Tandem Inverted Repeats in Mitochondrial DNA of Petite Mutants of *Saccharomyces cerevisiae**

(unimolecular renaturation/electron microscopy/molecular configuration/
denaturation mapping/repeat spacing)

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ABSTRACT Denatured mitochondrial DNA (mtDNA) from a grande (wild-type) yeast strain and a series of derived genetically characterized cytoplasmic petite mutants was examined in the electron microscope as DNA-protein monolayers prepared under conditions that permitted little bimolecular renaturation. In the grande and some petite strains, the mtDNA remained predominantly single-stranded. However, in several petite strains, a large proportion of molecules contained double-stranded segments indicative of unimolecular renaturation due to the presence of inverted repeat sequences. The length of the double-stranded segments of strain E41 was compared to the periodicity seen on denaturation maps. A repeat spacing twice the length of the inverted repeats was observed in the denaturation map. Inverted repeat length was similar to contour length of circular mtDNA molecules in this strain. On the basis of these observations most of the mtDNA from petite strain E41 appeared to consist of polymers of tandem inverted repeats interspersed with a small single-stranded "spacer" sequence between the repeat segments. In contrast, petite strain F13 mtDNA had few or no inverted repeats and showed a regular periodicity of 0.14 μm in the denaturation map, similar in length to the 0.13-μm circles present in the isolated mtDNA.

In contrast to higher eukaryotes, isolated yeast mitochondrial DNA (mtDNA) preparations contain linear molecules of heterogeneous size (1). A few 25-μm circles have been noted only in electron micrographs of osmotically shocked mitochondria (2). The mtDNA of yeast cytoplasmic petite (respiratory deficient) mutants is altered by large deletions resulting in changes in buoyant density and reduced kinetic complexity (1, 2–7). There may also be base sequence changes (8, 9) and tandem repetition of petite mtDNA sequences (3, 5, 7, 10). Petite mtDNA nonetheless may retain antibiotic resistance markers (3, 11) and cistrons for mitochondrial tRNAs (12–14), ribosomal RNA (15), and DNA sequences that are homologous with grande mtDNA (8–10, 16).

While in grande mtDNA only rare circular molecules are isolated, and none corresponding to the presumed 25 μm genome length (2), circular molecules have been much more readily obtained from petites (3, 5–7). We have shown recently in a series of genetically defined petite strains, that the size distribution of the circular molecules follows a regular pattern indicating the presence of a multimeric series of lengths (3, 5–7). We noted an excellent linear correlation between the contour length of the monomeric circular DNA molecules and the kinetic complexities of total mtDNA, even though the circles represent a small fraction (2–30%) of the mtDNA. Thus the DNA sequences appear to be the same in linear and circular molecules. The linear molecules appear to be polymers of the same unique sequence that defines the unit circle length.

In this study we have tried to analyze the arrangement of unit DNA sequences on linear mtDNA molecules in several petite strains. A considerable fraction of denatured mtDNA in some petites renatured almost instantly upon removal from denaturing conditions. Analysis of the configuration of duplex segments, together with the denaturation maps of mtDNA in two strains, indicate that the petite mitochondrial genomes probably contain repeating sequences either in tandem or inverted tandem arrangement.

MATERIALS AND METHODS

Yeast Strains. Studies were carried out on a haploid grande strain, IL8-8C, and a series of isogenic cytoplasmic petites induced from it by ethidium bromide mutagenesis. The strains were kindly provided by Dr. P. P. Slonimski (Centre de Génétique Moléculaire de CNRS, Gif-sur-Yvette, France). The parent and derived petite strains have been genetically analyzed with respect to resistance (R) and sensitivity (S) to chloramphenicol (C), erythromycin (E), and oligomycin (O) by Slonimski's group (11). The superscript 0 refers to the absence of genetic information. The strains are: grande, IL8-8C $(C^R E^R O^S)$; and petites F13 $(C^0 E^0 O^0)$; E41 $(C^0 E^R O^0)$; C42, D61, D41 $(C^R E^0 O^0)$; F11 $(C^R E^R O^0)$; and F12 $(C^0 E^R O^S)$ (11). The strains have been maintained in our laboratory over more than 2 years.

Isolation of mtDNA. The technique used to isolate high-molecular-weight mtDNA from yeast strains is described in detail elsewhere (5, 6). Briefly, [³H]adenine-labeled mtDNA is isolated directly from protoplast lysates by isopyknic banding 3–4 times in CsCl gradients until no nuclear DNA was detected. The mtDNA was free of nuclear DNA on isopyknic CsCl centrifugation in the analytic ultracentrifuge. The sensitivity of these procedures is sufficient to detect 1–2% nuclear DNA contamination. The average alkaline S values

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Abbreviation: mtDNA, mitochondrial DNA.
* Part of this work has been previously presented in preliminary form (refs. 7 and 20).
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of these DNA preparations were 25–27 S for strains IL8-8C, F12, F11, D41, D61, C42; and 8–11 S for strains E41 and F13.

Electron Microscopy of DNA. DNA (in 0.1 M NaCl, 0.01 M Tris, pH 8.5, and 0.001 M EDTA) was denatured by incubating in 0.2 N NaOH for 5 min at room temperature. The solution was cooled to 0° and neutralized with at least 10 volumes of cold 0.2 M Tris buffer, pH 8.5, and 0.02 M EDTA. The final DNA concentration was about 1 μg/ml. To 55 μl of DNA solution, 40 μl of formamide and 5 μl of cytochrome c (Calbiochem, Los Angeles, Calif.), 1 mg/ml in 0.02 M Tris, pH 8.5, 0.02 M EDTA, were added. Fifty micro-liters of the solution was examined and measured by the formamide-basic protein technique described by Davis, Simon, and Davidson (17). DNA protein monolayers were prepared using DNA solutions in 40% formamide spread onto a hypophase containing 10% formamide.

Denaturation Mapping. Denaturation mapping was carried out by a procedure modified from that of Inman (18). DNA-protein monolayers were prepared at formamide concentrations sufficiently high to achieve partial denaturation at room temperature, a concept adopted from Davis and Hyman (19). It was determined empirically that spreading in 80% formamide onto a 50% hypophase gave optimum partial denaturation for a vareity of grande and petite mtDNAs.

RESULTS

Self-renaturing Petite mtDNA. On examination of electron micrographs of denatured petite mtDNA, much of the mtDNA of some strains (71–83%) contained double-stranded regions, but only 11% was in this form in strain F13, the petite DNA having the lowest kinetic complexity (Table 1). Using the formamide spreading technique we were able to differentiate duplex DNA from single-stranded DNA since it is both straighter and thicker (17). Estimates of bimolecular renaturation during the preparation of DNA protein mono-layer predict no more than 10–15% duplex regions (5). The presence of complementary sequences in the same strand of petite mtDNA is strongly supported by the observation that the incidence of duplex regions was independent of DNA con-
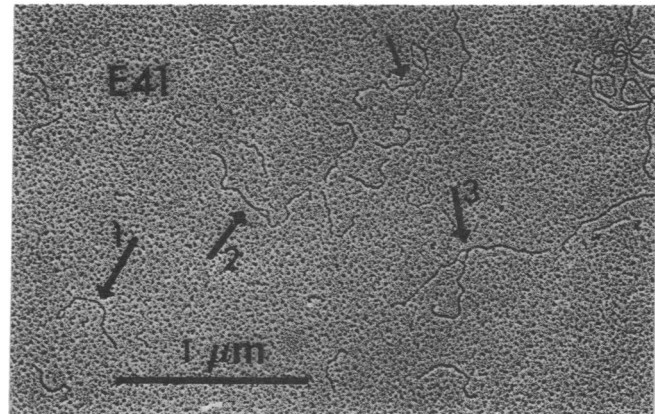

FIG. 1.　Denatured mtDNA from petite E41. DNA was denatured, neutralized, and prepared for electron microscopy (17) as described in the *text*. Most of the mtDNA is formed into duplex structures. The small *arrow* indicates a single-stranded molecule. Of the molecules with duplex regions, large *arrow 1* indicates a "hairpin" molecule, large *arrow 2* a molecule with a substitution loop, and large *arrow 3* a "spider" molecule. Single-stranded DNA is discriminated from duplex DNA by its thinner diameter and more collapsed configuration, as well as by the configuration of the branches in individual molecules.

centration. Denatured E41 mtDNA prepared for electron microscopy at both 1 and 10 μg/ml had duplex regions in about 85% of the molecules (Fig. 1, Table 1). Similar results were obtained on analysis of strain C42 and D41 mtDNA.

Electron micrographs of denatured E41 and F13 mtDNA are shown in Figs. 1 and 2, respectively. While F13 mtDNA is almost entirely single-stranded, except for the small duplex circles, most of the E41 mtDNA molecules contain long duplex regions and relatively few purely single-stranded molecules were present.

The conclusion that the duplex regions are formed from unimolecular renaturation of neighboring complementary sequences is further corroborated by the configuration of many of the duplex-containing molecules. The simple hairpin

TABLE 1.　*Properties of grande and petite mitochondrial DNAs*

Strain	Monomer circle length, μm (5, 6)	Relative k_2* (5, 6)	Calculated genome size, μm†	Molecules containing duplex segments‡ %	Molecules containing duplex segments‡ fraction	Inverted repeat length, μm
Grande:						
IL8-8C	25 (2)	1	25	2	7/415	—
Petite:						
F13	0.13	0.012	0.3	11	21/199	—
E41	0.5	0.018	0.45	83	111/134	0.4–0.5
				85	172/202§	
C42	0.8	0.031	0.8	71	87/126	0.8
D61	1.3	0.046	1.2	11	14/109	—
D41	2.1	0.095	2.4	77	89/115	1.5–1.9
F11	4.4	0.17	4.2	6	7/113	—
F12	5.5	0.22	5.5	10	11/105	—

* Kinetic complexity is expressed as the ratio of the k_2 to that of the grande, where k_2 is the second order rate constant of renaturation. The renaturation of DNA sheared to 5.4 S was followed optically (5, 6).

† We calculated genome size from k_2, assuming 25 μm for grande mtDNA (2).

‡ Molecules were denatured and DNA-protein monolayers prepared under conditions that did not allow bimolecular renaturation, as described in *Materials and Methods*.

§ Molecules were denatured and DNA-protein monolayers prepared at a 10-fold greater dilution than the other preparations described in the table.
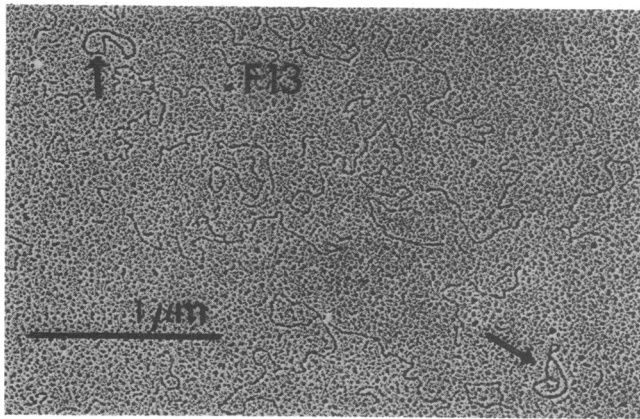
FIG. 2. Denatured mtDNA from petite F13. This DNA was denatured and prepared for electron microscopy in the same manner as in the E41 DNA shown in Fig. 1, and is almost completely single-stranded except for small duplex circles (*arrows*).

structures (Fig. 1 molecule 1) and the more complex molecules containing multiple duplex regions (Figs. 1 and 3) can be formed from unimolecular but not from bimolecular renaturation. In the ensuing description the term inverted repeat will be used for duplex DNA believed to be formed from the unimolecular renaturation of neighboring complementary sequences.

Length Measurements of Inverted Repeats. Only certain molecular configurations are suitable for the unambiguous measurement of the full length of an inverted sequence. A hypothetical sequence $a\ b\ c$ must coexist with its complement $c'\ b'\ a'$ in the same DNA strand to be an inverted repeat. A noncomplementary sequence x may be interposed between the two inverted sequences. Therefore, the sequence $y\ a\ b\ c\ c'$-$b'\ a'\ z$ will form the structure
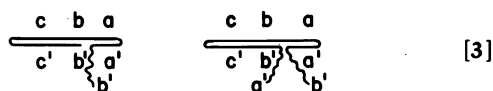
$$
\begin{array}{c}
c\quad b\quad a\quad {}^{y} \\
\hline
c'\quad b'\quad a'\quad {}_{z}
\end{array}
\qquad [1]
$$

and the sequence $y\ a\ b\ c\ x\ c'\ b'\ a'\ z$ will form the structure

$$
x\!\!\bigcirc\!\!
\begin{array}{c}
c\quad b\quad a\quad {}^{y} \\
\hline
c'\quad b'\quad a'\quad {}_{z}
\end{array}
\qquad [2]
$$

To define the full length of an inverted repeat it is necessary to measure a molecule having one end either duplex or a single-stranded loop, and the other end dividing into two single strands.

Some molecules, however, may contain only part of the complete inverted repeat sequence and others may contain more than one copy. The simplest cases of the latter will be

$$
\begin{array}{cc}
\begin{array}{c}
c\quad b\quad a \\
\hline
c'\quad b'\!\!\{\ a' \\
\qquad\quad b'
\end{array}
&
\begin{array}{c}
c\quad b\quad a \\
\hline
c'\quad b'\!\!\}\ a' \\
\quad a'\quad\ b'
\end{array}
\end{array}
\qquad [3]
$$

The length of the inverted repeat sequence is not defined when a molecule terminates in only one single strand, but it is defined when a single-stranded branch emerges from the side of an otherwise duplex region.

Larger numbers of inverted repeats in the same molecule will result in molecules with multiple renatured regions having a somewhat more complex configuration. Three such molecules are illustrated in Fig. 3; in two, configurations indicative of a

regular arrangement of inverted repeats are seen, and in one an irregular arrangement is observed. A few long single-stranded molecules were observed in E41 mtDNA preparations, indicating that some molecules had no inverted repeats.

In an E41 mtDNA preparation, 111 of 134 molecules examined contained duplex regions. Using the criteria described above to define the lengths of complete inverted repeats, 38% of the duplex-containing molecules, i.e., 42 molecules, were suitable for unambiguous measurement. The results of such measurements are shown in Fig. 4. More than 75% of the inverted repeats fall into the range from 0.43 to 0.50 μm, with a few slightly larger. Some regions were of double length. "Spacer" (i.e., single-stranded DNA between two duplex regions) length distribution appeared to be nonrandom with modes at 0.04 and 0.07 μm.

Of the 42 measurable inverted repeat molecules, 23 had multiple repeats, of which 19 showed a regular alternating arrangement, and four showed an irregular one. Of the original series of 134 molecules there were also four multiple-length single-stranded molecules that showed no inverted repeats, giving a total series of 27 multiple-length molecules. Thus 70% of the multiple-length molecules showed a regular, alternating arrangement of inverted repeats and spacer; 15% were long single-stranded molecules probably indicating a head-to-tail arrangement; and 15% were in a mixed arrangement.

The mtDNA of strain E41 has circle contour lengths similar to the inverted repeat lengths (3, 5–7). There were modes at 0.50 μm (slightly larger than the inverted repeat length), and 1.00 μm. There were also a few circles at 1.15 and 1.30 μm. Inverted-repeat and "spacer" lengths suggest a similar minor heterogeneity. It may be concluded that most inverted repeat sequences, and most circles, fall into a single-size class.

Petites C42 and D41 were similar to E41 in that their denatured mtDNA, prepared for electron microscopic examination under conditions that allowed minimal bimolecular renaturation, contained a relatively high percentage of duplex regions. Preliminary analysis showed inverted repeat lengths of 0.8 μm (the circles are 0.8 μm) for C42 mtDNA, and of 1.5–1.9 μm for D41 mtDNA (the circles are 2.1 μm). MtDNA from petites F11, F12, and D61 and the grande IL8-8C showed a low percentage (11% or less) of duplex DNA.

Denaturation Mapping. Denaturation mapping was carried out on mtDNA of strains F13 and E41. Mapping of F13 mtDNA has been previously described by Faye *et al.* (3) with similar results. From the denaturation map of E41 (Fig. 5) a recurring pattern of large and small loops was seen. The distance between recurring loop patterns was approximately 0.9 μm (twice the size of the unit length of the inverted repeat). This pattern was evident in 23 of 29 molecules, while in three a similar pattern but with some missing and fused loops was seen; two molecules had a similar pattern but with a larger spacing (about 1.10 μm), and in one molecule a regular spacing of about 0.5 μm was observed. The 0.9-μm period length on the denaturation map, which is twice the inverted repeat length, presumably represents two unit sequences attached in inverted tandem arrangement (head-to-head). Taken together with the analysis of inverted repeats, the denaturation maps indicate that most of E41 mtDNA (70–90%) consists of an orderly arrangement of tandem inverted repeats. A small percentage of the molecules had a slightly larger spacing and a few molecules may contain the same sequence arranged in tandem (head-to-tail) fashion, or in an irregular manner.
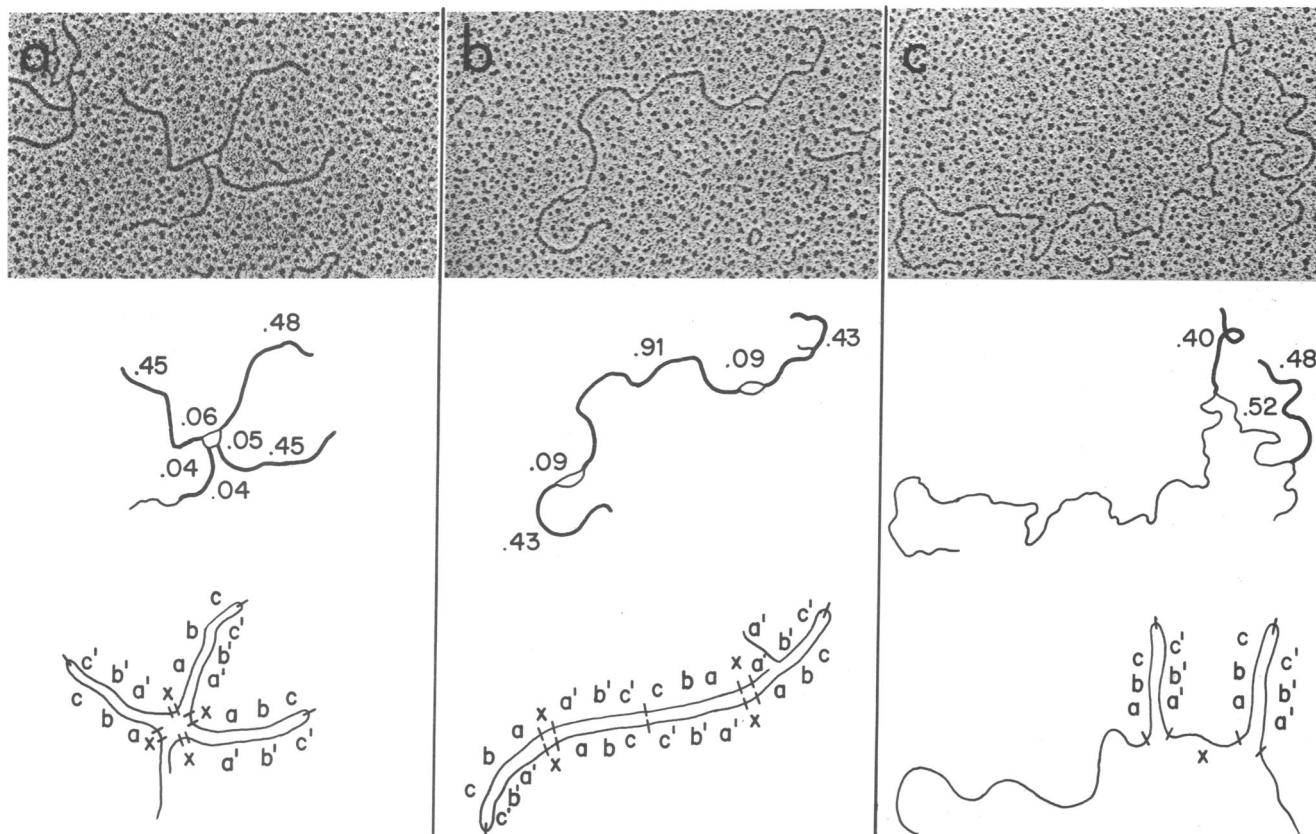
FIG. 3. Denatured mtDNA from petite E41 mtDNA containing multiple duplex regions per molecule. DNA was prepared as in Figs. 1 and 2. Below each electron micrograph are two diagrams; one illustrates the configuration of the molecule (dimensions indicated in μm) and the other outlines a hypothetical single-stranded molecule containing sequence a b c, its complement a'b'c', and nonrenaturing sequence, x. (a) A "spider" molecule, whose configuration we assume to be attributable to the presence of a regular arrangement of multiple inverted repeats in a single-stranded DNA molecule (see *text*). (b) A different configuration formed by the same regular arrangement of multiple neighboring complementary sequences in a single-stranded molecule as in (a). (c) A single-stranded molecule showing two self-renaturing complementary sequences, but with an irregular arrangement of sequences, in contrast to the molecules of (a) and (b).

In the denaturation map of F13 mtDNA (which has a negligible or very low (<11%) frequency of duplex regions and therefore of inverted repeats) a regular spacing of loops at 0.14 μm (Fig. 6) was observed as compared with a monomer circle size measured at 0.13 μm (3, 5–7). The denaturation maps indicate a regular tandem (head-to-tail) arrangement of the basic sequence defined by circle length in F13 linear mtDNA molecules.
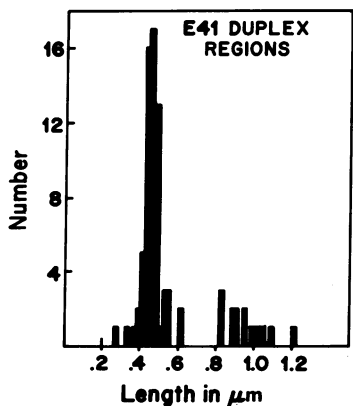

FIG. 4. Length measurements of E41 inverted repeats and "spacers". The lengths of 93 inverted repeats (single and multiple inverted repeats), as defined in the *text*, were measured from strain E41 and plotted on an 0.02-μm scale. The major unit sequence measured about 0.46 μm. There are also a small number of molecules having slightly longer unit sequences, and a few double lengths.

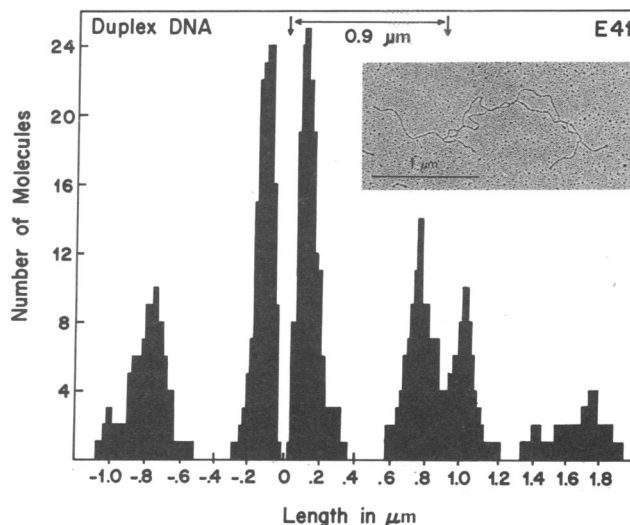FIG. 5. Frequency distribution of duplex DNA regions from E41 denaturation maps. The distribution is plotted on an 0.02-μm scale. A spacing of about 0.9 μm is apparent for the repeating unit. The *inset* shows a typically partially denatured molecule, from which the data were obtained.
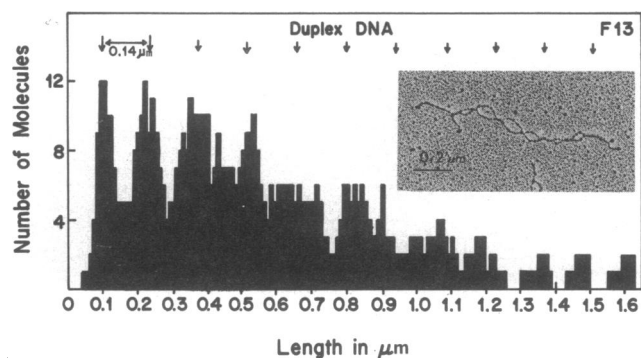
FIG. 6. Frequency distribution of duplex DNA from F13 mtDNA denaturation maps. The distribution based on data from 26 molecules was plotted on an 0.01-μm scale. A regular spacing of about 0.14 μm (*arrows*) is apparent. The *inset* shows a typical molecule from which the data were obtained.

DISCUSSION

The cytoplasmic petites used in this study are part of a series of genetically characterized mutants strains (3, 11, 15) induced by ethidium bromide. The petite mtDNA's presumably arise from the parental grande mtDNA primarily by a deletion process. The mtDNA of three petite strains examined from this series contained a large number of inverted repeat sequences. This conclusion is based on the following observations: (*1*) 60–85% of the mtDNA of these strains contained duplex regions when examined under conditions where not more than 11% bimolecular renaturation would be expected, (*2*) The formation of duplex regions was independent of DNA concentration, and (*3*) The configuration of many of the molecules was consistent with unimolecular and not with bimolecular renaturations. The conclusion is further corroborated by our observation that the lengths of the duplex regions were similar to the mitochondrial genome size estimated from renaturation kinetic analysis (4–6) or from the size of monomeric circular molecules (3, 5, 6) in the mtDNA. Furthermore, denaturation maps of E41 mtDNA, which contains many inverted repeats, shows a repeat spacing about twice the length of duplex regions, or of monomeric circles, a finding expected for inverted tandem (head-to-head) repetition of the mitochondrial genome. No other plausible explanation which would encompass these observations seems possible.

In these three petites unit sequences are arranged largely in a regular head-to-head, tail-to-tail fashion, as tandem inverted repeats. Molecules of this type cannot be derived from grande DNA by a simple deletion mechanism unless the inverted repeat sequence is already present in the grande genome. However, we detected virtually no unimolecular renaturation in grande mtDNA. If inverted repeats are not present in grande mtDNA, at least four steps may be involved in the formation of DNA of this type: (*1*) excision, (*2*) duplication (amplification I), (*3*) inversion of one of the duplicated segments, and (*4*) amplification II. Irregular arrangements of inverted repeats could be produced by occasional inversions in previously amplified DNA, but regular arrangement requires duplication and inversion prior to amplification. The head-to-head joining of a sequence to form an inverted repeat may require the terminal sequence of the DNA to be homologous with its complement $\left(\text{such as } \genfrac{}{}{0pt}{}{\text{TATATA} \ldots}{\text{ATATAT} \ldots} \text{ or}\right.$

$\left.\genfrac{}{}{0pt}{}{\text{AAATTT} \ldots}{\text{TTTAAA} \ldots}\right)$ to provide base pairing prior to joining; or may be independent of homology of base sequence.

The arrangement of unique sequences in mtDNA of petite F13 is strikingly different from that in E41 mtDNA. From the denaturation map of F13 mtDNA it may be concluded that the linear molecules are composed of tandem repeats of a sequence approximately 0.14 μm in length. Similar observations have been reported by Faye *et al.* (3) We have also found that the length of the monomer circles is similar, 0.13 μm (3, 5–7). Thus, petite F13 mtDNA consists primarily of linear molecules, which are arranged as regular tandem (head-to-tail) repeats of a unique 0.13- to 0.14-μm sequence. The formation of petite mtDNA of this type requires at least two separate steps: (*1*) excision (deletion), and (*2*) amplification.

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