Reprinted from *J. Mol. Biol.* (1972) **65**, 191–205

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The Mitochondrial Genome of Wild-type Yeast Cells

II. Investigations on the Compositional Heterogeneity of Mitochondrial DNA

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(*Received 26 July 1971*)

The compositional heterogeneity of yeast mitochondrial DNA was investigated by fragmenting it with spleen acid DNase (an enzyme known for its ability to split both strands at the same level) and by resolving the fragments on hydroxyapatite columns. When applied to mitochondrial DNA degraded to an $S_{20,w}$ value of 8·5 S, chromatography permitted the isolation of fragments having a G + C content of 26% (as opposed to the 18% value of unfractionated DNA) and molecular weight of 1 to 2 $\times$ 10^5 daltons. On the other hand, fragments having a G + C content of 14% and molecular weight of the order of 8×10^5 daltons were also isolated; these fragments formed 22% of mitochondrial DNA. Detailed investigations were carried out on the chromatographic fractions. This led to a better understanding of the heterogeneity of mitochondrial DNA and confirmed that the anomalous binding of silver ions and elution from hydroxyapatite of mitochondrial DNA is mainly due to its A + T-rich stretches.

Recurrent degradation of the A + T-rich fragments isolated by chromatography on hydroxyapatite permitted the preparation, in 12% yield, of fragments having a G + C content of 10% and molecular weights of $2{\cdot}5 \times 10^5$ daltons. This experiment showed that a large fraction, and possibly all the mitochondrial genome of yeast is formed by long G + C-rich and A + T-rich stretches which have molecular weights of the order of 10^5 to 10^6 daltons, and which are intermingled. While the former correspond in all likelihood to mitochondrial genes, like those coding for ribosomal RNA, the biological significance of the A + T-rich stretches is not clear.

1. Introduction

We have shown in the preceding paper (Bernardi, Piperno & Fonty, 1972) that mitochondrial DNA from wild-type (grande colonie) *Saccharomyces cerevisiae* shows an "intermolecular" heterogeneity in base composition at a size level of $1{\cdot}5 \times 10^6$ daltons when chromatographed on hydroxyapatite columns. Fragments having a molecular weight of about $1{\cdot}5 \times 10^6$ daltons and a G + C content of 22%, as opposed to the average value of 18% for unfractionated DNA, have been isolated, as have smaller fragments ($2{\cdot}5 \times 10^5$ daltons) having an even higher G + C content (24%). The present work was done in order to understand better the heterogeneous composition of yeast mitochondrial DNA and to test a model proposed for the structure of the mitochondrial genome (Bernardi *et al.*, 1972). According to this model, the mitochondrial genome contains A + T-rich stretches interspersed among G + C-rich

stretches. The A + T-rich stretches are thought to be responsible for the anomalous physical and chromatographic properties of yeast mitochondrial DNA.

Several different experimental approaches to this type of problem are possible. So far, we have restricted our choice to those involving: (a) fragmentation of native DNA molecules with preservation of their double-stranded structure; (b) separation of the fragments, obtained on the basis of their nucleotide composition and/or physical structure; and (c) chemical and physical characterization of the fragments.

In the present paper, we report results obtained using spleen acid DNase as the degrading agent; this enzyme is known for its ability to cut across both DNA strands at the same level (Bernardi, 1971; Bernardi & Sadron, 1961). Hydroxyapatite chromatography was used in order to separate the fragments obtained according to their A + T contents; it has been recently observed (Bernardi *et al.*, 1972) that yeast mitochondrial DNA owes its exceptionally high eluting molarity to its A + T-rich components, and that G + C-rich fragments are eluted at a lower molarity than the bulk of mitochondrial DNA.†

2. Materials and Methods

(a) *Degradation of mitochondrial DNA with spleen acid DNase*

Mitochondrial DNA from preparations IV or I + II (Bernardi *et al.*, 1972) was concentrated by rotary evaporation to an $A_{260} = 5{\cdot}0$, dialysed against 0·15 M-sodium acetate buffer, 0·001 M-EDTA, pH 5·0, and treated twice with chloroform–isoamyl alcohol (24:1 v/v). Digestion was performed with acid DNase from hog spleen (Bernardi, Bernardi & Chersi, 1966) in a 4-bulb viscometer using conditions already described (Bernardi & Cordonnier, 1965; Bernardi & Bach, 1968); the A_{260} of the DNA solution degraded in the viscometer was close to 3. In some cases, in which chromatographic fractions were further degraded, degradation was followed by sedimentation velocity analysis. In other cases, initial hyperchromic shifts were also investigated using a Zeiss spectrophotometer equipped with an automatic sample changer, a transmittance–absorbance converter, and a Multiriter recorder (Texas Instruments, Dallas, Texas).

(b) *Distribution of sedimentation coefficients*

The distributions of sedimentation coefficients were calculated from the equation $g(s) = 1/c_0 \times \mathrm{d}c/\mathrm{d}s$, where c_0 is the initial DNA concentration and s is the sedimentation coefficient. The dependence of $g(s)$ upon sedimentation time was found to be practically negligible in the range of S values under consideration. Sedimentation coefficients and distributions of sedimentation coefficients were calculated using a Programma 102 (Olivetti, Ivrea, Italy) desk computer equipped with a data storage attachment.

All other experimental methods and materials have been described in the preceding paper.

3. Results

Several samples of mitochondrial DNA derived from preparation IV (Bernardi *et al.*, 1972) and degraded by spleen acid DNase to different extents were chromatographed on hydroxyapatite columns. We will describe in section (a) below, the results obtained on the chromatographic fractions of a DNA sample degraded to an average S value of 8·5 S, which is almost the lowest size-level still compatible with preservation of secondary structure in all fragments. The chromatographic separation and the

† This work was presented at the International Meeting "Les Acides Désoxyribonucléiques des Eukaryotes", organized by the Centre National de la Recherche Scientifique at Port-Cros, France (5 to 9 May 1971).

characterization of other enzymic digests will be reported in sections (b) and (c); further investigations on the A + T-rich segments will be described in section (d).

(a) *The mitochondrial DNA fragments obtained by degradation with spleen acid DNase to* $S_{20,w} = 8{\cdot}5$ *s*

The chromatogram of mitochondrial DNA degraded by spleen acid DNase to 8·5 s (Fig. 1) is essentially identical to that of the starting DNA run under exactly the same experimental conditions (not shown here), except that its elution begins at a slightly lower molarity (0·24 M-sodium phosphate buffer (pH 6·8) instead of 0·25 M) and the front of the peak is eluted less abruptly.

Various properties of the chromatographic fractions were investigated, along with those of the unfractionated material.

(i) *Sedimentation coefficients*

The unfractionated 8·5 s DNA showed (Fig. 2(a)) a widespread distribution of sedimentation coefficients, $g(s)$. Sedimentation coefficients of the chromatographic fractions varied from 5 s for the low-eluting fragments to 10·5 s for the high-eluting fragments. The $g(s)$ of the former was narrower and more symmetrical (Fig. 2(b)) than that of the latter (Fig. 2(c)), which showed a relatively large lighter shoulder. The great heterogeneity of molecular sizes of the digested DNA, revealed by both its $g(s)$ and the S values of the chromatographic fractions, explains why the enzymic degradation cannot be pushed much beyond an average value of 8·5 s without causing some disruption of secondary structure in the more degraded molecules (see section (b)).

(ii) *Base composition*

This was found to vary from 26% G + C for early-eluting fractions to 14% G + C for the late-eluting ones. Base pairing was excellent in all cases. It is interesting that

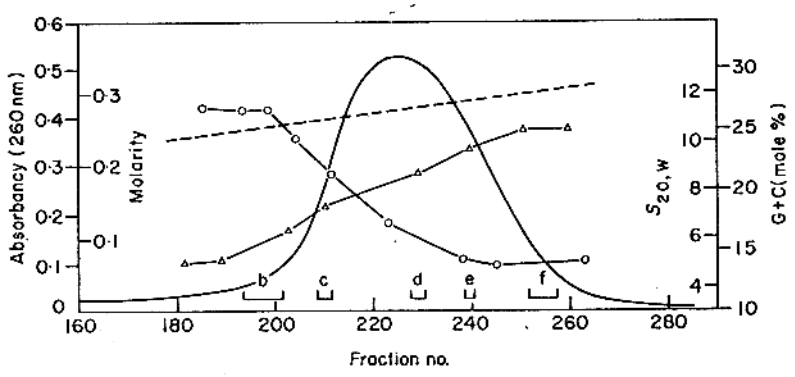

FIG. 1. Chromatography of mitochondrial DNA degraded to $S_{20,w} = 8{\cdot}5$ s by spleen acid DNase.

20 ml. of DNA solution in 0·1 M-sodium phosphate buffer (60 A_{260} units) were loaded on a 1·3 cm × 65 cm hydroxyapatite column equilibrated with the same buffer; the column was then washed with 50 ml. of 0·1 M-sodium phosphate buffer; elution was carried out with a 0·1 to 0·5 M gradient of sodium phosphate (1200 ml.). Fractions of 2·8 ml. were collected. The flow rate was 40 ml./hr. —○—○—, G + C content of single fractions as determined by direct nucleoside analysis. —△—△—, $S_{20,w}$ of pooled fractions (2 to 6 depending on the absorbance). The broken line indicates the molarity gradient. Horizontal bars indicate the pooled fractions utilized for the melting experiments.

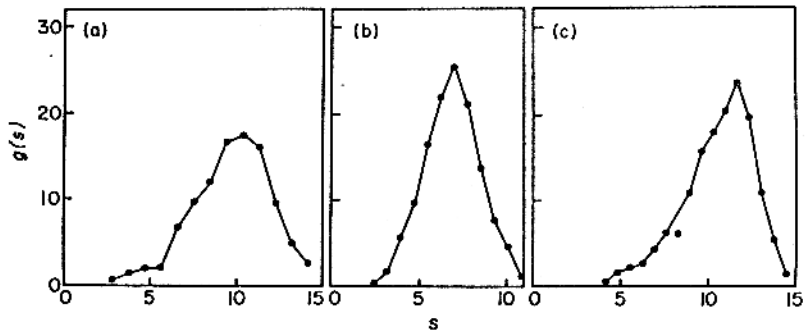

FIG. 2. Distribution of sedimentation coefficients of mitochondrial DNA degraded to $S_{20,w}$ = 8·5 s. (a) Unfractionated material; material from the chromatography shown in Fig. 1; (b) fractions 202 to 203; (c) fractions 250 to 251.

a constant base composition was found at both ends of the chromatogram. These two plateau regions have very different extents, the right-hand one involving a much more significant amount of DNA fragments. These two regions are separated by a main, intermediate region, where the G + C content regularly decreases from the left to the right of the chromatogram. The amount of DNA present in the late fractions and having a constant base composition equal to 14% G + C, represents 22% of the total mitochondrial DNA loaded on the column. The sedimentation coefficients of these chromatographic fractions are more than 10 s: they correspond therefore, to molecular weights of the order of 8×10^5 daltons or more (Bernardi & Sadron, 1964; Bernardi, 1968; see also footnote on p. 182 of the preceding paper). On the other hand, the low-eluting fractions having a G + C content of 26% represent about 3·5% of mitochondrial DNA; these fractions have sedimentation coefficients between 5 and 6 s, corresponding to molecular weights in the range 1 to 2×10^5 daltons. It may be interesting to note that one-third of the mitochondrial DNA fractions have a G + C content higher (21·3%) and two-thirds a G + C contents lower (15·5%), than the average value of 18%.

(iii) *Buoyant densities in CsCl density-gradients*

A value of 1·683 g/cm^3 was found for fraction 248. An extremely broad band was obtained for fractions 186 to 192, because of the very low molecular weight of the DNA fragments and their heterogeneity of composition (see section (iv)).

(iv) *Buoyant densities in Cs_2SO_4–Ag^+ density-gradients*

In contrast with the behaviour of "normal" DNA's, such as bacterial DNA's, but in agreement with findings already reported (Bernardi *et al.*, 1972), in all Cs_2SO_4–Ag^+ density-gradient experiments on degraded mitochondrial DNA, the high-density side of the peaks was always lower in G + C content than the low-density side. When examined in a Cs_2SO_4–Ag^+ preparative density gradient, the low-eluting fractions (204 to 207) showed a shoulder on the light side. The G + C content at the two far ends of the peak were 19·7% and 35·4% (Fig. 3(a)). The middle fractions showed a symmetrical peak (Fig. 3(b)); their base composition at the two far ends was 18% and 21·6% G + C, respectively. These values of the base compositions correspond to those found for the two extreme chromatographic fractions which were pooled together for the density-gradient experiment. An additional check on this sample was made by running another sample in a Cs_2SO_4–Hg^{2+} density gradient; in this case,

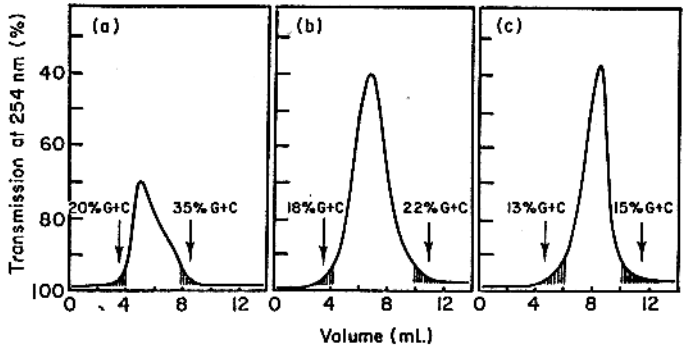

FIG. 3. Ag^+–Cs_2SO_4 preparative density gradients of DNA from pooled fractions of the chromatography shown in Fig. 1 as recorded by a Uvicord using a cell with an optical path equal to 0·3 cm. (a) Fractions 204 to 207; (b) 3·6 A_{260} units from fractions 212 to 222; (c) fractions 242 to 244. The hatched areas indicate the DNA fractions which were analysed. Bottom of the tube at left.

too, a single peak was found; and the far ends of the peak showed a base-composition practically identical to those found for the peak obtained in the Cs_2SO_4–Ag^+ density-gradient. Finally, the high-eluting fractions showed a symmetrical peak (Fig. 3(c)) with an even narrower distribution of base composition, namely between 13 and 15% G + C.

(v) *The first derivatives of the absorbance–temperature profiles*

Those given by the unfractionated material and the chromatographic fractions of mitochondrial DNA are shown in Figure 4. The degraded, unfractionated DNA (Fig. 4(a)) showed an almost symmetrical transition, shifted to lower temperature compared to the undigested DNA, with a maximum at about 67°C. The differential melting curves of the chromatographic fractions were found to be very different for the low- and the high-eluting materials. The low-eluting fractions (Fig. 4(b)) showed a very broad transition; the high-melting components were much more abundant than in the unfractionated material. The high-eluting fractions, in contrast, were characterized (Fig. 4(f)) by a main component melting at 67 to 71°C, and by the virtual absence of the high-melting components. The intermediate fractions, forming the largest part of the chromatogram, showed melting patterns (Fig. 4(c) to (e)) of an intermediate type, which gradually changed across the chromatogram; it is noteworthy, however, that patterns resembling those of the far right fractions were found at relatively low elution molarities. It appeared interesting to check the contribution of G · C base pairs to the melting of the high-eluting fractions of constant base composition. This was done by investigating the melting curve at 280 nm (Fig. 5). It is interesting that this melting curve, mainly reflecting the melting of G · C base pairs (Felsenfeld & Hirschman, 1965), is shifted to the left relative to that obtained at 260 nm, whereas the opposite was found to take place with undegraded, unfractionated DNA (Bernardi *et al.*, 1972).

(b) *The mitochondrial DNA fragments obtained by degradation with spleen acid DNase to* $S_{20,w} = 5{\cdot}5$ *s*

In this experiment the digestion of mitochondrial DNA (preparation IV) was simply pushed beyond the stage described in section (a), to an average S value of 5·5 s. At

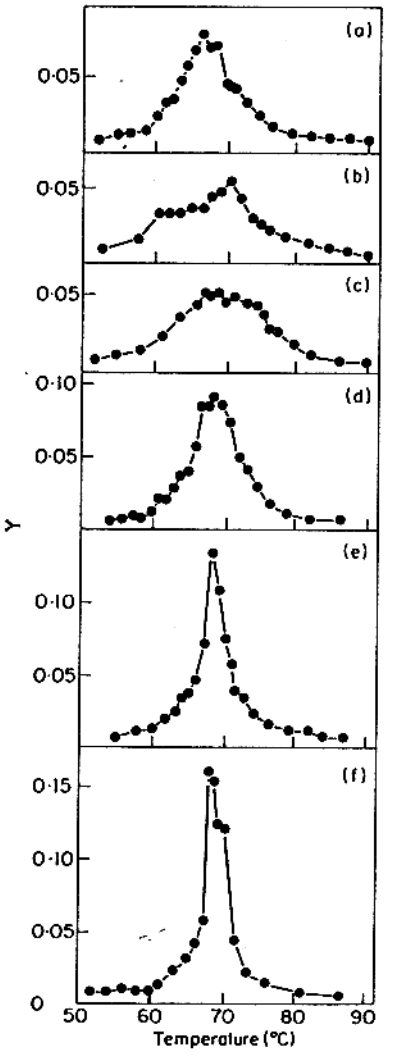

FIG. 4. Differential melting curves (see Fig. 4 of preceding paper) of mitochondrial DNA degraded to $S_{20,w} = 8{\cdot}5$ s. (a) Unfractionated material; (b), (c), (d), (e) and (f) material from pooled chromatographic fractions indicated by horizontal bars in Fig. 1.

this degradation level, about 22% of the material was not retained by the column equilibrated with 0·22 M-phosphate buffer (Fig. 6); this material had an estimated 23·4% G + C content and was shown, by additional chromatographic experiments, to consist of single-stranded DNA fragments. The rest of the DNA was eluted between 0·22 and 0·33 M-phosphate buffer with a pronounced shoulder on the low-eluting side. The sedimentation coefficients varied from 3·8 to 7 s across the chromatogram, the base composition from 23% to 12% G + C. It is noteworthy that no plateau region was found in the base composition of the low-eluting fractions, whereas a residual plateau of base composition formed by 13% of the total DNA was found for

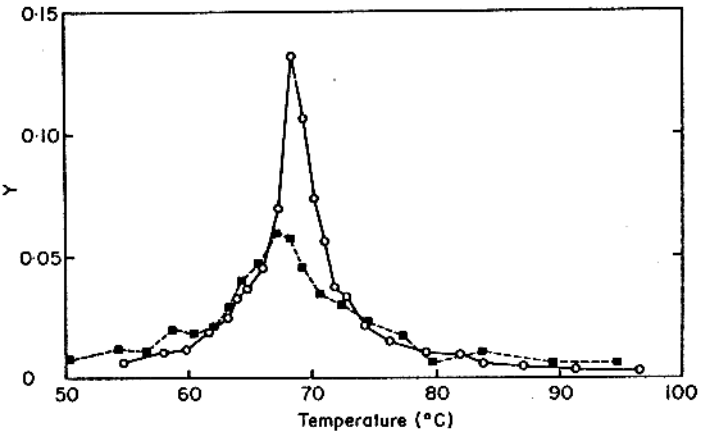

FIG. 5. Differential melting curves of mitochondrial DNA from the 14% G + C plateau region of the chromatography shown in Fig. 1. —○—○—, 260 nm; — ■ — ■ —, 280 nm.

the high-eluting fractions; in this case, fractions having less than 18% G + C formed 51% of the material and their average G + C contents were 14%.

(c) *The mitochondrial DNA fragments obtained by degradation with spleen acid DNase to a rather uniform size,* $S_{20,w} = 6{\cdot}6$ *s*

This experiment was undertaken to analyse the compositional heterogeneity of mitochondrial DNA after reducing the very strong heterogeneity in molecular weight of the enzymically degraded samples by further degradation of the high-eluting fractions. An additional object of this experiment was to obtain preliminary information on the nature of the fragments formed by further degradation of the intermediate fraction.

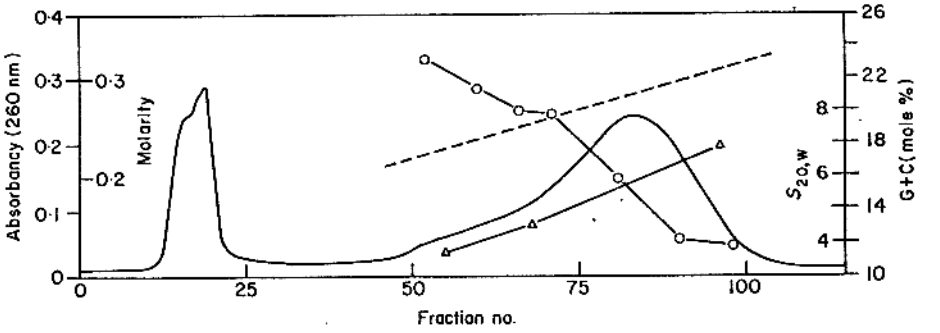

FIG. 6. Chromatography of mitochondrial DNA degraded to $S_{20,w} = 5{\cdot}5$ s.
14 ml. of DNA solution in 0·2 M-sodium phosphate buffer (pH 6·8) (19·5 A_{260} units) were loaded on a 1 cm × 50 cm hydroxyapatite column equilibrated with the same buffer; the column was then washed with 50 ml. of 0·2 M-sodium phosphate buffer; elution was carried out with a 0·2 to 0·6 M gradient of sodium phosphate buffer (480 ml). Fractions of 2·4 ml. were collected. The flow rate was 25 ml./hr. Circles and triangles indicate the G + C content and $S_{20,w}$, respectively as described in Fig. 1. The broken line indicates the molarity gradient.

A DNA sample (preparation IV) was first degraded with acid DNase to nearly the same viscosity as the sample used in the experiment of Figure 1. The degraded DNA was then chromatographed on hydroxyapatite. The chromatographic peak, identical to that of Figure 1, was divided into three parts. The low-eluting fractions (17%), showing $S_{20,w} = 6{\cdot}4$ s were kept as such; the central (50%), and the high-eluting fractions (33%), which had sedimentation coefficients of 8·4 and 11·4 s, respectively, were independently digested further. The sedimentation coefficients of the further degraded samples were 6·8 s and 6·6 s, respectively. At this point the three samples were pooled and chromatographed on a hydroxyapatite column (Fig. 7). The chromatogram, obtained under the same experimental conditions as those used in Figure 1, were more symmetrical than that of Figure 1. Expectedly, the variation in sedimentation coefficients of the chromatographic fractions was smaller than that found previously, since it ranged from 5 to 8 s instead of 5 to 11 s. The variation of base-composition through the chromatogram was similar to that found previously (section

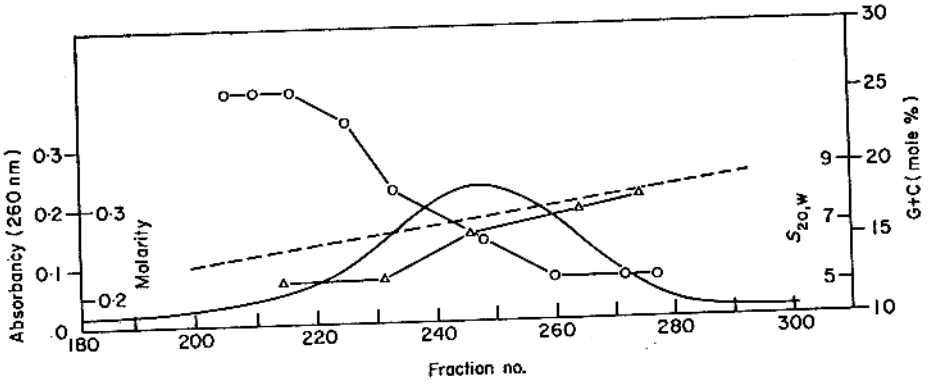

FIG. 7. Chromatography of mitochondrial DNA degraded to a rather uniform size; $S_{20,w} = 6{\cdot}6$ s. 55 ml. of DNA solution in 0·1 M-sodium phosphate buffer (28 A_{260} units) were loaded on a 1·3 cm × 65 cm hydroxyapatite column equilibrated with the same buffer; the column was then washed with 35 ml. of 0·1 M-phosphate buffer. The flow rate was 36 ml./hr. See the legend to Fig. 1 for the experimental conditions and symbols.

(a)), except that the G + C contents of the high-eluting fractions showed a plateau value of only 12·8%. It is interesting that the percentage of DNA fragments having this base composition was the same as that found in the corresponding region of the chromatogram shown in Figure 1, namely 22%, whereas the amount of material present in the low-eluting region showing a constant base composition of 26% G + C was significantly higher, about 6% instead of 3·5%.

(d) *The A + T-rich fragments of mitochondrial DNA*

The following experiment was designed to obtain information on the lowest G + C level attainable in A + T-rich fragments larger in size than about $2{\cdot}5 \times 10^5$ daltons. The A + T-rich fragments were obtained by recurrent degradation of the high-eluting fractions having a constant G + C composition. This procedure enabled us to follow the kinetics of the G + C decrease in the A + T-rich fragments, without interference from similar fragments formed by further degradation of the intermediate

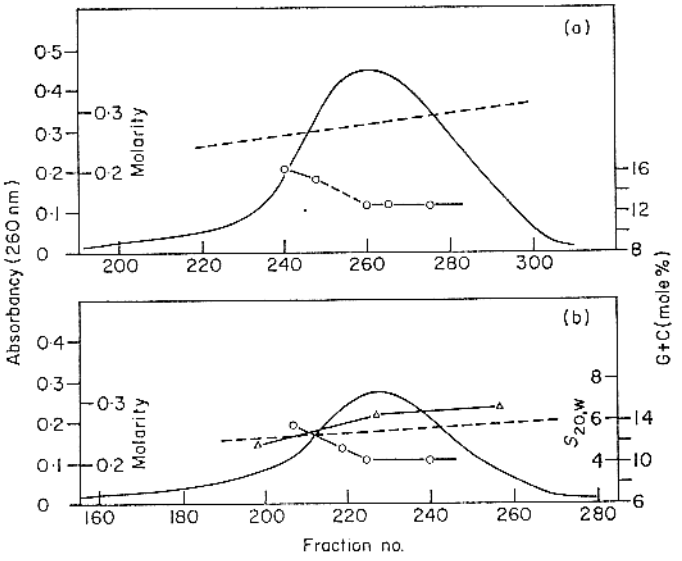

FIG. 8. Chromatography of A + T-rich fragments of mitochondrial DNA obtained by acid DNase degradation.

Frames (a) and (b) show chromatograms 2 and 3 of Table 1. See the legend to Fig. 1 for the experimental conditions and symbols.

fraction. Information was also obtained in this experiment concerning the order of magnitude of the A + T-rich stretches in mitochondrial DNA.

The starting material was, in this case, preparation I + II (Bernardi *et al.*, 1972). This was degraded from its original S value of 12·2 to 9·0 s and chromatographed. The A + T-rich fractions of the plateau regions were further digested and rechromatographed. This procedure was repeated once more. The last two chromatograms are shown in Figure 8. Table 1 shows the results for base composition along with the sedimentation data and the amounts of fractions actually recovered. A decrease in size of the A + T-rich fractions from $9{\cdot}2 \times 10^5$ to $2{\cdot}4 \times 10^5$ daltons was accompanied

TABLE 1

Preparation of A + T-rich fragments from mitochondrial DNA by recurrent digestion with spleen acid DNase†

Chromatogram	DNA submitted to degradation		A + T-rich fragments isolated by chromatography			
	S	M	Starting DNA	S	M	G + C (%)
	12·2	$1{\cdot}26 \times 10^6$	100			
1	9·0	$0{\cdot}57 \times 10^6$	39 (39)§	10·8	$0{\cdot}92 \times 10^6$	15·3
2	7·6	$0{\cdot}37 \times 10^6$	23 (59)		$(0{\cdot}6 \times 10^6)$‡	12·7
3	5·5	$0{\cdot}16 \times 10^6$	12 (52)	6·5	$0{\cdot}24 \times 10^6$	10·4

† See under Results, section (d), for further explanations.

‡ Estimated from the molecular weight of the corresponding unfractionated DNA ($0{\cdot}37 \times 10^6$).

§ Percentage of material recovered from each step.

by a decrease in G + C from 15 to 10%. The actual recovery of the fragments having a G + C content of 10% was 12% of the initial DNA. The molecular weights of the A + T-rich fragments from the plateau region were systematically higher than those of the degraded material as measured before chromatography. This finding is not surprising in view of the results of the chromatograms shown in Figures 1 and 7, and leads to the conclusion that the G + C-rich stretches of yeast mitochondrial DNA are preferentially split by spleen acid DNase. Evidence pointing to the same conclusion has also been obtained in an investigation carried out by different methods (A. Prunell & G. Bernardi, manuscript in preparation).

(e) *Other experiments*

The following experiments were performed in order to shed more light on two questions raised by the enzymic degradation and the fractionation procedure.

(1) The initial rate of hydrolysis of several DNA's by acid DNase was checked using identical experimental conditions (0·05 M-ammonium acetate, pH 5·5; 25°C). This experiment, carried out by following the hyperchromic shift caused by the enzyme, showed that *H. influenzae* DNA (38% G + C) was split at 1·23 times the rate of mitochondrial DNA (18% G + C). Synthetic poly[d(A - T) · d(A - T)] † was split at 0·8 times the rate of mitochondrial DNA, and poly(dA · dT) was resistant to the enzyme.

(2) A control experiment was performed in order to assess the relative importance of base composition (and/or structure) and molecular weight in causing the chromatographic separation of mitochondrial DNA fragments. The effect of molecular size upon elution molarity was investigated using poly[d(A - T) · d(A - T)] in order to avoid any influence of base composition. Half of the polymer loaded on the column was slightly degraded with acid DNase to increase the spread of molecular sizes. Figure 9 shows that in this case a marked fractionation according to molecular size takes place on the column, the small fragments being eluted before the large ones.

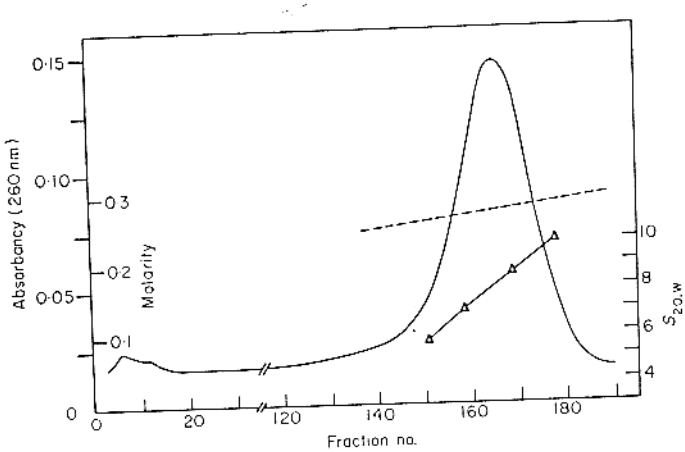

FIG. 9. Chromatography of biosynthetic poly[d(A - T) · d(A - T)] 9·5 ml. of polymer solution in 0·003 M-phosphate buffer (11 A_{260} units) were loaded on a 1·3 cm × 66 cm hydroxyapatite column equilibrated with 0·1 M-phosphate buffer. A 0·1 to 0·5 M gradient of phosphate buffer (1200 ml.) was applied. Fractions of 2·8 ml. were collected. The flow-rate was 36 ml./hr. The molarity gradient is shown by the broken line, $S_{20,w}$ values by the triangles.

† See footnote on p. 174 of paper I.

4. Discussion

(a) *Chromatography of enzymically degraded DNA on hydroxyapatite columns*

The chromatography on hydroxyapatite columns of mitochondrial DNA fragments showed fractionation according to their size and A + T level. The fundamental importance of this fractionation of structural features associated with the A + T level is shown by the following findings: (1) Chromatography of synthetic poly-[d(A-T) · d(A-T)], poly(dA · dT) and *Cancer pagurus* A + T-rich satellite has shown that these polydeoxyribonucleotides have peculiar elution molarities (Bernardi *et al.*, 1972); (2) G + C-rich fragments were also resolved from the bulk of mitochondrial DNA when they had nearly the same molecular weight as all other fragments (Bernardi *et al.*, 1972); proof that mitochondrial DNA fragments having the same size can be resolved on hydroxyapatite according to their A + T level has been subsequently obtained (A. Prunell & G. Bernardi, manuscript in preparation); (3) the A + T-rich fractions from the plateau region of the chromatogram of Figure 1 were very heterogeneous in molecular weight and contained very short fragments, as shown by the distribution of the sedimentation coefficients; since these fractions were remarkably homogeneous in base composition, as judged from the analyses of the Cs_2SO_4–Ag^+ density-gradients, it appears that some short A + T-rich fragments are eluted after longer fragments that are richer in G + C. Direct evidence on this point has been obtained in current investigations (A. Prunell & G. Bernardi, manuscript in preparation).

On the other hand, the experiment on poly[d(A-T) · d(A-T)], mentioned in section (e) of Results, demonstrates unequivocally the ability of the columns to resolve double-stranded polydeoxyribonucleotides according to their molecular weight; since the elution molarity of DNA's of different molecular weights is practically the same (Bernardi, 1965;1969), this resolution is very probably due to the displacement of the smaller fragments by the larger ones (an effect already observed, in a very different range of molecular weights, with mixtures of DNA's; Bernardi, 1969). Therefore, the fact that the G + C-rich fragments are, on the average, shorter than the A + T-rich fragments (owing to the preference of the enzyme for the G + C-rich stretches; see section (e) of Results) acts as an additional factor shifting them to lower elution molarities.

A comparison of the results shown in Figure 3 of the preceding paper (Bernardi *et al.*, 1972), and Figures 1 and 6 of the present one indicates that, as the enzymic digestion proceeds, the material eluted at constant G + C content, simultaneously decreases in amount and in G + C level: in the starting DNA almost 100% of the material it eluted at a G + C level of 18%; in the 8·5 s DNA 22% of the material has a constant G + C level of 14%; finally, in the 5·5 s DNA 13% of the material has a G + C level of 12%. This finding is compatible with the idea that, in order to be eluted in the A + T-rich plateau region, mitochondrial DNA fragments must: (1) have a ratio of A + T-rich to G + C-rich stretches above a certain threshold, and (2) be of sufficiently large size. When either one or both of these conditions are not fulfilled, DNA fragments move to lower elution molarities.

The experiment in which the size polydispersity of mitochondrial DNA was reduced by further degradation of the intermediate and high-eluting fractions (Fig. 7) is interesting in that it shows that these fractions give rise, upon further degradation, to both G + C-rich and A + T-rich fragments. This is shown by the fact that

the amount of material eluted in the high eluting region is the same (22%) as in the case of the first experiment of Figure 1; in addition, its G + C level is lower (12·8% *versus* 14%). Since it is clear from the comparison of Figures 1 and 6 that pushing the enzymic degradation reduces the amount of material eluting in the right-hand plateau region of the chromatogram, this indicates that fragments richer in G + C, which were shifted to lower eluting molarities after digestion, were compensated for by other fragments, richer in A + T, deriving from the intermediate region. At the same time, as pointed out in the Results, there is an increase, by a factor of two, of the material eluting in the low-eluting plateau region of high G + C contents. The experiment of Figure 7 leads, therefore, to the conclusion that more G + C-rich and more A + T-rich fragments are formed by further degradation of the intermediate fraction, suggesting a bimodal distribution of G + C contents in mitochondrial DNA fragments.

(b) *The G + C-rich fragments*

Fragments having a higher G + C content (26%) and a smaller size (1 to 2 $\times$ 10^5) than those seen previously (Bernardi *et al.*, 1972) have been found at the low-eluting end of the chromatograms shown in Figures 1 and 7. In both cases these fragments are eluted in a region of constant G + C level; they form 3·5 and 6% of total mitochondrial DNA in the experiments of Figures 1 and 7, respectively. When centrifuged in a Cs_2SO_4–Ag^+ density-gradient, fractions close to the plateau region were shown to contain fragments having a G + C content as high as 35%. A note of caution should be expressed here, however, because of the presence of 1% contaminating nuclear DNA in the starting, undegraded mitochondrial DNA. It is not inconceivable that nuclear DNA, a better substrate for acid DNase because of its higher G + C level, has been degraded further than the mitochondrial DNA (see section (a) above), and has been eluted before the G + C-rich fractions of mitochondrial DNA: in this case G + C levels above the average 26% might belong to mitochondrial DNA fragments.

(c) *The A + T-rich fragments*

The investigations on the A + T-rich fragments summarized in Table 1 provide useful information on the heterogeneity of mitochondrial DNA. (i) The large difference between the average molecular weight of the degraded, unfractionated DNA and the A + T-rich fragments (as eluted at high molarity and constant base composition) confirms that most scissions took place in the G + C-rich stretches of mitochondrial DNA. More important, the A + T-rich fragments have a size corresponding to a half to two thirds of that of the parent molecules and are recovered in 40 to 60% yields. This means that one can recover fragments as large as, or larger than, half the parent "molecules" with a remarkably high A + T content by causing a relatively small number of scissions which preferentially fall in the G + C-rich stretches of the parent fragments. Clearly, this can only occur if the A + T-rich "molecules" are formed by segments of widely different base composition and if the length of such segments is of the same order of magnitude as the fragments formed. A model in which the A + T-rich and the G + C-rich fragments would be shorter than, say, $0{\cdot}5 \times 10^5$ is not consistent with our results. (ii) The lowest level of G + C in the A + T-rich segments is definitely lower than the value, 10%, found for the fragments isolated

from the chromatogram of Figure 8(b), since the decrease in G + C does not show the least indication of approaching a plateau value. It is possible that the actual G + C level of the A + T-rich segments of mitochondrial DNA is as low as, or lower than, the lowest G + C level, 4%, found so far (Bernardi *et al.*, 1968; Mehrotra & Mahler, 1968) in DNA's from some cytoplasmic petite mutants.

The investigations on the A + T-rich fragments, as obtained from the chromatogram of Figure 1, confirm and extend the conclusions just presented. (i) The differential melting curves of the A + T-rich fragments, as obtained from the chromatogram of Figure 1, showed that they are much less heterogeneous in base composition than the starting DNA. In spite of their variation in G + C content between 13 and 15%, as opposed to the constant value of 18% for undegraded DNA, their differential melting curve extends over a narrower temperature range than that of the latter, and resembles that of the main melting component of unfractionated, undegraded DNA. (ii) The first derivative of the melting curve at 280 nm, mainly reflecting the melting of G · C base pairs, is shifted to the left relative to that obtained at 260 nm, whereas the opposite is true for undegraded, unfractionated DNA (Bernardi *et al.*, 1972). This suggests that the G + C-rich stretches still present in the A + T-rich fragments are probably shorter than the A + T-rich stretches. (iii) The similarity between the main components of differential melting curves obtained with the unfractionated, undegraded mitochondrial DNA and with the A + T-rich fraction suggests that the main component is actually due to the A + T-rich stretches of mitochondrial DNA. If this identification is correct, the extent of the A + T-rich stretches in mitochondrial DNA may be almost 50% (Bernardi *et al.*, 1972).

(d) *Implication of the A + T-rich stretches in the abnormal physical properties of mitochondrial DNA*

The implication of the A + T-rich stretches in causing the abnormally high buoyant density of mitochondrial DNA is indicated by the finding that the buoyant density of the A + T-rich fraction from the chromatogram shown in Figure 1 deviates from the value expected on the basis of the empirical relationship described by Schildkraut, Marmur & Doty (1962) more than does the undegraded, unfractionated DNA. Indeed, the buoyant density of the A + T-rich fractions is equal to, or slightly higher than, that of the latter, in spite of the fact that their base composition is lower in G + C by 4%. The prediction that the G + C-rich fractions should tend to have a "normal" buoyant density (Bernardi *et al.*, 1972) could not be verified because of the extremely low molecular weight of those fractions.

The buoyant density of degraded mitochondrial DNA in Cs_2SO_4–Ag^+ density-gradients revealed that the high density side of the peak was richer in A + T than the low density one. This is in contrast with the behaviour of normal DNA, since the G + C-rich molecules bind more Ag^+ and therefore band at a higher density than A + T-rich molecules. Since the binding of Ag^+ by alternating dA-T · dA-T sequences is practically non-existent at the Ag^+/DNA-phosphate ratio used (Davidson *et al.*, 1965), this abnormal behaviour appears to be due to the binding of Ag^+ by non-alternating dA · dT sequences.

The implication of the A + T-rich fragments in determining the anomalous chromatographic behaviour of yeast mitochondrial DNA has already been discussed (Bernardi *et al.*, 1972 and in section (a), above).

(e) *General comments*

The present work has shown that, upon degradation with spleen acid DNase, yeast mitochondrial DNA yields fragments having sizes in the range 1 or 3 $\times$ 10^5 daltons, which are highly enriched in A + T and G + C, respectively, over the average base composition of the unfractionated material. The enzymic degradation and the fractionation experiments lead to the conclusion that the mitochondrial genome of yeast is formed by interspersed G + C- and A + T-rich stretches. The average size of these stretches appears to be between 10^5 and 10^6 daltons. While the base composition of the G + C-rich stretches is entirely compatible with their coding ability for gene products like mitochondrial ribosomal RNA, the base composition of the A + T-rich stretches (and the possible presence of short repeated sequences in them; see Ehrlich, Thiery & Bernardi, 1972) suggests either that they are not transcribed or that they code for some peculiar gene product.

It should be stressed that the real levels of the A + T-rich and, even more, of the G + C-rich fragments in the mitochondrial genome are greatly underestimated by their actual recoveries for two reasons: (1) the yield of A + T-rich fragments, after recurrent degradation, obviously does not include all A + T-rich fragments which would have arisen from the main, intermediate fraction; the much lower recovery of G + C-rich fragments seems to be due to the specificity of acid DNase and to the separation method used in the present work (see the Discussion above); (2) the fragments obtained are the result of a practically random degradation and therefore originate from different regions of the genome and represent much more of it than is apparent from the figures of the recoveries. These two sets of considerations make it conceivable that the mitochondrial genome is entirely made up of G + C- and A + T-rich sequences, although, admittedly, rigorous evidence for this belief is not yet available.

This is the first time that a genome has been reported to contain, beside functional genes, a very large number of long nucleotide sequences with a very extreme base composition. Nothing can be said at the present time about the physiological role of the A + T-rich stretches found in yeast mitochondrial DNA. If the A + T-rich stretches contain relatively short repeating sequences, a possibility compatible with the depurination results reported in the following paper (Ehrlich *et al.*, 1972), the large extent of homologous nucleotide sequences might easily lead to recombinational processes and to the elimination of segments of the genome by internal crossing-over. It is tempting to believe that such a deletion process might have a role in the cytoplasmic petite mutation.

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