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Studies of *Schizosaccharomyces pombe* DNA polymerase α at different stages of the cell cycle

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ABSTRACT

The status of *Schizosaccharomyces pombe* (fission yeast) DNA polymerase α was investigated at different stages of the cell cycle. *S.pombe* DNA polymerase α is a phosphoprotein, with serine being the exclusive phosphoamino acid. By *in vivo* pulse labeling experiments DNA polymerase α was found to be phosphorylated to a 3-fold higher level in late S phase cells compared with cells in the G_2 and M phases, but the steady-state level of phosphorylation did not vary significantly during the cell cycle. Tryptic phosphopeptide mapping demonstrated that the phosphorylation sites of DNA polymerase α from late S phase cells were not the same as that from G_2/M phase cells. DNA polymerase α partially purified from G_1/S cells had a different mobility in native gels from that from G_2/M phase cells. The partially purified polymerase α from G_1/S phase cells had a higher affinity for single-stranded DNA than that from G_2/M phase cells. Despite the apparent differences in cell cycle-dependent phosphorylation, mobility in native gels and affinity for DNA, the *in vitro* enzymatic activity of the partially purified DNA polymerase α did not appear to vary during the cell cycle. The possible biological significance of these cell cycle-dependent characteristics of DNA polymerase α is discussed.

INTRODUCTION

Studies of DNA replication in *Escherichia coli*, phage λ and simian virus 40 (SV40) have identified proteins that are essential for initiation of DNA replication (1–3). Among the host proteins that are required and sufficient for SV40 viral DNA replication *in vitro* is DNA polymerase α/primase (4–7), which functions primarily for synthesis of a RNA–DNA primer for initiation at the SV40 origin and for priming each Okazaki fragment (2,3). *In vitro* SV40 replication studies have shown that stringent species-specific interactions between DNA polymerase α and other replication proteins are required for initiation of DNA synthesis (8). A domain of the DNA polymerase α catalytic polypeptide which interacts with SV40 large T antigen in a species-specific manner has been identified and the interaction is thought to be essential for the transition from the pre-initiation to the initiation stage (9,10). Genetic studies have shown that human DNA polymerase α expressed in budding yeast fails to either rescue several different conditional lethal alleles of the budding yeast *POL1* gene at the restrictive temperature or to complement a budding yeast *POL1* null allele in germinating spores and in vegetatively growing cells. Moreover, despite the fact that human DNA polymerase α can be expressed in fission yeast with catalytic function *in vitro*, the human enzyme cannot complement the disrupted *S.pombe polα::ura4*$^+$ allele in germinating spores (11). These findings strongly suggest that DNA polymerase α requires stringent protein–protein and/or protein–DNA interactions for initiation of chromosomal DNA synthesis and for priming lagging strand DNA synthesis.

We have shown that *S.pombe* DNA polymerase α is constant at the levels of transcript, protein and enzymatic activity throughout the cell cycle, similarly to human DNA polymerase α (12,13). This indicates that *S.pombe* DNA polymerase α is not regulated at the level of en masse synthesis or degradation during the cell cycle. Given the critical role played by DNA polymerase α in initiation and priming of lagging strand DNA synthesis, it is reasonable to anticipate that this enzyme may be regulated by post-translational modification, protein–protein and/or protein–DNA interactions. In this report we examined the status of DNA polymerase α in *S.pombe* during the cell cycle.

MATERIALS AND METHODS

Strains and media

The haploid *cdc25* strain carrying the *cdc25-22* allele (14) was grown in either rich medium (YE) or Edinburgh minimum medium (EMM). For *in vivo* labeling with [^{32}P]orthophosphate cells were grown in EMM plus phosphate (EMMP) containing either 100 μM or 1 μM phosphate as described (15). Haploid *cdc10* cells carrying the *cdc10-129* allele were used as a G_1 phase cell cycle marker for flow cytometry analysis.

Antibodies, preparation of cell lysates, immunoblotting, polyacrylamide gel electrophoresis and protein quantitation

Polyclonal chicken IgY antibodies B18, specific for *S.pombe* DNA polymerase α catalytic subunit, was used for immunoblot-

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ting. Preparation of the cell lysates, protein quantitation, gel electrophoresis and immunoblotting were performed as previously described (13).

Synchronization of *S.pombe* cells

An overnight culture of *cdc25* strain grown in YE medium at 25°C was inoculated into EMM medium and grown overnight at 25°C to a cell density of 5–10 × 10^6 cells/ml. This overnight cell culture was used to inoculate a culture in EMM medium at a cell density of 1.0 × 10^6 cells/ml and grown at 25°C to a cell density of 2 × 10^6 cells/ml. Cells were first shifted to 37°C for 4.5 h to arrest them at G_2, followed by shifting back to 25°C and then propagation for one cell cycle (14). Cells (100 µl) were collected every 15 min into 37% formaldehyde (11 µl) and stored at 4°C for septation count. Cell samples (3 ml) were collected at each time point and fixed in 1 ml 30% ethanol in phosphate-buffered saline (PBS) for flow cytometry analysis and cytology analysis.

Flow cytometry analysis

Cells (1 × 10^6 cells/ml) fixed in 30% ethanol/PBS were sonicated for ten 30 s pulses, washed once in PBS containing 50 mM $MgCl_2$ and stained overnight with 100 µg/ml Chromomycin 3A (Sigma) in PBS with 50 mM $MgCl_2$ at 4°C in a light-sealed container. Before analysis the cells were collected and resuspended in fresh Chromomycin 3A and analyzed with a Coulter EPIC 753 Flow Cytometer with 457 nm excitation and 510 nm fluorescence.

DAPI staining

Cell samples fixed in 30% ethanol as described above were collected, washed once in PBS and stained for 1 h in 5 µg/ml 4′,6′-diamidino-2-phenylindole (DAPI). Stained cells were washed in PBS twice and examined with an epifluorescence microscope.

In vivo [^{32}P]orthophosphate labeling of the asynchronous *S.pombe* cells

S.pombe cdc25 strain was grown in EMMP medium containing 1 mM phosphate overnight to a cell density of 5 × 10^6 cells/ml. Cells were collected, inoculated in EMMP medium containing 100 µM phosphate at a cell density of 1.0 × 10^6 cells/ml and grown for 3 h at 25°C. [^{32}P]Orthophosphate (10 µCi) was then added to the medium and the cells grown for an additional 3 h at 25°C.

In vivo [^{32}P]orthophosphate labeling of *S.pombe* synchronized by temperature block and release

In vivo ^{32}P-labeling of synchronized *cdc25* cells was performed as described (15) with a few modifications. A *cdc25* culture grown overnight in EMMP containing 1 mM phosphate was collected, resuspended in EMMP with 100 µM phosphate at a cell density of 1.0 × 10^6 cells/ml. Cells were further grown at 25°C for 4 h to a cell density of 2 × 10^6 cells/ml and then shifted to 37°C for 4.5 h to arrest at G_2. [^{32}P]Orthophosphate (NEN, Boston, MA) was added to the culture medium as follows:

For steady-state phosphorylation a 300 ml *cdc25* culture was labeled with 15 mCi [^{32}P]orthophosphate immediately after shifting the culture to 37°C. The cells were allowed to grow at 37°C for one cell cycle period (4.5 h) and were then released from G_2 by shifting from 37 to 25°C. Cell samples (20 ml) were collected every 15 min for a period of one cell cycle.

For the pulse labeling experiment a *cdc25* culture was arrested at G_2 phase as described above. Immediately after cell culture release from G_2 arrest 20 ml cell samples were collected every 15 min and pulse labeled with 1 mCi [^{32}P]orthophosphate for 15 min.

Labeled cells were washed and lysed as described (13). In both steady-state and pulse labeling experiments parallel cell cultures without [^{32}P]orthophosphate labeling were used to monitor cell synchronization by septation index, DAPI staining and flow cytometry.

DNA polymerase α phosphorylation analysis

Equal amounts of protein (1–1.5 mg) from [^{32}P]orthophosphate-labeled cell lysates were immunoprecipitated with preimmune IgY or B18 antibodies (13). Proteins immunoprecipitated by the preimmune IgY or B18 antibodies were fractionated by 8% SDS–PAGE, silver stained and autoradiographed.

Phosphoamino acid analysis and phosphopeptide mapping

Phosphoamino acid analysis was performed as described (16). For tryptic phosphopeptide mapping two 20 ml cell samples (1.2 × 10^8 cells) taken at 15 and 30 min after release from the temperature arrest were pulse labeled with 2 mCi [^{32}P]orthophosphate for 15 min and used as G_2/M cells. Two 20 ml cell samples (1.2 × 10^8 cells) taken 15 and 30 min after the peak of septation were pulse labeled with 2mCi [^{32}P]orthophosphate for an additional 15 min and used as late S phase cells. Cells were then lysed and the proteins were immunoprecipitated by B18 antibodies and separated by 4% SDS–PAGE. The immunoprecipitates were then transferred onto Immobilon-P membrane (Millipore Inc.) and autoradiographed. The ^{32}P-labeled DNA polymerase α protein band were excised, eluted and digested from the membrane with 50 mM ammonium bicarbonate buffer containing 20 µg trypsin (TPCK-treated trypsin, Worthington Biochemical Corp., Freehold, NJ) for 8 h at 37°C. This elution and digestion process was repeated twice. The tryptic phosphopeptides were subsequently lyophilized and resuspended in distilled H_2O repetitively several times. The phosphopeptides were then separated by thin layer electrophoresis with a Hunter thin layer electrophoresis unit (CBS Scientific Inc., Del Mar, CA) for 30 min at 1000 V in 0.1% ammonium carbonate buffer, pH 8.9, followed by chromatography for 10 h in phosphochromatography buffer as described (16). The migration pattern of the phosphopeptides was analyzed either by autoradiography or by phosphorimager (Molecular Dynamics).

Partial purification of *S.pombe* DNA polymerase α

A 1 l culture of *cdc 25* strain was grown at 25°C in EMM plus adenine to a density of 2.2 × 10^6 cells/ml. The culture was synchronized as described above, harvested at different time points after release from G_2 arrest, rinsed once with ice-cold stop buffer (15), then quickly frozen in liquid nitrogen and stored at –80°C until use. Cell pellets were thawed, resuspended in 30 µl lysis buffer (50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 60 mM 2-phosphoglycerate, 20 mM disodium *p*-nitrophenylphosphate, 20 µg/ml aprotinin, 40 µg/ml leupeptin and 1 mM DTT) and lysed as described (15). The cell lysates were

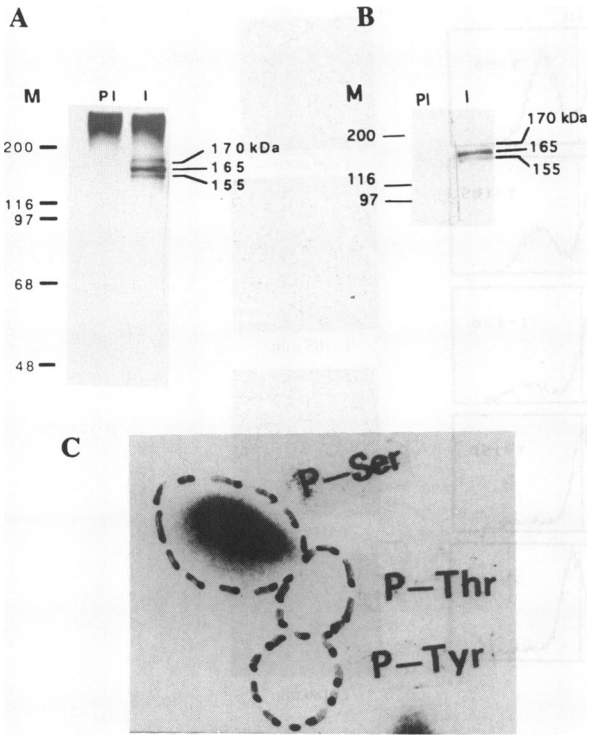

Figure 1. *S.pombe* DNA polymerase α is a phosphoprotein. Actively cycling *S.pombe cdc25* cells at a density of 1–2 × 10^6 cells/ml were labeled in medium containing 10 μCi/ml [^{32}P]orthophosphate as described under Materials and Methods. Cells were lysed as described (13). Immunoprecipitation was performed by incubating 1 mg ^{32}P-labeled cell extracts with either 10 μg preimmune (PI) or 10 μg B18 (I) antibodies. (**A**) An autoradiogram of the immunoprecipitates by preimmune antibodies (PI) or B18 antibodies (I) from crude cell lysates of [^{32}P]orthophosphate-labeled *cdc25* strain. (**B**) An immunoblot of the immunoprecipitates by B18 antibodies from cell lysates of ^{32}P-labeled *cdc25* strain with either preimmune (PI) or B18 antibodies (I). Due to the presence of either preimmune or B18 IgY in the immunoprecipitates that reacted strongly with the secondary antibody, only a portion of the immunoblots containing the *S.pombe* DNA polymerase α proteins are shown. Molecular weight markers are depicted under M. The molecular mass of *S.pombe* DNA polymerase α protein is marked as 170, 165 and 155 kDa. (**C**) *S.pombe* DNA polymerase α protein was immunoprecipitated from cell lysates from mid log phase ^{32}P-labeled *cdc25* cells. Phosphoamino acids were analyzed as described in Materials and Methods. The positions of each phosphoamino acid are indicated.

centrifuged at 15 000 r.p.m. for 15 min at 4°C. The soluble extract was adjusted to an ionic strength of 0.1 M and batch adsorbed onto 150 μl phosphocellulose pre-equilibrated with lysis buffer diluted to the same ionic strength (0.1 M). After rotating end over end for 30 min at 4°C the phosphocellulose was washed three times with 1 ml lysis buffer diluted to 0.1 M ionic strength. DNA polymerase α was eluted from the phosphocellulose with 200 μl 0.6 M potassium phosphate, pH 7.5, containing 2.0 μg/ml aprotinin and 4.0 μg/ml leupeptin. Eluates were adjusted to the same protein concentration by addition of 0.3 M potassium phosphate, pH 7.5.

Assay of partially purified *S.pombe* DNA polymerase α

Partially purified extracts (75 μl) from cell samples at each time point in duplicate were diluted with 400 μl Tris-buffered saline (TBS), adsorbed onto 100 μl of a 10% slurry of B18 antibodies cross-linked to Sephadex 4B and rotated end over end at 4°C for 45 min. The immunobeads were collected and washed. Forty microliters of DNA polymerase α assay mixture was added directly to the immunocomplex-containing beads and then incubated at 37°C with shaking for 10 min as described (17).

Native gel analysis of partially purified *S.pombe* DNA polymerase α

Both 0.7% agarose and 4% polyacrylamide (1:80) gels were utilized to assess the relative mobility of the partially purified native DNA polymerase α and the DNA polymerase α–single-stranded DNA complex at different stages of the cell cycle. Gels were cast and run in Tris–glycine buffer composed of 50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5.

To analyze the size of the partially purified enzyme 3 μl partially purified DNA polymerase α were mixed with 17 μl binding buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM EDTA, 20% glycerol), loaded immediately on the gels and run at either 30 mA for the polyacrylamide gel or 50 V for the agarose gel for 2–4 h at 4°C. Proteins were transferred onto Immobilon-P membrane and probed with B18 antibody (13). To analyze the affinity of polymerase α for DNA a partially purified enzyme fraction from an asynchronous cell culture was first titrated with increasing amounts of a single-stranded DNA (0–20 pmol 50mer oligodeoxynucleotide) (18). The amount of single-stranded DNA (2.5–3 pmol) that resulted in 50% of the polymerase α being bound was used for the affinity assay. Enzyme fractions (3 μl) partially purified from different stages of the cell cycle were incubated with 2.5 pmol single-stranded DNA in 17 μl binding buffer for 20 min at room temperature and then fractionated on a 0.7% agarose gel and immunoblotted as described above. To determine the affinity of DNA polymerase α for single-stranded DNA throughout the cell cycle 3 picomol single-stranded DNA in 18 μl binding buffer were pre-incubated with 3 μl partially purified enzyme fraction for 20 min at room temperature. The samples were then fractionated on a 0.7% native agarose gel, followed by immunoblotting.

RESULTS

S.pombe DNA polymerase α is a phosphoprotein

An asynchronous *cdc25* culture was labeled with [^{32}P]orthophosphate as described in Materials and Methods. DNA polymerase α was immunoprecipitated from ^{32}P-labeled cell lysates with preimmune and B18 antibodies and analyzed on an SDS gel (Fig. 1). The immunoprecipitate showed a triplet of phosphoprotein bands of 170, 165 and 155 kDa, corresponding to the mass of intact and proteolytically degraded *S.pombe* DNA polymerase α proteins, respectively (13). In contrast, the preimmune IgY did not immunoprecipitate any detectable phosphoprotein (Fig. 1A). To verify that the triplet phosphoprotein bands were indeed *S.pombe* DNA polymerase α the immunoprecipitates were immunoblotted either with preimmune antibodies or B18 antibodies. Again, a protein triplet with molecular masses of 170, 165 and 155 kDa was observed in the B 18 immunoprecipitates, but not in the immunoprecipitates with preimmune antibodies (Fig. 1B). Comparison of the autoradiogram and silver staining of the SDS gel exposed to the film showed that the extent of phosphorylation was proportional to the intensity of protein bands shown on the SDS gel (data not shown). Analysis of the

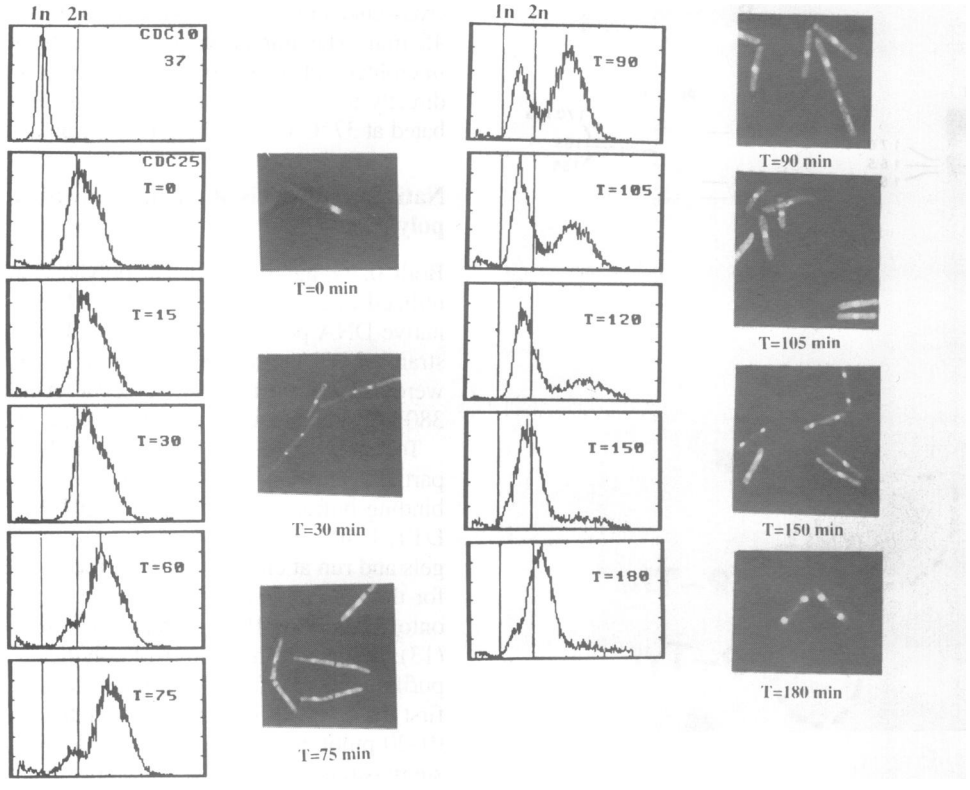

Figure 2. DNA contents and nuclear morphology of synchronized *cdc25*. *S.pombe* strain *cdc25* was synchronized by temperature block–release. Cell samples at different stages of the cell cycle were analyzed by flow cytometry and DAPI staining as described in Materials and Methods.

phosphoamino acids of *S.pombe* DNA polymerase α indicated that the DNA polymerase α catalytic subunit was phosphorylated exclusively at serine residues (Fig. 1C).

Synchronization of *cdc25* by temperature block and release

To determine the status of *S.pombe* DNA polymerase α at different stages of the cell cycle we synchronized *S.pombe cdc25* strain by the temperature block–release method. A culture was synchronized as described under Materials and Methods and analyzed by three criteria: (i) septation index; (ii) DAPI stain; (iii) flow cytometry. Cells synchronized by temperature block and release reproducibly had 60–80% of the cells in synchrony, as judged by septation index. After growing at 37°C for one generation cells were uniformly arrested in G_2, showing elongated morphology with 2*n* DNA content (Fig. 2, $T = 0$ min). Flow cytometry analysis showed that 30 min after release from G_2 arrest a large population of the cells had 2*n* to >2*n* DNA content. DAPI staining showed that most of the cells had two nuclei with elongated phenotype, indicating that these cell samples were progressing into M phase. Seventy five minutes post-release from G_2 arrest 60–80% of the cell population were septated (Fig. 2, $T = 75$ min). Flow cytometry showed that a large percentage of the cell population had >2*n* DNA content, with a small percentage of cells having <2*n* DNA content. DAPI staining of these cells showed that most had a septum with a single nucleus in each septated cell. Furthermore, a majority of the cell population had two unseparated cells (Fig. 2, $T = 75$ min). Since fission yeast has a short G_1 phase and also has a delay of about one quarter of a cell cycle between mitosis and cytokinesis, G_1 phase is completed before cytokinesis and cytokinesis overlaps with the S phase of the subsequent cell cycle. Thus at the peak of septation (75 min after release from G_2 arrest) 60–80% of the cell population had entered S phase of the subsequent cell cycle, even before completion of the previous cell division. This large population of septated but undivided cells in S phase thus displayed a >2*n* DNA content (Fig. 2, $T = 75$ min). A percentage of the population of cells with <2*n* DNA contents were cells in S phase that had completed cell division. Between 90 and 105 min a prominent population of S phase cells with completed cell division and 1.5*n* DNA content appeared and, as a result, the population of cells with >2*n* DNA (undivided S phase cells) proportionally decreased. DAPI staining of cells indicated that at 90–105 min after release from G_2 arrest most of the cells had gone through cytokinesis and appeared as single cells with normal (not elongated) phenotype and a single nucleus (Fig. 2, $T = 105$ min). Due to the abbreviated G_1 phase and the delay of cytokinesis of fission yeast after mitosis, cell samples collected 105 min after release from temperature block consisted of cells in mid S phase of the cell cycle. Two hours after release from the G_2 arrest cells were in late S phase and progressing into G_2 and M phase (Fig. 2, $T = 150$ and 180 min). DAPI staining showed a mixed population of cells, with most of these cell samples having a single nucleus in G_2 phase and some with two nuclei, as found in M phase. These data indicate that *cdc25* synchronized by temperature block–release provides a reliable synchronized cell culture for the study of the status of polymerase α at different stages of the cell cycle.

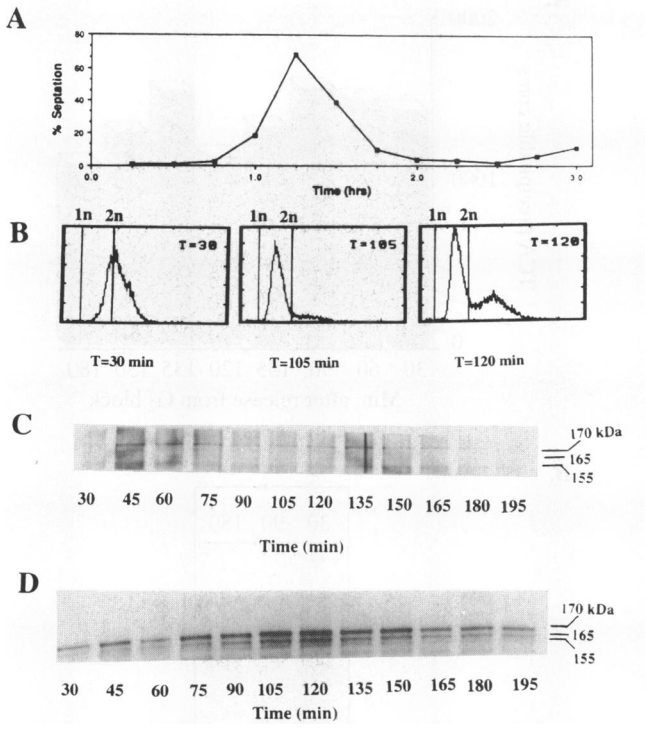

Figure 3. Hyperphosphorylation of *S.pombe* DNA polymerase α at late S phase of the cell cycle. *cdc25* cells were synchronized and pulse labeled with [^{32}P]orthophosphate for 15 min for one cell cycle. *S.pombe* DNA polymerase α protein was immunoprecipitated as described under Materials and Methods. (**A**) Percentage of septated cells in synchronized culture for one cell cycle. (**B**) FACS profiles of cells 30, 105 and 120 min after release from G_2 arrest. (**C**) SDS gel of the silver stained *S.pombe* DNA polymerase α proteins immunoprecipitated with B18 antibodies from each pulse labeled cell sample. Time (h) indicates time points when each cell sample was analyzed and was 15 min after each cell sample was collected. (**D**) Autoradiograph of the SDS gel shown in (C). Molecular masses of intact and degraded *S.pombe* DNA polymerase α proteins are indicated.

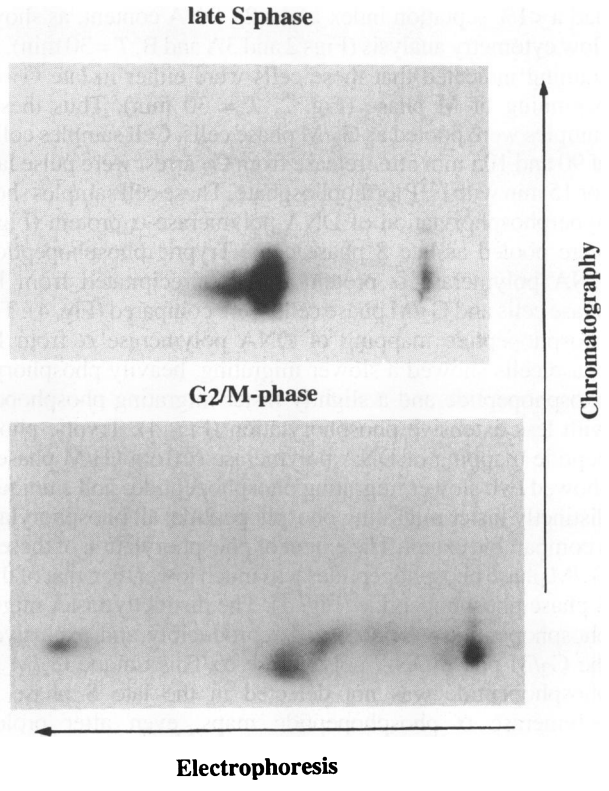

Figure 4. Tryptic phosphopeptide analysis of *S.pombe* DNA polymerase α from late S phase and G_2/M phase cells. DNA polymerase α proteins immunoprecipitated by B18 antibodies from [^{32}P]orthophosphate pulse labeled *cdc25* strain 120 or 30 min after release from G_2 arrest were processed for two-dimensional tryptic peptide analysis as described under Materials and Methods. The tryptic phosphopeptides were separated by horizontal two-dimensional thin layer electrophoresis with the cathode at the left, followed by ascending chromatography.

DNA polymerase α is hyperphosphorylated in late S phase

DNA polymerase α proteins immunoprecipitated from steady-state labeled cell samples were analyzed on SDS gels. Silver staining of the gels showed two protein bands of 170 and 165 kDa, representing the typical intact and degraded forms of *S.pombe* DNA polymerase α protein in each cell sample (13). The equal intensity of the protein bands on the gel indicated that comparable amounts of protein from each cell sample were analyzed. An autoradiogram of the same gel showed two phosphoprotein bands with molecular masses ranging from 170 to 165 kDa and similar intensity throughout the cell cycle (data not shown). The equal intensity of the two phosphoprotein bands indicates that the steady-state levels of DNA polymerase α protein phosphorylation did not vary significantly during the cell cycle.

We next investigated the phosphorylation of DNA polymerase α by pulse labeling (Fig. 3). Due to the 15 min pulse labeling, the cell samples analyzed on gel were collected 15 min earlier (Fig. 3C and D). Immunoprecipitates of B18 antibodies from each cell sample displayed a major protein band of 170 kDa, with two minor proteolytically degraded DNA polymerase α proteins of 165 and 155 kDa (13). Autoradiography of the same gel showed a cluster of three phosphoproteins. One predominant band is a phosphoprotein of 170 kDa, a minor phosphoprotein of 165 kDa and a faint phosphoprotein of 155 kDa (Fig. 3D). Densitometry showed that DNA polymerase α from cell samples collected 90 and 105 min after release from G_2 block followed by pulse labeling for an additional 15 min (105 and 120 min after release from G_2 block) displayed an ~3-fold higher phosphorylation than did early G_2 (150–165 min after release from G_2 arrest) or G_2/M cells (within 30 or 180–195 min after release from G_2 arrest). Flow cytometry data indicated that *S.pombe* cell samples analyzed at 105 min after release from G_2 block were indeed cells in S phase, having completed cytokinesis (Fig. 2, T = 105 min and Fig. 3B), and cells 120 min after release from G_2 arrest were in late S phase, at the onset of G_2 phase (Fig. 2, T = 120 min and Fig. 3B, T = 120 min). Thus DNA polymerase α is hyperphosphorylated in late S phase or at the onset of G_2 phase.

Phosphorylation sites of DNA polymerase α from S phase cells differ from those of G_2/M phase cells

To investigate the phosphorylation sites in *S.pombe* DNA polymerase α at different stages of the cell cycle *cdc25* cells were synchronized by the temperature block–release method. Within 30 min after release from 37°C two 20 ml cell samples were pulse labeled for 15 min with [^{32}P]orthophosphate. The cell samples

had a <1% septation index and a 2*n* DNA content, as shown by flow cytometry analysis (Figs 2 and 3A and B, $T = 30$ min). DAPI staining indicated that these cells were either in late G_2 or the beginning of M phase (Fig. 2, $T = 30$ min). Thus these cell samples were pooled as G_2/M phase cells. Cell samples collected at 90 and 105 min after release from G_2 arrest were pulse labeled for 15 min with [^{32}P]orthophosphate. These cell samples showing hyperphosphorylation of DNA polymerase α protein (Fig. 3D) were pooled as late S phase cells. Tryptic phosphopeptides of DNA polymerase α protein immunoprecipitated from late S phase cells and G_2/M phase cells were compared (Fig. 4). Tryptic phosphopeptide mapping of DNA polymerase α from late S phase cells showed a slower migrating, heavily phosphorylated phosphopeptide and a slightly faster migrating phosphopeptide with less extensive phosphorylation (Fig. 4). Tryptic phosphopeptide mapping of DNA polymerase α from G_2/M phase cells showed two slower migrating phosphopeptides and a unique and distinctly faster migrating phosphopeptide, all phosphorylated to a comparable extent. The extent of phosphorylation of these three G_2/M phase phosphopeptides was much lower than that of the late S phase phosphopeptide (Fig. 4). The distinctly faster migrating phosphopeptide was observed reproducibly and exclusively in the G_2/M phase DNA polymerase α. This unique G_2/M phase phosphopeptide was not detected in the late S phase DNA polymerase α phosphopeptide maps, even after prolonged exposure of the thin layer chromatogram.

In vitro DNA polymerase α activity is constant throughout the cell cycle

Phosphorylation of DNA polymerase α can have a variety of effects, such as activating or inactivating the enzymatic activity or enhancing or weakening the enzyme's affinity for interacting with DNA and/or other replication proteins. We thus tested the enzymatic activity of DNA polymerase α at different stages of the cell cycle. DNA polymerase α was first partially purified from synchronized *cdc25* cell samples at every 15 min for one cell cycle by phosphocellulose column chromatography and then immunoprecipitated with antibodies B18. DNA polymerase α activity measured with gapped calf thymus DNA showed no significant variations throughout the cell cycle (Fig. 5A), suggesting that phosphorylation does not affect the *in vitro* enzymatic activity.

Partially purified DNA polymerase α has different electrophoretic mobility on native gels at different stages of the cell cycle

We next analyzed whether DNA polymerase α associated with different proteins or had different charges at different stages of the cell cycle. Equal amounts of partially purified DNA polymerase α enzyme fractions from synchronized *cdc25* culture harvested 30, 90 and 180 min after release from G_2 arrest were fractionated on a 4% native acrylamide gel, followed by immunoblotting with antibodies B18 to identify the DNA polymerase α in the protein complex (Fig. 5B). Partially purified DNA polymerase α enzyme fractions harvested from cells 90 min after release from G_2 arrest (in S phase) reproducibly had slower mobility than those harvested 30 (G_2/M) and 180 min (M phase) after release from G_2 arrest. This suggests that DNA polymerase α in S phase either has a different charge or is associated with different proteins from the polymerase α in G_2/M phase.

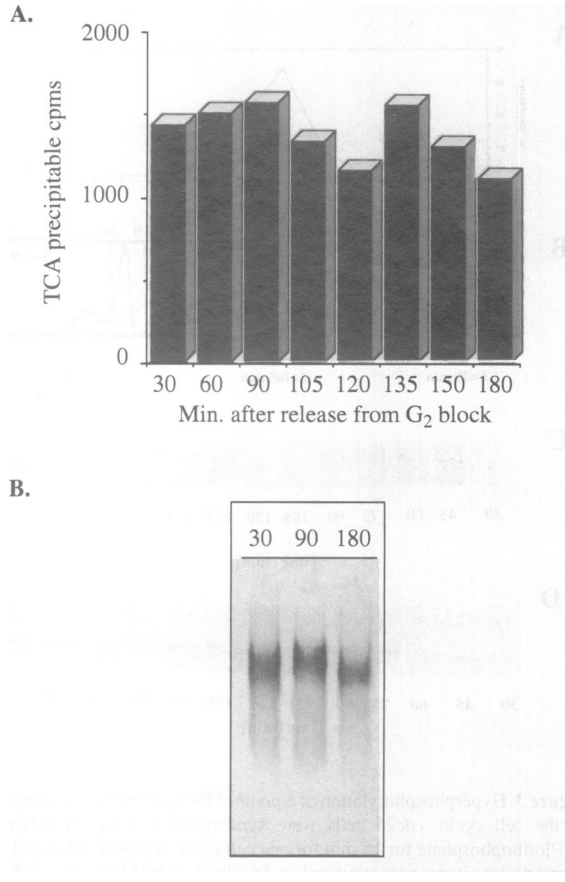

Figure 5. Purified DNA polymerase α enzymatic activity does not change during the cell cycle but partially purified DNA polymerase α enzyme fractions from G_2 and S phase cells show differential migration in native gels. (**A**) Duplicates of DNA polymerase α immunoprecipitated from partially purified enzyme fractions at the times indicated were assayed as immunocomplex as described in Materials and Methods and the averaged results plotted. (**B**) Partially purified DNA polymerase α enzyme fraction from cells 30, 90 and 180 min after release from G_2 arrest were fractionated by electrophoresis on a 4% native polyacrylamide gel, transferred onto a membrane and probed with DNA polymerase α-specific polyclonal antibodies B18 as described under Materials and Methods.

DNA polymerase α from S phase cells has higher affinity for single-stranded DNA than that from G_2/M phase cells

Partially purified DNA polymerase α enzyme fractions were pre-incubated with a single-stranded DNA (a 50 nt oligodeoxynucleotide). The optimal concentration of DNA used was pre-determined as described in Materials and Methods. Due to the mass of the protein–DNA complex we analyzed the DNA–protein complex on a 0.7% native agarose gel, followed by immunoblotting with B18 antibodies to identify the DNA polymerase α in the DNA–protein complex (Fig. 6). Partially purified DNA polymerase α enzyme fractions from a synchronized *cdc25* culture harvested 30, 90 and 180 min after release from G_2 arrest (G_2, S and M phases, respectively) had different affinity for the single-stranded DNA. In the absence of DNA (Fig. 5B) the partially purified DNA polymerase α enzyme fraction from S phase cells (harvested 90 min after release from G_2 arrest) had a slower electrophoretic mobility in native agarose gels than the

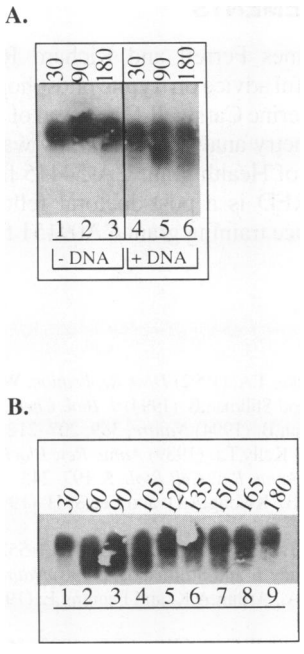

Figure 6. Partially purified DNA polymerase α enzyme fractions have differential affinity for single-stranded DNA at different stages of the cell cycle. (**A**) Partially purified DNA polymerase α enzyme fractions from cells 30, 90 and 180 min after release from G_2 arrest were incubated for 20 min at room temperature either without DNA (–DNA; lanes 1–3) or with 2.5 pmol single-stranded 50mer oligodeoxynucleotides (+DNA; lanes 4–6). The resultant protein–DNA complexes were fractionated by electrophoresis on a 0.7% agarose gel. The DNA-bound (lower band) and free DNA polymerase α complexes (upper band) were detected by immunoblot with B18 antibodies. (**B**) DNA polymerase α partially purified from cells released from G_2 arrest at the times indicated was incubated with 3 pmol single-stranded 50mer oligodeoxynucleotides and analyzed as described above.

enzyme fraction partially purified from G_2/M phase cells (cells harvested 30 and 180 min after release from G_2 arrest) (Fig. 6A, lane 2 compared with lanes 1 and 3). Upon addition of single-stranded DNA the enzyme fractions from S phase cells showed higher affinity for single-stranded DNA than the enzyme fractions from G_2/M phase cells (Fig. 6A). To verify this finding we synchronized 1 l of *cdc25* culture by temperature block–release. Under the conditions used for this scale-up synchronization of *cdc25* we reproducibly observed 75% of cells septated (peak of septation) at 105 min after release from G_2 arrest. DNA polymerase α enzyme fractions partially purified from cells of the synchronized cell culture harvested every 15 or 30 min after release from G_2 arrest were analyzed for their affinity for single-stranded DNA on native agarose gel (Fig. 6B). Again, DNA polymerase α partially purified from cells at the onset of S phase (90 min) or S phase (105 min) showed higher affinity for single-stranded DNA than those from G_2 or M phase cells (30 and 180 min after release from G_2 arrest). These results suggest that at the onset of S phase DNA polymerase α binds either directly or indirectly to single-stranded DNA with higher affinity than DNA polymerase α partially purified from G_2/M phase cells.

DISCUSSION

To pave the way to elucidate how DNA polymerase α is modulated to be competent for initiation at the origin, for priming Okazaki fragments and for other biological functions we analyzed *S.pombe* DNA polymerase α for its *in vivo* phosphorylation status, its association with other proteins and its affinity for single-stranded DNA at different stages of the cell cycle.

A temperature block–release method was used to synchronize cells in this study, instead of arresting various cell division cycle (*cdc*) strains to obtain cell samples at different stages of the cell cycle. Each *cdc* strain arrests cells at a unique point of the cell cycle, depending on the functional defect of each particular *cdc* mutant gene. For example, *cdc22* arrests cells at an early sub-stage of S phase at the non-permissive temperature (19). Studies of *S.pombe* DNA polymerase α phosphorylation in *cdc22* at 37°C can only reveal the phosphorylation status at this particular sub-stage of the S phase. As shown in this study, the phosphorylation status of DNA polymerase α at the early stage of S phase is different from that of late S phase. Furthermore, [^{32}P]orthophosphate incorporation efficiency of different *cdc* strains arrested at their respective stages of the cell cycle differs in each strain. Therefore, comparison of results of cell cycle-regulated phosphorylation of DNA polymerase α in different *cdc* strains should be interpreted with caution. By temperature block–release of *cdc25*, a synchronized cell population derived from a single strain was obtained. Therefore, the status of DNA polymerase α can be meaningfully compared at different stages of the cell cycle.

The observed constant steady-state level of phosphorylation of DNA polymerase α may be the net result of an equilibrium of kinase and phosphatase activities during the cell cycle. The vast intracellular phosphate pool of *S.pombe* did not allow us to study cell cycle-specific DNA polymerase α phosphatases. However, by pulse labeling, the extent of DNA polymerase α phosphorylation in late S phase cells was found to be 3-fold higher than that of G_2 or M phase cells (Fig. 3). Further studies are required to determine whether this difference is due to a variation in intracellular kinase activities, phosphatase activities or phosphate pool during the cell cycle. Tryptic phosphopeptide maps reproducibly showed a unique and distinctively faster migrating phosphopeptide from G_2/M phase cells under the electrophoresis condition (Fig. 4), indicating that it carries a highly negative charge. Comparison of the tryptic maps (Fig. 4) to a tryptic phosphopeptide map predicted by the programs PEPTIDE SORT and MOBILITY (16) at pH 8.9 suggested that this fast migrating, unique and distinct G_2-specific phosphopeptide contained several serine residues located near the N-terminus.

It is not known whether the steady-state level of human DNA polymerase α phosphorylation is similar to or different from that of *S.pombe* DNA polymerase α. The observed hyperphosphorylation of *S.pombe* polymerase α in late S and/or the onset of G_2 phase is not entirely concordant with the finding of human DNA polymerase α being hyperphosphorylated in G_2/M phase (20).

Despite the apparent cell cycle-regulated phosphorylation of this enzyme, the polymerase enzymatic activity measured *in vitro* does not vary significantly during the cell cycle (Fig. 5A). This suggests that differential phosphorylation of DNA polymerase α is not essential for DNA polymerization activity *in vitro*, as measured with optimally gapped calf thymus DNA. This is in agreement with the previous finding of no significant variation in DNA polymerizing activity of human DNA polymerase α *in vitro*, despite the observed cell cycle-regulated phosphorylation (20). Efforts to dephosphorylate either human or fission yeast polymerase α with phosphatase did not significantly affect the polymerizing activity (data not shown). It is possible that the

phosphorylation sites in the native enzyme are not accessible to the phosphatase. Alternatively, cell cycle-regulated phosphorylation might have a role in protein–protein or protein–DNA association. We thus explored the size, charge and affinity for single-stranded DNA of DNA polymerase α from partially purified enzyme fractions at different stages of the cell cycle. The partially purified DNA polymerase α enzyme fraction from S phase cells reproducibly showed slower electrophoretic mobility in a native gel than the enzyme fraction from G_2/M phase cells (Figs 5B and 6A). The DNA polymerase α enzyme fraction partially purified from cells at the onset of and during S phase also had higher affinity for single-stranded DNA than the enzyme fraction partially purified from late S phase or G_2/M phase cells (Fig. 6A and B). Despite the observations that at different stages of the cell cycle DNA polymerase α (i) is phosphorylated to different extents and at a different site(s), (ii) associates with different proteins or with proteins of different charges and (iii) has different affinity for single-stranded DNA, it is difficult to correlate the phosphorylation status of DNA polymerase α with the mobility of the polymerase α protein in native gels or association with DNA.

Genetic studies of fission yeast genes *cdc18*, *cut5/rad4* and *cdt1* have suggested that gene products required for the onset of S phase also play a role in restraining M phase if S phase is incomplete (21–25). DNA polymerase α is an essential enzyme for the onset of S phase. Recently a multicopy suppressor of a fission yeast temperature-sensitive DNA polymerase α mutant swi7-H4 was isolated. The suppressor, *cds1*$^+$, encodes a protein with a typical protein kinase motif and is thought to be a component of the S/G_2 checkpoint system. Genetic data suggest that the primary role of *cds1*$^+$ is in a pathway monitoring DNA synthesis and sending a signal to block the onset of mitosis while DNA synthesis is in progress. It has been proposed, although not yet biochemically proven, that *cds1*$^+$ physically interacts with DNA polymerase α (25). If DNA polymerase α is indeed a substrate of cds1 kinase, hyperphosphorylation of DNA polymerase α at S phase by cds1 kinase could be one of the many ways to ensure completion of DNA replication prior to entering mitosis. Future studies on DNA polymerase α phosphorylation status in cells with a *cds1*$^-$ background might provide insight into whether cds1 is the kinase that phosphorylates polymerase α at different stages of the cell cycle.

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