

## Rolling circle replication of DNA in yeast mitochondria

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**The conformation of mitochondrial DNA (mtDNA) from yeasts has been examined by pulsed field gel electrophoresis and electron microscopy. The majority of mtDNA from *Candida (Torulopsis) glabrata* (mtDNA unit size, 19 kb) exists as linear molecules ranging in size from 50 to 150 kb or 2–7 genome units. A small proportion of mtDNA is present as supercoiled or relaxed circular molecules. Additional components, detected by electron microscopy, are circular molecules with either single- or double-stranded tails (lariats). The presence of lariats, together with the observation that the majority of mtDNA is linear and 2–7 genome units in length, suggests that replication occurs by a rolling circle mechanism. Replication of mtDNA in other yeasts is thought to occur by the same mechanism. For *Saccharomyces cerevisiae*, the majority of mtDNA is linear and of heterogeneous length. Furthermore, linear DNA is the chief component of a plasmid, pMK2, when it is located in the mitochondrion of baker's yeast, although only circular DNA is detected when this plasmid occurs in the nucleus. The implications of long linear mtDNA for hypotheses concerning the ploidy paradox and the mechanism of the petite mutation are discussed. Key words: mitochondrial DNA/pulsed field gel electrophoresis/replication/yeast**

### Introduction

According to established belief all known mitochondrial genomes in budding yeasts (with only two exceptions) consist of circular DNA molecules, which range in size from 19 kb in *Candida (Torulopsis) glabrata*, henceforth *T. glabrata*, to >100 kb in *Brettanomyces custersii* (O'Connor *et al.*, 1976; Hoeben and Clark-Walker, 1986). Copy number has been estimated to vary from 20 to 100 genome equivalents per cell, representing 5–20% of total cellular DNA (Dujon, 1981; Conrad and Newlon, 1982). The evidence for circularity of mitochondrial genomes comes from physical mapping with restriction endonucleases, genetic data and visualization by electron microscopy (Dujon, 1981). However, it has been noted by a number of authors that even under very mild extraction conditions the majority of mitochondrial DNA (mtDNA) in baker's yeast, *Saccharomyces cerevisiae*, appears as linear molecules (Hollenberg *et al.*, 1970; Clark-Walker, 1972; Petes *et al.*, 1973; Locker *et al.*, 1974; Christiansen and Christiansen, 1976). The rarity

of circular molecules has been interpreted to mean that linear molecules result from random fragmentation of native circles by mechanical shearing (Petes *et al.*, 1973; Dujon, 1981). However, an alternative interpretation is that linear molecules need not be an artefact. Indeed, these structures could provide insight into the mechanism of mtDNA replication in yeasts, a subject that has remained problematical.

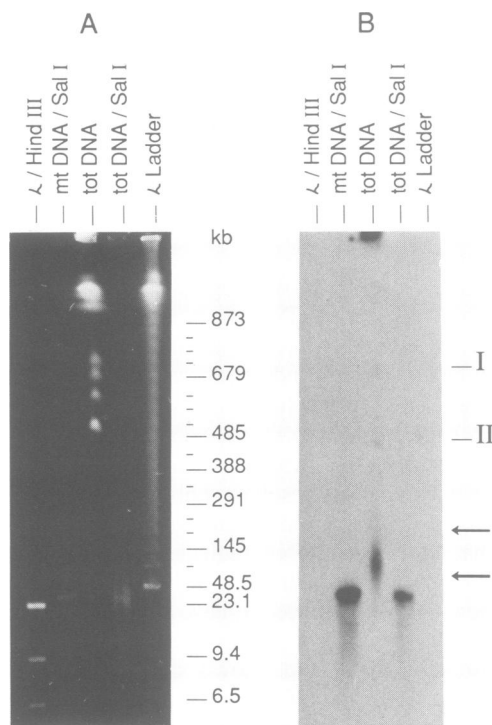
The advent of pulsed field gel electrophoresis (PFGE) has yielded a new means of resolving chromosome-sized DNAs as well as providing a convenient method for studying the conformation of intact DNA molecules, including circular forms (Schwartz and Cantor, 1984; Beverly, 1988; Simske and Scherer, 1989). Hence we have used this technique to separate *T. glabrata* and *S. cerevisiae* mitochondrial and chromosomal DNAs and shown that mtDNAs of unit genome size 19 kb and 80 kb respectively can be recovered from a region corresponding to the position of ~50–100 kb linear molecules (Skelly and Maleszka, 1989). In accord with our observations, Bendich and Smith (1990) have reported that similar PFGE resolution patterns of mtDNAs from *S. cerevisiae* and plants could best be interpreted as the migration of linear molecules. These results suggest that molecules other than circles are part of the DNA population in yeast mitochondria *in vivo*.

The aim of the present study has been to clarify conformation of mtDNA in yeasts by focusing on the small (19 kb) genome in *T. glabrata*. Our results show clearly that a majority of mitochondrial DNA consists of long, linear molecules having heterogeneous size while the presence of circular molecules with tails (lariats) raises the possibility that replication involves a rolling circle. In addition, by employing isogenic strains of *S. cerevisiae* harbouring a recombinant plasmid in different cellular compartments, we demonstrate that production of linear, tandemly repeated molecules is confined to mitochondria. Furthermore, replication of mtDNA by a rolling circle mechanism offers an explanation for the ploidy paradox, whereby the measured quantity of mtDNA per cell exceeds the number of genetical units (Gingold, 1988) and in addition, provides a new way of considering the production of respiratory deficient (petite) mutants.

### Results

#### Long linear molecules

The resolution of total DNA from a wild type (Rho<sup>+</sup>) strain of *T. glabrata* is shown in Figure 1. Under these conditions, only small chromosomal DNA molecules (<1 Mb) are resolved. Although the wild type has at least 13 chromosomes, ranging in size from ~500 kb to >2000 kb (not illustrated), bigger chromosomal DNA molecules (>1 Mb) are compressed into a single band, but nevertheless they enter the gel. When the electrophoretically separated DNA is transferred to a nylon membrane and



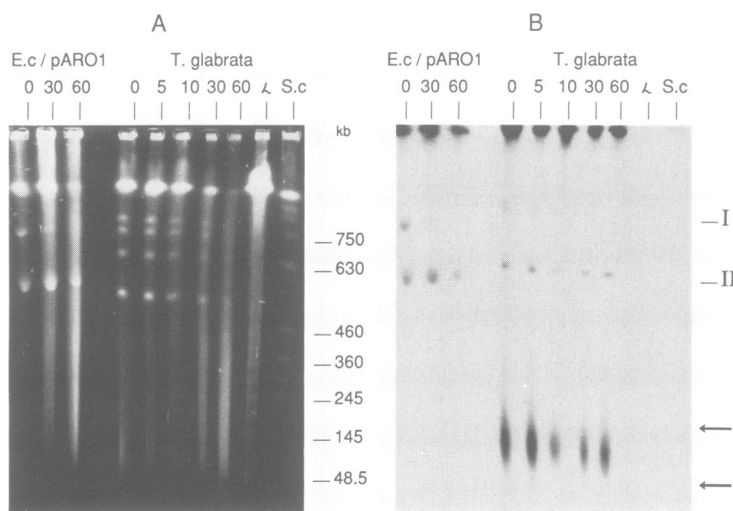
**Fig. 1.** Ethidium bromide stained agarose gel (A) and autoradiograph (B) of separated DNA samples (from left to right): *Hind*III digested DNA of phage lambda, *Sal*I digested mtDNA from *T. glabrata*, total DNA from *T. glabrata*, *Sal*I digested total DNA from *T. glabrata* and phage lambda ladder. Total DNA was prepared by the agarose plug method, mtDNA was CsCl-gradient purified (see Materials and methods for details). The autoradiograph has been obtained by hybridization with CsCl-gradient purified mtDNA. Size estimates correspond to phage lambda concatamers and *Hind*III fragments of lambda DNA. Each step in the lambda ladder represents an increment of 48.5 kb. Conditions for PFGE: pulse time 30 s at 200 V for 1 h, then 50 s at 150 V for 4 h and 50–85 s (linear ramp) at 150 V for 20 h. Total run time: 25 h. Arrows indicate the fast migrating, linear DNA; I, position of the supercoiled DNA; II, position of relaxed circles.

probed with labelled, CsCl-purified mtDNA from *T. glabrata*, a complex hybridization pattern is found (Figure 1B). All hybridizing DNA is of mitochondrial origin, since there is no detectable reaction in a  $\text{Rho}^0$  strain which lacks mtDNA (Skelly and Maleszka, 1989; not illustrated). There are two regions hybridizing strongly with the mtDNA probe: a fast migrating diffuse region corresponding in size to the 50–150 kb linear markers (Figures 1 and 2, between arrows) and a fraction at the top of the gel that shows very little or no mobility. In addition, two minor bands, designated I and II, are also detected.

The nature of each DNA component was investigated by varying the PFGE conditions. In comparison with linear markers, the relative mobility of the diffuse, fast migrating component does not change with different PFGE conditions (varying pulse and run times and agarose concentration). This property is consistent with the migration of linear molecules having heterogeneous sizes. By contrast, the apparent sizes of the two bands, designated I and II, vary considerably depending on the electrophoretic regime. Under conditions shown in Figure 1, bands I and II co-migrate with 680 kb and 485 kb linear DNA respectively, while they are located at 800 kb (band I) and 630 kb (band II) under the different electrophoretic regime shown in Figure 2. This change in relative mobility under different conditions suggests that these bands contain circular molecules (see below).

The DNA at the top of the gel remains there under a wide range of conditions. It is important to note that a certain amount of trapped material is always detected at the top of PFGE gels regardless of the DNA used, bacterial, plasmid or eukaryotic (see Figure 2, which contains plasmid pAR01). This most likely reflects the properties of the PFGE technique which leads to some non-specific trapping of DNA molecules.

Calibration of the diffuse, fast migrating region against phage lambda concatamers leads to a size of ~50–150 kb, with the mean around 75 kb (although in some samples the upper limit of molecules in this component might be as long



**Fig. 2.** Ethidium bromide stained agarose gel (A) and autoradiograph (B) of separated total DNA (agarose plugs) from an *E. coli* strain harbouring plasmid pAR01 and *T. glabrata* treated for 0, 5, 20, 30 and 60 min with  $^{60}\text{Co}$   $\gamma$ -ray source, equivalent to 0, 20, 40, 120 and 250 Gray respectively. The autoradiograph was obtained by hybridization with a *Hind*III–*Hind*III fragment of pAR01 for *E. coli* DNA and mtDNA (see Figure 1) for *T. glabrata*. The size estimates correspond to phage lambda concatamers and *S. cerevisiae* chromosomal DNAs. Conditions for PFGE: pulse time, 20 s for 10 min at 20 V, then 50 s for 20 h at 150 V, then 100 s for 6 h at 100 V and 150 s for 16 h at 90 V. Total run time: 42 h. Arrows indicate the fast migrating, linear DNA; I, position of supercoiled DNA; II, position of relaxed circles.

as 250 kb). This indicates that the majority of molecules are longer than the 19 kb unit. To examine this notion we digested an agarose plug containing total DNA with *SalI* and compared its migration with a similar digest of CsCl-purified mtDNA. Digestion with *SalI*, which cleaves once in mtDNA (Clark-Walker *et al.*, 1980) yields a single, hybridizable band that is the same size as the *SalI* digested CsCl-purified mtDNA (Figure 1). It is also clear that this band runs further than the diffuse component in the undigested DNA, which indicates that the majority of linear forms are longer than unit genome size. Digestion with restriction enzyme also results in removal of mtDNA from the top of the gel. It is noteworthy that the *SalI* digested mtDNA is located at approximately the same position as the largest *HindIII* fragment of phage lambda DNA (23.1 kb). The discrepancy between the reported unit size (19 kb) and the observed size under the present conditions results most likely from the anomalous electrophoretic mobility of relatively AT-rich molecules (Maniloff, 1989) such as yeast mtDNAs.

### Circular DNA

To examine further the identity of bands I and II we tested the effect of  $\gamma$ -ray treatment on their behaviour. As a control we have used an *Escherichia coli* strain harbouring a recombinant plasmid pAR01 (16.1 kb) which has a similar contour length to *T. glabrata* mtDNA. Gamma radiation causes 100 times more single-strand breaks than double-strand breaks (Block and Loman, 1973) and since single-strand breaks have no known effect on the mobility of linear DNA molecules, it is possible to study the degradation of DNA in a dose dependent fashion and to determine the location of circular and linear structures (van der Blik *et al.*, 1988). Moreover, the transition from supercoiled to relaxed circles should occur at low doses of radiation. The effect of gamma radiation on mtDNA in *T. glabrata* and plasmid DNA in *E. coli* is shown in Figure 2. In both organisms increasing radiation leads to a gradual disappearance of band I, while band II shows an increase (Figure 2B). This indicates in each case that band I is the supercoiled, monomeric DNA, while band II is the relaxed circular form. Furthermore, with the bacterial plasmid it can be seen that the radiation dosage does not produce linear DNA from the nicked circles. In addition, under these experimental conditions there is no detectable change to the mobility of the fast migrating diffuse region of mtDNA (Figure 2, between arrows). This result is consistent with the view that the fast migrating component is composed of linear 50–150 kb molecules as insufficient radiation has been used to create double-stranded breaks in molecules of this size. Some variation in the intensity of hybridization signal in this area results from differences in the amount of DNA (agarose plugs) per lane.

### Conformation of plasmid pMK2 in nuclei or in mitochondria

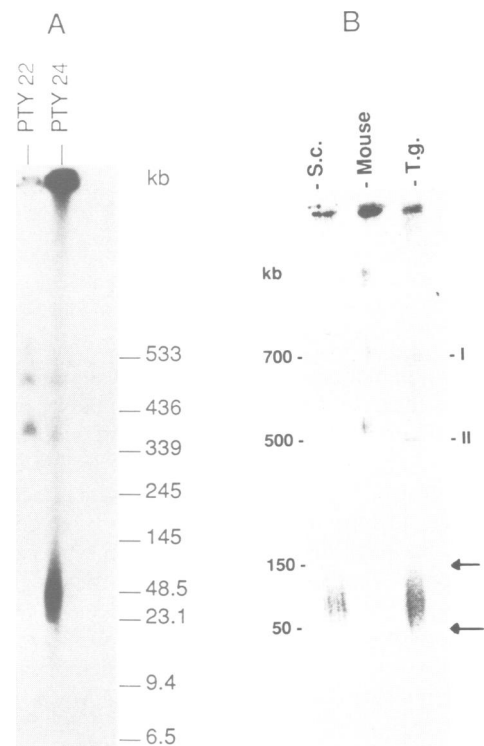
Yeast organelles, lacking mtDNA, can be transformed by high-velocity micro-projectile bombardment with *in vitro* constructed plasmids (Butow and Fox, 1990).

Recent, successful construction of an *S. cerevisiae* strain with mitochondria that contain only sequences derived from a defined plasmid (Fox *et al.*, 1988; Thorsness and Fox, 1990) offers a unique opportunity to study the behaviour of the same DNA molecule in two different cellular compartments. Plasmid pMK2 can be maintained in mitochondria

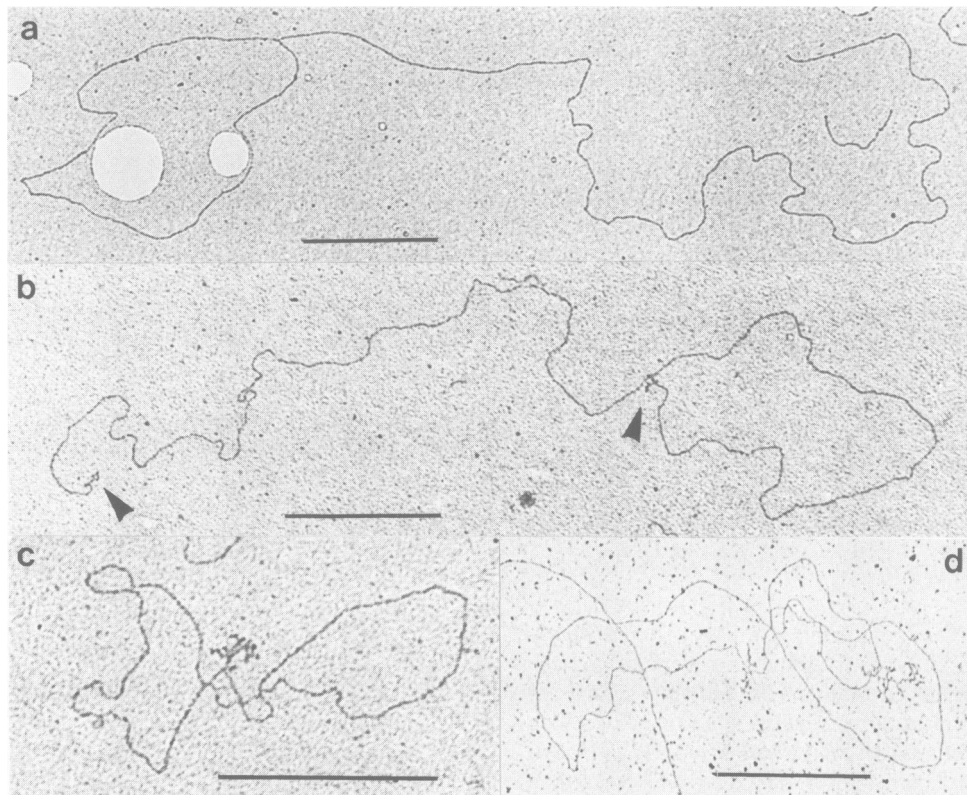
and in nuclei as shown previously (Thorsness and Fox, 1990). Depending on its intracellular localization, this 'synthetic' DNA behaves either like baker's yeast mtDNA or as a multicopy 2  $\mu$ m based recombinant plasmid. The results shown in Figure 3A, which compare two isogenic strains harbouring the plasmid in mitochondria (PTY24) or in nuclei (PTY22), demonstrate clearly that plasmid pMK2, while replicating in the organelle, exhibits a typical 'mitochondrial' conformation with a significant portion of the DNA consisting of linear, non-uniform molecules of 23–100 kb. This form of DNA is not detectable when the plasmid replicates in the nucleus where it is present as circular molecules.

### Conformation of mtDNA in the mouse

To see if the electrophoretic behaviour of mtDNA in yeasts is a universal property of mitochondrial genomes, we have examined the conformation of mouse mtDNA. This organism was chosen because the mechanism of mtDNA



**Fig. 3.** Autoradiographs of separated total DNAs. (A) Two isogenic *S. cerevisiae* strains harbouring plasmid pMK2 in the nucleus (PTY22) and in mitochondria (PTY24). The probe was plasmid pUC19. Size estimates correspond to *S. cerevisiae* chromosomal DNAs and *HindIII* digested phage lambda DNA. (B) *S. cerevisiae* Dip2, mouse and *T. glabrata* DNA. The probe is a mixture of mtDNA from *T. glabrata* and a 3.4 kb cloned fragment of bovine mtDNA (see above). Size estimates correspond to *S. cerevisiae* chromosomal DNAs. Conditions for PFGE: A: pulse time, 50 s at 150 V for 27 h, then 70 s at 150 V for 4 h; total run time, 31 h. B: pulse time, 50 s at 150 V for 20 h, then 100 s at 150 V for 16 h; total run time, 36 h. The amount of mtDNA in panel B (representing a computer generated image of a Southern blot) was quantified using the Phosphor-imaging technology from Molecular Dynamics (see Materials and methods). The distribution of each component of *T. glabrata* mtDNA shown in (B) is: top, 37%; supercoiled DNA (band I), 3%; open circles (band II), 6% and linear mtDNA (between arrows), 53%. In the case of mouse mtDNA, 78% of molecules did not enter the gel, while in the *S. cerevisiae* sample 37% of mtDNA is trapped.



**Fig. 4.** Molecules from a mtDNA preparation from *T. glabrata*: **a**, monomer circle with a double-stranded tail (lariat). **b**, lariat with collapsed single-stranded regions at the end of the tail and at the junction of the tail with the circle (arrows). **c**, monomer circle with a collapsed single-stranded tail. **d**, dimer circle with two single-stranded tails. The bar in each case represents 1  $\mu\text{m}$ .

replication in mammals is well known (Clayton, 1984). We compared the electrophoretic pattern of mtDNA from mouse tissue with the mtDNA pattern from *T. glabrata* and *S. cerevisiae* (Figure 3B). It is clear that mtDNA from the mouse consists of circular molecules which lack the fast-migrating, diffuse band (Figure 3B). It is curious that although the mitochondrial genome in *S. cerevisiae* is four times bigger than that in *T. glabrata*, the lengths of the linear forms are similar in these two species. We have no explanation for this result; however, we have found in other yeasts that the size range of linear molecules varies considerably between species (Skelly and Maleszka, 1991).

#### **Rolling circles**

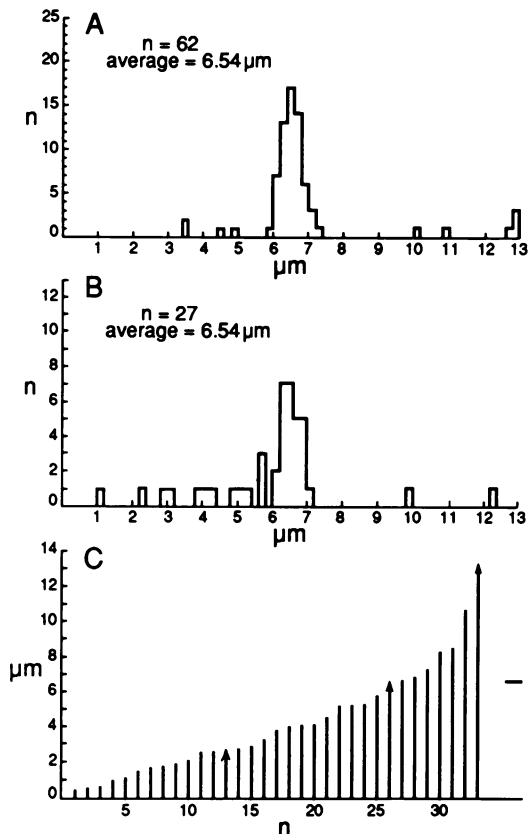
Examination of *T. glabrata* mtDNA by electron microscopy reveals a number of forms (Figures 4 and 5, Table I). In addition to the supercoiled and relaxed circular DNA previously described (O'Connor *et al.*, 1976), there are circular molecules with either single- or double-stranded tails. Approximately 15% of relaxed circular monomers have single-stranded tails which appear as collapsed branching structures (Figure 4c). Circular molecules with double-stranded tails (lariats) comprise ~30% of all monomeric forms, while tail length varies considerably to beyond unit size (Figure 4a and b, Figure 5). Some lariats have single-stranded DNA at the junction of the circle to the double-stranded tail (Figure 4b). This observation, together with smaller circle size of some lariats with single-stranded DNA at the junction (Figure 5B), suggests that the template circle and possibly the tail can be single-stranded at the

junction region. In addition, some of the double-stranded tails have small single-stranded regions at the free end (Figure 4b) while other molecules have single-stranded regions between the end of the tail and the circle (not illustrated). Relaxed circular dimers can also have single-stranded and double-stranded tails. In the example illustrated in Figure 4d, the dimer has two single-stranded tails.

Other topological forms of DNA have been observed, such as Y forms, H forms (Table II) and linear molecules ranging in size from  $< 1 \mu\text{m}$  (Figure 4a) to three or four monomer equivalents (not illustrated). The Y forms could arise from breakage of lariats, while H forms could be generated by cleavage of a dimeric circle having two double-stranded tails. As expected from gel electrophoresis, linear molecules are the most abundant class. However, we have not attempted to measure the quantity of linear molecules observed in the electron microscope as we believe that the milder conditions of gel electrophoresis give a more accurate estimate of the *in vivo* occurrence of these forms.

A class of molecule that has not been observed is a theta structure representing circular DNA with a bubble. Although one theta molecule was seen it is believed to have been an artefact formed by a double-stranded tail falling back onto the circle during spreading of the DNA for electron microscopy. This interpretation is supported by arms of the bubble being unequal in length (not illustrated). Likewise, tail-less circular molecules outside the monomer or dimer size classes may have arisen from linear forms by chance association of free ends.

More complex networks of DNA have been seen that cannot be placed into any of the above classes. It is unclear



**Fig. 5.** Distribution of *T. glabrata* circle contour length. (A) Circular molecules without tails. (B) Molecules with either single- or double-stranded tails. (C) Tail length of lariats. In (A) and (B) the number of molecules and average contour lengths are indicated. The horizontal line in (C) marks the unit length of the *T. glabrata* circle ( $6.54 \mu\text{m}$ ) and the arrowheads indicate tails that are obscured by grid bars.

whether these represent chance aggregates or networks formed by replicating or recombinant molecules.

#### Quantitative analysis

Computer generated images of eight Southern blots of separated total DNA from *T. glabrata* were analysed using the ImageQuant software from Molecular Dynamics. These blots represent different samples as well as different electrophoretic regimes. The amount of material trapped in wells is sample dependent and varies from 16 to 44%; linear, fast migrating molecules represent 50–70% of the total mtDNA, while the rest is distributed between supercoiled circles (1–8%) and relaxed circles (2–6%). Detailed analysis of one gel is given in the legend to Figure 3B. The electrophoretic regime does not influence the amount of trapped or released material, suggesting that insufficient lysis of cells might be partially responsible for hybridization in the wells.

#### Discussion

Both gel electrophoresis and electron microscopy have shown that mtDNA in *T. glabrata* and *S. cerevisiae* can exist in various topological forms. In gel electrophoresis the major component (50–70%) migrates as linear DNA of heterogeneous size, while minor components consist of supercoiled and relaxed circular forms which together

**Table I.** Frequency ( $n$ ) of DNA forms in *T. glabrata* mtDNA observed in the electron microscope<sup>a</sup>

Supercoiled monomers	Relaxed circular monomers			Y	H
	No tail	With single-stranded tails	With double-stranded tails		
7	49	13	27	50	5

<sup>a</sup>The predominant class of linear molecules has been excluded from the analysis.

comprise ~3–15% of the mtDNA in *T. glabrata*. Electron microscopy has confirmed that supercoiled and relaxed circular molecules are present and revealed the existence of circular forms with single- or double-stranded tails.

A key question raised by results from both techniques centres on the conformation of mtDNA *in vivo*. A principle concerned in this matter is the degree of damage to DNA resulting from the analytical procedures. For instance, the majority of mtDNA appears to be linear by both techniques but much longer molecules are found by gel electrophoresis than by electron microscopy. Hence the former procedure may reflect more closely the *in vivo* conformation of mtDNA. This view is reinforced firstly by the observation that chromosomal sized DNA molecules can be separated by gel electrophoresis and secondly by the demonstration that no linear molecules are present when the mitochondrial plasmid is replicating in the nucleus. Hence it can be concluded that gel electrophoresis does not fragment DNA. Unfortunately this technique does not yield information about the status of circles with tails. These molecules, because of their hybrid nature, are likely to be distributed between the linear and relaxed circular regions of the gel or alternatively they could be trapped at the top of the gel.

Damage to DNA during isolation and preparation for electron microscopy is also suggested by the preponderance of Y forms over lariats (Table I). Thus, the absence of circular molecules with bubbles, in DNA examined by electron microscopy, may be an artefact. While it is not clear whether theta forms exist, it seems reasonable to conclude that the majority of mtDNA replication takes place by a rolling circle mechanism. Data supporting this proposal are (i) the occurrence of lariats, some with tails longer than circles, (ii) the observation that the majority of DNA exists as linear molecules longer than unit size (50–150 kb) and (iii) the presence of circles with single-stranded tails. Persistence of this latter species suggests that mitochondria lack a 5' exonuclease that can act on single-stranded DNA. This notion stems from the observation that synthesis of long, linear molecules for plasmids of the ColE1 type, which normally yield only circular progeny, occurs in mutants of *E. coli* that do not have RecBC activity (exonuclease V), which is apparently essential for inhibition of the production of linear multimers (Cohen and Clark, 1986). Similarly in yeasts, the composition of the replication machinery, rather than DNA structure, has an important influence on the mode of replication. By comparing the conformation of the same plasmid in either mitochondria or nuclei it has been found that linear molecules are present only in the former compartment. This demonstrates that the replication machinery differs in the two environments, so that in mitochondria a rolling circle mechanism is permitted while conventional  $2 \mu\text{m}$  DNA type replication occurs

in nuclei. Furthermore, differences are likely to exist between components of the mtDNA replication machinery in yeasts and vertebrates as the latter do not employ a rolling circle mechanism.

The presence of circular molecules with single-stranded tails also suggests that lagging strand synthesis has not kept pace with leading strand replication on the circle. Thus a rate limiting step in organelle DNA synthesis may be priming on the single-strand tail rather than initiation of rolling circle replication.

As indicated above, it is not known whether mtDNA replication occurs exclusively by a rolling circle mechanism. However, to explain the occurrence of circular DNA in the absence of an alternative expanding-bubble process, it is necessary to postulate that circles are formed from tails by recombination. Recombination may be facilitated by DNA ends as well as by recombinogenic sequences which occur in baker's yeast mtDNA (Clark-Walker, 1989). Indeed, production of circles from linear molecules also provides a different perspective for viewing the formation of deleted genomes in petite mutants. Hitherto it has been believed that defective genomes are excised from intact circular molecules. In the present context, formation of deletions is seen as an aberration of the circularization process.

The high proportion of linear, tandemly repeated mtDNA may also help to explain the ploidy paradox whereby the number of mitochondrial genome units in baker's yeast is less than the measured quantity of mtDNA (Gingold, 1988). As replication appears to be confined to circular DNA (either by rolling circle or expanding bubble), it is postulated that these few molecules are the genetic units.

## Materials and methods

### Strains and media

*T. glabrata* CBS 138 (O'Connor *et al.*, 1976) and *S. cerevisiae* Dip2 (Evans *et al.*, 1985) are the same strains as previously described. *S. cerevisiae* PTY22 (*MAT $\alpha$* , *ura3-52*, *ade-2*, *leu 2-3,112*, *Rho<sup>0</sup>*) and *S. cerevisiae* PTY24 (isogenic to PTY22, but contains plasmid pMK2 in mitochondria) were obtained from P.E. Thorsness. Strain PTY24 is equivalent to PTY17 (Thorsness and Fox, 1990). The presence of plasmid pMK2 in mitochondria was tested by patching PTY24 colonies to a lawn of TF145 (*MAT $\alpha$* , *ura3 $\Delta$* , *ade-2*, *Rho<sup>+</sup> mit cox2-17*), mating for 2 days and then printing the diploids to ethanol/glycerol media. To obtain a strain that contains pMK2 only in the nucleus we introduced this plasmid into PTY22 by electroporation, using a Gene Pulser (Bio-Rad) set at 1000 V and 25  $\mu$ F. *E. coli* AG-1 (Stratagene) harbours plasmid pAR01 (Skrzypek *et al.*, 1990). Yeast cultures were grown in standard liquid media containing 1% Bacto-Peptone, 0.5% yeast extract and 2% glucose, at 30°C to near stationary phase. Strain PTY22, containing plasmid pMK2 in the nucleus, was grown in a selective minimal medium (0.67% Difco Yeast Nitrogen Base and 2% glucose), lacking uracil. *E. coli* was grown in L broth at 37°C.

### Pulsed field gel electrophoresis

We employed the LKB hexagonal electrode array (CHEF configuration) with the Pulsaphor Plus control unit as described previously (Maleszka and Clark-Walker, 1989). Agarose was IBI Electrophoresis grade. The electrophoresis buffer (25 mM Tris-borate, 1 mM EDTA, pH 8.3) was cooled to 9°C. Details for switching intervals are in the figure legends. Yeast and bacterial DNA plugs were prepared in 0.7% FMC InCert agarose, treated with Zymolyase 20T from Kirin (yeasts) and lysed in 100 mM EDTA, 1% SDS, pH 8. Agarose plugs containing mouse total DNA were prepared from a sample of mouse sarcoma cells GT180, embedded in 0.7% InCert agarose and lysed in 1% SDS, 0.1% Triton X-100, 100 mM EDTA at 55°C. Digestions with restriction enzymes were carried out for at least 6 h at 37°C in a buffer recommended by the supplier with at least a 10-fold excess of enzyme. Before digestion, plugs were washed in 10 mM NaCl, 10 mM Tris-HCl, pH 8, for 2–3 h at 55°C.

### Degradation of DNA with $\gamma$ -rays

Small agarose plugs were placed in Eppendorf tubes in  $\sim$ 100  $\mu$ l of 125 mM EDTA and 0.5% SDS, inserted into a rotating sample holder and exposed to a <sup>60</sup>Co  $\gamma$ -ray source (CSIRO, Plant Industry, Canberra). The dosage variation within the holder was 2.5%.

### mtDNA isolation and electron microscopy

*T. glabrata* mtDNA was isolated by centrifugation in CsCl bis-benzamide H33258 as described previously (Clark-Walker *et al.*, 1981). To minimize physical damage, mtDNA was collected with a wide-mouthed Pasteur pipette. DNA was prepared for electron microscopy by the protein-film technique of Kleinschmidt (1968), as described previously (Clark-Walker, 1973). Samples were spread within 30 min of removal from the gradient without extraction of the dye or CsCl. Grids were stained with uranyl acetate and examined in a Jeol 2000EX electron microscope at 5000 $\times$  magnification. Measurements were made from prints using a Sigma-Scan software from Jandel.

### Labelling and hybridization

Preparation of <sup>32</sup>P-labelled DNA probes was by the random priming method (Feinberg and Vogelstein, 1983). Transfer of DNA to nylon membranes was achieved by exposure of ethidium bromide-stained agarose gels to short wave UV light for 2–3 min, followed by treatment in 0.5 M NaOH for 30 min and blotting directly onto Pal Biodyne nylon membrane without the neutralization step. The DNA was fixed by baking at 80°C for 1 h. Hybridization was carried out at 58°C in 2  $\times$  SSC (0.3 M NaCl, 0.03 M sodium citrate) for 18 h.

### Quantitative analysis

Hybridized Southern blots of PFGE gels were exposed to a Phosphor-storage screen and scanned with a Molecular Dynamics 400S PhosphorImager. Computer generated images of individual gels were analysed using ImageQuant software v. 3.0 from Molecular Dynamics.

### Plasmids and molecular probes

pAR01 is a 16.1 kb yeast-*E. coli* recombinant plasmid harbouring the *S. cerevisiae* *LEU2* marker, the bacterial *Amp<sup>R</sup>* marker, a segment of the 2  $\mu$ m circle and a segment of cloned yeast DNA (Maleszka and Skrzypek, 1990). A *HindIII*-*HindIII* fragment containing only yeast sequences was used as a probe. Plasmid pMK2 (9.5 kb) contains the yeast *URA3* marker, 2  $\mu$ m origin of replication, the bacterial *Amp<sup>R</sup>* marker and *S. cerevisiae* mitochondrial *COX2* gene (Thorsness and Fox, 1990). Plasmid pBMT11 contains a 3.4 kb fragment of bovine mtDNA inserted into *BamHI*-*EcoRI* cut pAT153 (Young and Anderson, 1980). pUC19 is an *E. coli* vector (Yanisch-Perron *et al.*, 1985).

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