Heterogeneity of mitochondrial DNA from *Saccharomyces carlsbergensis*.
Denaturation mapping by electron microscopy

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ABSTRACT

Electronmicroscopic observation of the denaturation pattern of 130 partially denaturated linear mitochondrial DNA molecules from *Saccharomyces carlsbergensis* was used to investigate the distribution of AT-rich sequences within the mitochondrial genome. The molecules were observed after heating to 43°C in the presence of 12% formaldehyde. These conditions resulted in an average denaturation per molecule of 21%. The average length of the molecules was 10 μm , and a few molecules had a length corresponding to the size of the complete genome. The undenaturated regions varied in length from 0.1 to 5.0 μm with denaturated regions of length 0.02 to 0.1 μm in between. A denaturation map was constructed by use of one of the long molecules (28.7 μm) as a master molecule for positioning of all other molecules. This map shows distinct regions corresponding to the position of easily denaturated sequences in the mitochondrial DNA. These sequences which presumably correspond to the very AT-rich regions, known to exist in the yeast mitochondrial DNA, were found at intervals of about 0.5 - 3 μm on the map.

INTRODUCTION

The DNA in wild-type mitochondria of the yeasts *Saccharomyces cerevisiae* and *S. carlsbergensis* is circular with a contour length of about 26 μm ¹. The average base composition of the DNA is 18% G+C, if determined by chemical methods, but calculated from buoyant density, 1.684 g/cm³, and the thermal denaturation temperature, 74.0°C, the G+C content is 24% and 12%, respectively². This discrepancy in G+C content, determined by the different methods, is explained by the presence of an appreciable fraction of both (dAT:dAT) and (dA:dT) structures in the yeast mitochondrial DNA^{3,4}. These AT-rich stretches interfere with the determination of the genome size from quantitative renaturation experiments^{5,6}. The ribosomal RNA in yeast mitochondria is known to be a transcript of the mitochondrial DNA. It has a G+C content of about 25%^{7,8} and molecular weights of 1.3×10^6 daltons

and 0.7×10^6 daltons for the large and the small species of RNA, respectively⁹. It is thus evident that the yeast mitochondrial DNA is very heterogenous in base composition.

Intramolecular heterogeneity is also inferred from the properties of yeast mitochondrial DNA fractionated on hydroxy apatite¹⁰⁻¹² and from density gradient centrifugation of sonicated yeast mitochondrial DNA¹³. The relative positions of the pure poly-AT or very AT-rich sequences within the mitochondrial genome are not known.

To obtain further information on the extent and distribution of the very AT-rich sequences in yeast mitochondrial DNA, we have studied this DNA by electronmicroscopy after partial denaturation of the DNA in the presence of formaldehyde.

MATERIALS AND METHODS

Saccharomyces carlsbergensis NCYC 74S was grown, converted to spheroplasts and the mitochondrial DNA isolated. The purity was confirmed by analytical ultracentrifugation and thermal denaturation, as described⁶.

ELECTRONMICROSCOPY

a) Denaturation and spreading of partially melted DNA

A small sample (20 μ l) of mitochondrial DNA in 0.01 M Na_2HPO_4 , 1 mM EDTA, pH 7.0 at a concentration of approximately 10 μ g/ml was mixed with 10 μ l of 35% formaldehyde (Merck), neutralized with 2 M NaOH at 0°C. This mixture was incubated for 10 min in an ultrathermostate (Heto, Denmark) to the temperature decided ($\pm 0.02^\circ\text{C}$). The denaturation was stopped by adding 250 μ l of icecold 1 M ammoniumacetate, pH 7.5, and 75 μ l 0.04% cytochrome c (Fulka AG, Buchs, Germany)¹⁴. The partially melted DNA was spread onto 0.25 M ammoniumacetate, pH 7.5, contained in a plastic pethridish 90 mm in diameter, by letting one drop of the spreading solution fall gently to the surface of the hypophase. The boundaries of the DNA-cytocrone c monolayer were visualized by a little talc on the surface of the hypophase.

b) Transfer and staining

The DNA cytochrome c monolayer was picked up on 3 mm copper grids covered by a thin formvar (1595 E) film stabilized with carbon. The monolayer, after transfer to the carbon surface,

was stained with uranyl acetate (RP Carlo EBBA, Italy) (5×10^{-5} M in 90% ethanol) for 30 sec. and finally rinsed for 10 sec. with isopentane¹⁵.

c) Electronmicroscopy

The preparations were photographed with a Jeol 100 B electronmicroscope (60 KV) at magnifications of 6,000 or 9,000 times using high contrast specimen holders. The negatives (Kodak electron image plates) were enlarged five times to make paper copies.

d) Measurements

The pictures of the DNA molecules on the paper copies were traced by using a Hewlett-Packard digitizer, model 9108, and the lengths of the molecules calculated by a Hewlett-Packard calculator, model 9100 A. Both of the single-stranded branches in the denaturated regions were measured. When a difference existed between the length of the two branches, the one of greatest length was chosen for the following presentation of results.

RESULTS AND DISCUSSION

a) Electronmicroscopy of partially denaturated mitochondrial DNA

In Fig. 1 are shown examples of electronmicroscopic pictures of yeast mitochondrial DNA molecules, partially denaturated in 0.01 M sec. phosphate (pH 7.0), 1 mM EDTA containing 12% formaldehyde by heating at 43°C for 10 min followed by rapid cooling. In other experiments, the DNA was heated at 40°C, 41°C and 42°C. Also at these temperatures practically all molecules contained one or more denaturated regions. Nuclear DNA was investigated under similar conditions and no denaturated sites were observed. The total fraction of a mitochondrial DNA molecule denaturated was found to increase with increasing temperature used for denaturation of the DNA (Table 1). This circumstance seemed to be an effect of the appearance of new denaturated regions rather than an effect of an increase in length of already existing denaturated sites. The increase in number of denaturated sites at higher temperatures may be explained either by a greater probability for denaturation of regions with a certain fixed base composition (the AT-rich regions) or by a successive melting of regions with an increasing G+C content. The experiments reported here cannot differentiate between these possibilities.

TABLE 1: Degree of denaturation per DNA molecule at different temperatures^{a)}

Temperature (°C)	Fractional length denaturated per molecule with standard deviations (%) ^{b)}	
40	5 ± 3	(5)
41	9 ± 4	(6)
42	9 ± 6	(27)
43	21 ± 6	(130)

a) Measured by electronmicroscopy after heating to different temperatures in 12% formaldehyde (see text).

b) Number in paranthesis gives number of molecules measured.

As seen from table 1 the fraction of a molecule melted at different temperatures increases from 5% at 40°C to 21% at 43°C. The last temperature corresponds to the mean denaturation temperature at that salt and formaldehyde concentration, so one might expect at an average 50% of a molecule to be denaturated. The 21% denaturation actually found may be explained by a reformation of native DNA when the denaturation conditions are withdrawn. At temperatures above 43°C the denaturation proceeded to very extensive strand separation. From these considerations it was suggested that a denaturation temperature of 43°C might give optimal conditions for a denaturation mapping of the mitochondrial DNA. Accordingly, this temperature was chosen for the following experiments.

The distribution of the length of the 130 individual DNA molecules studied after partial denaturation at 43°C in the presence of 12% formaldehyde is shown in Fig. 2. The length of a molecule was calculated as the sum of the length of undenaturated and denaturated regions. Because a single-stranded DNA branch of a denaturated loop has a certain probability of collapsing or folding up on itself, the longest branch was always used as the length of a denaturated region. However, the average difference in the length of the branches of all denaturated loops measured was only 0.02 μm or about 5%. It is seen from Fig. 2 that the distribution in length of the DNA molecules is

Fig. 1. Electron micrographs of mitochondrial DNA partially denatured at 43°C in the presence of 12% formaldehyde. The DNA cytochrome c monolayer was picked up on formvar covered and carbon stabilized grids and stained by uranyl acetate. The preparations were photographed at a magnification of 9,000. The total length of each molecule is: (a) 11.50 μm.

Fig. 1. Electron micrographs of mitochondrial DNA partially denatured at 43°C in the presence of 12% formaldehyde. The DNA cytochrome c monolayer was picked up on formvar covered and carbon stabilized grids and stained by uranyl acetate. The preparations were photographed at a magnification of 9,000. The total length of each molecule is: (b) 10.95 μ m.

Fig. 1. Electron micrographs of mitochondrial DNA partially denatured at 43°C in the presence of 12% formaldehyde. The DNA cytochrome c monolayer was picked up on formvar covered and carbon stabilized grids and stained by uranyl acetate. The preparations were photographed at a magnification of 9,000. The total length of each molecule is: (c) 13.13 μm.

Fig. 2. Length distribution of 130 mitochondrial DNA molecules partially denaturated at 43°C in the presence of 12% formaldehyde. The length of each molecule was calculated as the sum of the length of undenaturated and denaturated regions. The x-axis shows per cent of molecules, the y-axis length of the molecules in μm.

fairly even around an average of about 10 μm . Three of the molecules fall outside the distribution. The length of these molecules is 25, 26 and 28.7 μm and may therefore represent the linear broken forms of intact mitochondrial genomes. One of these molecules is used below as a master molecule for alignment of all smaller molecules in the construction of a denaturation map of the mitochondrial genome.

b) Construction of a denaturation map of the mitochondrial genome

An attempt to derive a denaturation map of a genome from a collection of individual partially denaturated DNA molecules raises several both theoretical and technical problems. One of the main problems is related to the question whether one has a homogenous population of DNA molecules. In the case of the bacterial phage DNA's, λ and P_2 ^{16,17}, and the mammalian Ad 2 virus DNA¹⁴, it is possible to obtain a population of DNA molecules which are all the linear form of the complete genome, and which have all been opened up precisely at the same position, that is at the site for the cohesive ends of these molecules. The proper orientation of these observed molecules was possible from the denaturation patterns at specific locations. These circumstances also allowed a normalization of the molecules to an average unit length with a corresponding adjustment of the length of all denaturated and undenaturated regions.

It is obvious that most of these conditions are not obtained with yeast mitochondrial DNA. From the results of work in several laboratories over the last years, it seems that the yeast mitochondrial DNA is particularly fragile, and that it has not been possible to obtain DNA preparations which contained more than a very small proportion of molecules having a length corresponding to the total genome. As mentioned earlier, the molecules observed in this study had an average molecular length of about 10 μm . These linear molecules presumably originate from a random breakage of the 26 μm long circular yeast mitochondrial genome. This complicates the alignment necessary for comparisons of the denaturation pattern between the individual molecules. However, if each molecule contained one or more clearly recognizable regions, such an alignment might still have been fairly easy.

As seen from the denaturation pattern of the molecules in Fig.3, such unambiguously recognizable regions are not easily found. The absence of such regions or pattern of regions presumably indicates that the easily denaturated AT-rich sequences are highly dispersed all over the genome but is naturally also a function of the stocastical nature of the denaturation process.

From the considerations given above follows that for the construction of the denaturation map we had to rely on purely quantitative measures of similarity. For that purpose one of the three long linear molecules thought to represent the complete genome was chosen as a master molecule. The position relative to the master molecule of all other molecules was then found by comparisons of the denaturation pattern of the master with that of all other molecules (see below). In Fig. 3 is shown the relative positioning of all the 130 molecules partially denaturated at 43°C. The master molecule relative to which all other positions are calculated, is marked with the capital letter M and an arrow. The position of an individual molecule was calculated by the aid of a computer programme. The molecule was divided in 0.01 μ m intervals and moved from one end along the master in 0.01 μ m steps . This procedure was repeated with the other end first. The over-all adaption at a given position was calculated as the ratio between the number of agreements (denaturated or undenaturated region at corresponding points in the two molecules) and the number of disagreements (denaturated region at one and undenaturated region at the other at a given point) at that position. The best fit indicated by this method was suggested to correspond to the proper orientation and position of a molecule relative to the master. As a restriction to the procedure a minimum overlap of at least 3 μ m was required for a position to be accepted. In the best position the number of agreements between two molecules were from 5 to 20 fold the number of disagreements, and in most cases the best position was clearly defined with a 1.2 to 3 fold higher proportion between agreement and non agreements than the nex best position.

The results of the positioning of the partially denaturated molecules in Fig. 3 are summarized in Fig. 4 and Fig. 5 which represent two different illustrations of the denaturation map of

FIG. 3.

Fig. 3. Denaturation map for 130 mitochondrial DNA molecules heated to 43°C in the presence of 12% formaldehyde. The position and extent of denaturated regions in each molecule are illustrated by black rectangles on horizontal lines (undenaturated regions). The molecules were electronmicrographed and measured as described in Materials and Methods. The position and orientation of each molecule relative to a master molecule were calculated as described in the text. The master molecule used is indicated by the capital letter M and an arrow. This molecule ($28.7\ \mu\text{m}$) is suggested to have a length corresponding to a complete mitochondrial genome.

Fig. 4. Histogram derived from data in Fig. 3. It shows the fraction of molecules carrying a denaturated site at a given position on the x-axis of that figure. This fraction was calculated for each 0.01 μm interval along the x-axis of Fig. 3. The two arrows indicate the beginning and the end of the master molecule in Fig. 3 used for positioning of all other molecules (see legend to Fig. 3 and text).

FIG.5.

Fig. 5. Denaturation map of the mitochondrial genome derived from data in Fig. 4. The circle has the same length as the master molecule (28.7 μm) indicated in Fig. 3, and is obtained by joining the points corresponding to the ends of the master molecule. The marks on the inside of the circle indicate distances in μm . The black areas on the circle show the position and extent of regions with a high probability for denaturation under the experimental conditions used (see legend to Fig. 3). These regions were drawn when more than 25% of the molecules transversing a point (each 0.01 μm) were observed as denatured (columns above 0.25 in Fig. 4), and only the part of Fig. 4 that corresponds to the master molecule has been considered. The arrow indicates the point for joining of the ends of the master molecule.

the mitochondrial genome. The significance of the calculated positioning of the molecules appears from the average denaturation pattern in Fig. 4. If the positioning had been random, the fraction of the molecules denaturated in each region would have been distributed around 21% (Table 1). It is seen that the background is lower, in the region for the position of the master molecule, about 10%. In this region several distinct peaks corresponding to coincidence of denaturated sites in about 70% of the molecules are seen. The distance between most of these easily denaturated regions varies from 0.5 to 3 μm . The increasing background for the regions extending each end of the position of the master molecule is to be expected because of the decreasing number of molecules placed in these regions. The distribution and extent of the easily denaturated presumably very AT-rich regions on the mitochondrial genome are visualized in Fig. 5. The circle is obtained by joining the ends of the region corresponding to the master molecule in Fig. 4 and is thus 28.7 μm long. With the reservation referring to the less well defined denaturation pattern of the regions extending the ends of the master molecule mentioned above, the closure of the circle seems justified also by a certain similarity of the denaturation pattern around the ends of the master molecule. The localization and extent of the easily denaturated regions (black areas) corresponds to the cross-section of the black columns in Fig. 4 at a level of 25% denaturation.

The calculation, which transfer the observations of single molecules to a denaturation map of the mitochondrial genome, are essentially a set of optimization procedures. It is therefore important to realize that a nonsense picture of the mitochondrial genome might be derived. However, several facts can be stated to support the validity of the map: Repetition of the whole calculation procedure by use of one of the other long molecules as a master, revealed a map, that by rotation and mirroring was similar to the map in Fig. 5.

If the pattern of the observed molecules is not reflecting distinct features of a common sequence, one would expect a map that strictly if anything reflected the structure of the master

molecule - in particular no new denaturation sites would be expected on the map. This is in contrast to our map, where five new sites can be identified. Moreover, in regions with several closely placed sites on the master, we can see different patterns of modifications: Some sites are combined to one larger site, - some sites remain as clearly defined entities.

The extent of the denaturated regions in Fig. 5 is naturally influenced by the conditions for denaturation of the DNA, lower denaturation temperatures presumably giving narrower regions. It is obviously also dependent on the way in which the figure is constructed. If the size of the easily denaturated regions in Fig. 5 can be taken as an indication for the extent of the very AT-rich sequences, these do not vary much. In conclusion, it appears as if the very AT-rich sequences known to exist in yeast mitochondrial DNA are highly dispersed all-over the genome at intervals from about 0.5 μm to about 3 μm and that these sequences are not widely different in size.

The intramolecular heterogeneity of yeast mitochondrial DNA found in this study is in agreement with results obtained by others using hydroxyapatite fractionation of partially degraded DNA^{10,11} and with reassociation and sedimentation studies⁶. The fractionation studies showed, that A+T and G+C rich sequences were interspersed in the mitochondrial DNA. The average size of both types of sequences was at least 10^5 to 10^6 daltons, and the A+T rich sequences might constitute almost 50% of the DNA^{10,11}.

It is known that mitochondrial DNA codes for one copy of r-RNA, several transfer RNAs and some hydrophobic inner membrane proteins¹⁸. None of these genes, of course, can be placed on the denaturation map. However, it is seen from Fig. 5 that the map contains stretches relatively resistant to denaturation (i.e. high G+C content) and of a length able to accomodate, for example, a r-RNA cistron (1.5-2 μm). A definite positioning of the mitochondrial genes on the denaturation map must await further studies on mitochondrial genetics and eventually electron microscopy of hybrids between known mitochondrial RNAs and mitochondrial DNA.

The principal findings in this study support the results of intramolecular heterogeneity of yeast mitochondrial DNA obtained

by hydroxyapatite fractionation of partially degraded DNA^{10,11}. These authors found A+T and G+C rich sequences interspersed in the mitochondrial DNA. The average size of both types of sequences was at least 10^5 - 10^6 daltons. It was further concluded that the A+T rich sequences might constitute almost 50% of the mitochondrial DNA. Very recent studies on the degradation of yeast mitochondrial DNA with micrococcal nuclease¹⁹ and with restriction enzymes^{20,21} show that specific fragments of the mitochondrial genomes from different wild type yeasts add up to a size in the order of 40 to 50 megadaltons. Furthermore these studies show differences between different wild-types in the specific fragmentation pattern and the absence of major repeated components. The G+C rich stretches are very heterogenous in base composition¹⁹ and with an average size of about $1-2 \times 10^6$ daltons. These results are in good agreement with the present work, and further studies of the denaturation pattern of specifically cleaved fragments of mitochondrial DNA may further improve the resolution of our map and help in placing the fragments in proper order.

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