6$; smaller fragments ($M_r = 4\times10^4$), having a G + C level as high as 65%, have been isolated in a yield of 10%. Both results are perfectly consistent with the melting curve of mitochondrial DNA (Bernardi *et al.*, 1970). In addition, the analysis of the small fragments released from the A + T-rich stretches is consistent with the circular

dichroism spectra (Bernardi & Timasheff, 1970) and compatible, like the pyrimidine isostichs (Ehrlich *et al.*, 1972), with the idea that they contain repeated, short nucleotide sequences.

It should be pointed out that our interest in the mitochondrial DNA of yeast is, to a considerable extent, due to the fact that it represents a very simple model of eukaryotic nuclear DNA. The special attention we have paid to the methodology used in this work is justified by the possibility of using it in studying the organization of genetic material in more complex systems.

2. Materials and Methods

(a) *Materials*

Yeast mitochondrial DNA was preparation I + II, already described and investigated in detail elsewhere (Bernardi *et al.*, 1972; Piperno *et al.*, 1972; Ehrlich *et al.*, 1972).

Crab DNA was prepared from *Cancer pagurus* according to Kay *et al.* (1952) and purified by hydroxyapatite chromatography. Its G + C content was found to be 33%.

Crab satellite DNA was purified by preparative centrifugation in a Cs_2SO_4–Ag^+ density gradient at pH 8·0, and a Ag^+/nucleotide ratio of 0·3. Its G + C content is 3%.

"50%-satellite" crab DNA was prepared by mixing crab DNA and crab satellite DNA so as to have a satellite level of about 50%†. Its G + C content was 20·7%.

Micrococcal nuclease was purchased from Worthington (Freehold, N.J., code NFCP-7FA, 13,000 units/mg). An enzyme stock solution was prepared by dissolving 0·1 mg of nuclease in 300 μl of distilled water. The enzyme solution was diluted 20- or 50-fold, immediately before use, with a 0·1% bovine serum albumin (Sigma, St Louis, Mo.) solution in water.

(b) *Enzymic digestions*

DNA solutions were dialysed against 0·1 M-borate, pH 8·8, and adjusted to an $A_{260} = 1$ and to 2·5 mM-$CaCl_2$.

(i) *Digestions at 6 or 25°C*

1-ml DNA samples were introduced in Teflon-stoppered, jacketed quartz cuvettes having a 1-cm optical path, thermostatically controlled at the desired temperature. Enzyme solution was added (10 to 25 μl of the 1 : 20 dilution, at 6°C; 15 to 30 μl of the 1 : 50 dilution, at 25°C) and digestion was allowed to proceed for 5 to 30 min in a Zeiss spectrophotometer, until the desired hyperchromicity was attained. Digestion was stopped by adding 50 μl of 0·75 N-acetic acid and 50 μl of 0·1 M-EDTA. Digests were then transferred into test tubes containing 1 vol. of cold (0°C) chloroform/isoamyl alcohol (24 : 1, v/v) mixture and swirled for 5 min on a Whirlimixer (Fisons Scientific Apparatus, Loughborough, England). Tubes were then centrifuged at 4°C in order to obtain phase separation, and stored at −20°C until use.

(ii) *Digestions at the melting temperature*

1-ml DNA samples were introduced in the cuvettes and their A_{260} values were measured at room temperature. Circulation of water at 69°C (in the case of mitochondrial DNA) or at 67·5°C (in the case of 50%-satellite) through the jacket of the stoppered cuvette was then started. The ultraviolet absorbance was determined again to measure the hyperchromic shift caused by the temperature increase. 1 to 10 μl of enzyme solution (1 : 50 dilution) were then added, and digestion was allowed to proceed for 5 to 10 min until the desired hyperchromic shift was attained.

Enzymic digestions were stopped by immersing the cuvettes in an ice bath and by adding, after 1 min, 50 μl of 0·75 N-acetic acid and 50 μl of 0·1 M-EDTA (pre-cooling was necessary to prevent more extended melting subsequent to the removal of stabilizing Ca^{2+}). After an A_{260} reading at room temperature, digests were processed as described above.

† This mixture will be referred to as 50%-satellite.

(c) *Sedimentation coefficients*

Coefficients were measured as described elsewhere (Prunell & Bernardi, 1973) using the band centrifugation method. "Double-" and "single-stranded" molecular weights were calculated using the relationships of Prunell & Bernardi (1973).

(d) *Melting curves*

Temperature–absorbance experiments were done using a Zeiss PMQII spectrophotometer equipped with an automatic sample exchanger. The temperature of DNA samples was controlled by circulating water through the cuvette jackets and the cuvette holder, using a Haake (Berlin, Germany) thermostat equipped with a linear programming system. Under our conditions, the temperatures in the 5 sample-containing cuvettes were the same within 0·1 deg. C; the sixth cuvette contained a thermocouple (Philips, Eindhoven, Holland) immersed in paraffin oil. A digital millivoltmeter, a multi-channel Data Transfer Unit (Solartron, Farnborough, England) and a tape-puncher (Facit, Stockholm, Sweden) were used in order to record temperature and ultraviolet transmission of the samples. The Zeiss sample exchanger was modified in order to start temperature and transmission recording a few seconds before each cuvette holder movement. In some cases, melting curves were simultaneously recorded at two wavelengths, 260 and 280 nm. A Fortran program was used (1) to calculate temperatures and absorbances; (2) to correct absorbances for the temperature expansion of DNA solutions; (3) to eliminate the effects of electric noise on punched tape. The first derivatives of the melting curves were calculated as already described (Bernardi *et al.*, 1970, 1972).

(e) *Gel filtration*

Gel filtration of the DNA digests obtained at the melting temperature and at 6 or 25°C was done on Sephadex G100 (Pharmacia, Uppsala, Sweden) or Sephadex G25 columns, respectively. As a rule, 1 ml DNA solutions were loaded on 0·8 cm × 40 cm columns equilibrated with 0·05 M-ammonium acetate, pH 5·5. Flow rate was kept at 12 ml/h using a Technicon (Chauncey, N. J.) peristaltic pump. A Uvicord (LKB, Stockholm, Sweden) equipped with a 0·3-cm cell was used to monitor the chromatograms. The excluded and the retarded fractions were collected manually, and their volume and absorbancy at 260 nm were measured.

The relative amounts of the two fractions, on a nucleotide basis, were estimated as follows. Since the G + C content of the excluded and the retarded fractions were widely different and the sum of the two fractions has the average base composition of DNA, the percentage of the excluded fraction was calculated from the ratio $(G + C)_t - (G + C)_r/(G + C)_e - (G + C)_r$, where the subscripts t, r and e refer to the total, the retarded and the excluded DNA, respectively.

Some experiments in which gel filtration of enzymic hydrolysates was carried out on agarose columns were performed as described elsewhere (Prunell & Bernardi, 1973).

(f) *DEAE-cellulose chromatography*

1 or 2 ml of DNA digests as obtained at 6°C (starting A_{260} equal to 1) were loaded on 0·4 cm × 12 cm DEAE-cellulose (Serva, Heidelberg, Germany) columns. Enzymic digestions were stopped as described above, except that EDTA was not added, since it was found to bind to DEAE-cellulose and to disturb the adsorption–desorption process. A linear gradient of ammonium acetate (0·025 M to 0·5 M, pH 6·0; total vol. 150 ml) was applied using a 5·5 ml/h flow rate. The transmission at 271 nm (corresponding approximately to the isosbestic point of adenylic and thymidilic acids) of the effluent was monitored with a Zeiss spectrophotometer equipped with a flow microcell and a recorder (Ehrlich *et al.*, 1972). 1-ml fractions were collected. The relative amount of each component was estimated by integration of the area under the peak (Ehrlich & Prunell, manuscript in preparation).

The early eluting components were identified by degradation with: (a) acid phosphomonoesterase B (Chersi *et al.*, 1971); (b) acid exonuclease (Bernardi & Bernardi, 1968) plus acid phosphomonoesterase B; and (c) acid phosphomonoesterase B, followed (after inactivation) by exonuclease. Separation of nucleotides and nucleosides and analysis of

the latter on Aminex A6 columns (see below), after the enzymic degradations just mentioned, allowed identification of mononucleotides, dinucleotides, and of A-T and T-A isomers, respectively. It should be pointed out that the high molecular weight (excluded) DNA fraction present in the digests remained on the column under the conditions used.

Base composition of DNA fractions was determined, after enzymic degradation to nucleosides (Carrara & Bernardi, 1968) by chromatography on Aminex A6 (Bio-Rad, Richmond, Calif.) columns (Thiery *et al.*, 1973; Ehrlich & Prunell, manuscript in preparation).

3. Results

(a) *Degradation of yeast mitochondrial DNA with micrococcal nuclease*

When yeast mitochondrial DNA is degraded by micrococcal nuclease, the size distribution of the digests is bimodal, as shown by chromatography on Sephadex columns (Fig. 2). This leads to a separation of high molecular weight, excluded, and low molecular weight, retarded, material at all stages of the digestion and at each temperature (6, 25, 69°C) used. Digests obtained at 69°C (which is the melting temperature (t_m) of mitochondrial DNA in the solvent used) showed satisfactory separations on G100 columns (Fig. 2(a)), but not on G25 columns (not shown); the reason for the latter result was the presence of relatively large oligonucleotides in the digest (see below). Digests obtained at 6°C (Fig. 2(b)) or 25°C (not shown), in contrast, were perfectly resolved by Sephadex G25 columns; in this case the retarded material was subfractionated into two fractions, the first one containing small oligonucleotides (mostly dinucleotides) and T, the second one the other three nucleotides and some G-containing dinucleotides.

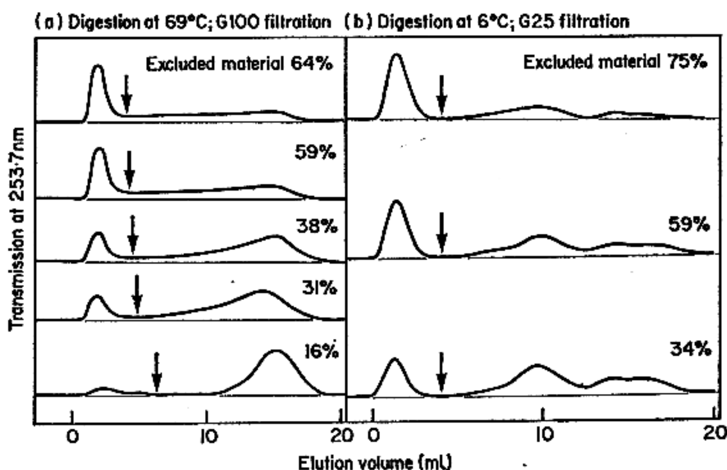

FIG. 2. Recorded chromatograms at 253·7 nm on Sephadex G100 (a) and G25 (b) of micrococcal digests of mitochondrial DNA obtained at 69°C (a) and 6°C (b). Arrows indicate the elution volume at which the excluded material was cut from the retarded one. For the estimates of excluded material and the experimental conditions see Materials and Methods. Zero elution volume corresponds to the beginning of fraction collection.

As expected, the hyperchromicity elicited by heating undigested mitochondrial DNA at the t_m, 22%, was immediately and completely reversed by cooling to room temperature. In contrast, the reversibility upon cooling to 25°C of the hyperchromicity caused by heating *plus* enzymic degradation (total hyperchromicity at the t_m,

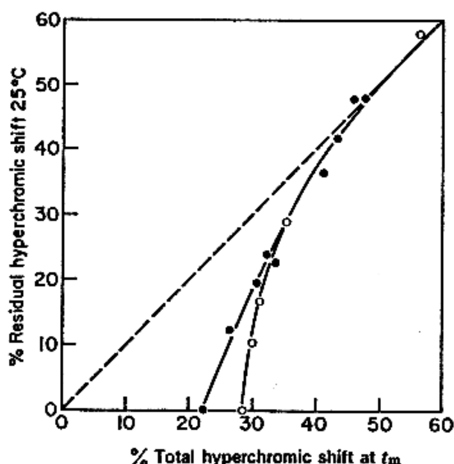

FIG. 3. Plot of the residual hyperchromicity (after enzymic digestion at 69°C and 67·5°C, respectively, of mitochondrial DNA (●) and 50%-satellite (○), and cooling to 25°C) *versus* the total hyperchromicity (enzymic + thermal) as measured at 69°C and 67·5°C (see Text). Total hyperchromicities were corrected for volume expansion of DNA solution.

Fig. 3), though still immediate, was less and less complete as digestion progressed. In Figure 3, the difference, along the ordinate axis, between the curve through the experimental points and the broken line of slope 1 corresponds to the hypochromicity caused by cooling from t_m to 25°C. Needless to say in digestions carried out at 6°C or 25°C only an "enzymic" hyperchromicity was observed.

Figure 4 shows the G + C content of the excluded and retarded fractions as obtained from mitochondrial DNA degraded by micrococcal nuclease. The retarded

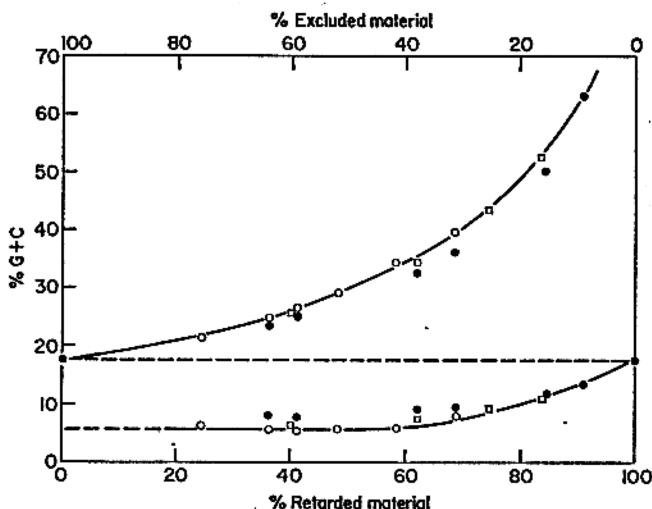

FIG. 4. Plot of the G + C content of the excluded and retarded fractions of mitochondrial DNA as degraded at 6°C (○), 25°C (□) and 69°C (●), against the relative amount of excluded or retarded material. Sephadex G25 columns were used for digests obtained at 6°C and 25°C. G100 columns for digests obtained at 69°C.

material, as obtained at 69°C, had a constant G + C content of about 7 to 8% up to a point where it represented about 50% of the starting material†. In digests obtained after further degradation, the G + C contents of the retarded material slowly increased. At the same time, the G + C contents of the excluded fractions also increased, reaching levels higher than 60% when 90% of the DNA was retarded. Interestingly, slight differences could be seen in the G + C contents of fractions derived from digests obtained at 6°C, 25°C, and 69°C, the lower digestion temperatures leading to lower G + C values (5 to 6%) of the retarded fraction in the initial plateau region. A detailed characterization of the two fractions is presented below.

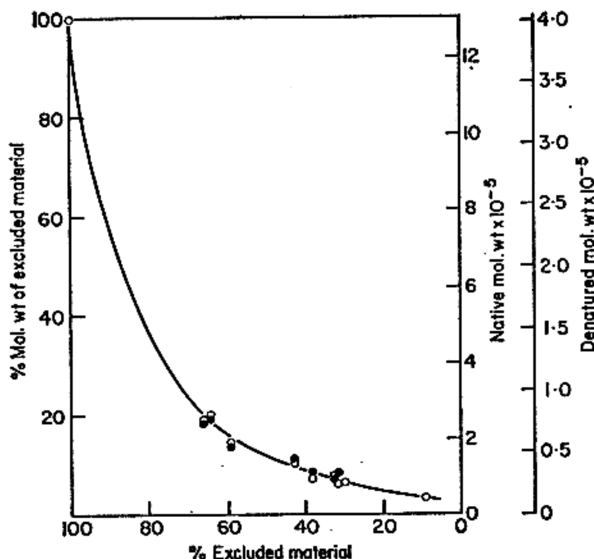

FIG. 5. Plot of native (○) and denatured (●) molecular weights of excluded fractions of mitochondrial DNA, as degraded at 69°C, *versus* their relative amounts.

(b) *Properties of excluded material*

Figure 5 shows the native and denatured molecular weights of the excluded material, as obtained at 69°C, as a function of the extent of degradation. Interestingly, the native molecular weight of the excluded fraction at 50% degradation is about $1 \cdot 6 \times 10^5$ and at 90% degradation still is 4×10^4. A comparison of native and denatured molecular weights shows that the starting DNA contains a small number of nicks; this number does not increase during degradation, as shown by the fact that the native and denatured molecular weights (as determined during enzymic degradation) fit the same curve. Both native and denatured molecular weights of excluded material from digests obtained at 6°C were found to be considerably lower, by a factor of about 5, than those found at 69°C at a comparable level of degradation.

Figure 6 shows the differential melting curves of the excluded material after different degrees of degradation at 69°C. In all cases, the t_m of the degraded material (Fig. 6(b) to (d)) was higher than that of undegraded mitochondrial DNA (Fig. 6(a)), conclusively showing that the excluded material had a native structure. The dif-

† Extents of degradations are always expressed as percentages of retarded material, which, in turn, are given on a nucleotide basis (see Materials and Methods).

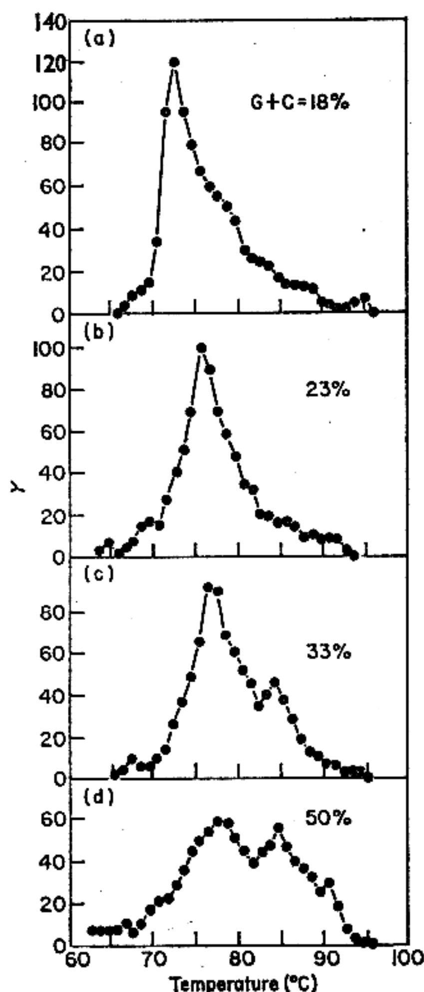

FIG. 6. Differential melting curves of excluded fractions of mitochondrial DNA as degraded at 69°C. The ordinate indicates the increment in relative absorbance per degree

$$Y = \frac{1000(At_1 - At_2)}{A_{100} - A_{25}}/t_1 - t_2,$$

where At_1, At_2, At_{100}, A_{25} are absorbances at temperatures t_1, t_2, 100 and 25°C, respectively. The G + C contents of the fractions are indicated in the Figure.

ferential melting profiles, as enzymic degradation progressed, showed a gradual disappearance of the low-melting components and a regular increase of the high-melting ones. It is evident from Figure 6(b) and (c), that the material corresponding to the first, main melting component of Figure 6(a) is largely decreased in the degraded DNA of Figure 6(b) and practically ceases to exist in the sample of Figure 6(c). The main peak of Figure 6(b) and (c) essentially corresponds to the first shoulder of Figure 6(a) (see also the comments on Figure 7(a) and (b), below). An evident shift of the melting temperature towards lower values is shown by the most degraded material (Fig. 6(d)). The average t_m of this material, which only represents 16% of

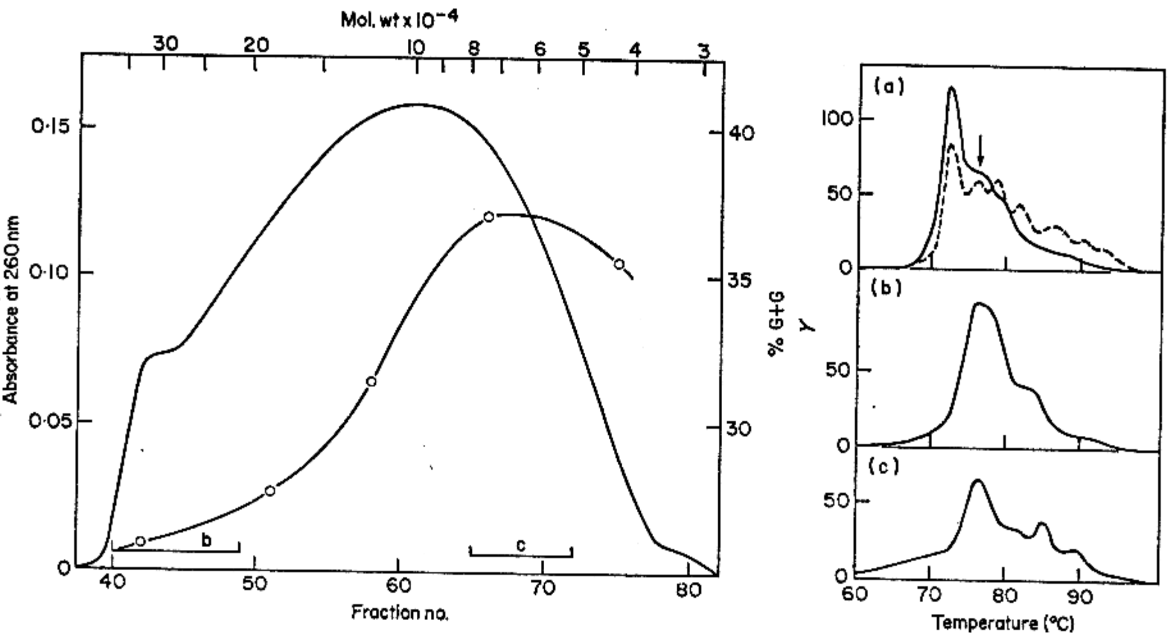

FIG. 7. Chromatogram of 2·5 A_{260} units of degraded mitochondrial DNA, on a 1·2 cm × 57·1 cm Sepharose 6B column. Flow rate was 6 ml/h; 0·61 ml fractions were collected. DNA was degraded at 69°C to 57% degradation, and filtered on a Sephadex G100 column. The excluded fraction was concentrated, dialysed *versus* SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate), and loaded on the Sepharose column equilibrated with the same solvent. The differential melting curves of the starting undegraded DNA (in this case the broken line refers to measurements done at 280 nm) and of pooled chromatographic fractions b and c are shown in inserts (a), (b) and (c), respectively. See text.

the starting DNA, is lowered by about 5 to 6 deg. C (compare the t_m of Figure 6(d) with the t_m corresponding to the highest melting 16% of Figure 6(a)).

The distribution of sizes and compositions in the G + C-rich fragments was studied as follows. Excluded material obtained by digesting mitochondrial DNA at 69°C up to 57% degradation (G + C = 32%; $M_r = 1{\cdot}2\times10^5$) was chromatographed on a Sepharose column and the properties of the fractions were investigated (Fig. 7). The largest fragments ($M_r > 3\times10^5$), partially excluded from the gel, had a G + C content of 26%; smaller fragments had increasingly higher G + C levels, which reached a maximum of 37%.

Upon melting, the early eluting material showed a major component with a t_m of 77°C and a minor component with a t_m of 83°C (Fig. 7(b)). Since in this case the molecular weight was high enough not to cause any depression in t_m, the major component of the large fragments could be identified with the shoulder of the undegraded mitochondrial DNA (Fig. 7(a); arrow); in view of the presence of the higher-melting minor component, the G + C content of the major component can be estimated as equal to 23 to 24%. As expected, the large fragments did not contain any material corresponding to the main component of undegraded DNA.

The late eluting material showed a more complex melting pattern, characterized by the presence of low-melting (62 to 72°C) material and by a number of components melting at 77°C, 81·5°C, 85°C and 89°C (Fig. 6(c)). In this case, molecular weight was low enough to cause a t_m depression. Assuming that the material melting at 62 to 72°C corresponded to a contamination by the main component of Figure 7(b), the G + C of the majority of the material can be estimated at 40% G + C with about half of it reaching values in the vicinity of 50%.

(c) *Properties of retarded material*

The retarded fractions, obtained by gel filtration on Sephadex G100 of mitochondrial DNA digests at 69°C, were analysed on DEAE-cellulose columns using ammonium acetate–7 M-urea, pH 5·5, as the eluant. The isostich pattern did not vary, at least between 30% and 50% degradation; it was characterized by the presence of mono- to heptanucleotides and was centred on the di- and trinucleotide peaks (not shown), with about 60% material larger than dinucleotides.

A more detailed study was done on mitochondrial DNA as degraded at 6°C, in which case the DEAE-cellulose chromatography was performed without urea in the eluting buffer (Fig. 8). Under these conditions, the major nucleotide components, T,

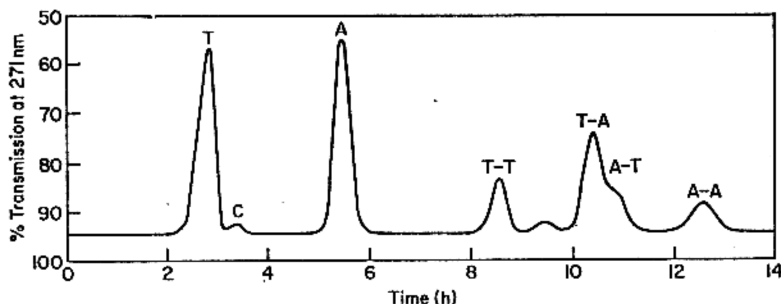

Fig. 8. DEAE-cellulose chromatogram of a mitochondrial DNA digest obtained at 6°C and showing a degradation level of 63%. The peak following T-T is formed by C-C + C-T + T-C; G is eluted with A, and G-containing dinucleotides with A-A.

A, T-T, A-T + T-A, and A-A are well separated; C and a peak probably corresponding to C-C + C-T + T-C were also separated, whereas G was eluted with A, and G-containing dinucleotides with A-A; tri- to pentanucleotides were also seen in the chromatogram (not shown), but their amount was about 7% of the retarded material. A major difference in the retarded material as released at 6°C compared to that released at 69°C is, therefore, the smaller average size of the former.

TABLE 1

Composition of mono- and dinucleotides as released by micrococcal nuclease at 6°C

	Hyperchromic shift (%)	Degradation† (%)	T	A	T-T	A-T + T-A	A-A	C	C-T
Mitochondrial DNA	20	25	35·0	31·2	6·7	21·2	4·6	0·3	1·0
	38·5	49	32·9	31·1	7·1	22·3	5·1	0·6	0·9
	49·7	63	31·2	31·1	6·9	22·0	6·2	1·3	1·4
	52·6	66	32·1	30·7	7·2	20·3	6·5	1·5	1·5
Crab satellite DNA	32	40	34·2	34·7	0·7	29·8	0·6	0	0
	43	54	34·2	34·2	0·5	30·6	0·4	0	0
	63	80	33·8	33·1	0·7	31·7	0·7	0	0
50%-satellite	19	24	34·0	36·0	0·8	28·2	0·8	0	0
	31·5	40	34·3	33·6	0·8	30·8	0·4	0·2	0
	45·5	58	31·6	31·7	1·4	33·2	1·7	0·5	0

† % of retarded fraction as calculated from the hyperchromic shift.

The results obtained by studying the composition of the resolved mono- and dinucleotides, as released at different digestion levels, are given in Table 1. No changes in the relative amounts of the components were observed up to 50% degradation; the chromatograms obtained at farther degradation levels showed an increase in the peaks corresponding to C and to C-C + C-T + T-C. The lower amount of T-T + A-A (13%) compared to A-T + T-A (21%) suggests that the enzyme preferentially splits the non-alternating T and A sequences rather than the alternating ones, since pyrimidine tract analysis (Ehrlich *et al.*, 1972) had shown a larger amount of T_2, T_3 and T_4 compared to T.

At all temperatures, hyperchromic shift increased linearly with the amount of retarded material (Fig. 9). The slopes of the straight lines were different at different temperatures and the hyperchromic shifts extrapolated to 100% retarded material were 55%, 65% and 76%, when degradations were carried out at 69, 25 and 6°C, respectively. Interestingly, the same straight lines were obtained for 50%-satellite and mitochondrial DNAs from the petite mutants. The results of Figure 9 fit the finding just reported that isostich distribution of the retarded material is different at different degradation temperatures.

(d) *Crab satellite DNA and 50%-satellite*

Figure 10 shows the G + C content of excluded and retarded fractions of degraded 50%-satellite, as determined at different levels of enzymic degradation. The G + C level of the retarded material was constant and equal to 3% G + C (the average base

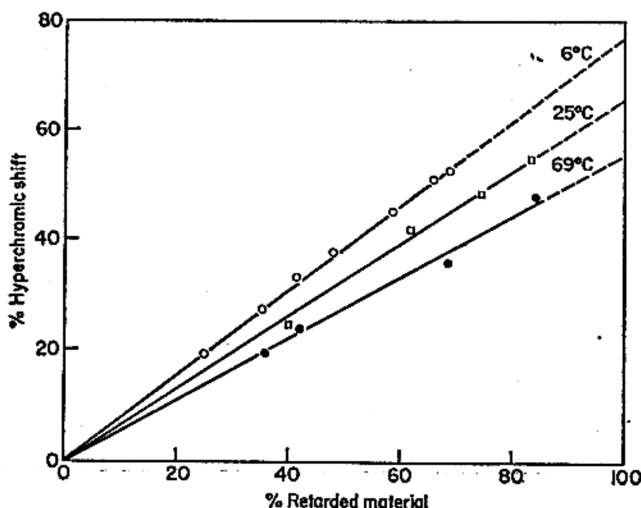

FIG. 9. Hyperchromic shifts (residual hyperchromic shift for degradation at 69°C) as obtained at 3 different digestion temperatures are plotted against the amount of retarded material.

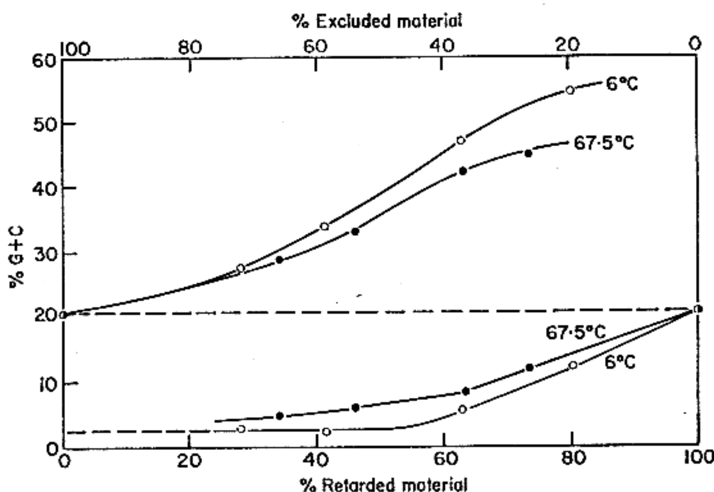

FIG. 10. Plot of the G + C content of excluded and retarded fractions of 50%-satellite crab DNA as degraded at 6°C (○) and 67·5°C (●).

composition of the crab satellite DNA) until 50% of the material was degraded at 6°C, whereas it was significantly higher and increased continuously when degradation was performed at 67·5°C, a temperature corresponding to the midpoint of the plateau region separating the melting transitions of the satellite and the main DNA component.

The composition of mono- and dinucleotides released at 6°C by micrococcal DNAase from both crab satellite DNA and 50%-satellite are shown in Table 1. When the data for crab satellite and 50%-satellite are compared, it can be seen that the relative amounts of components are the same up to about 50% degradation of the 50%-satellite and therefore to the total elimination of the satellite from the

mixture. Divergences appear further on, as a consequence of the degradation of main DNA, the increase of T-T and A-A in the hydrolysate being particularly striking. Tri- to pentanucleotides were also seen in the DEAE-cellulose chromatogram (not shown). Their distribution and relative amount (17% of retarded material) were constant and identical for satellite and 50%-satellite DNA at the degradation levels indicated in Table 1.

In the case of the crab satellite, the relative amounts of mononucleotides were very close to those found in mitochondrial DNA (adenylic acid being, however, slightly higher), but large differences were found in the dinucleotides: the alternating A-T's were much higher, whereas A-A and T-T were much lower in amount compared to mitochondrial DNA. This finding fits with the notion (Ehrlich *et al.*, 1973) that crab satellite DNA only contains 7% of clustered T and A (essentially as di- and trinucleotides), whereas mitochondrial DNA spacers contain (Ehrlich *et al.*, 1972) over 40% of clustered T and A (mostly di- to tetranucleotides).

Other results concerning 50%-satellite were presented in Figure 3. It was already mentioned that the 50%-satellite fitted the relationships of Figure 9.

4. Discussion

(a) *The G + C-rich stretches*

The essential points about the high-molecular-weight material released by micrococcal nuclease are shown in the present work to be: (i) the mitochondrial genes have a rather low average G + C content; this can be estimated as 31% or 33%, according to whether the G + C content of the spacers, which account for 50% of the genome, is taken as 5% or 3%, respectively.

(ii) The G + C-rich stretches are endowed with a large compositional heterogeneity; at 57% degradation, about half of the genome is formed by fragments, having an average molecular weight of $1{\cdot}2\times10^5$, which range from 25% to 50% in G + C levels. It would be interesting to compare this compositional heterogeneity with those of phage and bacterial DNAs; unfortunately this is not feasible because the data in the literature (Miyazawa & Thomas, 1965) concern larger fragment sizes and different size distributions.

(iii) The results in Figure 7 have several interesting implications. The fact that the largest gene fragments have the lowest G + C levels, in spite of the preference of the enzyme for sequences containing A and T, suggests that the genes containing these fragments are much longer than those with the highest levels of G + C. In other words the size of fragments with different G + C levels still reflects after degradation the size of the mitochondrial DNA genes from which they came. It is very tempting to think that the long fragments of 23 to 24% G + C, showing a main symmetrical component upon melting, originate from the large ribosomal cistrons since ribosomal RNA from yeast mitochondria has a G + C level of 23% (Reijnders *et al.*, 1972); on the other hand some of the fragments with a G + C level around 50% might come from transfer RNA cistrons.

(b) *The A+T-rich spacers*

The basic questions here concern the relative amount of the spacers, their G + C level and their average size in the genome.

(i) The best estimate about the amount of the spacer material comes from the base composition of the retarded material. This is constant (in both G + C content, Figure 4, and mono- and dinucleotide composition, Table 1) up to about 50% degradation; at higher degradation levels both the G + C content of the retarded material (Fig. 4) and the amounts of mono- and dinucleotides formed by, or containing, C increase (Table 1). The meaning of this result is quite clear if one looks at the behaviour of the 50%-satellite: in this case, too, the G + C content and the composition of mono- and dinucleotides of the retarded material, as released at 6°C, are constant and equal to the values of satellite DNA up to a degradation level of 50%; it is evident, therefore, that the satellite is totally released as retarded material before the main DNA. It should, therefore, be concluded that the amount of spacer material in mitochondrial DNA is equal to the amount of satellite in the 50%-satellite, namely 50%.

(ii) Concerning the G + C level of the spacer material the following can be said. In the mitochondrial DNA, the G + C level of the small fragments released in the first half of the degradation is higher, 7 to 8%, when degradation is done at 69°C, compared to that found in degradations done at 6°C or 25°C, which is only 5 to 6% (Fig. 4). An explanation for the higher G + C value at high temperature comes from the finding that when the 50%-satellite is degraded at 67·5°C, the G + C level of the retarded material also is higher compared to 6°C (Fig. 10). In this latter case, it is clear that the higher G + C level found in degradations carried out at high temperature is originated by "leakage" of short fragments from the main DNA. A similar phenomenon is certainly responsible for the higher G + C level of retarded material released at 69°C in the case of mitochondrial DNA, leading to the provisional conclusion that the "correct" G + C level of the spacers is not 7 to 8% but 5 to 6%.

In fact the G + C level of the spacers is very probably lower than 5% because a leakage from the G + C-rich stretches is, in all likelihood, not completely eliminated at 6°C. This conclusion is supported by a comparison of the melting curves of 50%-satellite and of yeast mitochondrial DNA: in the first case there is a wide gap between the melting of satellite DNA (3% G + C) and of main DNA (37% G + C), whereas in the second one there is a lack of resolution between the main melting component (corresponding to the spacers) and the higher melting component (corresponding to DNA as low as 23% in G + C) which may leak small fragments. Results obtained with mitochondrial DNA from petite mutants (work in preparation) also point in this direction.

(iii) The average size of the spacers can be estimated as follows. When degradation has reached 50%, practically all the spacers have been completely degraded to retarded material by a degradation which occurs without any base specificity when the spacers are in a single-stranded configuration (von Hippel & Felsenfeld, 1964). At this point the average size of the G + C-rich stretches is $1{\cdot}6\times10^5$. The average size of the spacers can only be higher than this value. This is a minimum estimate since the excluded material is not only fragmented by the removal of the spacers, but also by breaks introduced in the A + T-rich regions of the genes. This minimum estimate actually corresponds to the lowest values in the size estimates derived from previous results on spleen acid DNAase degraded material (Piperno *et al.*, 1972) and partial denaturation maps in the electron microscope (Christiansen *et al.*, 1974), which show a number of easily denatured regions, not widely different in size, inter-

spersed at intervals of 0·5 to 3 μm with more stable regions of length 0·1 to 5 μm.

(c) *Mechanism of action of mitochondrial DNA degradation by micrococcal nuclease*

The results obtained in the present work confirm and extend the conclusions of previous investigators (Dirksen & Dekker, 1960; von Hippel & Felsenfeld, 1964; Wingert & von Hippel, 1968) on the mechanism of action of micrococcal nuclease.

The hyperchromicity caused by digestion, the gel filtration and the DEAE-cellulose chromatography of the digests studied here all show that the fragment size distributions are different at 6 and 69°C, the typical bimodality being, however, always present, as is the case with other DNAs.

At 6°C, the degradation is accompanied by the release of mono- and dinucleotides. It is not clear, at the present time, whether this release is associated with all endonucleolytic breaks or not, nor whether mono- and dinucleotides, which are apparently formed from the beginning of the degradation, are directly formed from large double-stranded fragments or from short-lived oligonucleotides derived from the former by melting. It is evident, however, that the enzyme inserts a much larger number of breaks into the spacers compared to the genes, as a consequence of the fact that the short sequences recognized and split are much more frequent in the former than in the latter. Clearly, some leakage of material from the genes can be expected under such conditions.

At 69°C, the enzyme preferentially splits the spacer sequences, which are in a single-stranded configuration at this temperature corresponding to the t_m of mitochondrial DNA and therefore to 50% hyperchromic shift. A very small amount of gene stretches rich in A·T base pairs, and therefore in a single-stranded configuration at the t_m, are also split off at the same time, originating a more serious leakage of gene fragments compared to the 6°C degradations. The further degradation of the large single-stranded fragments so released originates oligonucleotides which are large enough, on the average, to require Sephadex G100 for their separation from the macromolecular fragments. The material which very rapidly reanneals upon cooling from 69 to 25°C, and which decreases in amount as degradation proceeds (Fig. 3) can be identified with A + T-rich single-stranded ends still attached to G + C-stretches.

(d) *Some general issues*

The major conclusions and implications of the present work are the following. The mitochondrial genome of yeast consists, to an extent of about 50%, of spacers having a G + C level lower than 5%. It is extremely unlikely that such spacers code for anything or that they are transcribed. If the circular 50×10^6 molecules seen in the electron microscope (Hollenberg *et al.*, 1970) correspond to a basic unit of information, only 25×10^6 of it are informationally meaningful, and therefore the five-fold difference in size of animal and yeast mitochondrial DNA cannot be equated with a five-fold difference in informational content. In this connection, it should be observed that much less than 10×10^6 of yeast mitochondrial DNA has been accounted for, so far, in terms of known gene products (ribosomal and transfer RNAs). Under such circumstances, it seems to be premature to believe that the "large" mitochondrial genomes of unicellular eukaryotes, like yeast, necessarily contain more genetical information than the "small" mitochondrial genomes of animal cells and that the

latter have evolved from the former through the loss of dispensable functions. Such views essentially rest on renaturation kinetics data. It is well known, however (Hollenberg *et al.*, 1970; Christiansen *et al.*, 1971,1974, and our unpublished results), that the renaturation kinetics of yeast mitochondrial DNA present a number of anomalies concerning the temperature and molecular weight dependence which are not well understood at the present time. The present availability of G + C-rich and A + T-rich stretches should help in clarifying these problems.

A very important question is whether the existence of spacer sequences in mitochondrial DNA is unique to yeast, an organism which does not have an absolute requirement for functional mitochondria, or is of general significance. A number of findings may be quoted in favour of the widespread, if not general, occurrence of spacer sequences (not necessarily of the A + T type) in mitochondrial DNAs: (a) we have been able to show (work in preparation) that about 20% of mitochondrial DNA from *Euglena gracilis* is formed by stretches having a G + C content of only 9%. (b) Very low melting components, corresponding to sequences extremely rich in A + T have been recently detected by Bultmann & Laird (1973) and by Polan *et al.* (1973) in the mitochondrial DNA of *Drosophila melanogaster*; these components account for 20 to 30% of the DNA. (c) Comparative investigations on the mitochondrial DNAs of *Xenopus laevis* and *Xenopus mülleri* by Dawid (1972) pointed to the possibility of rapidly evolving spacer sequences, accounting for about 20% of the mitochondrial genome. Incidentally, it should be stressed that the existence of spacers in mitochondrial DNA can be considered as a eukaryotic trait.

Concerning the nucleotide sequences of the spacers, the constancy in both base composition and composition of mono- and dinucleotides of the retarded material released during the first half of the degradation certainly indicates a high compositional homogeneity. They do not necessarily imply that the spacers are made up of perfectly repeating short sequences, nor that they do not show slight regional variations in sequence. The fact, however, that the spacers contain on the average one G·C base pair every 20 to 30 base pairs, which are in the form of short alternating and non-alternating A-T sequences makes highly probable, on statistical grounds alone, the existence in the spacers of a large number of homologous sequences long enough to allow stable duplexes to be formed by a recombination mechanism. This point, which may be the key of the genetical instability of the mitochondrial genome of yeast, is being investigated in our laboratory by sequence methods.

After this work was submitted for publication, Lamb & Rojanapo (1973) reported that mitochondrial RNA from two wild-type yeast strains showed G + C contents of 28·9% and 35·1%, respectively. These values are very close to the average G + C level of mitochondrial genes found in the present work (32%); it indicates that the A + T-rich spacers are not transcribed and suggests that all genes are transcribed.

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