Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast

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Summary

Cell growth and division require the doubling of cellular constituents followed by their equal distribution to the two daughter cells. Within a growing population, the ratio of mitochondrial to cellular volume is maintained, as is the number of mitochondrial genomes per cell. The mechanisms responsible for coordinating nuclear and mitochondrial DNA synthesis, and for balancing increases in cell and mitochondrial size are not well understood. In studies of the fission yeast *Schizosaccharomyces pombe* we quantified cellular and mitochondrial DNA content by both Southern blot analysis and flow cytometry of cells stained with a variety of DNA-binding fluorochromes, which we show are able to detect nuclear and mitochondrial DNA with different efficiencies. In the conditional cell division cycle mutant *cdc10*, which is unable to initiate nuclear DNA synthesis, we found that there was an increase in the mitochondrial DNA content in the absence of nuclear DNA replication. This demonstrates that mitochondrial and nuclear DNA synthesis are not obligately linked. We also show that mitochondrial DNA replication is not required for the increase in mitochondrial size that occurs as cells elongate, although this results in a decrease in the ratio of mitochondrial DNA to mitochondrial volume.

Key words: fission yeast, mitochondrial DNA, DNA replication, flow cytometry.

Introduction

For cells to progress through the mitotic cell cycle in an orderly manner, numerous events must be coordinately regulated to ensure that cellular constituents are doubled prior to cell division and apportioned equally to the daughter cells. A precise duplication of the nuclear DNA and a mechanism whereby each of the daughter cells receives a complete set of chromosomes is essential for cell survival. For other more abundant constituents such as ribosomes and mitochondria partitioning must occur, although a lower level of precision may be tolerated. The ratio of mitochondrial to cytoplasmic volume remains relatively constant during cell growth and division (Posakony *et al.* 1977) but little is known about the mechanisms that control the proliferation of mitochondria. Although mitochondrial DNA (mtDNA) content doubles during each cell cycle to maintain a constant number of genomes per cell in the population, and mtDNA polymerase is a nuclear encoded protein (Dujon, 1981; reviewed by Burgers, 1989) the weight of evidence supports the conclusion that mtDNA synthesis is not restricted to the S phase of the cell cycle during which nuclear DNA replication occurs (reviewed by Attardi and Schatz, 1988; Clayton, 1982; Dujon, 1981). In the presence of various drugs that inhibit protein or DNA synthesis and in a number of cell division cycle (cdc) mutants of *Saccharomyces cerevisiae* defective in the initiation of DNA synthesis mtDNA synthesis occurs in the absence of nuclear DNA synthesis (Newlon and Fangman, 1975, and references therein).

The budding yeast *Saccharomyces cerevisiae,* which is a facultative anaerobe, can survive in the absence of a functional mitochondrial genome when grown on fermentable carbon sources, as evidenced by the petite mutants ρ^- and ρ^0 in which, respectively, the mtDNA is largely deleted or is entirely absent. Even in the absence of mitochondrial protein synthesis DNA replication continues in the ρ^- mutant, because the polymerase responsible for mtDNA replication is encoded by a nuclear gene (Dujon, 1981). The fission yeast *Schizosaccharomyces pombe* has been described as a petite-negative yeast because mutants equivalent to the ρ^- and ρ^0 strains of *S. cerevisiae* have not been isolated (Heslot *et al.* 1970; Ahne *et al.* 1984). This organism may thus have a more precise requirement for regulation of the duplication of mitochondria and mtDNA than organisms that can survive in the absence of a functional mitochondrial genome. Studies in which *S. pombe* cells were treated with drugs that inhibit DNA or protein synthesis (Del Giudice and Wolf, 1980; Del

Giudice *et al.* 1981) resulted in the conclusion that mitochondrial and nuclear DNA synthesis are coordinately controlled in *S. pombe*. This is in contrast to *S. cerevisiae* in which mtDNA replication progresses under conditions inhibitory for nuclear DNA synthesis.

When various temperature-sensitive cell division cycle mutants of both *S. cerevisiae* (Russell *et al.* 1989) and *S. pombe* (Costello *et al.* 1986) were characterized by flow cytometry to quantify the cellular DNA content of cells stained with DNA fluorochromes it was noted that the fluorescence increases as these mutants are incubated at the restrictive temperature. This is in contrast to results obtained by biochemical methods, indicating that the total cellular DNA content of the blocked cells remains constant during a temperature arrest.

In the current study, we have analyzed the *S. pombe* cell division cycle mutant *cdc10* (Nurse *et al.* 1976). At the permissive temperature nearly all of the cells contain a G_2 DNA content typical of an exponentially growing wild-type population, whereas at the restrictive temperature the cells arrest in the G_1 phase of the cell cycle with half of the DNA content typical of an exponentially growing population. The temperature-sensitive defect in *cdc10* cells has not been characterized at the biochemical level. When incubated at the restrictive temperature this mutant elongates and undergoes a time-dependent increase in cellular DNA content as detected by flow cytometry. Analyses using a variety of DNA fluorochromes with different base specificities of binding, Southern blot analysis of mtDNA levels and microscopic examination of cells stained with DNA and mitochondrial specific fluorochromes have led us to conclude that in the absence of nuclear DNA synthesis cell elongation is paralleled by an increase in both mitochondrial volume and mtDNA content. Mitochondrial growth also occurs in cells undergoing elongation in the absence of both nuclear and mtDNA synthesis, indicating that mtDNA replication is not required for the growth of this organelle.

Materials and methods

Cells and cell culture

Wild-type *S. pombe* cells, strain 972 h^- (Gutz *et al.* 1974) and *cdc10*-129 h^- cells, which at the restrictive temperature arrest in the G_1 phase of the cell cycle (Nurse *et al.* 1976), were cultured in minimal media as previously described (Nurse, 1975). Hydroxyurea (Sigma) was added to cultures at a final concentration of 12 mM. In experiments involving a shift from growth at the permissive temperature to the restrictive temperature cells were initially grown to a density of approximately 4×10^6 cells ml^{-1} at 25°C, the permissive temperature of *cdc10* cells, before being shifted to the restrictive temperature of 35.5°C. Cell samples were withdrawn at the time of the shift and after a 4-h incubation at the restrictive temperature.

Flow cytometry

Fixation. Cells were pelleted, washed once and resuspended in sterile water at 4°C, then vortexed as −20°C 100% ethanol was added to 70%. Fixed cells were stored at 4°C.

Staining. Propidium iodide: ethanol-fixed cells were stained with propidium iodide (10 $\mu g\,ml^{-1}$) following digestion with RNase A essentially as described (Costello *et al.* 1986).

Hoechst 33258: ethanol-fixed cells were pelleted, washed once in PBS (0.2 $g\,l^{-1}$ KCl, 0.2 $g\,l^{-1}$ KH_2PO_4, 8 $g\,l^{-1}$ NaCl, 1.15 $g\,l^{-1}$ NaH_2PO_4, pH 7.5). The dye was dissolved in 40% ethanol in PBS and the cells were stained at room temperature for 30 min in 5 μM Hoechst 33258 (Calbiochem) in PBS.

Chromomycin A3: ethanol-fixed cells were pelleted, washed once in PBS containing 50 mM $MgCl_2$ and stained for 1 h at room temperature in 10 $\mu g\,ml^{-1}$ Chromomycin A3 (Sigma) in PBS with 50 mM $MgCl_2$.

4′,6-Diamidino-2-phenylindole (DAPI): ethanol-fixed cells were pelleted, washed once in PBS and stained for 1 h at room temperature in 5 $\mu g\,ml^{-1}$ DAPI (Sigma). The stock solution of DAPI was prepared by dissolving the dye in PBS.

Rhodamine 123: live cells were stained in growth media to which Rhodamine 123 (Sigma) was added to a concentration of 15 $\mu g\,ml^{-1}$. Cells were stained at their growth temperature for 15 min, washed twice and then destained for 30 min in temperature-equilibrated growth media prior to analysis. As a control for the specificity of the dye, one half of each sample was treated with 2,4-dinitrophenol (DNP; Sigma) during the destaining incubation, by adding this proton ionophore to a final concentration of 1 mM.

3,3′-Dihexyloxàcarbocyanine iodide ($DiOC_6(3)$): live cells were stained essentially as described above for Rhodamine 123 except that the stain (Eastman Kodak) was used at a final concentration of 0.5 $\mu g\,ml^{-1}$ in culture media and staining was carried out for 5 min, on the basis of the methods of Hayashi and Ueda (1989).

Flow cytometric analysis. Analysis was performed using either a Beckton Dickinson FACScan for propidium iodide- and Rhodamine 123-stained cells or the Coulter Epics 753 for Hoechst 33258, DAPI and Chromomycin A3-stained cells.

The FACScan fluorescence excitation is from a 15 mW argon–ion laser that excites at 488 nm. Emitted fluorescence was measured at 530 nm for Rhodamine 123 and at 585 nm for propidium iodide. Forward-angle light scatter (1–10°) was also measured and used in combination with fluorescence to gate DNA histograms to remove cellular debris. Linear fluorescence data were collected and analyzed using the Consort 30 hardware and software system (Beckton Dickinson).

On the Coulter Epics 753, fluorescence of all dyes was excited by a 5 W argon laser tuned to specific wavelengths for the different fluorochromes. Propidium iodide fluorescence was excited at 488 nm (400 mW) and emitted fluorescence measured above 600 nm. Chromomycin A3 fluorescence was excited at 457 nm (150 mW) and emitted fluorescence measured above 590 nm. Hoechst and DAPI fluorescence was excited at 329 nm and emitted fluorescence measured above 425 nm. Forward angle (1–19°) and 90° light-scatter signals were also measured and used in combination with fluorescence to gate DNA histograms to remove cellular debris. Linear fluorescence data were collected on an MDADS computer and analyzed with the EASY2 computer system (Coulter Electronics).

Microscopy

Fixation. Cells were fixed in 70% ethanol as described above.

Staining. DAPI: ethanol-fixed cells were pelleted, resuspended in PBS, and air dried on coverslips coated with a 1 $mg\,ml^{-1}$ solution of poly-L-lysine. The coverslips were then mounted on slides with 50% glycerol containing 1 μM DAPI and 1 $mg\,ml^{-1}$ paraphenylamine diamine.

Rhodamine 123 and $DIOC_6(3)$: live cells stained as described above were placed on a slide pre-coated with a layer of minimal agar in order to preserve cell viability and immobilize the cells for photomicrography.

Microscopy. Cells were observed with a Zeiss Axioskop Photomicroscope and photographed using XP-1 film (Ilford).

Mitochondrial DNA content

The amount of mtDNA per cell was determined by quantitative Southern blot analysis (Sambrook *et al.* 1989) in which the relative amounts of mitochondrial and nuclear DNA were compared. *S. pombe* DNA was prepared as described (Durkatz *et al.* 1985), digested with the restriction enzyme *Bam*HI and fragments were separated on a 1% agarose, TBE gel (Sambrook *et al.* 1989). Transfer to Gene Screen Plus Hybridization Transfer Membrane (DuPont) and hybridization were according to the manufacturer's recommendations. The filters were hybridized with the *Nde*I to *Bam*HI cDNA fragment of the nuclear gene *cdc2* (Gould and Nurse, 1989) in order to quantify the cellular DNA content and then rehybridized with plasmid pDG3 containing the

entire *S. pombe* mitochondrial genome cloned into the vector pBR322 (Del Giudice, 1981) to quantify the mtDNA content. The probe DNA was labelled with [α-^{32}P]dATP with the random priming method using the Prime-it Random Primer Kit (Stratagene) according to manufacturer's instructions. Filters were exposed to pre-flashed X-OMAT AR film (Kodak).

The relative amount of mitochondrial compared to nuclear DNA was determined by quantifying the relative intensity of the hybridization signals obtained using a Bio-Rad Scanning Densitometer.

Results

Mitochondrial and nuclear DNA replication are independently regulated in the cell division cycle mutant cdc10

DNA content and thus the position of cells within the mitotic cell cycle can be analyzed by staining cells with a fluorescent DNA binding dye and quantifying the level of fluorescence using flow cytometry. It has been observed that temperature-sensitive cell division cycle (cdc) mutants of both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* incubated at the restrictive temperature show an anomalous increase in DNA content when quantified using this method (Costello *et al.* 1986; Russell *et al.* 1989). To investigate this phenomenon we chose the *S. pombe* cell division cycle mutant *cdc10*, which, on the basis of biochemical quantification, has been shown to arrest with a G_1 DNA content that is half of the normal G_2 DNA content of an exponentially growing population (Nurse *et al.* 1976). As shown in Fig. 1, cells stained with Chromomycin A3 or propidium iodide have a G_2 DNA content at the 0 h time point when the cells are growing at the permissive temperature. At the 4 h point all of the cells are arrested with a G_1 DNA content which is approximately half of the G_2 level. In contrast, when stained with the DNA binding dye Hoechst 33258 the cells initially arrest with a DNA content more than half the G_2 level and show an additional 50 % increase in DNA content after 4 h at the restrictive temperature. An anomalous increase was also seen when the cells were stained with DAPI, a dye with DNA-binding properties similar to Hoechst 33258 (data not shown). There was no shift in the position of the G_1 or G_2 histogram peaks when cells were stained with either propidium iodide or Chromomycin A3. Experiments using wild-type cells and alterations in the fixation and staining procedures revealed that this shift was not simply a temperature effect nor was it due to changes in autofluorescence, non-specific cell wall staining or the accessibility of the DNA to the dye. By analyzing a number of cdc mutants that arrest in different stages of the cell cycle we were also able to eliminate differences in the position of the cells in the cell cycle and differences in chromatin conformation as being responsible for the anomalous shift in fluorescence intensity (data not shown).

The four DNA fluorochromes used have different specificities of binding to DNA. DAPI and Hoechst 33258 have a high affinity for AT base pairs whereas Chromomycin A3 binds preferentially to GC base pairs and propidium iodide is relatively non-sequence-specific in its interaction (reviewed by Shapiro, 1988). Because DAPI has previously been identified as a dye with high affinity for mtDNA that is often very A+T-rich (Williamson and Fennell, 1979) and 66 % of the mitochondrial genome of *S. pombe* consists of A·T base pairs (B. F. Lang, personal communication), we investigated the possibility that the apparent shift in DNA content was due to increased mtDNA. The genome of *S.pombe* is 18 000 kb (Smith *et al.* 1987) while the mitochondrial genome of the strains used in this study is 19.4 kb (B. F. Lang, personal communication). The multiple copies of the mitochondrial genome in each cell account for approximately 6 % of the total cellular DNA in growing cells (Bostock, 1969). A large increase in mtDNA during the 4 h of growth at the restrictive temperature would be required to account for changes in DNA content of the magnitude seen with flow cytometry if genomic and mtDNA are detected at equal efficiency. However, the binding of DAPI and Hoechst 33258 to DNA is greatly enhanced when the A·T base pairs are clustered (Muller and Gautier, 1975; Portugal and Waring, 1988). An examination of the sequence of the mtDNA of *S. pombe* (B. R. Lang, personal communication) reveals that it contains nearly 900 stretches of five or more A·T base pairs and that over 50 % of the A·T base pairs in the genome lie within these clusters. Thus an increase in mtDNA content could be overestimated when cells are stained with the DNA fluorochromes DAPI or Hoechst 33258.

In order to quantify directly the level of mtDNA in *cdc10* cells, DNA was prepared from cells grown at the permissive temperature and cells incubated at the restrictive temperature for 4 h. A Southern blot analysis was performed on these two samples in which the filter was sequentially hybridized with plasmids containing either the entire *S. pombe* mitochondrial genome or the nuclear gene *cdc2* (Fig. 2). The intensity of the hybridization signals was quantified using a scanning densitometer and the mitochondrial to nuclear DNA ratio for each sample determined. The results of three independent samples showed increases in mtDNA relative to nuclear DNA of 3.8-, 3.2- and 5.3-fold during the arrest. Thus, on average, there is an approximate fourfold increase in mitochondrial DNA relative to nuclear DNA. However, as the cells shift from a predominantly G_2 DNA content at the permissive temperature to a G_1 DNA content at the restrictive temperature, each arrested cell contains half as much nuclear DNA as a typical cell in the starting population. On a per cell basis this represents a twofold increase in the level of mtDNA during the arrest. This is in sharp contrast to the 10- to 15-fold increase in mtDNA which would be required to account for changes in fluorescence of the magnitude detected with the AT-binding dye Hoechst 33258. To ensure that there was no shearing or loss of mtDNA during the DNA isolation procedure, cells incubated at the permissive or restrictive temperature were embedded in agarose blocks, digested with restriction enzymes and subjected to Southern blot analysis according to methods routinely used for pulsed-field gel electrophoresis of high molecular weight DNA. The results obtained with this method are in agreement with those described above (data not shown).

The relatively small increase in mtDNA in cells incubated at the restrictive temperature can be detected when the DNA fluorochromes DAPI or Hoechst 33258 are used to quantify cellular DNA content, presumably due to their particularly high affinity for clusters of AT base pairs, which are abundant in *S. pombe* mtDNA. Although differences in nuclear size and chromatin conformation make a direct visual comparison of fluorescence intensity difficult, Fig. 3 clearly shows very bright particulate cytoplasmic staining when cells incubated at the restrictive temperature for 4 h are stained with DAPI and

examined by fluorescence microscopy. The increase in cytoplasmic fluorescence due to mtDNA seen in Fig. 3B relative to Fig. 3A, occurs as the cells elongate.

Mitochondrial growth in cdc10 *cells grown at the restrictive temperature*

To investigate the morphology of the mitochondria of

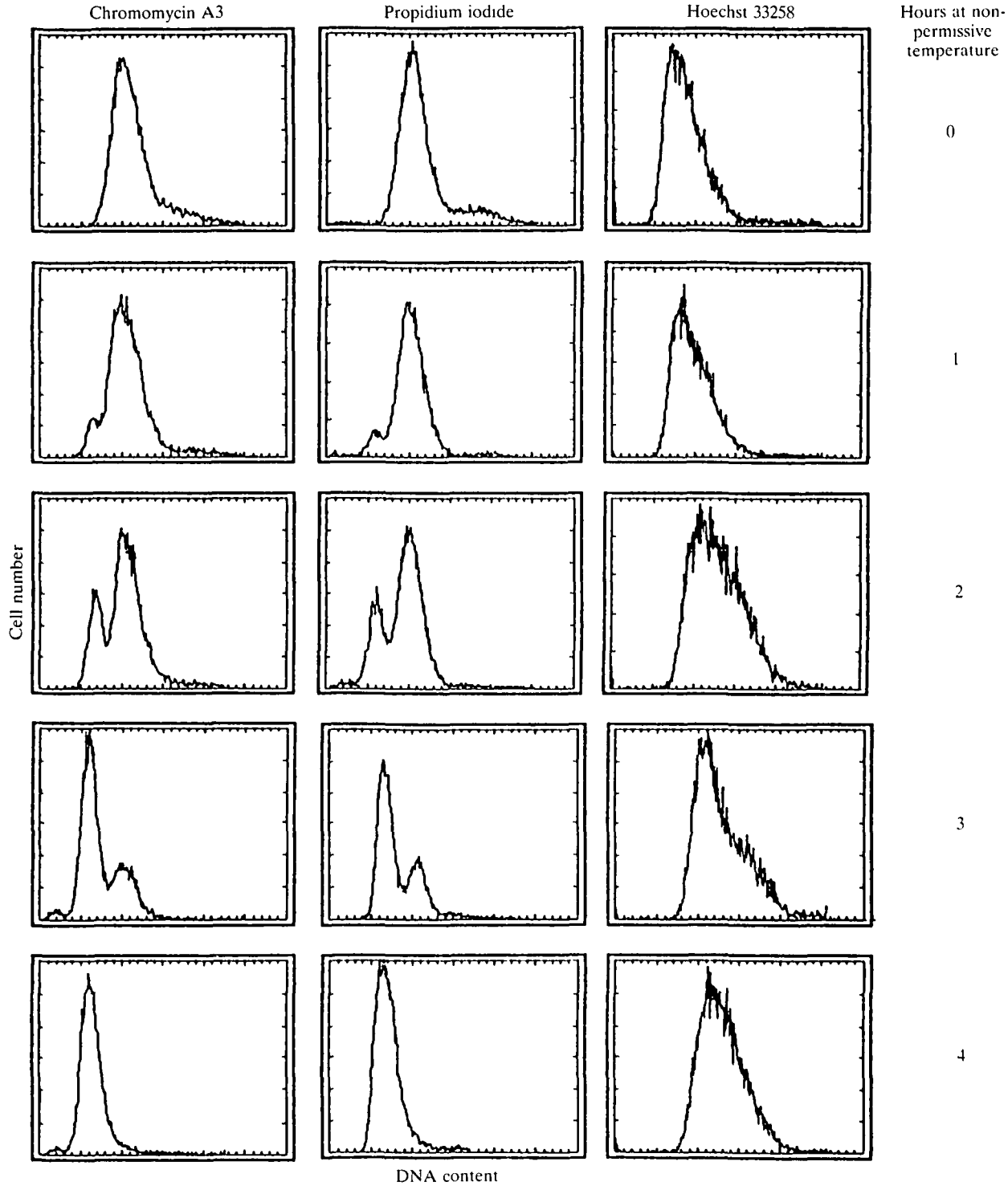

Fig. 1. Flow cytometric analysis of DNA content of *cdc10* cells at the permissive and non-permissive temperatures. *cdc10* cells were grown to mid-exponential phase at the permissive temperature of 25 °C and then shifted at $t=0$ to the restrictive temperature of 35.5 °C for 4 h. Samples were withdrawn hourly, fixed in ethanol and later stained with either propidium iodide, Chromomycin A3 or Hoechst 33258 as described in Materials and methods. Linear fluorescence histograms show relative DNA content (arbitrary units) on the horizontal axis and the cell number on the vertical axis. Flow cytometric analysis was performed on a Coulter Epics 753 Flow Cytometer. The positions of the histogram peak(s) at time 0, 1, 2, 3 and 4, respectively, for Chromomycin A3 are 100, 65 and 99, 63 and 107, 66; for propidium iodide are 100, 96, 59 and 96, 59 and 104, 61; for Hoechst 33258 are 71, 85, 114, 114, 122.

cdc10 cells at both the permissive and restrictive temperatures, living cells were stained either with Rhodamine 123 or with $DiOC_6(3)$. Both of these lipophilic cationic dyes are selectively accumulated in the mitochondria due to the difference in electrical potential across the inner mitochondrial membrane (reviewed by Shapiro, 1988; and Chen, 1988). Photomicrographs of cells stained with $DiOC_6(3)$ are presented in Fig. 3. The photomicrographs shown in Figs 3C and D of cells incubated at the permissive and restrictive temperatures, respectively, were taken and printed under identical conditions and show an increase in staining intensity at the higher temperature. In order to establish that the staining was specific to mitochondria, stained cells were treated with the proton ionophore dinitrophenol, which dissipates the mitochondrial membrane potential. Cells photographed at twice the exposure time used in Fig. 3C and D show no detectable mitochondrial staining after treatment with dinitrophenol, demonstrating the specificity of the stain (data not shown).

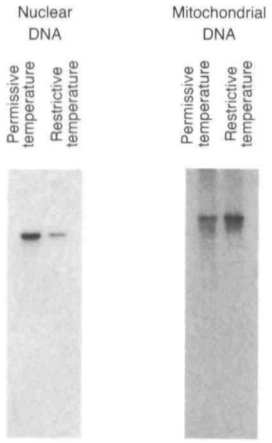

Fig. 2. Quantification of mitochondrial and nuclear DNA content of *cdc10* cells at the permissive and restrictive temperatures. Southern blot analysis of DNA isolated from *cdc10* cells grown at the permissive temperature or incubated for 4 h at the restrictive temperature. The hybridization filter was first probed with the nuclear *cdc2* gene (left panel) and subsequently re-hybridized with plasmid pDG3 containing the entire *S. pombe* mitochondrial genome (right panel).

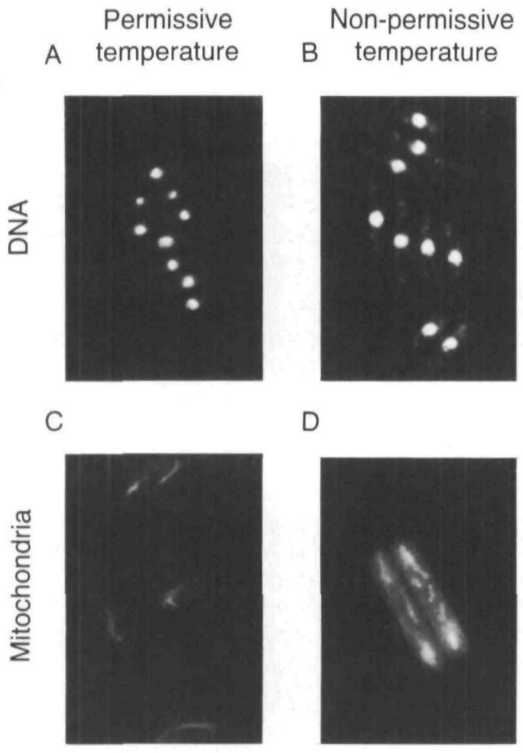

Fig. 3. Photomicrographs of *cdc10* cells at the permissive and restrictive temperatures stained with the DNA binding dye DAPI or the mitochondrial dye $DIOC_6(3)$. (A and B) Photomicrographs of *cdc10* cells at the permissive temperature or the restrictive temperature, respectively, stained with DAPI. (C and D) Photomicrographs of *cdc10* cells at the permissive or restrictive temperature, respectively, stained with $DIOC_6(3)$.

A change in the mitochondria also occurs during the temperature shift. At the permissive temperature (Fig. 3C), a reticulated structure (Davidson and Garland, 1977) is seen. The mitochondria are often found in the peripheral cytoplasm extending along the entire length of the cell, perhaps reflecting the association between the organelle and cytoplasmic microtubules (Kanbe *et al.* 1989). When cells are incubated at the restrictive temperature for 4 h (Fig. 3D) the same basic morphology can be seen but the volume of membrane and matrix is increased and the globular structures are larger. To quantify this change in mitochondrial volume, cells were incubated at the permissive and restrictive temperature, stained with Rhodamine 123, and the cellular fluorescence measured using a flow cytometer. There was a threefold increase in Rhodamine 123 staining during the 4-h incubation at the restrictive temperature (data not shown).

Mitochondrial growth occurs as cells increase in size

As discussed above, *cdc10* cells incubated at the restrictive temperature undergo cellular elongation, mtDNA replication and mitochondrial proliferation. To determine whether this mitochondrial growth depends on continued mtDNA replication, we investigated an experimental condition in which cells undergo elongation in the absence of DNA replication. We incubated wild-type *S. pombe* cells in 12 mM hydroxyurea, and measured mitochondrial proliferation and mitochondrial DNA content as described above. The results of this experiment confirmed the previous findings that both mitochondrial and nuclear DNA replication are inhibited in the presence of hydroxyurea and that during this inhibition the cells elongate (Novak and Mitchison, 1990; Del Giudice *et al.* 1981; K. Wolf, personal communication; data not shown). The Rhodamine 123 staining of the cells treated with hydroxyurea is twice that of untreated control cells (data not shown), but in this case the increase occurs in the absence of mtDNA synthesis. Photomicrographs in Fig. 4A and B of cells treated with hydroxyurea and stained with DAPI show that there is no increase in cytoplasmic DNA staining during the treatment. There is, however, a discernible increase in the mitochondrial staining and in the length of the cells as shown in Fig. 4C and D. Flow cytometric analysis of the DNA content of Hoechst-stained cells in Fig. 5 does not show the increase in fluorescence seen when *cdc10* cells are incubated at the restrictive temperature (compare with Fig. 1), lending further support to the conclusion that the fluorescence shift is due to mtDNA. This finding is inconsistent with the fluorescence increase resulting from a non-specific association of the dye with newly synthesized mitochondrial membrane. In both the *cdc10* temperature shift and the hydroxyurea

blocks there is an increase in mitochondrial membrane synthesis but only when mtDNA replication continues, in the *cdc10* mutant, is there an increase in fluorescence. Therefore we conclude that increased staining seen during the temperature shift represents an increase in mtDNA.

Discussion

The mitochondria of the fission yeast *S. pombe* are essential for the survival of this organism. This is in contrast to the budding yeast *S. cerevisiae*, which can survive in the absence of a functional mitochondrial genome when grown on fermentable carbon sources. The coordination between mtDNA and nuclear DNA replication differs between these two organisms. In the presence of protein synthesis inhibitors, the replication of both the mitochondrial and nuclear genomes of *S. pombe* is prevented, whereas mitochondrial DNA replication but not nuclear replication continues in *S. cerevisiae* (Del Guidice and Wolf, 1980). These and other results were interpreted as demonstrating that the replication of mitochondrial and nuclear DNA are strictly coordinated in *S. pombe* (Del Giudice and Wolf, 1980). While there may be significant differences in the coordination of mitochondrial and nuclear DNA synthesis between fission and budding yeasts, we have shown that in *S. pombe* these two processes can be independently regulated: in the cell division cycle mutant *cdc10* mitochondrial DNA replication continues in the absence of nuclear DNA synthesis.

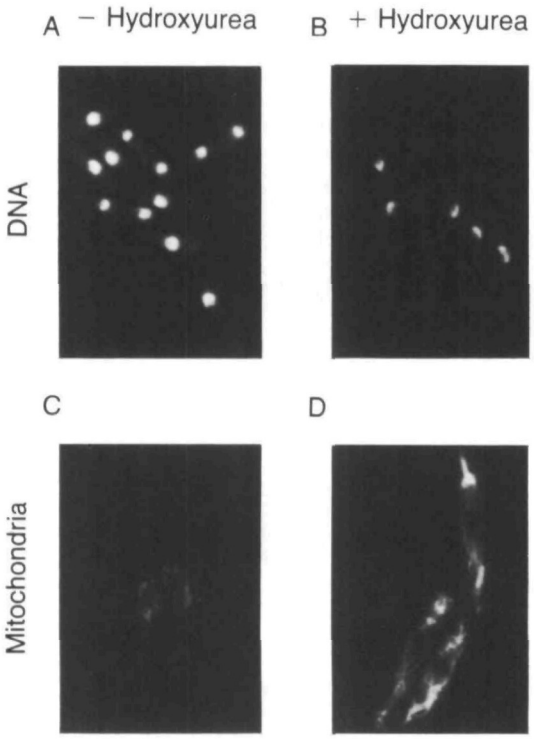

Fig. 4. Photomicrographs of wild-type cells treated with the DNA synthesis inhibitor hydroxyurea and stained with the DNA binding dye DAPI or the mitochondrial dye $DIOC_6(3)$. (A and B) Photomicrographs of wild-type cells treated in the absence (−Hydroxyurea) or presence (+Hydroxyurea) of hydroxyurea, respectively, and stained with DAPI. (C and D) Photomicrographs of wild-type cells incubated in the absence or presence of hydroxyurea, respectively, and stained with $DIOC_6(3)$.

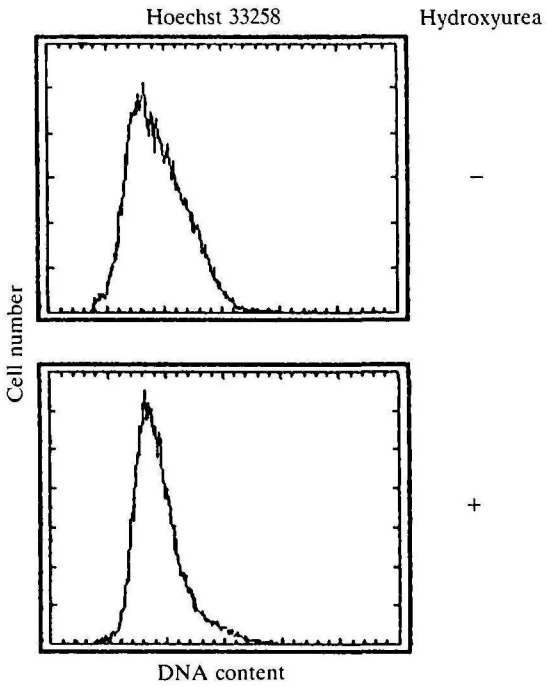

Fig. 5. Flow cytometric analysis of DNA content of wild-type cells treated with the DNA synthesis inhibitor hydroxyurea. Wild-type 972 h^- cells were grown to mid-exponential phase and half of the culture was treated with 12 mM hydroxyurea while the other half was untreated. After 4 h cell samples were fixed in ethanol and later stained with Hoechst 33258 as described in Materials and methods. Linear fluorescence histograms show relative DNA content (arbitrary units) on the horizontal axis and the cell number on the vertical axis. Flow cytometric analysis was performed on a Coulter Epics 753 Flow Cytometer.

Using various DNA-specific fluorochromes to measure cellular DNA content in *cdc10* mutants we observed, in addition to the cells with a G_2 DNA content characteristic of exponentially growing *S. pombe* cells seen at zero hour, the appearance of a population of cells with a G_1 DNA content, indicative of the temperature-sensitive defect in these cells, within 1 h after a shift to the restrictive temperature. When held at the restrictive temperature for up to 4 h cells continue to accumulate in the G_1 phase of the cycle but relatively little change in the fluorescence intensity of the G_1 or G_2 populations is seen in cells stained with propidium iodide and Chromomycin A3. In contrast, both DAPI and Hoechst 33258 dyes showed large time-dependent increases in total cellular staining. The fact that DAPI and Hoechst show high specificity for A+T-rich DNA, which is particularly abundant in *S. pombe* mtDNA, led us to the conclusion that the increased staining with DAPI and Hoechst 33258 reflects continued mtDNA synthesis during cell cycle arrest. Southern blot analysis results demonstrating a twofold increase in mtDNA per cell are consistent with this conclusion.

We believe that our ability to detect a relatively small increase in the mtDNA content using flow cytometry reflects the base specificity of the DNA fluorochromes DAPI and Hoechst 33258. The mitochondrial genome of *S. pombe* is very A+T-rich and these bases are most often found in short runs to which these dyes have a very high

affinity (Muller and Gautier, 1975; Portugal and Waring, 1988) This characteristic is advantageous when microscopically visualizing mtDNA (Williamson and Fennell, 1979).

The mechanisms controlling the coordination between the cell and the mitochondria in terms of growth and DNA replication are not well understood. It is, however, clear from the findings presented here that mitochondrial replication can proceed in the absence of nuclear DNA replication in *S. pombe*. Furthermore, the mitochondria increase in size under these conditions. Even in the absence of mtDNA replication, when cells are treated with the DNA synthesis inhibitor hydroxyurea, mitochondrial growth occurs as cells elongate. This is similar to the situation in *S. cerevisiae* in which ρ^0 mutants lacking mtDNA continue mitochondrial growth and proliferation (reviewed by Dujon, 1981).

Changes in the abundance and structure of mitochondria have been reported to occur in various organisms in response to changes in growth conditions (Stevens, 1977) and cell volume (Lee and Johnson, 1977; Posakony *et al.* 1977). In *S. pombe* changes in mitochondrial structure as cells progress through the mitotic cell cycle have been documented (Kanbe *et al.* 1989). In addition we have shown that mitochondrial proliferation in *S. pombe* parallels changes in cell length even in the absence of mtDNA replication.

The alteration in the structure of the mitochondria in *cdc10* cells incubated at the restrictive temperature compared with the permissive temperature perhaps represents changes that occur during proliferation of mitochondrial membranes in *S. pombe* and might be a model system in which to study this process. It might also be useful to study membrane proliferation that occurs in the absence of mtDNA replication in cells treated with hydroxyurea, as a way of investigating the effects of changing the balance between the amount of mtDNA and mitochondrial volume.

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