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Is the yeast Anaphase Promoting Complex needed to prevent re-replication during G_2 and M phases?

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The Anaphase Promoting Complex (APC) is required for anaphase progression and B-type cyclin proteolysis. The recent finding that inactivation of the APC allows 'over-replication' of DNA has led to the proposal that the APC might also be required for preventing reduplication of chromosomes during G_2 and M phases. In this report we re-investigate the phenotype of *apc* mutant cells and find that they do not re-replicate their DNA during the period taken for wild-type cells to traverse G_2 and M phases. *apc* mutants do, however, gradually increase their DNA content after long periods of cell cycle arrest. Such DNA synthesis occurs almost exclusively in the cytoplasm and neither occurs in cells lacking mitochondrial DNA nor depends on Cdc6, a protein which is essential for the initiation of chromosomal but not mitochondrial DNA replication. ARS1, a chromosomal replication origin, is not re-fired in cells deprived of APC function, confirming that the 'over-replicated' DNA in *apc* mutant cells is of mitochondrial origin. Furthermore, we find that APC function is required to promote but not to prevent re-replication in *ndc10* mutant cells. We therefore propose that the APC is not involved in preventing re-duplication of chromosomes during G_2 and M phases.

Keywords: APC/cyclin B-Cdk1/DNA replication/mitosis/pre-replication complex

Introduction

Cells synthesize most of their constituents from instructions that reside within the DNA sequences of their chromosomes. They must therefore take inordinate care in duplicating this information and in segregating the two copies known as sister chromatids to opposite poles of the cell during mitosis. To maintain a stable karyotype, cells never—or only rarely—fire replication origins more than once between successive rounds of sister chromatid separation. It is not fully understood how they achieve this delicate feat.

Recent work suggests that the initiation of DNA replication in eukaryotic cells is a two-step process, the first of which is the assembly of a large complex of 'initiator' proteins at future origins, known as pre-replication complexes (pre-RCs) (Diffley *et al.*, 1994), and the second of which is the activation of S phase-promoting cyclin-dependent protein kinases, known as S-CDKs (Epstein and Cross, 1992; Schwob and Nasmyth, 1993; Jackson *et al.*, 1995; Ohtsubo *et al.*, 1995; Strausfeld *et al.*, 1996; Krude *et al.*, 1997). A related set of CDKs that are activated later during the cell cycle, known as M-CDKs, promote the formation of the mitotic spindle and the separation of sister chromatids (Booher *et al.*, 1989; Moreno *et al.*, 1989; Surana *et al.*, 1991; Fitch *et al.*, 1992; Basi and Draetta, 1995). In both budding and fission yeast, M-CDKs can also promote the initiation of DNA replication when S-CDKs are inactivated by mutation (Schwob and Nasmyth, 1993; Fisher and Nurse, 1996; Mondesert *et al.*, 1996). S- and/or M-CDKs are active from the beginning of S phase through to the separation of sister chromatids at anaphase (for reviews see Nasmyth, 1993; Nurse, 1994).

It has been proposed that S- and M-CDKs not only promote origin firing in cells that have previously formed pre-RCs but also prevent their *de novo* assembly (Dahmann *et al.*, 1995). Thus, CDKs have opposing effects on the two steps of the initiation process, inhibiting the first step but activating the second. By this means, cells avoid entering a state in which they can both form pre-RCs and trigger origins that have formed them to initiate DNA replication. According to this hypothesis, each new round of origin firing depends on a cycle of CDK activity: a period of low activity that permits the assembly of pre-RCs followed by a period of high activity that permits origin firing. Re-firing of origins during S, G_2 and M phases is therefore prevented by S- and M-CDK activity present during these phases of the cell cycle. A new round of replication depends on the inactivation of S- and MCDKs at anaphase.

Budding yeast cells utilize two mechanisms to inactivate S- and M-CDKs during anaphase. First, they initiate ubiquitin-mediated proteolysis of B-type M-phase cyclins (Amon *et al.*, 1994; Zachariae and Nasmyth, 1996) and second they turn off ubiquitin-mediated proteolysis of a potent CDK inhibitor called Sic1p (Schwob *et al.*, 1994). Ubiquitination of B-type cyclins is mediated by an N-terminal destruction box (Glotzer *et al.*, 1991) and depends on a large multisubunit complex called the Anaphase Promoting Complex (APC) or cyclosome (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). The APC is involved in mediating the destruction, at anaphase, of many proteins besides cyclins, some of whose destruction may be necessary for the separation of sister chromatids (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996). Mutations in APC subunits prevent cyclin ubiquitination and degradation (Zachariae and Nasmyth, 1996). In several cases, such mutations also prevent the separation of sister chromatids at anaphase (Hirano *et al.*, 1988;

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Irniger *et al.*, 1995; Yamashita *et al.*, 1996). The budding yeast APC has at least 10 subunits: Apc1p, Cdc16p, Cdc23p, Cdc26p, Cdc27p in addition to unidentified 150, 100, 80, 79 and 78 kDa proteins (Irniger *et al.*, 1995; Zachariae *et al.*, 1996; W.Zachariae, personal communication), homologues of which have been found in fission yeast (Hirano *et al.*, 1990; Samejima and Yanagida, 1994; Yamashita *et al.*, 1996), *Aspergillus nidulans* (Engle *et al.*, 1990; O'Donnell *et al.*, 1991), *Xenopus laevis* (King *et al.*, 1995; Peters *et al.*, 1996) and humans (Starborg *et al.*, 1994; Tugendreich *et al.*, 1995).

It has recently been reported that mutations which inactivate the yeast APC and prevent both cyclin degradation and anaphase surprisingly do not prevent re-replication (Heichman and Roberts, 1996) as had previously been thought. This suggests that the APC, in addition to promoting anaphase, may be required to prevent re-initiation of DNA replication during G_2 and M phases (Juang *et al.*, 1997; for reviews see Heichman, 1996; Wuarin and Nurse, 1996). This hypothesis is interesting for at least two reasons. First, it implies that cells can after all assemble pre-RCs in the presence of high M-CDKs. Second, it implies that the APC has an as yet undefined role during G_2 and M phases in preventing the accumulation of factors that promote origin firing. The degradation of such factors by the APC might normally depend on S- and M-CDKs, which would explain how these kinases also prevent re-replication during G_2 and M phases. Alternatively, the hypothesis that CDKs inhibit assembly of pre-RCs might simply be wrong.

Because this new finding about the APC promises to shed important new insight into the mechanism of re-replication control, we have re-investigated the phenotype of yeast *apc* mutants. We find that such mutants, upon being shifted to the restrictive temperature, do indeed eventually accumulate DNA. However, this DNA synthesis occurs largely if not exclusively in the cytoplasm, it fails to occur in rho^0 strains lacking mitochondrial DNA, and it does not depend on the Cdc6 replication initiation protein. Furthermore, using a 5-bromodeoxyuridine (BrdU) incorporation assay capable of detecting replication of specific DNA sequences, we failed to detect DNA replication of even a small region surrounding the ARS1 origin of DNA replication. Our data suggest that the APC is not required to prevent re-replication of the nuclear genome during the G_2 and M phases of wild-type cells.

Results

Cells deprived of APC function do not re-replicate during the period taken by wild-type cells to traverse G_2 and M phases

In previously published work characterizing DNA replication in a *cdc23-1 apc* mutant (Irniger *et al.*, 1995), we isolated, by centrifugal elutriation, small unbudded daughter cells from a culture growing at 23°C and incubated them at 37°C. Using FACs analysis to measure cellular DNA content, we found that the mutant cells underwent a complete round of DNA replication at 37°C with kinetics that were similar if not identical to that of wild-type cells. They subsequently formed mitotic spindles but failed to separate sister chromatids. Under similar conditions, wild-type cells separated sister chromatids 30–

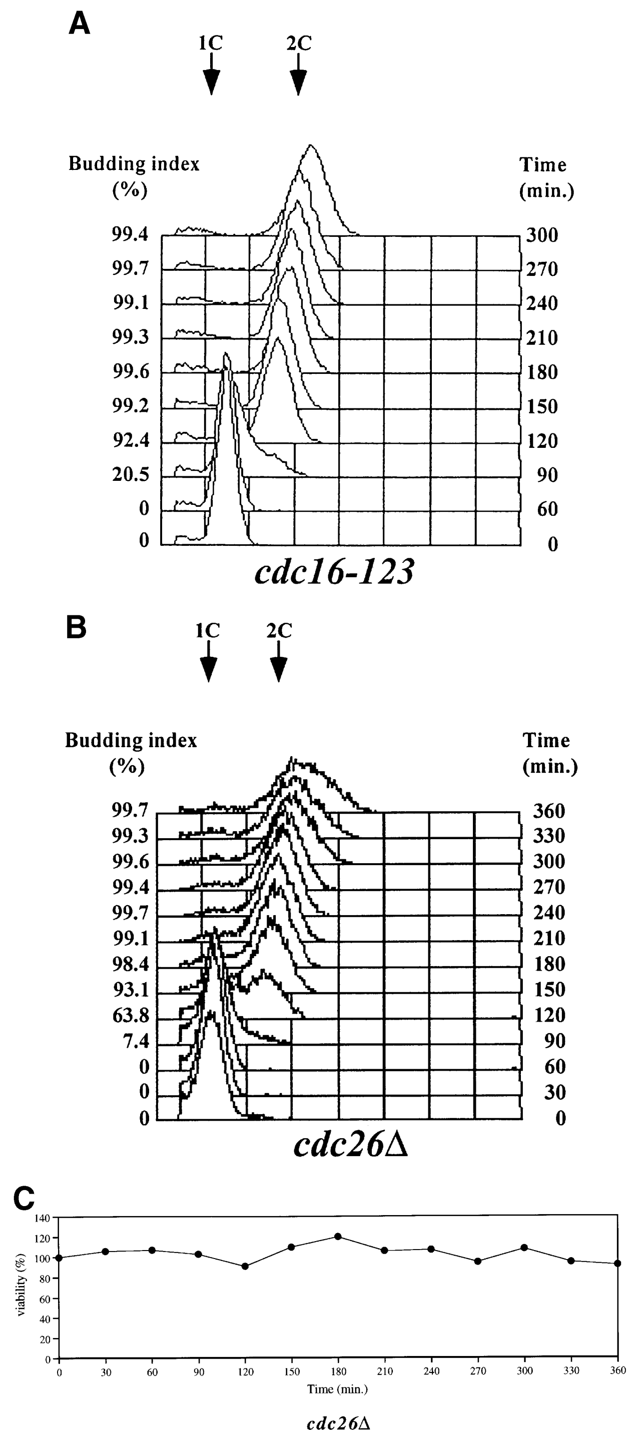

Fig. 1. *cdc16-123* and *cdc26Δ* mutant cells do not re-replicate DNA during the interval taken by wild-type cells to traverse G_2 and M phases. Small daughter cells of *cdc16-123* (K4103) (A) and of *cdc26Δ* (K7013) (B and C) were isolated by centrifugal elutriation (Time = 0) and incubated at 37°C in YEPD medium. (**A** and **B**) Budding index and DNA content were determined at the indicated time points. (**C**) Viability of *cdc26Δ* cells after incubation at the restrictive temperature for the indicated time periods.

40 min after the completion of DNA replication. The DNA content of *cdc23-1* mutant cells remained constant (at 2C) for 120 min after completion of S phase but thereafter gradually increased. We obtained very similar data with *cdc16-123* mutant cells (Figure 1A; for wild-type data see Zachariae *et al.*, 1996, Figure 5) and in

mutants lacking *CDC26* (Figure 1B), which encodes a component of the APC that is essential only at 37°C and is required to prevent the dissociation of Cdc16p and Cdc27p from the rest of the complex (Zachariae *et al.*, 1996; W.Zachariae, personal communication). Furthermore, all *cdc26* deletion mutant cells (*cdc26*Δ) remained viable (that is, capable of giving rise to colonies of haploid cells) for 4 h after completion of DNA replication at 37°C (Figure 1C). In the case of *cdc23-1* and *cdc26*Δ mutants, which clearly arrest at metaphase of the first cell cycle, few if any cells managed to separate sister chromatids during this period (Irniger *et al.*, 1995; also data not shown). The key point is that we detected little if any DNA accumulation in *cdc16-123*, *cdc23-1* or *cdc26*Δ mutants during the first 40 min after their completion of S phase; that is, during the interval taken by wild-type cells to traverse G_2 and M phases. The gradual increase in the apparent DNA content of *apc* mutant cells after prolonged incubation could be due to mitochondrial DNA replication or indeed to an increase in non-DNA background fluorescence (both of which only become appreciable when cells become very large) or to re-initiation of rare origins. Heichman and Roberts (1996) observed more extensive DNA accumulation in *cdc16* and *cdc27* mutants released from a pheromone-induced G_1 arrest at the restrictive temperature and proposed that it corresponds to re-replication of the nuclear genome. It is possible that the lesser DNA accumulation in our experiments (Figure 1A and B) is due to our having started with small cells that had not previously been cell cycle arrested.

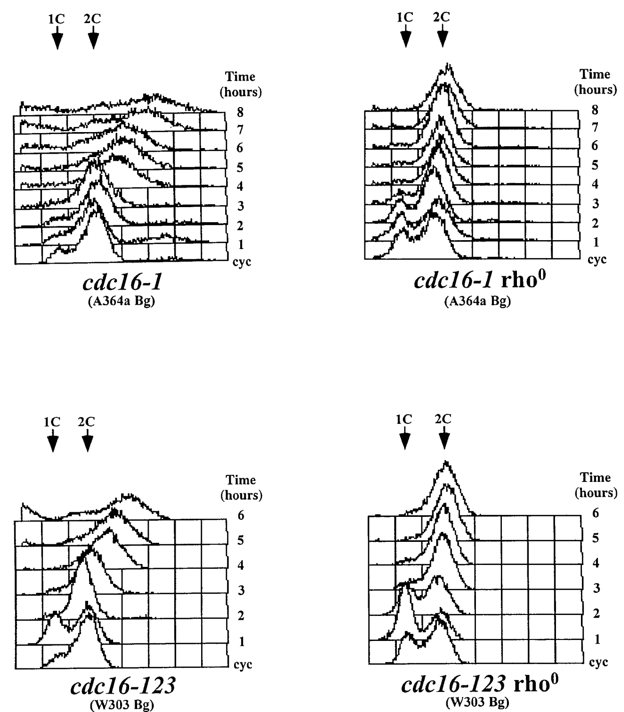

Fig. 2. Cells lacking APC function accumulate excess DNA after long periods of incubation at the restrictive temperature but fail to do so when mitochondria deficient. Rho^+ and rho^0 versions of *cdc16-1* (KHY201, K6114) and *cdc16-123* (K5600, K6823) strains were grown to logarithmic phase in YEPD medium at 23°C (cyc) and subsequently shifted to 37°C. After 3 and 6 h incubation at 37°C, the cultures were diluted 1:2 with prewarmed YEPD medium (37°C). At the indicated time points, samples were withdrawn for FACs analysis. Bg, background.

apc mutant cells gradually increase their DNA content after long periods of incubation at the restrictive temperature

To investigate further whether *apc* mutant cells re-replicate their nuclear genome while arrested in metaphase at 37°C, we analysed the DNA content of cells after shifting asynchronous cultures from 23°C to 37°C. We found that *cdc16-1* and *cdc16-123* mutant cells accumulated with a 2C DNA content within 60 to 120 min but started to accumulate greater than 2C DNA contents after 180 min (Figure 2). This phenomenon occurred in both the strain background used by Heichman and Roberts (A364a) and our strain background (W303). We obtained similar results with *cdc16-264* and *cdc27-22* mutants (data not shown).

The accumulation of excess DNA in cdc16 mutant cells occurs mainly in the cytoplasm

To address whether the increasing DNA content of *cdc16* mutants was due to re-replication of the nuclear genome, we monitored DNA synthesis within individual cells by immunodetection of BrdU incorporation (Neff and Burke, 1991). Thymidine kinase activity, which is required for BrdU labelling, was provided to the cells by introducing five copies of the thymidine kinase gene from herpes simplex virus under the control of the yeast *GPD* promoter [*GPD*-Tk construct; as described by Dahmann (1995)].

Asynchronous cultures of diploid wild-type and three different mutant strains were shifted from 23°C to 37°C for 2.5 h, after which they were incubated for a further 2.5 h at 37°C in the presence of BrdU. Most of the BrdU incorporation of wild-type cells was confined to regions of the cell that resemble the size and position of nuclei (it is not possible to localize BrdU incorporation and the position of nuclei in the same cells because the acid treatment needed to detect BrdU incorporation depurinates the DNA such that it can no longer be stained by DAPI). All wild-type cells contained staining consistent with replication of their nuclear DNA (Figure 3A–C). No staining was detected in control strains lacking *GPD*-Tk or when antibody against BrdU was omitted (data not shown). In contrast, the BrdU incorporation of *cdc13-1* mutant cells, which are thought to arrest in M phase with a 2C DNA content, was distributed in small patches throughout the cell's cytoplasm and at the cell's periphery (Figure 3A). These patches presumably belong to mitochondria (see below). The pattern of BrdU incorporation of *cdc16-123* and *cdc16-264* mutants resembled that of *cdc13-1* mutants (Figure 3A). Very few, if any, *cdc16* mutant cells had nuclear staining similar to that of wild-type cells. The lack of nuclear staining was not due to dissolution of nuclei in the mutants because their nuclei were readily detected by DAPI staining of cells not treated with acid (Figure 3B).

To confirm that we were in fact capable of detecting re-replication using our BrdU incorporation assay, we performed the same experiment with an *esp1-1* mutant, which is known to re-replicate the entire nuclear genome despite failing to undergo nuclear division (Baum *et al.*, 1988). One-quarter or less of the *esp1-1* nuclei accumulated with a 4C DNA content during the 5 h incubation at the restrictive temperature (Figure 3C) and a similar

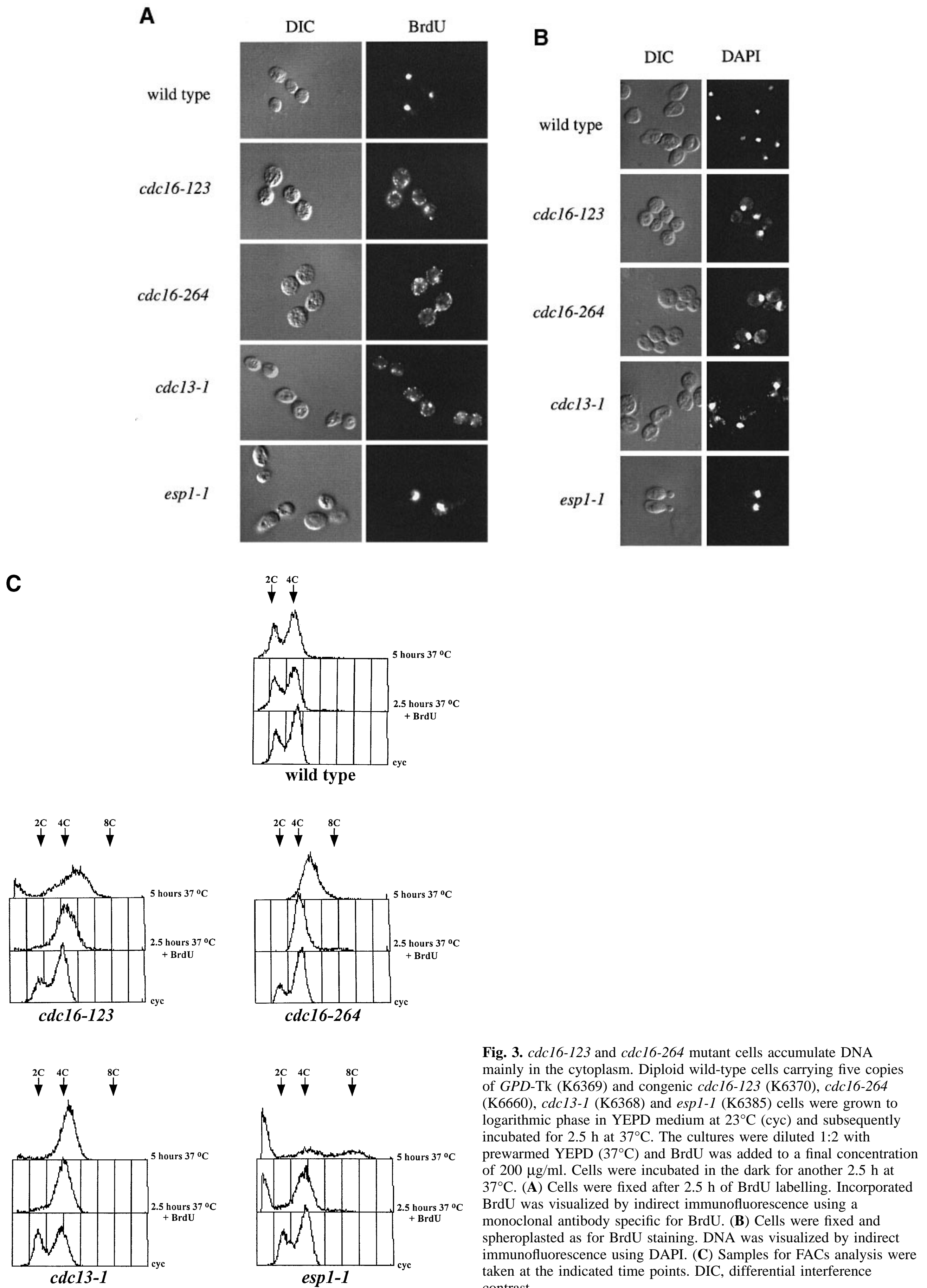

Fig. 3. *cdc16-123* and *cdc16-264* mutant cells accumulate DNA mainly in the cytoplasm. Diploid wild-type cells carrying five copies of *GPD*-Tk (K6369) and congenic *cdc16-123* (K6370), *cdc16-264* (K6660), *cdc13-1* (K6368) and *esp1-1* (K6385) cells were grown to logarithmic phase in YEPD medium at 23°C (cyc) and subsequently incubated for 2.5 h at 37°C. The cultures were diluted 1:2 with prewarmed YEPD (37°C) and BrdU was added to a final concentration of 200 μg/ml. Cells were incubated in the dark for another 2.5 h at 37°C. (**A**) Cells were fixed after 2.5 h of BrdU labelling. Incorporated BrdU was visualized by indirect immunofluorescence using a monoclonal antibody specific for BrdU. (**B**) Cells were fixed and spheroplasted as for BrdU staining. DNA was visualized by indirect immunofluorescence using DAPI. (**C**) Samples for FACs analysis were taken at the indicated time points. DIC, differential interference contrast.

number of nuclei had nuclear BrdU staining (Figure 3A and B). Our assay is therefore clearly capable of detecting genuine re-replication. We conclude that very little nuclear DNA is replicated between 2.5 and 5 h after shifting *cdc16* mutants to 37°C. The bulk of DNA synthesized during this period has a cellular distribution characteristic of mitochondrial DNA.

apc mutant cells do not 'over-replicate' their DNA when mitochondria-deficient

To investigate whether the DNA synthesized by cells lacking Cdc16 function belongs to mitochondria, we compared by FACs analysis the DNA accumulation of *apc* mutants containing mitochondrial DNA with congenic rho^0 strains lacking it. We found that rho^0 strains arrested permanently with a 2C DNA content. This result was obtained with *cdc16-1*, *cdc16-123*, *cdc23-1*, *cdc27-22*, *cdc27-1* and *apc1-1* mutants and, in the case of *cdc16-1*, in both the W303 and the A364a backgrounds (Figure 2 and data not shown). *cdc27-22* was found to be a leaky mutation capable of going through multiple cell cycles before finally arresting in mitosis. Similar results were obtained when we repeated this experiment using medium containing 4% glucose (instead of the customary 2%); this was to test whether the failure of rho^0 strains to synthesize excess DNA might be due to their less efficient utilization of glucose (data not shown). These data, like those obtained using BrdU staining, suggest that the bulk of the DNA synthesized by *apc* mutants shifted to the restrictive temperature is due to mitochondrial DNA.

The chromosomal replication origin ARS1 does not re-fire in *cdc16* mutant cells

Our failure to detect appreciable BrdU incorporation within the nuclei of rho^+ cells does not exclude the possibility that small but appreciable regions of the genome, for example those surrounding replication origins, might in fact be re-replicated in *apc* mutants. Our technique would probably not be sensitive enough to detect such small amounts of re-replicated DNA (10% of the genome or less). To investigate the possible re-replication of specific origin sequences, we used antibodies directed against BrdU to immunoprecipitate DNA containing BrdU and measured the quantity of specific DNA sequences in such immunoprecipitates using PCR (see Materials and methods). We used four sets of primers that amplified four different intervals in the region surrounding ARS1 (one of which contained ARS1 itself). Diploid *cdc13-1*, *cdc16-123*, *cdc16-264*, *esp1-1* and wild-type (wild-type +Tk) cells containing *GPD*-Tk and wild-type cells lacking *GPD*-Tk (wild-type –Tk) were shifted to 37°C for 3 h and then incubated in the presence of BrdU for a further 2.5 h. All four fragments including ARS1 and its surroundings were amplified from the immunoprecipitates derived from wild-type and *esp1-1* mutant cells expressing thymidine kinase from the *GPD* promoter but not from immunoprecipitates derived from wild-type cells lacking *GPD*-Tk (Figure 4). Using this technique, we failed to detect any incorporation of BrdU into ARS1 region sequences of *cdc13-1*, *cdc16-123* and *cdc16-264* mutants cells. These data suggest that ARS1, at least, does not re-fire in *cdc16* mutant cells.

Cdc6 is not required for the continuous DNA synthesis in *cdc16* mutant cells

Yet another way of addressing whether the increased DNA content of cells lacking APC function is due to nuclear DNA replication is to determine whether it depends on proteins, like Cdc6p, which are known to be required for chromosome duplication (Bueno and Russel, 1992; Kelly *et al.*, 1993; Piatti *et al.*, 1995). We therefore constructed wild-type and *cdc16-123* strains, which were deleted for *CDC6* and kept alive by an HA3-tagged version of the *CDC6* gene driven by the *GAL1,10* promoter (*cdc6*::*his*G *GAL-CDC6* strains). These strains proliferate normally in medium containing galactose, because the HA3-tagged Cdc6 protein is fully functional, but they fail to replicate their genomes and undergo a 'reductional' anaphase when shifted to medium containing glucose and lacking galactose, which represses transcription from the *GAL1,10* promoter (Figure 5B; as described in Piatti *et al.*, 1995). Wild-type and *cdc16-123* mutant cells containing either a wild-type *CDC6* gene or an HA-tagged version expressed from the *GAL1,10* promoter (growing in the presence of galactose) were shifted to 37°C for 2.5 h and then transferred (still at 37°C) to a glucose-containing medium lacking galactose. Western blotting showed that the HA3-tagged Cdc6 protein disappeared within 30 min in both wild-type and *cdc16-123* mutant cells (Figure 5A). FACs analysis, on the other hand, showed that the DNA content of *cdc16-123* mutant cells gradually increased from 90 to 240 min after transfer to glucose medium, whether or not the cells contained Cdc6 protein (Figure 5B); that is, the gradual increase in the DNA content was similar if not identical in the *CDC6* and *cdc6::his*G *GAL-CDC6* strains. Furthermore, the accumulation of DNA in *cdc16-123 cdc6::his*G *GAL-CDC6* cells transferred to glucose was similar to that of the same cells kept in galactose medium (Figure 5B). These data suggest that the accumulation of DNA in *cdc16-123* cells incubated at the restrictive temperature is not dependent on the presence of Cdc6 protein. The Western data also suggest that proteolysis of Cdc6p is not mediated by the APC. This is an equally important point, because it has been suggested that the 'over-replication' of *cdc16* mutants might arise due to their failure to degrade Cdc6p (Heichman and Roberts, 1996). Other data (Piatti *et al.*, 1996) in fact suggest that Cdc6p proteolysis is mediated instead by the Cdc4/Cdc34/Cdc53/Skp1 ubiquitination system (known as the S phase Promoting Complex; for review see King *et al.*, 1996).

The re-replication of *ndc10* mutant cells depends on APC function

Ndc10p is a component of a kinetochore binding complex needed for the attachment of microtubules to chromosomes (Doheny *et al.*, 1993; Goh *et al.*, 1993; Jiang *et al.*, 1993). Like *esp1-1* mutants, *ndc10-1* mutants fail to move sister chromatids to opposite poles of the cell during mitosis but nevertheless proceed to re-replicate their nuclear genome. If the APC had an important role in preventing re-replication during G_2 and M phases, then one might expect that it would not be required for the re-replication of mutants that fail to undergo mitosis, such as *ndc10-1* and *esp1-1*. If on the other hand, the APC were important for inactivating M-CDKs and thereby for generating a state of low CDK activity necessary for forming pre-

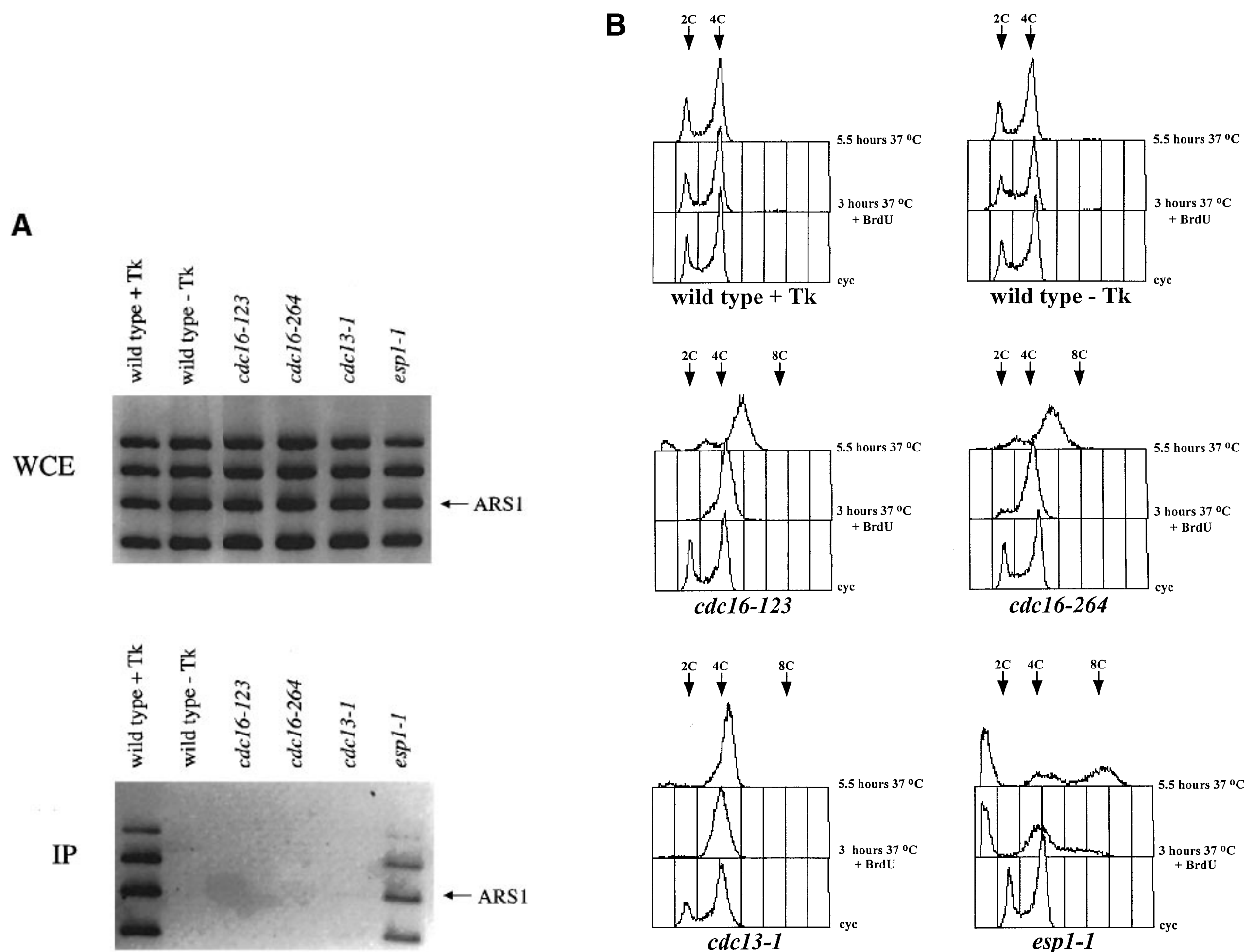

Fig. 4. ARS1 is not re-fired in *cdc16* mutant cells. Congenic diploid *cdc16-123* (K6370), *cdc16-264* (K6660), *cdc13-1* (K6368), *esp1-1* (K6385) and wild-type (wild-type +Tk) (K6369) cells containing *GPD*-Tk and wild-type cells lacking *GPD*-Tk (wild-type –Tk) (K6380) were grown to logarithmic phase in YEPD medium at 23°C (cyc) followed by 3 h of incubation at 37°C. The cultures were diluted 1:2 with prewarmed YEPD medium (37°C), 200 µg/ml BrdU were added and cultures were incubated at 37°C for another 2.5 h (in the dark). (**A**) DNA was isolated and immunoprecipitated with antibody specific for BrdU. DNAs of immunoprecipitates (IP) and of whole cell extracts prior to immunoprecipitation (WCE) were subjected to PCR using four primer pairs to amplify ARS1 and three different surrounding sequences. The PCR products were separated by agarose gel electrophoresis. (**B**) At the indicated time points the cellular DNA contents were determined by FACs analysis.

replication complexes, then one might expect that the re-replication of *ndc10-1* mutants would be abolished by mutating *CDC16*. According to the first hypothesis, *cdc16-1 ndc10-1* double mutants should accumulate DNA in a pattern resembling *ndc10-1* single mutants, whereas according to the second hypothesis, the double mutant should resemble *cdc16-1* mutants in its pattern of DNA accumulation. We therefore compared, by FACs analysis, the pattern of DNA accumulation of *cdc16-1*, *ndc10-1* and *cdc16-1 ndc10-1* double mutants following their release (at 37°C) from a G_1 arrest induced by mating pheromone (Figure 6). All mutants underwent a complete round of DNA replication within 80 min. About 50% of *ndc10-1* cells underwent a second complete round between 120 and 200 min. Most if not all *cdc16-1* mutant cells arrested initially with a 2C DNA content but thereafter gradually accumulated DNA, presumably of mitochondrial origin. The key point is that the pattern of DNA accumulation in the *cdc16-1 ndc10-1* double mutant resembled that of the *cdc16-1* single mutant. DNA accumulation occurred only gradually as in the *cdc16-1* single mutant and not as a discrete doubling from 2C to 4C as in the *ndc10-1* mutant. The gradual accumulation of DNA in *cdc16-1 ndc10-1* double mutants was in fact somewhat more rapid than that seen in *cdc16-1* single mutants, which could be due to the greater cell size of the double mutant cells and their greater number of mitochondrial DNAs. Very similar results were obtained when we compared accumulation of DNA in *esp1-1* and *cdc16-1 esp1-1* mutants (data not shown). These data demonstrate that the re-replication of *ndc10-1 and esp1-1* mutants depends on APC function.

Discussion

APC function is needed for anaphase progression and cyclin destruction

The Anaphase Promoting Complex (APC) is required for ubiquitin-mediated destruction of a number of proteins during mitosis. Its substrates in yeast include: B-type cyclins (Irniger *et al.*, 1995); Pds1p and Cut2, that are inhibitors of sister chromatid separation (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996); Ase1p, a protein that is important for and associates with mitotic spindles (Juang *et al.*, 1997); and Cdc5p, a protein kinase of the Polo

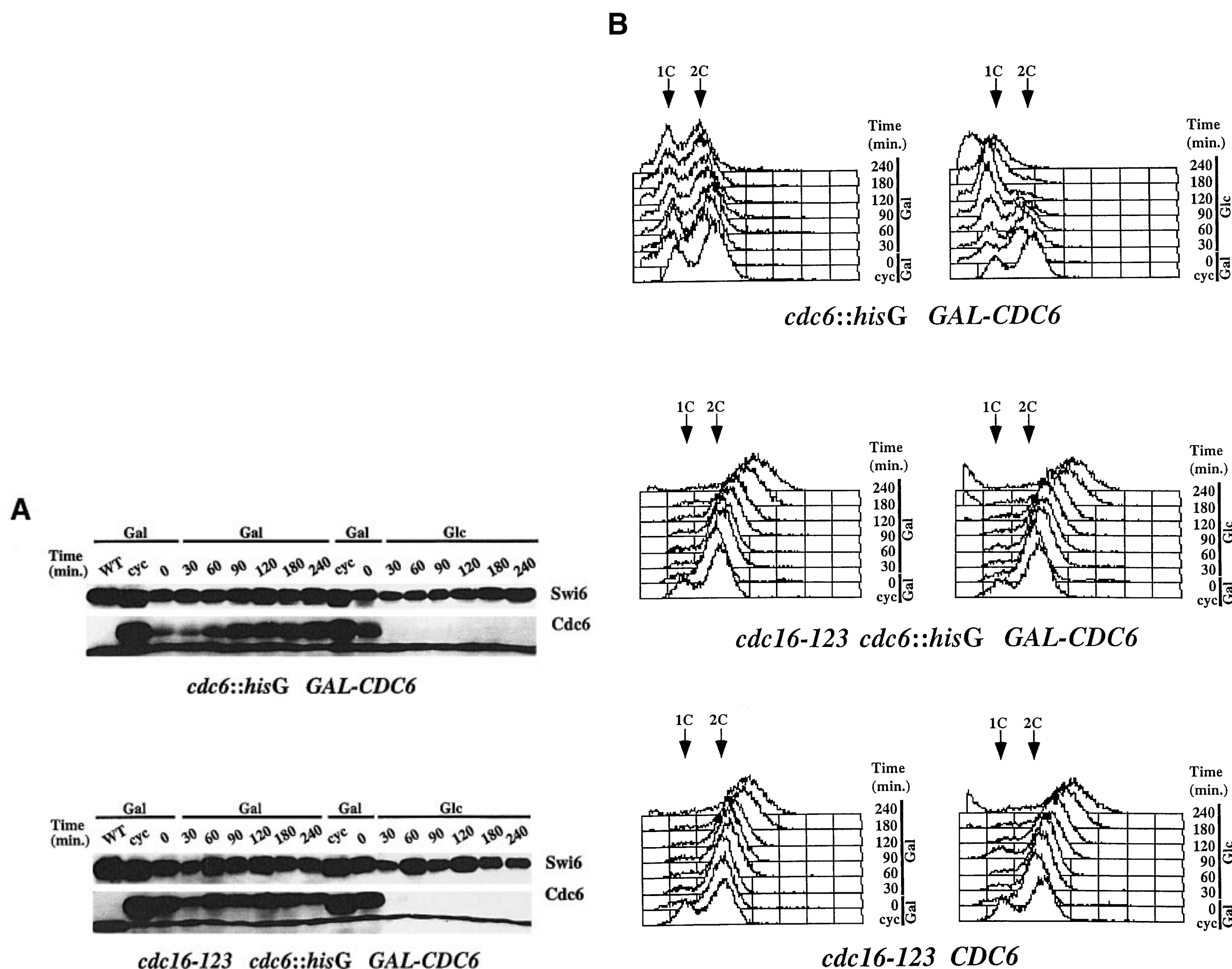

Fig. 5. The continuous DNA synthesis in *cdc16-123* mutant cells is not dependent on Cdc6p. Wild-type *cdc6::his*G *GAL-CDC6* (K6428), *cdc16-123 CDC6* (K5600) and *cdc16-123 cdc6::his*G *GAL-CDC6* (K6433) strains were grown to logarithmic phase in YEPRafGal medium at 25°C (cyc) and subsequently shifted to 37°C for 2.5 h. Cells were harvested by filtration (Time = 0), resuspended in either YEPRafGal (Gal) or YEPD (Glc) medium (37°C) and incubated at 37°C for another 4 h. At the indicated time points, samples were collected for (**A**) Western blot and (**B**) FACs analysis. In (A) Swi6 was used as an internal loading control. Cdc6 was detected using the 12CA5 antibody. K699 (WT) was used as negative control for the 12CA5 antibody.

family (M.Shirayama and K.Nasmyth, in preparation). The APC is composed of at least 10 different subunits (Engle *et al.*, 1990; Hirano *et al.*, 1990; O'Donnell *et al.*, 1991; Samejima and Yanagida, 1994; King *et al.*, 1995; Tugendreich *et al.*, 1995; Peters *et al.*, 1996; Yamashita *et al.*, 1996; Zachariae *et al.*, 1996; W.Zachariae, personal communication). Cells carrying temperature-sensitive mutations in APC subunits fail to destroy B-type cyclins, Pds1p, Ase1p and Cdc5p when shifted to the restrictive temperature. They also fail to separate sister chromatids and arrest in a metaphase-like state with intact mitotic spindles and high cyclin B/Cdk1 kinase activity (Irniger *et al.*, 1995; Yamashita *et al.*, 1996). Early work suggested that *apc* mutants also failed to enter a new cell cycle and to re-duplicate their chromosomes (Irniger *et al.*, 1995), which fits with the current notion that M phase CDKs must be inactivated for chromosomes to be prepared for the next round of DNA replication (Broek *et al.*, 1991; Hayles *et al.*, 1994; Dahmann *et al.*, 1995).

A role for the APC in re-replication control?

It has been recently proposed, however, that certain *apc* mutants accumulate large amounts of nuclear DNA during their cell cycle arrest with high M-CDK, which suggests that the APC might be required to prevent re-firing of origins during G_2 and M phases (Heichman and Roberts, 1996). This proposal is of considerable interest, first, because it suggests that cells might after all be able to re-initiate DNA replication in the presence of high M-CDK activity and, second, because it suggests that the APC has an important cell cycle function not only during G_1 and anaphase but also during G_2. In this paper, we have re-investigated the phenotype of several *apc* mutants and found no evidence that a significant fraction of their nuclear genome is re-duplicated during their cell cycle arrest. We confirmed that *apc* mutants do indeed accumulate DNA after long periods of cell cycle arrest but showed that this corresponds to duplication of their mitochondrial genomes. Using an assay capable of measuring BrdU

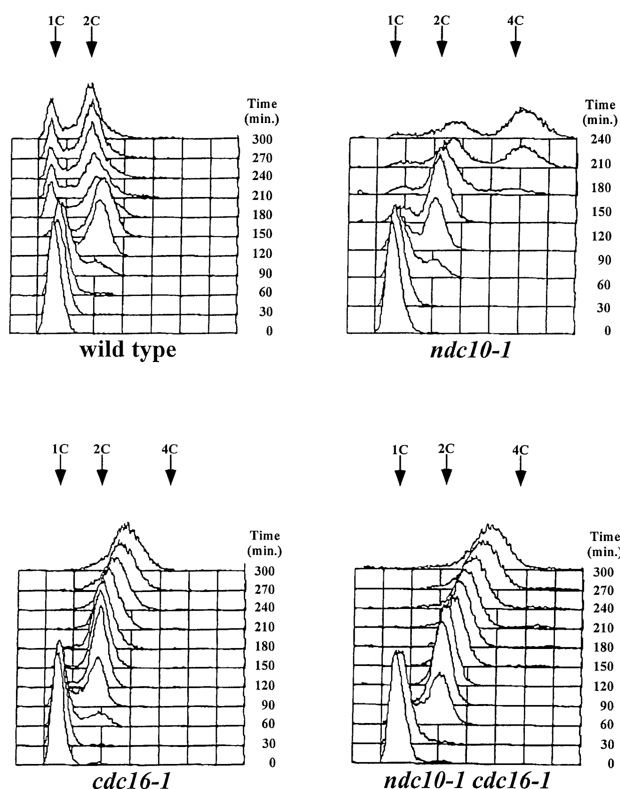

Fig. 6. Cdc16 function is needed for re-replication in *ndc10-1* mutant cells. Wild-type (K699), *ndc10-1* (K4610), *cdc16-1* (K2529) and *ndc10-1 cdc16-1* (SP365) cells were arrested by α-factor for 2.5 h at 25°C (Time = 0), collected by centrifugation, washed with YEPD medium (37°C) and then released into YEPD at 37°C. Samples for FACs analysis were taken every 30 min.

incorporation into specific DNA sequences, we showed that even small regions of the genome in the vicinity of replication origins are not re-replicated in *apc* mutants.

Our data are inconsistent with the conclusion that the APC is required to prevent origin re-firing during G_2 and M phases. The discrepancy between our conclusion and that of Heichman and Roberts (1996) is unlikely to be due to our use of different APC alleles or due to different strain backgrounds, because we have obtained similar results with several different mutant alleles in two different strain backgrounds, including those used by Heichman and Roberts. In some cases, we have performed somewhat different experiments to come to our conclusions. Instead of using density shift experiments to analyse incorporation of nucleotide tracers into DNA, we used *in situ* immunodetection of BrdU incorporation to monitor DNA replication in individual cells and into specific regions of the genome. Our data do not exclude the possibility that, under very particular conditions that we have clearly been unable to reproduce, certain *apc* mutants do indeed reduplicate their nuclear genomes. Our data do however cast severe doubt on the robustness of this phenomenon. Our results suggest instead that APC activity is necessary for re-replication of the yeast genome. Whether this is because re-replication depends on ubiquitination of cyclins or that of some yet to be discovered inhibitor of re-replication is not yet certain.

Materials and methods

Strains and media

All strains were isogenic or at least backcrossed three times to W303 (K699) (*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ssd1-d*) except for KHY201 (*MAT***a** *cdc16-1*) and K6114 (*MAT***a** *cdc16-1* rho^0) which were isogenic to strain A364a (Heichman and Roberts, 1996). All strains were haploid unless otherwise indicated.

Standard techniques were used for genetic crosses (Mortimer and Hawthorne, 1969) and DNA manipulations (Maniatis *et al.*, 1982). In strain K7013 (*MAT***a** *cdc26Δ*), *CDC26* was replaced by the *Klyveromyces lactis URA3* gene (Zachariae *et al.*, 1996). All strains carrying five copies of *GPD*-Tk (K6369, K6370, K6660, K6368, K6385) were derived from K5601 (*MAT***a** *GPD*-Tk, five copies, described by Dahmann, 1995). K6428 (*MAT***a** *cdc6*::*his*G *ura3*::*URA3-GAL-HA3CDC6*, abbreviated by *cdc6*::*his*G *GAL-CDC6*) and K6433 (*MAT*α *cdc16-123 cdc6*::*his*G *ura*::*URA-GAL-HA3CDC6*, abbreviated by *cdc16-123 cdc6*::*his*G *GAL-CDC6*) were derived from K5763 (*MAT***a** *cdc15-2 cdc6*::*his*G *ura3*::*URA3-GAL-HA3CDC6*, described in Piatti *et al.*, 1996). SP365 (*MAT***a** *ndc10-1 cdc16-1*) was derived from parental strains K4610 (*ndc10-1*) and K2529 (*cdc16-1*).

Rho^0 strains K6114 and K6823 were generated by growing KHY201 (*MAT***a** *cdc16-1*) and K5600 (*MAT*α *cdc16-123*), respectively, on YEPD plates supplemented with ethidium bromide. The loss of mitochondria was checked by DAPI staining and death on YEP plates supplemented with 3% glycerol as carbon source.

Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with either 2% glucose (YEPD), 2% raffinose (YEPRaf) or 2% galactose and 2% raffinose (YEPRafGal).

Growth conditions, cell cycle arrests and cell synchronization techniques

Prior to each arrest, cells were grown to logarithmic phase (OD_{600} 0.3–1.0) at the permissive temperature (20–25°C) and then diluted to OD_{600} 0.2.

In the experiment described in Figure 2, cycling cultures were shifted to 37°C for 8 h. After 3 and 6 h incubations at 37°C, the cultures were diluted 1:2 with prewarmed (37°C) YEPD medium to prevent cells from starvation.

In the experiments described in Figures 3 and 4, cultures of cells were diluted 1:2 with prewarmed YEPD medium (37°C) before addition of BrdU to provide fresh nutrients to the cells. Cells were labelled with BrdU (200 μg/ml) for 2.5 h in the dark at 37°C.

Cells in which Cdc6 synthesis was controlled by the *GAL1,10* promoter (K6428 and K6433, Figure 5), were grown to log phase in YEPRafGal medium at 25°C and then incubated for 2.5 h at 37°C. Cultures were divided, cells harvested by filtration and then resuspended in either YEPD or YEPRafGal medium (prewarmed at 37°C).

α-factor was used to prepare synchronous cultures by growing cells to logarithmic phase at 25°C in YEPD medium and arresting them with pheromone (2 μg/ml) for 2.5 h. Uniformly arrested, cells were collected by centrifugation, washed with 3 vol. of YEPD and resuspended in fresh YEPD medium lacking α-factor and prewarmed at 37°C.

To isolate small, unbudded daughter cells, strains were grown at 23°C in YEPRaf medium and subsequently subjected to centrifugal elutriation as described previously (Schwob and Nasmyth, 1993).

Determination of viability

The concentration of cells was determined using a cell counter device (Casy 1, Schärfe System). Then 10^4, 10^3 and 3×10^2 cells of each time point were plated in duplicates on YEPD medium and incubated at 25°C for 3 days.

Detection of BrdU by indirect immunofluorescence

Incorporation and detection of BrdU was performed as described by Neff and Burke (1991). Incorporated BrdU (Sigma) was detected by a monoclonal anti-BrdU antibody (Becton-Dickinson, #7580) at a 1:50 dilution, and the signal was visualized by indirect immunofluorescence with CY3-conjugated anti-mouse antibody (1:400). Pictures were taken with a charge-coupled device camera mounted on a Zeiss Axiophot epifluorescence microscope equipped with a 100 W mercury lamp.

Detection of re-replication of specific chromosome sequences

The procedures are based on the methods described elsewhere (Vassilev and Russev, 1988; Anachkova and Hamlin, 1989; Vassilev and Johnson, 1989, 1990).

Two hundred ml of cells (0.3–0.4×10^7 cells/ml) were labelled with 200 μg/ml BrdU (Sigma) for 2.5 h in the dark. All subsequent steps were either performed in the dark or by the use of light safety tubes to prevent light-induced damage to nascent BrdU–DNA strands. Cells were harvested by centrifugation and washed with PBS (0.14 M NaCl; 10 mM phosphate, pH 7.2). Cell breakage was performed in lysis buffer 1 (0.2% sodium dodecyl sulfate, 1 M NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) by the use of glass beads (1.5–2×10^8 cells/300 μl lysis buffer 1). Cell extracts were sonicated five times for 15 s each using a micro-ultrasonic cell disrupter (Kontes) to shear DNA to an average size of 500 bp. After 2 h of incubation with 0.2 mg/ml proteinase K (Merck) and 0.1–0.2 μg/ml RNase A (Boehringer-Mannheim) at 37°C, DNA was isolated by phenol/chloroform extraction and ethanol precipitation, denatured in 0.2 M NaOH, neutralized with HCl in the presence of 0.1 M Tris–HCl, pH 8.0, precipitated with ethanol and dissolved in 30 μl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Magnetic beads coated with rat monoclonal anti-mouse immunoglobulin (Dynabeads M-450, Dynal) were incubated with anti-BrdU antibody (Becton-Dickinson, #7580) for 6–12 h at 4°C (1 μg antibody/ 8×10^6 beads). One μg DNA and 250 μg salmon sperm DNA (sonicated as described above) were dissolved in 300 μl of PBS containing 0.1% Triton X-100, incubated for 5 min at 95°C and subjected to immunoprecipitation with 0.9 μg anti-BrdU antibody for 1 h at room temperature with gentle agitation. Precipitates were washed twice with TBSE (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA), four times with TBSE containing 1% Triton X-100, and once with TE. Specifically bound DNA was eluted from the beads by incubation of beads in 50 μl of elution buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate) for 10 min at 65°C with agitation. Eluted DNA was deproteinized by 2 h incubation (37°C) in 200 μl lysis buffer 2 (50 mM Tris–HCl, 10 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate) containing 0.2 mg/ml proteinase K. After phenol/chloroform extraction, DNA was precipitated with ethanol using glycogen (Boehringer-Mannheim) as carrier and dissolved in TE.

PCRs were carried out in 50 μl total volume with one-quarter of the material immunoprecipitated by the magnetic bead-coupled anti-BrdU antibody (this amount was optimized to amplify PCR products without reaching a plateau phase in the PCR cycles used) and 0.1 μg of whole cell extract DNA prior to immunoprecipitation, respectively (when 10 ng of whole cell extract DNA were used for PCR a similar result was obtained). Primers were used to amplify the genome sequences located 454.5 bp, 458.5 bp, 462.5 bp (containing ARS1) and 466.5 kb from the left telomere of chromosome IV with the sizes of 350 bp, 310 bp, 270 bp and 228 bp, respectively. The four pairs of primers were used together in each PCR as described by Tanaka *et al.* (1997).

Thirty per cent of PCR products were separated in 2.3% agarose gels and visualized with 0.2 μg/ml ethidium bromide. Pictures were taken using Gel Print 2000i (Biophotics).

Western blot analysis

Western blot analysis was performed as described by Surana *et al.* (1993) with the exception that the enhanced chemiluminescence detection system (ECL, Amersham) was used instead of labelled iodide. One hundred μg of total protein was transferred to Immobilon P membranes (Millipore). HA-tagged Cdc6 was detected with 12CA5 monoclonal antibody at a 1:100 dilution. Anti-Swi6 antibody was used at 1:100 00 dilution. Secondary antibodies were purchased from Amersham.

Other techniques

Flow-cytometric DNA quantitation was determined according to Epstein and Cross (1992) on a Becton-Dickinson FACscan using CellQuest software.

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