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DNA polymerase alpha, a component of the replication initiation complex, is essential for the checkpoint coupling S phase to mitosis in fission yeast

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SUMMARY

Genetic analysis in the yeast *Schizosaccharomyces pombe* has shown that three genes *cdc18*, *cut5*, and *cdt1*, are essential for DNA synthesis and also for the checkpoint control that couples completion of DNA replication to the onset of mitosis. To test whether assembly of the replication initiation complex is an important element in the checkpoint control pathway we have investigated if DNA polymerase alpha (*pol1*), a component of the initiation complex, is essential for the S-phase checkpoint control. We show that germinating *S. pombe* spores disrupted for the *pol1* gene enter mitosis despite defects in DNA synthesis. This is shown by monitoring septation index, DNA content, and by direct immunofluorescence of mitotic spindles using antibodies to alpha-tubulin. In addition we have isolated six temperature sensitive mutants in the *pol1* gene that cause cell cycle arrest when grown at the non-permissive temperature. Our experiments support a model in which DNA polymerase alpha, in addition to being part of the initiation complex, is required for a checkpoint signal that is activated as cells traverse *START*, and is essential to prevent mitosis until S phase has been completed. In contrast, proteins responsible for the elongation of DNA may not be necessary for this checkpoint signal.

Key words: *Schizosaccharomyces pombe*, DNA polymerase alpha, S phase, mitosis, replication initiation complex

INTRODUCTION

Initiation of eukaryotic chromosome replication is thought to involve step-wise assembly of a multi-protein complex at a replication origin (Stillman, 1989; Challberg and Kelly, 1989; Hurwitz et al., 1990; Borowiec et al., 1990). The first step in the assembly of a replication initiation complex in vitro involves binding of an origin-binding protein to a site of initiation, or origin (Dean et al., 1987), followed by binding of RP-A, which facilitates origin unwinding (reviewed by Stillman, 1989), and DNA polymerase alpha/primase, which is required for primer synthesis (Gannon and Lane, 1987; Collins and Kelly, 1991; Dornreiter et al., 1990; Melendy and Stillman, 1993; Schneider et al., 1994). Assembly of RF-C, DNA polymerase delta, and PCNA are then required for processive leading- and lagging-strand DNA synthesis (Lee et al., 1991; Tsurimoto and Stillman, 1989, 1991a,b). DNA polymerase delta is responsible for both lagging and leading strand synthesis, whilst DNA polymerase alpha is only required for synthesis of short RNA-DNA primers essential for initiation (Waga and Stillman, 1994). DNA polymerase alpha is also a target for post-translational modifications in vivo, some of which are cell cycle specific (Nasheuer et al., 1991). How these modifications might regulate the activity of DNA polymerase alpha or events leading to the initiation of DNA synthesis is currently unknown.

Once cells are committed to the mitotic cycle and enter S phase, a checkpoint control ensures that mitosis and cell division do not occur until DNA replication is completed (reviewed by Hartwell and Weinert, 1989; Murray, 1992). In fission yeast this is thought to involve post-translational modifications of the mitotic kinase, $p34^{cdc2}$ (Enoch and Nurse, 1990, 1991; Enoch et al., 1991). A similar checkpoint also exists in *Xenopus* (Dasso and Newport, 1990). Mutations in *S. pombe* that abolish dependency of mitosis on completion of S phase have been identified (Enoch et al., 1992). These mutants called *hus*, for hydroxyurea sensitive, enter mitosis when DNA replication is blocked by treatment with hydroxyurea. Eight *hus* genes were identified, three of which were shown to be allelic to the previously identified *rad 1*, *3*, and *17* genes known to be essential for radiation-induced cell cycle arrest (Al-Khodairy and Carr, 1992; Rowley et al., 1992). Three additional genes, *cdc18*, *cut5*, and *cdt1*, have been shown to be required for DNA synthesis as well as for the checkpoint that couples mitosis to the completion of S phase (Kelly et al., 1993; Saka and Yanagida, 1993; Saka et al., 1994; Hofmann and Beach, 1994), potentially linking the replication apparatus to the checkpoint. However, other genes such as DNA polymerase delta ($pol3^+$; Francesconi et al., 1993), and PCNA ($pcn1^+$; Waseem et al., 1992) are not required for this checkpoint control. Although these two genes encode components of the replication complex, they are only thought to be required for the switch from an intiation complex to a more processive elongation complex (Waga and Stillman, 1994). It is therefore possible that the initiation complex, distinct from the elongation complex, provides a signal for the checkpoint

that inhibits mitosis when cells are delayed or arrested in S phase. To test this possibility, we have investigated the phenotype of germinating spores deleted for the gene encoding the catalytic subunit of DNA polymerase alpha. This gene has been cloned from *S. pombe* by hybridization to a fragment of the *Saccharomyces cerevisiae* POL1 gene (Damagnez et al., 1991) and also by complementation of a *swi7* mutant (Singh and Klar, 1993). In the following study we will refer to it as *pol1*. We show that disruption of the *pol1* gene from *S. pombe* leads to abortive entry into mitosis in the absence of a complete round of DNA synthesis, suggesting DNA polymerase alpha is directly involved in or is required for the assembly of the S-phase checkpoint signal.

MATERIALS AND METHODS

S. pombe methods

All strains were derived from 972h^-, 975h^+, and 968h^{90} using standard genetical procedures (Leupold, 1970). A diploid strain heterozygous for the *pol1* disruption and the *pol1* gene were a gift from A. Klar. Details on the construction of the disruption and the cloning of the *pol1/swi7* gene have been published (Singh and Klar, 1993). All media and growth conditions unless otherwise noted were as described (Moreno et al., 1991). Cells were stained with DAPI (4,6, diaminidino-2-phenylindole) as previously described (Moreno et al., 1991). Cells were prepared for FACS and stained with propidium iodide as previously described (Sazer and Sherwood, 1990). For cell number determination cells were fixed with formal saline as described (Moreno et al., 1991). Cells were prepared for immunofluorescence using methanol fixation as previously described (Moreno et al., 1991). Tat antibodies (a gift from Keith Gull) and a goat anti-mouse Texas Red-conjugated secondary antibody (Jackson Immunoresearch Laboratories) were used for visualization of alpha tubulin.

Isolation of temperature sensitive mutants

Method 1

We transformed pools of a bank of 2,000 ts lethal mutants with a plasmid carrying the *pol1* gene. ts$^+$ transformants were selected at the restrictive temperature, with concomitant selection for the auxotrophic marker on the plasmid. Approximately 10,000 transformants were screened and those showing a ts$^+$ phenotype were subjected to plasmid linkage analysis to determine if the ts$^+$ phenotype is dependent upon the presence of the plasmid. This was carried out by streaking the transformants onto non-selective medium at the permissive temperature to allow plasmid loss, then replica plating them onto both selective medium at the permissive temperature, and non-selective medium at the restrictive temperature. Mutants that require the plasmid carrying the *pol1* gene for growth at the restrictive temperature were retransformed both with the original plasmid and the vector to confirm that the *pol1* gene rescues the ts phenotype.

Method 2

Plasmid pWH5 (Wright et al., 1986) containing the *pol1* gene was digested with *Bam*HI/*Xma*I and the 7.5 kb insert containing the complete *pol1* gene was inserted into *Bam*HI/*Xma*I digested pJK148 (Keeney and Boeke, 1994). The resulting plasmid, pJK-*pol1* was mutagenized by treatment with hydroxylamine as described (Busby et al., 1982) and following mutagenesis, DNA was amplified by transforming *E. coli* strain *ung*$^-$ by electroporation. Approximately 100,000 colonies were pooled, and mutant plasmid DNA prepared. Following linearization with *Nde*I, which cuts at a single site within the *leu1* gene, the mutant DNA bank was transformed into the *S. pombe* diploid strain *h^{90}/h^{90} leu1-32/leu1-32 ura4-d18/ura4-d18 adeM216/adeM210 pol1::ura4$^+$/pol1$^+$*. Linearization within the *leu1* gene greatly facilitates homologous integration at the *leu* chromosomal locus (Keeney and Boeke, 1994). Stable *leu1* integrants were then selected, pooled, washed once with sterile distilled water, and approximately 2×10^7 cells were inoculated into sporulation media (A_{595} =0.005) and incubated for 2-3 days at 30°C or until a high percentage of azygotic asci were observed. Cells were then collected by centrifugation, and resuspended in 50 ml sterile distilled water containing helicase (Biosepra SHP, 1:500 dilution). Following digestion, spores were plated on minimal agar supplemented with adenine. After growth at 25°C, approximately 50,000 colonies were replica plated to yeast extract containing phloxin B and shifted to 35.5°C. Cells defective for growth at the high temperature were selected as putative *pol1* mutants. To determine if the mutation was in the *pol1* gene, we tested whether the mutation was linked to the *leu1* gene. Linkage analysis was performed as follows. Ts mutants were crossed to *ade-M210* or *M216 leu1-32 ura4-d18*. Following conjugation, stable diploids were isolated by selecting on minimal agar plates. Diploids were then sporulated on malt extract plates, and spores collected following treatment with helicase. Spores were then plated on minimal agar supplemented with adenine, selecting for leucine and uracil prototrophy (to select for both the *pol1* disruption and for the *pol1* allele integrated at *leu1*). Colonies that grew at 25°C were then replica plated to 35°C. Those mutants that only gave ts progeny are linked to leu1 and the integrated pol1 allele. If, however, the mutation is not linked to the *pol1* gene, 50% of the colonies should be wild type at the restrictive temperature. This analysis confirmed that greater than 80% of the mutations isolated are linked to the *pol1* gene.

RESULTS

Cells deleted for polymerase alpha undergo defective mitosis

To investigate the role of DNA polymerase alpha in the checkpoint operating in S-phase we investigated the phenotype of germinating spores that had been disrupted for the *pol1* gene by insertion of a single copy of the *ura4* gene (Singh and Klar, 1993). Following sporulation of a *pol1::ura4$^+$/pol1$^+$ ura4-D18/ura4-D18 h^{90}/h^{90}* diploid, spores were collected and inoculated into minimal media lacking uracil. This selects for spores carrying the *pol1* disruption which is marked with *ura4$^+$*, and therefore only 50% of the spores germinate under these conditions. Following inoculation, cells were collected and monitored for septation, aberrant mitoses, cell number, and DNA content every hour for 12 hours. As a control, spores were made from a *ura4-d18/ura4$^+$ h^{90}/h^{90}*. At the time of inoculation FACS analysis confirmed that all spores have a 1C content of DNA as expected for haploid cells (Fig. 1A, 2 hours). Six hours following inoculation approximately 50% of the control spores have germinated and some cells have completed S phase and have a 2C content of DNA (Fig. 1A, 6 hours, control). At later times, all germinated spores have completed at least a single round of DNA synthesis and show the expected 2C content of DNA which is normal for exponentially growing *S. pombe* (Fig. 1A, 6-12 hours, control). The first sign of septation is observed 6 hours following inoculation (Fig. 1B), and all cells undergoing cell division (septated cells) are binucleate and phenotypically normal (Fig. 1C). Cell number in the control increases exponentially after a lag (Fig. 1D), with a doubling time similar to wild-type cells. Control cells stained with the DNA binding dye DAPI 12 hours following inoculation (Fig. 2A-B) show that most cells are in

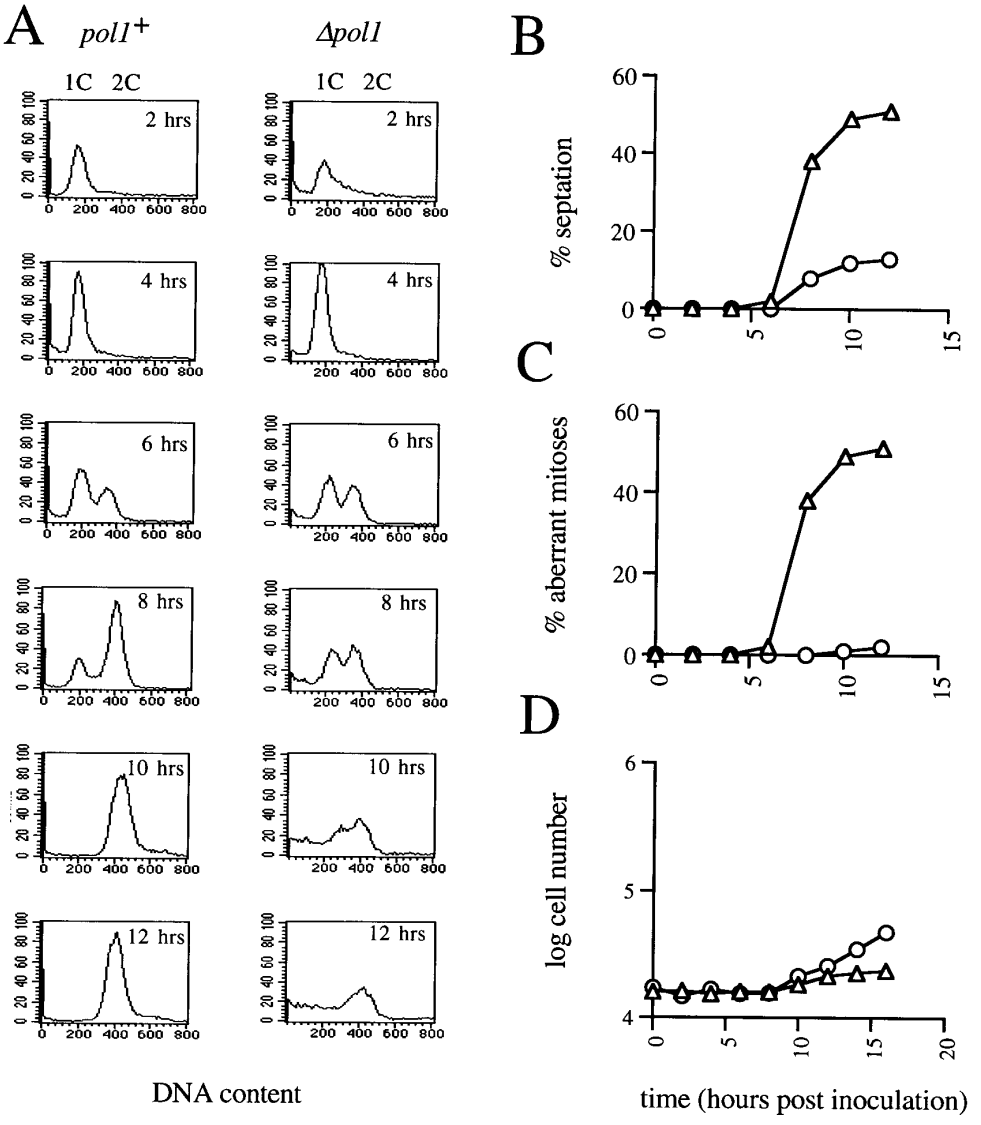

Fig. 1. Spores disrupted for the *pol1* gene enter mitosis following a defective round of DNA synthesis. (A) DNA content of *pol1*$^+$ and *Δpol1* spores by flow cytometry. The position of 1C and 2C controls are shown. Note that spores disrupted for *pol1* are delayed in S phase and arrest with variable amounts of DNA from less than 1C to 2C. (B) Septation index of *pol1*$^+$ (O), and *Δpol1* spores (Δ) at 2, 4, 6, 8, and 10, and 12 hours following inoculation into minimal media without uracil. Under these conditions only 50% of the spores germinate (see text). Septation index is defined as the percentage of germinated spores containing a septum. For each point at least 500 cells were counted. (C) Percentage of germinated *pol1*$^+$ and *Δpol1* spores that have undergone an aberrant mitosis (see Fig. 2B) (D) Increase in cell number for *pol1*$^+$ and *Δpol1* spores.

interphase with a single nucleus, with some cells undergoing septation with two nuclei and a septum.

Germinating spores disrupted for *pol1* have a different phenotype. At the time of inoculation spores have a 1C content of DNA similar to the control but 8 hours following inoculation, while control spores have nearly completed S phase, DNA synthesis in spores disrupted for *pol1* is delayed (Fig. 1A, *Δpol1*). Although these spores lack a functional *pol1* gene, they apparently have enough residual *pol1* protein inherited during sporulation to allow some DNA synthesis to occur. This is consistent with observations in *S. cerevisiae* that DNA polymerase alpha (*POL1*) is a stable enzyme and cells are capable of undergoing more than one round of cell division in the absence of *POL1* transcription and protein synthesis (Falconi et al., 1993). *S. pombe* cells disrupted for *pol1* delay in S phase and arrest with variable amounts of DNA up to 2C indicating that some of these cells have undergone an incomplete round of DNA synthesis (Fig. 1A, 6-12 hours, Δpol1). This is consistent with previous experiments showing that *pol1* is essential for viability (Park et al., 1993; Singh and Klar, 1993; Francesconi et al., 1993). The terminal phenotype for spores disrupted for *pol1* was reported to be small dead cells, consistent with these spores having a growth defect (Francesconi et al., 1993).

Our results confirm this observation, and in addition indicate that cells fail to grow as a consequence of being blocked in M phase after undergoing an aberrant mitosis (see below). Septation occurs in the *pol1* disruptant at the same time as the wild-type control, but the septation index increases to a high level reaching a maximum value of 60% at 10 hours following inoculation, compared to 10% for the control (Fig. 1B). At 10-12 hours after inoculation DAPI stained cells disrupted for pol1 show that a high percentage (approximately 60%) of cells are anucleate, or have the nucleus cut by assembly of a septum (Fig. 2C-D). All the cells that have septated by twelve hours following inoculation show mitotic defects (Fig. 1C). Although most of the *Δpol1* germinated spores form septa, they are generally unable to complete cell division, leading to high levels of septation and a reduced increase in cell number (Fig. 1D). The small increase in cell number shown for *Δpol1* was found to vary slightly from experiment to experiment and in some cases was negligible.

We confirmed that disruption of the *pol1* gene leads to

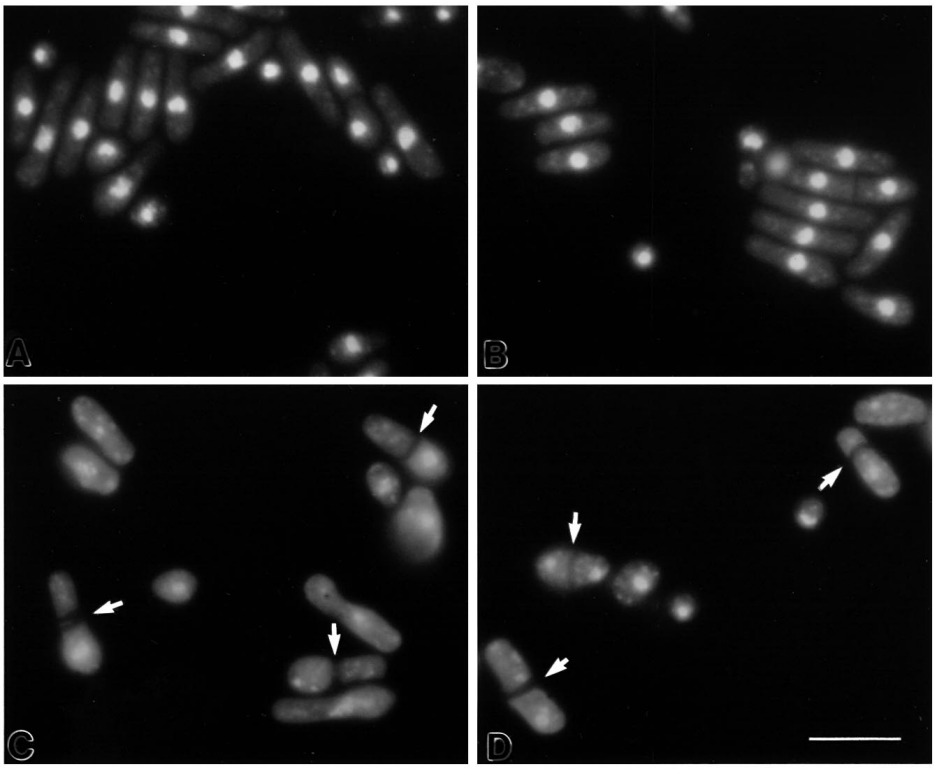

Fig. 2. Spores disrupted for *pol1* undergo an aberrant mitosis. Examples of germinating *pol1*$^{+}$ spores (A-B), and *Δpol1* spores (C-D) stained with the DNA binding dye DAPI 10 hours after inoculation. Arrows indicate *Δpol1* cells that have undergone an aberrant mitosis. Bar, 10 μm.

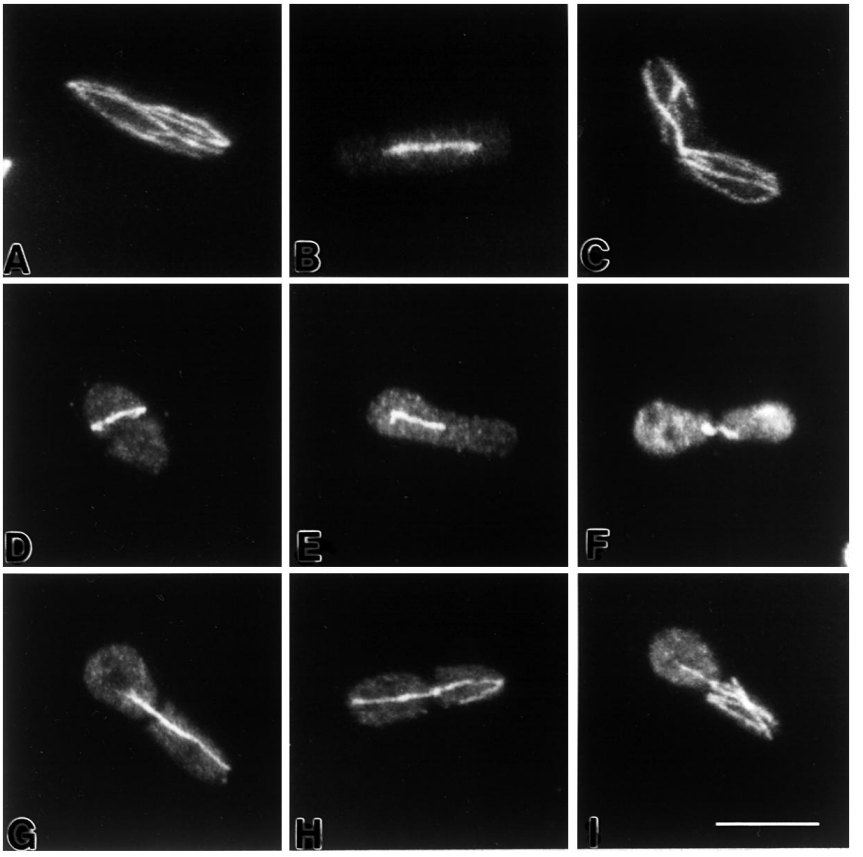

Fig. 3. Assembly of mitotic spindles occurs in spores deleted for *pol1*. Wild-type cells in interphase (A), mitosis (B), and during cytokinesis (C) stained with anti-alpha tubulin antibodies and visualized by confocal microscopy. In mitotic cells (B) a mitotic spindle is observed. (D-I) *Δpol1* germinating spores 10 hours following inoculation. A number of examples are shown where a mitotic-type spindle has formed in spores disrupted for *pol1*. In D-F, the assembly of a short mitotic spindle has occurred and in some cases this has been bisected by a septum (F). In other examples the spindle has elongated and is bisected by a septum (G-I). (I) Cells that have undergone an aberrant mitosis, and have began to assemble an interphase array of microtubules. Bar, 10 μm.

aberrant entry into mitosis by staining cells with antibodies to alpha-tubulin to detect assembly of mitotic spindles. As a control, tubulin staining of wild-type cells in either interphase (Fig. 3A), metaphase (Fig. 3B), or undergoing cytokinesis (Fig. 3C) are shown. Interphase cells or cells undergoing cytokinesis show the characteristic telophase microtubule array

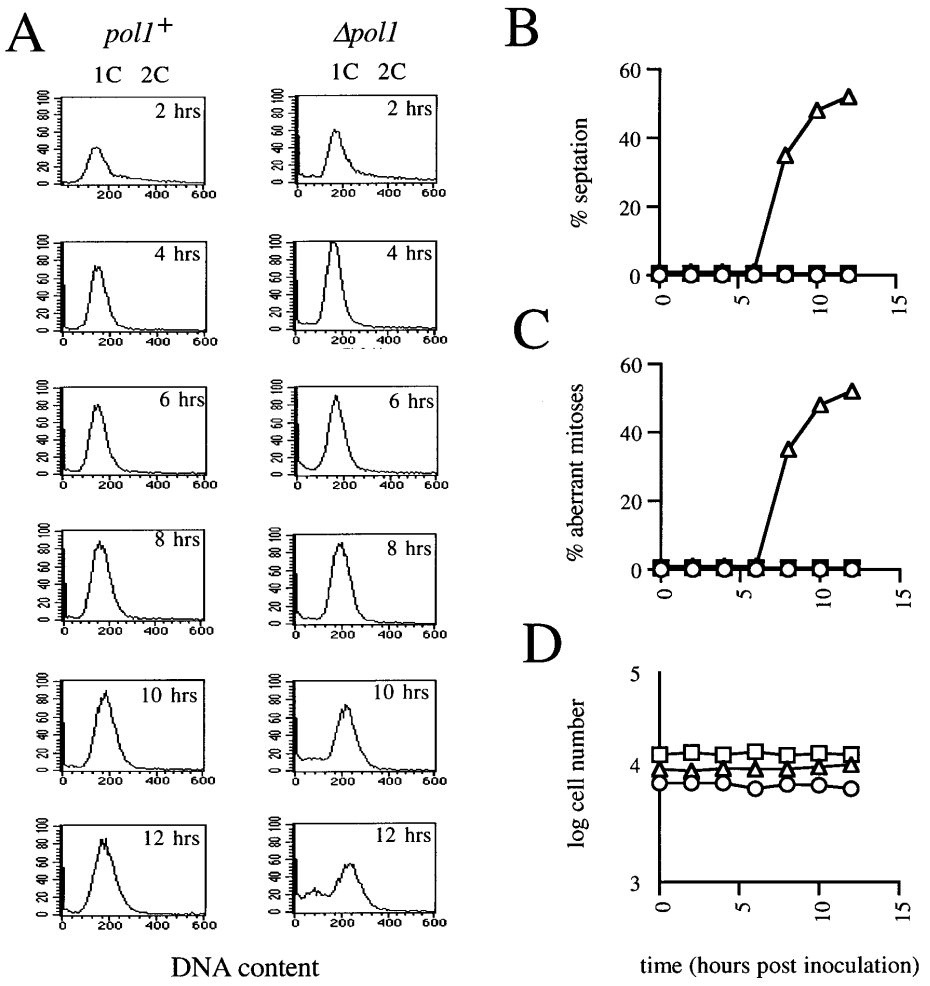

Fig. 4. Spores disrupted for *pol1* enter mitosis in the presence of hydroxyurea. (A) DNA content of *pol1*$^+$ and *Δpol1* spores following inoculation into minimal media minus uracil containing hydroxyurea. Hydroxyurea (12 mM) was added at two points during the timecourse, at 0 and 6 hours following inoculation. This is sufficient to inhibit DNA synthesis for at least 8 hours and, in the wild-type control, completely inhibits septation and cell division for up to twelve hours (see below). Note that spores disrupted for *pol1* show a small 1/2 C peak of DNA at 12 hours post-inoculation consistent with some cells completing cytokinesis in the absence of DNA synthesis. (B) Septation index of *pol1*$^+$ (○) *Δpol1* (△) and Δpcn (□) spores at 4, 6, 8, 10, 12 hours. (C) % germinated *pol1*$^+$, *Δpol1*, and *Δpcn* spores undergoing an aberrant mitosis. (D) Increase in cell number for *pol1*$^+$, *Δpol1*, and *Δpcn1* spores.

extending from the poles (Fig. 3A and C, respectively), and metaphase cells show assembly of a short mitotic spindle (Fig. 3B). At 8 hours following inoculation germinated Δpol1 cells show a variety of defects in the mitotic spindle as visualized by confocal microscopy. Cells with spindles with varying lengths can be visualized (Fig. 3D-I), and in some instances the spindle is disorientated (Fig. 3D), or is severed by formation of a septum (Fig. 3F-I).

These data indicate that deletion of *pol1* leads to an aberrant mitosis and suggest that DNA polymerase alpha is necessary for the checkpoint signal preventing mitosis if DNA synthesis is incomplete or defective.

Spores disrupted for polymerase alpha enter mitosis in the presence of hydroxyurea

Our results show that disruption of the *pol1* gene leads to aberrant mitosis following incomplete DNA synthesis. However, the Δ*pol1* cells synthesize a substantial amount of DNA, and so one possibility is that cells enter mitosis after almost completing DNA replication. To test whether spores disrupted for *pol1* would still enter mitosis in the complete absence of DNA synthesis, we treated germinating spores with the DNA synthesis inhibitor hydroxyurea at 0 and 6 hours following inoculation. In the presence of hydroxyurea cells remain arrested with a 1C content of DNA for up to 8-10 hours post-inoculation (Fig. 4A), and in control cells, septation is completely inhibited for up to 12 hours (Fig. 4B). In contrast, spores disrupted for *pol1* begin to septate at 6 hours post-inoculation showing that the presence of hydroxyurea does not delay the onset of mitosis (Fig. 4B). Germinating spores stained with either DAPI or Caluflour show that in the presence of hydroxyurea, control cells elongate and show no signs of entering mitosis (Fig. 5A-B), whilst many of the cells disrupted for *pol1* are wild type in size and have undergone an aberrant mitosis (Fig. 5C-D). The percentage of aberrant mitoses in the *pol1* disruptant continues to accumulate reaching nearly 60% at 12 hours post inoculation (Fig. 4B).

We had previously demonstrated that PCNA (*pcn1*), a regulatory subunit of DNA polymerase delta, is essential for viability, and following spore germination the *Δpcn1* disruption undegoes a first cell cycle arrest and elongates with a cdc phenotype (Waseem et al., 1992). Similar to the *pol1* disruption, significant DNA synthesis occurs in the *Δpcn1* disruptant, however, cells fail to divide. We tested whether *Δpcn1* would also arrest with a *cdc* phenotype in the presence of hydroxyurea, and found that *Δpcn1* spores behave identically to the control, failing to enter mitosis in the absence of DNA replication (Fig. 4B-D, see also Fig. 5A-B). This experiment further supports a specific role for DNA polymerase alpha in the S phase checkpoint control.

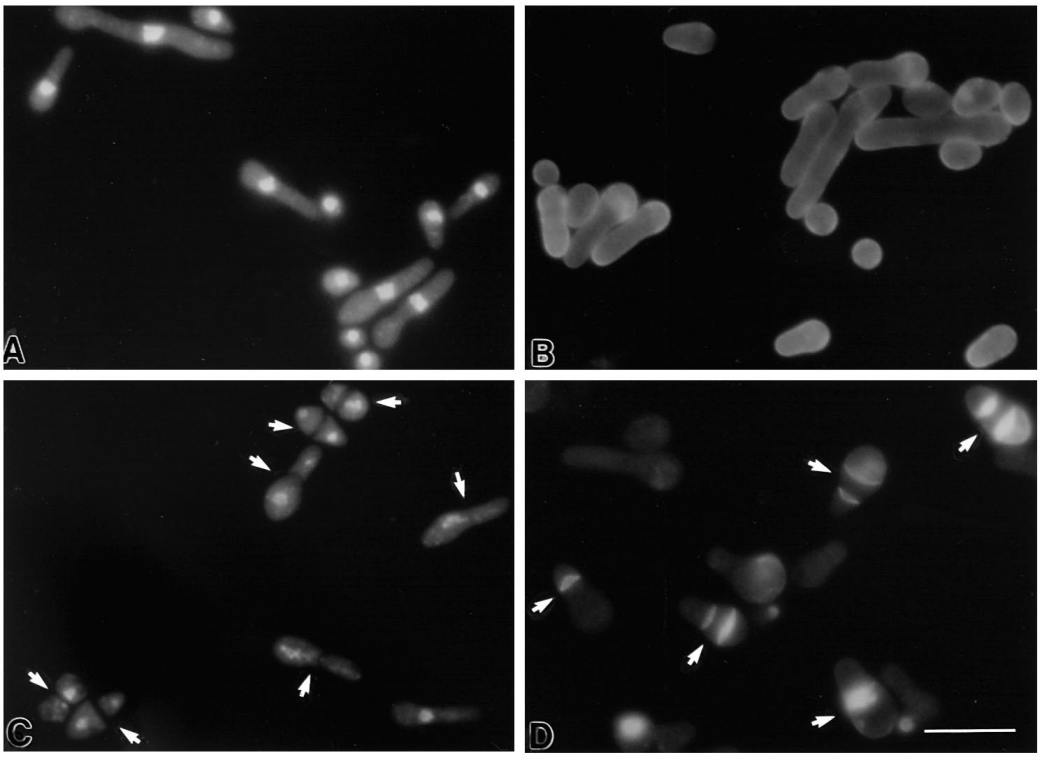

Fig. 5. Spores disrupted for *pol1* undergo an aberrant mitosis in the presence of hydroxyurea. Examples of germinating *pol1*$^+$ spores (A-B), and *Δpol1* spores (C-D) stained with the DNA binding dye DAPI (A and C) or Calcofluor, (B and D) 10 hours after inoculation in the presence of 12 mM hydroxyurea. Arrows indicate cells that have undergone an aberrant mitosis. DAPI and Calcofluor staining of spores disrupted for *pcn1*$^+$ is identical to the wild-type control (*pol1*$^+$; data not shown). Bar, 10 μm.

Isolation of temperature sensitive mutations in DNA polymerase alpha

To extend our studies on the role of DNA polymerase alpha in the checkpoint control we isolated conditional mutant alleles in the *S. pombe pol1* gene. From our previous experiments we anticipated finding three different classes of mutations. First, mutations that would completely inactivate the *pol1* protein might lead to a failure to initiate DNA synthesis as well as a disruption of the checkpoint control. This would result in a *cut* phenotype, as cells enter mitosis in the absence of DNA replication. Second, we might find mutations that do not impair the replication activity of *pol1*, but disrupt the ability to establish the checkpoint signal. These type of mutants would be viable under normal growth conditions, but would be expected to enter mitosis when DNA synthesis is blocked by addition of hydroxyurea. This phenotype would be identical to the phenotype reported for the *hus* mutations. Finally, mutants that disrupt the replication activity of *pol1*, but leave the checkpoint intact would lead to a *cdc* or elongated cell phenotype.

For the isolation of mutations in *pol1* two methods were used. First, a bank of temperature sensitive lethal mutants were screened for mutants that could be complemented by the *pol1* gene, and this method yielded one mutant, *pol1-1*. As a second approach a plasmid DNA containing the *pol1* gene was mutagenized with hydroxylamine and then integrated at low copy at the *leu1* chromosomal locus. Integrants were then screened for cells unable to grow at high temperature or for failure to arrest in S phase in the presence of hydroxyurea. This approach yielded five mutants, *pol1-2* to *pol1-6*. For details on the procedures used for isolation of mutants please see Materials and Methods.

Our attempt to isolate mutations in *pol1* that cause a failure in the checkpoint were unsuccessful. All the mutants, *pol1-1* to *pol1-6*, show a *cdc* or elongated cell phenotype when grown at the non-permissive temperature or when treated with hydroxyurea and therefore we interpret these mutants as not being defective for the checkpoint control. However, in addition to showing a *cdc* phenotype, one mutant, *pol1-1*, did show a small number of aberrant mitoses when grown at the non-permissive temperature (Fig. 6A, as indicated). However, the penetrance of this phenotype is very low suggesting that in the majority of these cells the checkpoint control is still functional. Analysis of DNA content by flow cytometry (Fig. 6B) is shown for *pol1-1*. Some cells are significantly delayed through S phase, however, most cells appear to have completed substantial DNA synthesis. Similar results were found for the remaining five mutants, *pol1-2*, to *pol1-6* (data not shown). We therefore conclude that in these mutants *pol1* is partially defective and unable to properly complete DNA replication, but is still capable of generating a checkpoint signal blocking mitosis.

DISCUSSION

In fission yeast, if cells are arrested in S phase a checkpoint control ensures that cells do not enter mitosis in the absence of a completed round of DNA replication by preventing activation of the mitotic kinase, p34^{cdc2} (Enoch et al., 1992; Gould and Nurse, 1989; reviewed by Nurse, 1990). The biochemical signal generated during S phase that ultimately inhibits p34^{cdc2} activation has yet to be identified. The observation that *cdc18* (Kelly et al., 1993), *cut5* (Saka and Yanagida, 1993; Saka et al., 1994), and *cdt1* (Hofmann and Beach, 1994) are all required for both DNA synthesis and the checkpoint control suggests that these components interact together for both of

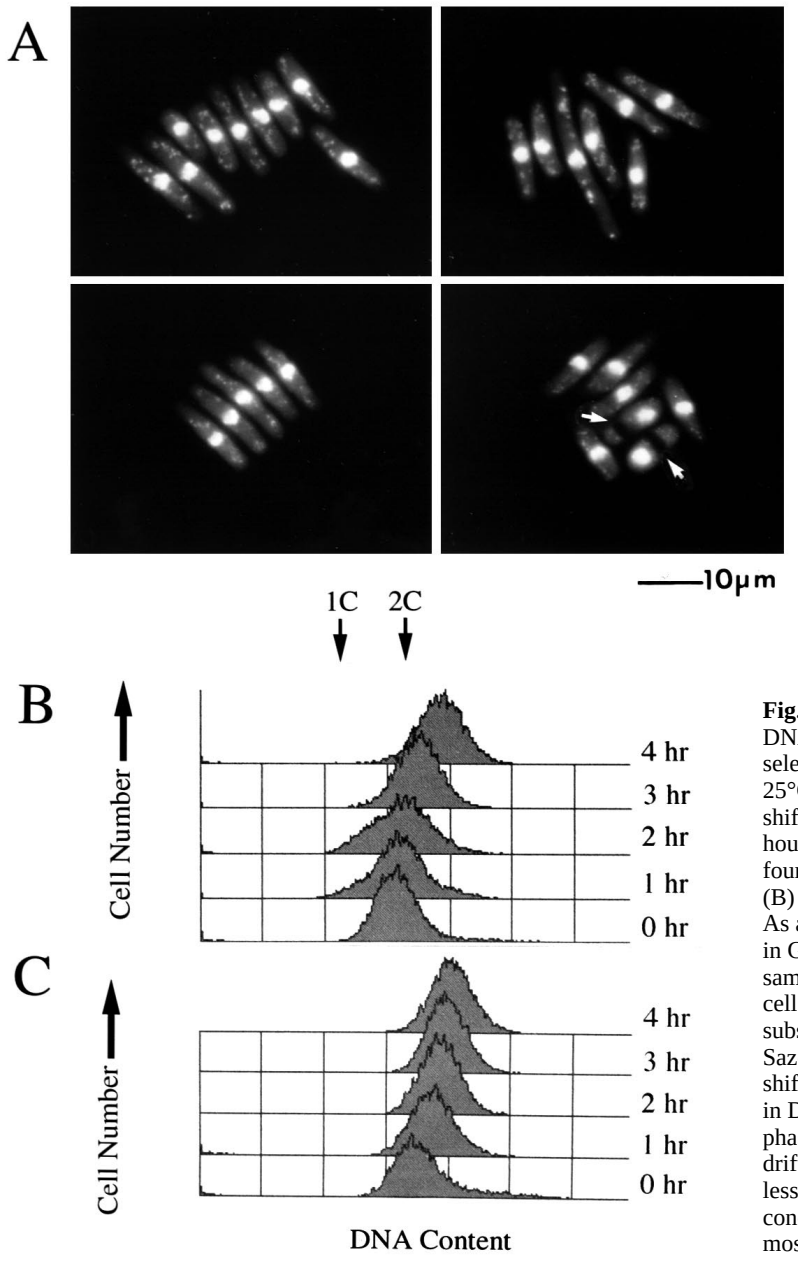

Fig. 6. Analysis of *pol1-1*, a temperature sensitive mutant in DNA polymerase alpha. A single *pol1-1* yeast colony was selected and grown in yeast extract medium (YE) overnight at 25°C. Cells were then diluted in YE to an A_{595} of 0.15 and shifted to the non-permissive temperature of 35.5°C for four hours. (A) Four different examples of DAPI stained *pol1-1* cells four hours following shift to the non-permissive temperature. (B) DNA content of *pol1-1* cells at the restrictive temperature. As a control, *cdc25-22* (Nurse et al., 1976) a mutant that arrests in G_2 was shifted to the non-permissive temperature and samples were prepared for FACS. As is normally observed for cell cycle mutants that arrest in G_2 the 2C peak drifts substantially to the right as cells elongate (Fig. 6C, see also Sazer and Sherwood, 1990). In contrast, when *pol1-1* cells are shifted to the restrictive temperature some cells show a decrease in DNA content suggesting that these cells are delayed in S phase (Fig. 6B, 1-2 hours). At later times the peak does begin to drift to the right (Fig. 6B, 3-4 hours), but the amount of drift is less than that observed for cdc25 (Fig. 6C, 4-6 hours). We conclude that *pol1-1* is defective for DNA synthesis and cells most likely arrest in late S phase.

these functions. Given the similar phenotype we have described here for the *pol1* disruption and the fact that DNA polymerase alpha is required for initiation of DNA replication, we propose that the proteins encoded by *cdc18*, *cut5*, *cdt1*, and *pol1* are components of the replication intiation complex. This complex would then provide the signal required to prevent cells from entering mitosis when either arrested or delayed in S phase. In contrast, neither DNA polymerase delta (*polIII*) or PCNA (*pcn1*), which are components of the elongation complex are required for this checkpoint (Francesconi et al., 1993; Waseem et al., 1992).

In our spore germination experiments, some cells have clearly synthesized DNA even in the absence of a functional *pol1* gene. This can be explained by residual amounts of *pol1* being left in spores following sporulation. However, when spores are germinated in the presence of hydroxyurea to inhibit DNA synthesis, spores disrupted for *pol1* still enter mitosis in the absence of DNA replication. Control cells, which contain an intact *pol1* gene arrest normally with an elongated *cdc* phenotype. This provides further evidence that *pol1* is required to maintain the checkpoint inhibitory signal when cells are arrested during S phase.

Interestingly, our observations concerning the checkpoint signal in fission yeast, may differ from those reported in budding yeast. In *S. cerevisiae*, it has been proposed that the C-terminal domain of DNA polymerase epsilon (*pol2*), plays a critical role in a checkpoint signal operating in S phase. These conclusions are based on analysis of mutant alleles of DNA *pol2* that fail to arrest in S phase and prematurely enter mitosis following treatment with hydroxyurea (Navas et al., 1995). Other *S. cerevisiae* mutants, in either *pol1* (DNA polymerase alpha) or *polIII* (DNA polymerase delta) do not show check-

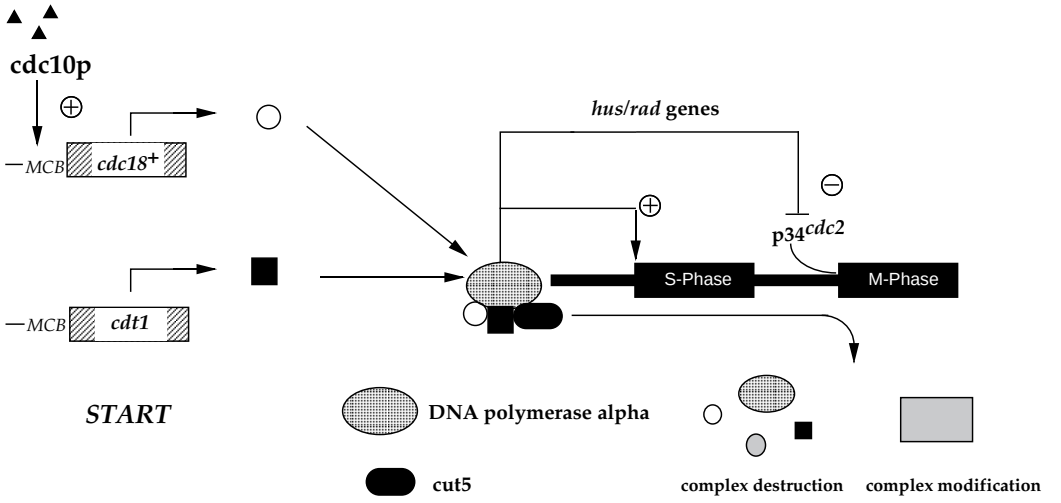

Fig. 7. Model for S phase checkpoint control in *S. pombe*. At START, cells are committed to the mitotic cycle and begin to assemble an active replication initiation complex. We suggest that some feature of this complex, possibly involving DNA polymerase alpha, acts as a signal that ultimately leads to inhibition of the p34^{cdc2} kinase. This signal might involve other components including Cdc18, Cut5, and Cdt1, however the biochemical function of these proteins is still unknown. This checkpoint would prevent cells from entering mitosis prior to the completion of DNA synthesis, or when cells are arrested or delayed in S phase. After DNA replication is finished, the replication complex is either modified or disassembled allowing activation of p34^{cdc2} and entry into mitosis.

point defects, and are therefore not thought to be essential for this control. These observations led the authors to conclude that *pol2* is the checkpoint sensor. As we show in this report, in *S. pombe* the replication initiation complex is also linked to the checkpoint control; in this case, however, the sensor might be DNA polymerase alpha. One possibility to reconcile these differences is that, in *S. cerevisiae*, DNA polymerase epsilon interacts with DNA polmerase alpha at the replication fork, and that this interaction may be mediated through DNA polymerase epsilon's C-terminal tail. The POL2 alleles described by Navas et al. (1995) may alter this interaction in such a way as to block the checkpoint control signal generated by the initiation complex, possibly involving POL1. Alternatively POL2 may be the replication sensor, but in order to function as such must first bind to the replication origin, and this might require the prior binding of POL1. Assembly of these two polymerases at a replication origin might then represent the true initiation complex in vivo, and this complex would then provide the signal needed to establish the checkpoint. To help clarify the situation, it will be important to determine if, in *S. cerevisiae*, the checkpoint is defective in the complete absence of DNA polymerase epsilon using a strain deleted for the POL2 gene. Likewise, it would also be interesting to address the role of DNA polymerase epsilon in the checkpoint control in *S. pombe*.

A model for checkpoint controls in fission yeast is shown in Fig. 7. Prior to *START*, cells are maintained in the pre-*START* G_1 interval by a *rum1* dependent checkpoint control (Moreno and Nurse, 1994; D'Urso and Nurse, 1995). Following passage through *START*, genes required for initiation of DNA replication including *cut5*, and the periodically transcribed *cdc18* and *cdt1* genes promote onset of S phase by facilitating the assembly of an active replication initiation complex including the products of the *cdc18*, *cut5*, *cdt1*, and *pol1 genes*. Initiation of DNA replication in vitro requires the function of DNA polymerase alpha/primase and this enzyme is thought to be one of the first components that binds a replication origin. It is also possible that assembly of the complex might occur at another point in the cell cycle, and is only modified at the G_1 to S phase transition to become active as a replication initiation enzyme and to generate the checkpoint signal. In this model, existence of an initiation complex would be sufficient to generate the checkpoint signal, and ongoing replication would not be required. The number of active replication initiation complexes required to establish the checkpoint is not clear, but a critical number may be necessary to generate a sufficient signal to inhibit mitosis during S phase. Termination of DNA replication would then lead to disruption or modification of the complex(s) and loss of the checkpoint signal. This might occur by direct interaction between two colliding replication complexes, or when a replication complex terminates at either centromeric or telomeric DNA sequences. This would allow the checkpoint control to be tightly linked to completion of DNA replication, and would be a quick and efficient way of removing the block to mitosis following DNA synthesis.

The signal generated by either assembly or modification of the replication initiation complex may be catalytic to allow sufficient amplification to inhibit onset of mitosis. One possibility is that an accessory protein interacting with the complex may somehow generate high levels of an effector molecule that ultimately inhibits the mitotic kinase. Some candidates in higher eukaryotes might be an *RCC1*-related factor (Nishimoto et al., 1978) or other GTP-GDP exchange factors, or a protein kinase which is only active when complexed with a replication initiation complex. Interestingly, *RCC1* is essential for DNA synthesis in *Xenopus* cell free extracts (Dasso et al., 1992), and has a potential role in the checkpoint coupling S phase to mitosis in mammalian cells (Nishimoto et al., 1978; Nishitani et al., 1991). However, it is not known whether *RCC1* interacts with any components of the replication initiation complex. Although a structural homologue of RCC1, *pim1/dcd1* has been identified in fission yeast and has been implicated in the checkpoint control (Matsumoto and Beach, 1991), other experiments have suggested it may not be required for the checkpoint (Sazer and Nurse, 1994).

In this study we have also isolated six temperature-sensitive mutants in DNA polymerase alpha which cause cell cycle arrest. FACS analysis showed that DNA synthesis still occurs in these mutants, and visual observation demonstrated that the mitotic control was still intact. Possibly in the temperature

sensitive mutants the DNA polymerase alpha protein allows assembly of the initiation complex and thus generation of the checkpoint signal whilst its catalytic activity is compromised leading to defects in DNA synthesis.

A similar checkpoint to mitotic progression has been identified in cell free extracts from *Xenopus* eggs (Dasso and Newport, 1990), and this checkpoint also involves inhibition of p34^{cdc2} by tyrosine phosphorylation (Smythe and Newport, 1992). These authors concluded that some feature of unreplicated DNA is required to establish the checkpoint control. They speculated that either unreplicated DNA itself, unused replication origins, or a complex bound to unreplicated DNA is responsible for the checkpoint signal (Dasso and Newport, 1990). Our observations in fission yeast are consistent with the latter, suggesting that *pol1* and perhaps other proteins important for assembly of the initiation complex associated with unreplicated DNA are required to block activation of p34^{cdc2}, preventing premature entry into mitosis. It would be interesting to know whether DNA polymerase alpha in the *Xenopus* cell free system is required to delay the onset of mitosis under conditions where a checkpoint prevents activation of p34^{cdc2}.

Further biochemical studies are required to determine if the structure of the polymerase/primase complex changes during the cell cycle and, if so, whether any of these changes are important for establishing the checkpoint. In addition, genetic analysis using the conditional alleles of *pol1* isolated in this study should help to identify factors that interact with *pol1*, or are important in the signal transduction pathway that inhibits mitosis during S phase.

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