Molecular Biology of the Cell
Vol. 4, 145–157, February 1993

Cell Cycle Expression of Two Replicative DNA Polymerases α and δ from *Schizosaccharomyces pombe*

Hyunsun Park, Stefania Francesconi, and Teresa S.-F. Wang

Laboratory of Experimental Oncology, Department of Pathology, and Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305-5324

Submitted October 12, 1992; Accepted December 3, 1992

We have investigated the expression of two *Schizosaccharomyces pombe* replicative DNA polymerases α and δ during the cell cycle. The *polα*$^+$ and *polδ*$^+$ genes encoding DNA polymerases α and δ were isolated from *S. pombe*. Both *polα*$^+$ and *polδ*$^+$ genes are single copy genes in haploid cells and are essential for cell viability. In contrast to *Saccharomyces cerevisiae* homologs, the steady-state transcripts of both *S. pombe polα*$^+$ and *polδ*$^+$ genes were present throughout the cell cycle. Sequence analysis of the *polα*$^+$ and *polδ*$^+$ genes did not reveal the *Mlu* I motifs in their upstream sequences that are involved in cell cycle-dependent transcription of *S. cerevisiae* DNA synthesis genes as well as the *S. pombe cdc22*$^+$ gene at the G_1/S boundary. However, five near-match *Mlu* I motifs were found in the upstream region of the *polα*$^+$ gene. *S. pombe* DNA polymerases α and δ proteins were also expressed constantly throughout the cell cycle. In addition, the enzymatic activity of the *S. pombe* DNA polymerase α measured by in vitro assay was detected at all stages of the cell cycle. Thus, these *S. pombe* replicative DNA polymerases, like that of *S. pombe cdc17*$^+$ gene, are expressed throughout the cell cycle at the transcriptional and protein level. These results indicate that *S. pombe* has at least two regulatory modes for the expression of genes involved in DNA replication and DNA precursor synthesis.

INTRODUCTION

DNA replication and mitosis are two major events in the cell cycle. These two events occur in a temporal and interdependent order that is maintained by multiple control pathways. A prerequisite to understanding the molecular basis of the cell cycle progression is the elucidation of the mechanisms that regulate DNA replication and mitosis. Fission yeast *Schizosaccharomyces pombe* has served as a useful model system in unraveling the regulatory mechanisms of mitosis. However, the regulation of DNA replication in fission yeast is not well understood. In S phase, initiation and termination of DNA synthesis are precisely regulated to ensure that DNA replication occurs only once in the cell cycle. To fully understand the molecular basis of entry into, progression through, and exit from S phase, it is necessary to elucidate the regulation of the essential components involved in these processes. Seven cellular proteins involved in eukaryotic DNA replication were identified using a cell-free simian virus 40 DNA replication system (Challberg and Kelly, 1989; Stillman, 1989; Hurwitz *et al.*, 1990). Two replicative DNA polymerases, α and δ, are involved in the viral DNA replication. DNA polymerase α plays a key role in nascent DNA synthesis during initiation of the viral DNA replication (Tsurimoto and Stillman, 1991; Wang, 1991). DNA polymerase δ is thought to play a role in elongation of the nascent DNA strand synthesized by DNA polymerase α (Tsurimoto and Stillman, 1991). The cDNAs and genes encoding DNA polymerases α and δ were cloned in mammalian cells and in budding yeast (Johnson *et al.*, 1985; Pizzagalli *et al.*, 1988; Wong *et al.*, 1988; Boulet *et al.*, 1989; Zhang *et al.*, 1991). Genetic studies using *Saccharomyces cerevisiae* conditional lethal mutants indicate that both DNA polymerases α and δ are required for cellular DNA replication (Budd *et al.*, 1989; Sitney *et al.*, 1989).

Expression of the human DNA polymerase α gene has been studied extensively (Wang, 1991). As cells enter the cell cycle from quiescence (G_0), the expression of human DNA polymerase α is induced (Wahl *et al.*, 1988; Pearson *et al.*, 1991). When cells exit from the cell cycle during differentiation, the expression of hu-

man DNA polymerase α is down-regulated (Moore and Wang, unpublished data). In actively cycling cells, however, the transcript, protein, and enzymatic activity of human DNA polymerase α are constitutively expressed throughout the cell cycle. Similar findings have been observed in several mammalian replication proteins (Sherley and Kelly, 1988; Wahl *et al.*, 1988; Morris and Mathews, 1989; Tseng *et al.*, 1989). It was also shown that in actively cycling cells the human DNA polymerase α protein is phosphorylated in a cell cycle-dependent manner (Nasheuer *et al.*, 1991).

In budding yeast, the transcription of at least 17 genes encoding enzymes for DNA synthesis or deoxynucleotide triphosphate precursor production is coordinately induced at the G_1/S boundary. The proteins encoded by these genes include DNA polymerase α (*POLI* or *CDC17*) (Johnston *et al.*, 1987), DNA polymerase δ (*POLIII* or *CDC2*) (Bauer and Burgers, 1990), DNA polymerase ϵ (*POLII*) (Araki *et al.*, 1992), two subunits of DNA primase (*PRI1* and *PRI2*) (Foiani *et al.*, 1989; Johnston *et al.*, 1990b), three subunits of replication factor-A (*RFA1, RFA2,* and *RFA3*) (Brill and Stillman, 1991), proliferating cell nuclear antigen (*POL30*) (Bauer and Burgers, 1990), DNA ligase (*CDC9*) (White *et al.*, 1986), ribonucleotide reductase (*RNR1*) (Elledge and Davis, 1990), thymidylate kinase (*CDC8*) (White *et al.*, 1987), and thymidylate synthase (*TMP1* or *CDC21*) (Storm *et al.*, 1984). A conserved DNA sequence element, 5′-A/TPuACGCGTNA/T-3′, was found in the upstream regions of these genes. This DNA element contains an *Mlu* I restriction site and is named the *Mlu* I cell cycle box (MCB) (Andrews and Herskowitz, 1990; Gordon and Campbell, 1991; McIntosh *et al.*, 1991). The MCB elements are suggested to play a role in coordinating the expression of these DNA synthesis genes at the G_1/S boundary. A trimer of the *Mlu* I hexamer sequences was shown to be able to induce the periodic transcriptional expression of a reporter gene. A protein complex was identified that bound to MCB motifs with a periodicity similar to that of transcriptional activation of the gene (Lowndes *et al.*, 1991). Recent reports suggest that the *SWI6* protein plays a role in the MCB-mediated cell cycle-dependent expression of DNA synthesis genes in *S. cerevisiae* (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a). These data suggest that the cell cycle-dependent transcription of the DNA synthesis genes in *S. cerevisiae* may be coordinated by a common MCB-binding factor.

The expression of two genes involved in DNA synthesis was studied in *S. pombe*. Transcriptional expression of the *cdc22*$^+$ gene encoding a subunit of ribonucleotide reductase was shown to be induced at the G_1/S boundary, similar to the budding yeast DNA synthesis genes (Gordon and Fantes, 1986). Two MCB elements and an MCB-binding activity were also identified in the promoter region of the *cdc22*$^+$ gene (Lowndes *et al.*, 1992b). Moreover, the *S. pombe cdc10*$^+$ gene product was identified as a component of the binding activity (Lowndes *et al.*, 1992b). Interestingly, the C-terminal half of the cdc10 protein, which is sufficient to execute the start of the cell cycle, has sequence similarity to the *SWI6* protein (Breeden and Nasmyth, 1987). Thus, the regulatory pathway mediated by MCB–MCB binding activity required for cell cycle-dependent transcription of budding yeast DNA synthesis genes appears to be conserved in *S. pombe cdc22*$^+$ gene expression. In contrast, the steady-state transcript of *S. pombe cdc17*$^+$ gene encoding DNA ligase and the enzymatic activity of DNA ligase are expressed constitutively throughout the cell cycle (White *et al.*, 1986).

Because the regulatory mode of the cell division cycle of *S. pombe* differs from that of *S. cerevisiae* (Forsburg and Nurse, 1991), it is important to understand how these *S. pombe* DNA polymerases that are essential for DNA replication are regulated during the cell cycle. We have isolated the genes encoding DNA polymerases α and δ (*polα*$^+$ and *polδ*$^+$) from *S. pombe* and demonstrated in this report that the transcripts, proteins, and enzymatic activities of *S. pombe* DNA polymerases were present throughout the cell cycle in a manner similar to that of the *S. pombe cdc17*$^+$ gene. Therefore, *S. pombe* has at least two different regulatory modes for the expression of genes involved in DNA synthesis.

MATERIALS AND METHODS

Strains and Media

Two *S. pombe* gene banks, GB*Sau*3A and GB*Hin*dIII, were in *Escherichia coli* strain JA226. *E. coli* strain SURE (Stratagene, La Jolla, CA) was used to propagate the plasmid pGT-polα and to produce glutathione S-transferase (GST) and polymerase α fusion protein. *E. coli* strain JM101 was used to propagate the plasmid pGT-polδ and to produce GST-polδ fusion protein. *S. pombe* wild-type strain 972h$^-$ (Gutz *et al.*, 1974) was used for cell cycle synchronization by counterflow elutriation. *S. pombe* haploid strain (*cdc25*) carrying *cdc25-22* allele (Fantes, 1979) was used for synchronization by block and release. *S. pombe* haploid strains (*cdc10, cdc22, cdc25,* and *cdc2*) carrying *cdc10-129* (Nurse *et al.*, 1976), *cdc22-m45* (Nasmyth and Nurse, 1981), *cdc25-22* (Fantes, 1979), and *cdc2-33* (Nurse *et al.*, 1976) alleles were used to synchronize the cells at discrete stages of the cell cycle. *S. pombe* cells were grown in Edinburgh minimum medium (EMM) as described (Moreno *et al.*, 1991). During elutriation, the original cell culture medium was collected and used as conditioned medium to propagate the synchronized cells. *E. coli* cells were grown in 2× YT medium (Maniatis *et al.*, 1982).

Materials

Glutathione-agarose was from Sigma (S-linkage; St. Louis, MO). Cyanogen bromide-activated Sepharose 4B was from Pharmacia (Piscataway, NJ), and protein cross-linking was performed according to manufacturer's suggestion. Rabbit anti-chicken IgG was from Sigma. DEAE-Sephacel was from Pharmacia.

Protein Quantitation and Gel Electrophoresis

Protein concentration was measured using Bradford assay (Bio-Rad, Richmond, CA) with bovine serum albumin (BSA; Sigma) as protein standards. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) (Laemmli, 1970) was carried out with 8% acrylamide gels.

Cloning of S. pombe DNA Polymerases α and δ

Gene banks, GB*Sau*3A and GB*Hin*dIII, were constructed with *Sau*3A and *Hin*dIII partially digested *S. pombe* genomic DNA in the vector pWH5 (Wright *et al.*, 1986) and were generous gifts from Paul Young (Queens University, Ontario, Canada). To isolate the DNA polymerase α genomic clone, a *Sac* I (2462 nt)-*Hin*dIII (3394 nt) fragment of *S. cerevisiae* homolog *POLI* gene (Pizzagalli *et al.*, 1988) was used as a probe to screen the two *S. pombe* gene banks. This fragment was ^{32}P-labeled by random hexamer labeling (Boehringer-Mannheim, Indianapolis, IN). Hybridization was carried out in 20% formamide, 6× SSPE, 5× Denhardt's solution, 0.1% SDS, and 0.5 mg/ml yeast tRNA at 42°C. Washing was done in 0.2× SSC and 0.1% SDS at 40°C. Six overlapping positive clones were isolated after screening 1.6×10^4 colonies of each genomic library. A clone (pWHpolα) containing a 5.3-kilobase (kb) DNA insert was further characterized and sequenced.

To isolate DNA polymerase δ genomic clone, a 365-base pair (bp) fragment of *S. cerevisiae POLIII* gene (1660–2024 nt) generated by polymerase chain reaction (PCR) was used as a probe to screen the genomic libraries (Boulet *et al.*, 1989). This DNA fragment was ^{32}P-labeled during PCR, which was done using 5′-ATGAATTCGA-GTCTGTGTTCTCTTCGAAGGC-3′ and 5′-ATGTCGACTGTTGAC-CACGAGCTAGT-3′ as the upstream and downstream primers, respectively. PCR reaction conditions are 94°C for 1 min, 55°C for 1 min, and 73°C for 1 min, for 25 cycles. An additional five cycles were done in the presence of ^{32}P-α-dCTP (Amersham, Arlington Heights, IL). Hybridization was carried out at 40°C under the same conditions used for the *S. pombe* DNA polymerase α cloning. The washing was performed in 0.5× SSC and 0.1% SDS at 45°C. Five overlapping positive clones were isolated after screening 1.6×10^4 colonies of each genomic library. A genomic clone (pWHpolδ) containing a 7.3-kb insert was further characterized and sequenced.

DNA Sequencing

DNA inserts from positive clones of *S. pombe* DNA polymerases α and δ were subcloned into either M13mp18 or pUC18 vector (Yanisch-Perron *et al.*, 1985) in both orientations. A series of deleted plasmids was generated using the cyclone deletion kit (IBI, New Haven, CT). The resulting plasmids were sequenced by dideoxynucleotide method using a T7 DNA polymerase sequencing kit (Pharmacia) (Maniatis *et al.*, 1982).

Cell Cycle Synchronization

Three methods were used to synchronize *S. pombe* cell culture for cell cycle studies.

1) Counterflow centrifugal elutriation. Wild-type *S. pombe* strain 972h$^-$ was grown to a cell density of 5×10^6 cells/ml in EMM at 29°C. A total of 1×10^{10} cells was loaded into the cell chamber of a Beckman (Palo Alto, CA) J21 centrifuge equipped with a JE-6 rotor at a rotor speed of 3600 rpm and at a flow rate of 100 ml/min. During the centrifugation, cells were maintained at 29°C and continuously flushed with fresh EMM medium. Small cells were collected between 3400 and 3500 rpm, diluted in conditioned medium to 2×10^6 cells/ml, and propagated for two cell cycles at 29°C. One hundred milliliters of cell samples was collected by filtering onto a Millipore membrane (pore size 0.45 μM; Millipore, Bedford, MA) every 20 min after propagation. Cells were washed with deionized water and stored at −80°C. At each time point, a 1-ml aliquot of cells was fixed in 2% formaldehyde and stored at 4°C to monitor septation index.

2) Induction synchronization using *cdc* strains. Cultures of isogenic *cdc* mutant strains were grown to early log phase at 25°C and then shifted to 37°C for one cell cycle (4.5 h). The arrested cells were collected by centrifugation, washed with stop buffer (Moreno *et al.*, 1991), and stored at −80°C.

3) Synchronization by block and release. A culture of *cdc25* strain grown at 25°C to 2×10^6 cells/ml was shifted to 37°C for 4.5 h to arrest the cells in G_2 phase of the cell cycle (Fantes, 1979). The cells were then released from G_2 arrest by shifting the culture back to 25°C and propagated for two cell cycles. Due to the larger size of the arrested cells, the subsequent cell division after release occurs in about half of the normal cell cycle time at 25°C (Mitchison, 1988). Therefore, 100 ml of cell samples was collected every 15 min after release by filtering the cells onto a Millipore membrane (pore size 0.45 μM; Millipore). The cells were washed with stop buffer (Moreno *et al.*, 1991) and stored at −80°C. At each time point, a cell aliquot was prepared as described in 1) to monitor the septation index.

Construction of DNA Templates for Antisense RNA Probe Synthesis

A 510-bp *Xba* I-*Hin*dIII fragment of the *S. pombe pola*$^+$ gene was subcloned into pGEM3 vector (Promega, Madison, WI). To generate an antisense RNA probe for *pola*$^+$ transcript, this plasmid was linearized with *Pvu* II and transcribed with SP6 RNA polymerase. A 480-bp *Hin*dIII-*Hin*dIII fragment of the *S. pombe polδ*$^+$ gene was subcloned into pGEM3 vector. To generate an antisense RNA probe for *polδ*$^+$ transcript, this construct was linearized with *Nhe* I and transcribed with T7 RNA polymerase. A 310-bp *S. pombe ura4*$^+$ gene fragment from 561 to 870 nt (Grimm *et al.*, 1988) was produced by PCR and subcloned into the *Sma* I site of pGEM3 vector. To generate an antisense RNA probe for *ura4*$^+$ transcript, this construct was linearized with *Nhe* I and transcribed with T7 RNA polymerase. A 1.3-kb *Hin*cII-*Hin*cII fragment of *S. pombe* histone *H2A1* gene (Matsumoto and Yanagida, 1985) was subcloned into the *Sma*I site of pGEM3 vector. To produce an antisense RNA probe for histone *H2A1* transcript, this construct was linearized with *Hin*dIII and transcribed with T7 RNA polymerase. To generate ^{32}P-labeled antisense probes, all of the in vitro transcription reactions were performed in the presence of ^{32}P-α-CTP (Amersham) using the in vitro transcription buffer system from Stratagene.

RNase Protection Analysis

Total RNAs were prepared as described by Moreno *et al.* (1991). For RNase protection analysis, equal amounts of total RNA (5–10 μg) from each cell fraction were mixed with each ^{32}P-labeled antisense RNA probe. The RNase protection analysis of the *S. pombe pola*$^+$, *polδ*$^+$, *ura4*$^+$, and histone *H2A1* transcripts were performed separately as previously described (Wahl *et al.*, 1988).

Constructs for GST-Fusion Proteins

A recombinant plasmid, pGT-polα, was constructed to produce a GST-fusion protein with *S. pombe* DNA polymerase α polypeptide from amino acid residue K^{118} to L^{634}. The pGT-polα was generated by cloning the end-repaired *Dra* I (925 nt)-*Xba* I (2479 nt) fragment of *S. pombe pola*$^+$ DNA into the end-repaired *Eco*RI site of pGEX-2T vector (Pharmacia). A recombinant plasmid, pGT-polδ, was constructed to produce a GST-fusion protein with *S. pombe* DNA polymerase δ polypeptide from amino acid residue Q^{101} to V^{352}. A fragment of *polδ*$^+$ DNA was amplified by PCR using 5′-TATGGATCCCAGCAAATCGATAGT-GAGGAG-3′ as upstream primer and 5′-AATGGATCCTACTT-GTGTTCCACCGATTTG-3′ as downstream primer. The PCR was performed under the same conditions used for generating the probe to isolate *S. pombe* DNA polymerase δ genomic clones. This PCR fragment was digested with *Bam*HI and cloned into the *Bam*HI site of pGEX-2T to generate pGT-polδ. Both constructs were checked for correct reading frame with GST protein by DNA sequencing.

Expression and Purification of GST-Fusion Proteins

The pGT-polα and pGT-polδ were transformed into *E. coli*. The expression and purification of GST-polα and GST-polδ fusion proteins were carried out as described by Smith and Johnson (1988). Overnight cultures of *E. coli* strains transformed with either pGEX-2T or one of the fusion constructs described above were diluted 1:10 in 2× YT containing 100 μg/ml of ampicillin and incubated at 37°C with shaking. After 1 h of growth, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The bacterial culture producing GST-polα protein was then incubated for 5 h, and the culture producing GST-polδ protein was incubated for 3 h at 37°C with vigorous shaking. The bacterial cultures were pelleted by centrifugation at 5000 × *g* for 5 min at 4°C and resuspended in a 1:10 volume of MTPBS (Smith and Johnson, 1988). The bacteria were then lysed on ice by mild sonication and centrifuged at 10 000 × *g* for 15 min at 4°C. The bacterial crude cell lysates were incubated with glutathione-agarose beads (Sigma) for 15 min at room temperature. The glutathione-agarose beads were then washed three times with MTPBS. The bound GST-fusion proteins were eluted by incubating the beads in equal volume of 20 mM reduced glutathione, pH 7.5.

Generation and Purification of Polyclonal Chicken IgY Antibodies

GST-polα or GST-polδ fusion protein (400 μg) was used as an antigen to immunize two separate chickens. Twenty days after initial immunization, the chickens were boosted three times with 200 μg of each antigen at 20-d intervals. Polyclonal IgY antibodies produced with each antigen were purified from egg yolks as described (Gassmann *et al.*, 1990). The antibodies produced with GST-polα and GST-polδ fusion proteins were named B18 and DNH2, respectively. B18 antibodies were first passed through a GST-Sepharose 4B column at room temperature to remove the antibodies that recognized the GST portion of the fusion protein. The flow-through fraction from the GST-Sepharose 4B column was loaded onto a 2-ml column of Sepharose 4B containing purified GST-polα fusion proteins at room temperature. The column was washed extensively until no protein was detected in the flow through. The DNA polymerase α-specific antibodies were eluted with 4 ml of 4.5 M $MgCl_2$ solution, dialyzed immediately in 250× volume of Tris-buffered saline (TBS), and then in 100× volume of TBS containing 50% glycerol at 4°C. The DNA polymerase δ-specific antibodies were affinity-purified as described (Pringle *et al.*, 1991). The DNH2 antibodies were first passed through a GST-Sepharose 4B column to remove the antibodies against GST protein at room temperature. The flow through from the GST-Sepharose 4B column was incubated with nitrocellulose membrane, which was blotted with GST-polδ fusion proteins for 2 h at room temperature. The nitrocellulose membrane was then washed twice with TBS. The DNA polymerase δ-specific antibodies were eluted from the membrane with 1 ml of 4.5 M $MgCl_2$ solution for 20 min. The eluted antibodies were dialyzed in TBS as described above.

DEAE-Sephacel and Phosphocellulose Chromatography of S. pombe Whole Cell Lysates

S. pombe whole cell lysates were isolated as described by Moreno *et al.* (1991). For cell cycle studies of *S. pombe* DNA polymerase δ protein, *S. pombe* whole cell lysates from each cell fraction were partially purified by DEAE-Sephacel chromatography. Equal amounts of proteins (350 μg) from each whole cell lysate were incubated with equal volumes (70 μl) of DEAE-Sephacel resins that were equilibrated in 25 mM KPO_4, pH 7.0, at 4°C. Under these conditions, the amount of applied proteins did not exceed the capacity of the resins. After incubation for 1 h, the resins were collected by centrifugation and washed once with 500 μl of equilibrating buffer at 4°C. The bound proteins were then eluted from the resins with equal volume of 0.4 M KCl for each sample. The entire volume of eluted protein fraction was loaded onto an SDS polyacrylamide gel and used for analysis of *S. pombe* DNA polymerase δ protein by immunoblotting. The flow-through fractions from each DEAE-column were also analyzed by immunoblotting and did not reveal *S. pombe* DNA polymerase δ protein. To investigate the *S. pombe* DNA polymerase α protein during the cell cycle, the whole cell lysates from each fraction were partially purified by phosphocellulose chromatography. The experimental condition was same as DEAE-Sephacel chromatography described above except that the phosphocellulose beads were equilibrated with 50 mM KPO4, pH 7.5, before incubating with whole cell lysates and the bound proteins were eluted with 0.4 M KPO4, pH 7.5.

Immunoblotting

Affinity-purified B18 antibodies were used to detect GST-polα and *S. pombe* DNA polymerase α proteins. Affinity-purified DNH2 antibodies were used to detect GST-polδ and *S. pombe* DNA polymerase δ proteins. Anti-PSTAIR antibodies (Solomon *et al.*, 1990) were used to detect $p34^{cdc2}$ protein. The proteins were separated by 8% SDS-PAGE and transferred onto Problot membrane (Applied Biosystems, Foster City, CA) as described (Hsi *et al.*, 1990). The membrane was incubated with the respective antibodies in TBS containing 3% BSA (Sigma) at 4°C overnight. The membrane was then washed three times with TBS and incubated with secondary antibody, either rabbit anti-chicken or rabbit anti-mouse horseradish peroxidase conjugated IgG (Sigma), in 1:3000 dilution in TBS for 1 h at room temperature. After five washes with TBS, the membrane was incubated in 3',3'-diaminobenzidine and peroxide as described (Lane and Harlow, 1988).

Immunoprecipitation of S. pombe DNA Polymerase α

One milligram protein from whole cell lysates was precleared by incubating with Sepharose 4B cross-linked to preimmune IgYs for 1 h at 4°C with rotation. After centrifugation, the supernatant was collected, incubated with 4 μg of affinity-purified B18 antibodies for 1 h at 4°C, and then incubated with Sepharose 4B beads containing rabbit anti-chicken IgG for 40 min at 4°C. The immunobeads were collected by centrifugation, washed four times with 50 mM tris(hydroxymethyl) aminomethane-HCl, pH 7.5, 0.5 M NaCl, 1% NP-40, and washed once with TBS. The immunobeads were boiled in SDS sample loading buffer and loaded directly onto the gel to analyze immunoprecipitated proteins. To assay the DNA polymerase α enzymatic activity, the immunobeads were incubated in a reaction mixture (Wang *et al.*, 1984) for 30 min at 37°C with shaking.

RESULTS

Genes and Primary Structures of S. pombe DNA Polymerases α and δ

Five overlapping genomic clones containing the gene encoding *S. pombe* DNA polymerase α (*pol*α^+) were isolated as described under MATERIALS AND METHODS. A clone, pWHpolα, containing a 5.3-kb insert was further characterized. This clone has a 4306-bp *pol*α^+ gene containing an open reading frame (ORF) flanked by a 486-bp upstream and a 0.7-kb downstream sequence (Figure 1A). In agreement with the results of Damagnez *et al.* (1991), the ORF of our *pol*α^+ gene starts from an inframe ATG to a termination codon TGA at nucleotide 4307, which is interrupted by a 91-bp intron from nucleotide 302–392 (Figure 1A). The coding sequence of the *pol*α^+ gene encodes a protein of 1405 amino acids with a predicted molecular weight of

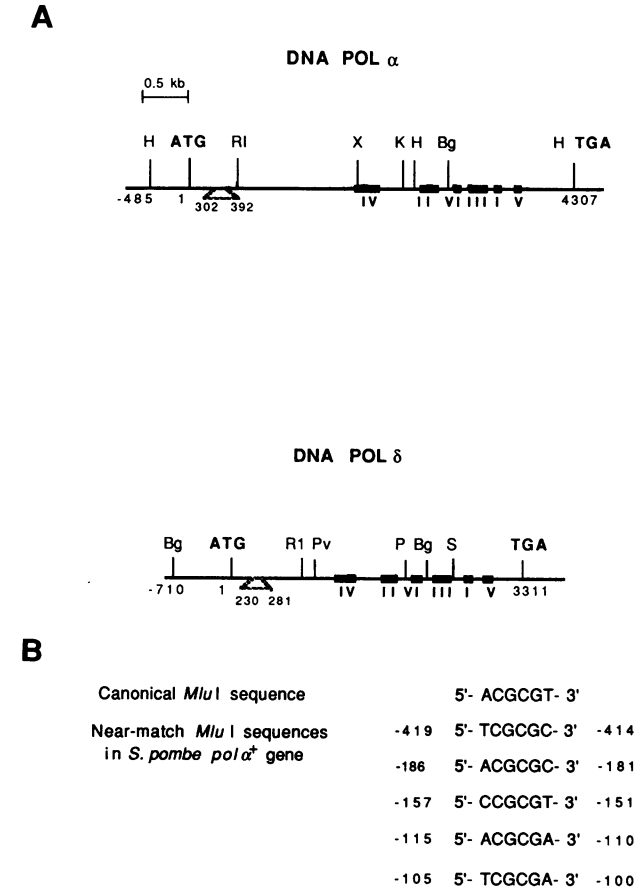

Figure 1. *S. pombe* $pol\alpha^+$ and $pol\delta^+$ genes. (A) Restriction maps of *S. pombe* $pol\alpha^+$ and $pol\delta^+$ genes. Solid lines represent genomic DNA containing coding and upstream sequences. Solid black boxes represent the six conserved regions in α-like DNA polymerases, which are numbered according to the extent of similarity among the α-like DNA polymerases (I > II > III > IV > V > VI) (Wong *et al.*, 1988). Broken inserts represent the location of introns. ATG indicates translation start codon. TGA indicates translation termination codon. Abbreviations: H, *Hin*dIII; RI, *Eco*RI; X, *Xba* I; K, *Kpn* I; Bg, *Bgl* II; P, *Pst* I; Pv, *Pvu* II; S, *Sal* I. (B) Near-match *Mlu* I sequences in the upstream sequence of *S. pombe* $pol\alpha^+$ gene. *S. pombe* $pol\delta^+$ sequence genBank accession number is L07734.

159 347 Da. Four overlapping genomic clones containing the gene encoding *S. pombe* DNA polymerase δ ($pol\delta^+$) were isolated as described under MATERIALS AND METHODS. A positive clone, pWHpolδ, containing a 7.3-kb insert was further characterized. This clone has a 3310-bp $pol\delta^+$ gene containing an ORF flanked by a 3.3-kb upstream and a 0.7-kb downstream sequence (Figure 1A). The ORF of the $pol\delta^+$ gene starts from an inframe ATG to a termination codon TGA at nucleotide 3311, which is interrupted by a 52-bp short intron located from 230 to 281 nt (Figure 1A). The coding sequence of the $pol\delta^+$ gene encodes a protein of 1086 amino acids with a predicted molecular weight of 123 985 Da. The predicted amino acid sequence of $pol\delta^+$ gene isolated by us revealed several discrepancies from that reported by Pignede *et al.* (1991). Our DNA polymerase δ sequence has Q^{102} and T^{419} in contrast to E^{102} and S^{419}. In addition to these differences, our predicted amino acid sequence from the residue 777 to 784 showed KLEFEKVY containing two additional amino acids, which was entirely different from the sequence NWSF-T- in this region reported by Pignede *et al.* (1991). It is possible that the differences in these amino acid residues are due to a genetic heterogeneity of the *S. pombe* strains from which the genomic DNAs were originally isolated. Nevertheless, our full-length $pol\delta^+$ gene was able to functionally complement a deleted $pol\delta^-$ allele in an *S. pombe* diploid strain. This verified that the $pol\delta^+$ gene that we isolated encoded a functional *S. pombe* DNA polymerase δ (Francesconi and Wang, unpublished data).

Analysis of the 486-bp upstream sequence of the $pol\alpha^+$ gene did not reveal any canonical *Mlu* I motif (MCB), but five near-match *Mlu* I sequences were found from −100 to −449 nt. The upstream sequence of the $pol\delta^+$ gene up to −341 nt also did not contain any *Mlu* I motif (Figure 1B). Genomic Southern analysis indicates that both $pol\alpha^+$ and $pol\delta^+$ genes are single copy genes in haploid cells. Results from one-step gene disruption indicate that both $pol\alpha^+$ and $pol\delta^+$ genes are essential for cell viability (Francesconi, Park, and Wang, unpublished data). The predicted primary amino acid sequences for both *S. pombe* DNA polymerases α and δ revealed the six conserved regions that are present in α-like DNA polymerases (Figure 1A) (Wong *et al.*, 1988; Wang, 1991). Sequence comparison of the DNA polymerase α between *S. pombe* and *S. cerevisiae* showed 39% identity and 58% similarity in the primary amino acid sequences. Sequence comparison of DNA polymerase δ between these two yeasts showed 55% amino acid identity and 71% amino acid similarity.

Expression of the Transcripts of S. pombe $pol\alpha^+$ and $pol\delta^+$ Genes During the Cell Cycle

We examined the level of steady-state transcripts of $pol\alpha^+$ and $pol\delta^+$ genes in *S. pombe* for two cell cycles. The culture of *S. pombe* wild-type strain $972h^-$ was synchronized by counterflow elutriation as described under MATERIALS AND METHODS. Cell cycle synchrony was monitored based on the percentage of septated cells in the total cell population (Figure 2A). Due to the low abundance of the transcripts of both $pol\alpha^+$ and $pol\delta^+$ genes, the level of transcripts was measured by RNase protection analysis. Histone *H2A1* gene was used as a control of periodic expression, whereas $ura4^+$ gene was used as a control of constant expression during the cell cycle. The transcripts of $pol\alpha^+$, $pol\delta^+$, histone *H2A1*, and $ura4^+$ genes in total RNAs were detected by RNase protection. The results indicated that $ura4^+$ steady-state

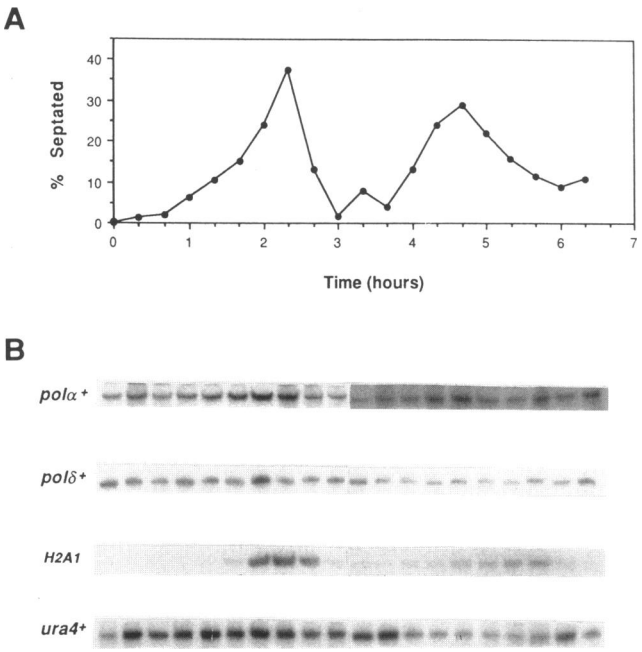

Figure 2. Level of steady-state transcripts of *S. pombe* $pol\alpha^+$ and $pol\delta^+$ genes during the cell cycle. *S. pombe* wild-type strain 972h$^-$ was grown and synchronized by counterflow elutriation as described under MATERIALS AND METHODS. (A) Percentage of septated cells in synchronized culture for two cell cycles. The percentage of septated cells was calculated by counting ≥200 cells at each time point after synchronization. Time (in hours) indicates time points when cell samples were collected. (B) Level of steady-state transcripts of $pol\alpha^+$, $pol\delta^+$, histone *H2A1*, and *ura4*$^+$ genes in *S. pombe* during the cell cycle. The $pol\alpha^+$, $pol\delta^+$, *H2A1*, and *ura4*$^+$ indicates the steady-state transcripts of respective genes detected by RNase protection analysis on total RNAs isolated from each synchronized cell fraction. Transcripts are shown below each time point where the cell fractions were collected as indicated in A.

transcript was present throughout two cell cycles, whereas the steady-state transcript of histone *H2A1* gene was expressed periodically, reaching a peak in S phase of the cell cycle. The steady-state transcripts of the $pol\alpha^+$ and $pol\delta^+$ genes were present throughout two cell cycles. The steady-state transcript of $pol\delta^+$ gene was expressed at a constant level during the cell cycle, but a slight increase in the amount of the $pol\alpha^+$ transcript was observed during S phase (Figure 2B). To further investigate this observation, the steady-state transcripts of $pol\alpha^+$ and $pol\delta^+$ genes were measured at discrete stages of the cell cycle by RNase protection analysis. Four isogenic *S. pombe cdc* strains (*cdc10, cdc22, cdc25,* and *cdc2*) were used to arrest the cells at discrete stages of the cell cycle (G_1, S, G_2, and G_2/M) for 4.5 and 6.5 h. The result from RNase protection analysis demonstrated that the $pol\alpha^+$ transcript was expressed at a constant level at all stages of the cell cycle, similar to the level of $pol\delta^+$ and *ura4*$^+$ transcripts. These results indicate that the transcripts of *S. pombe* $pol\alpha^+$ and $pol\delta^+$ genes are expressed throughout the cell cycle.

Polyclonal Antibodies for S. pombe Polymerases α and δ

Polyclonal IgY antibodies for *S. pombe* DNA polymerase α as well as for DNA polymerase δ proteins were generated to study the expression of these enzymes. GST-polα fusion protein expressed in *E. coli* transformed with pGT-polα (Figure 3A) was used as an antigen to produce B18 antibodies. GST-polδ fusion protein expressed in *E. coli* transformed with pGT-polδ (Figure 3C) was used as an antigen to produce DNH2 antibodies.

To test the immunoreactivity of B18, affinity-purified B18 antibodies were used for immunoblotting GST-polα fusion protein, GST protein, and proteins from *S. pombe* whole cell lysates. The B18 antibodies recognized a cluster of proteins in the whole cell lysates isolated from actively growing wild-type 972h$^-$, *cdc10,* and *cdc25* strains. The protein cluster ranged from 155 to 170 kDa with a predominant 165-kDa protein (Figure 3B, lanes 4–6) and was not recognized by preimmune IgYs (Figure 3B, lanes 1–3). The intensity of the protein cluster detected by B18 antibodies was enhanced after partial purification of whole cell extract by phosphocellulose chromatography (see MATERIALS AND METHODS). Affinity-purified B18 antibodies readily recognized the GST-polα fusion protein but were not able to recognize GST protein (Figure 3B, lanes 7 and 8). B18 antibody was also able to immunoprecipitate native DNA polymerase α protein from *S. pombe* whole cell lysates. Protein cluster ranging from 155 to 170 kDa was detected by SDS-PAGE fractionation of the immunoprecipitate (see Figure 5A). These proteins were identical in mass to those detected by immunoblotting with B18 antibodies. The DNA polymerase α protein in the immunoprecipitate retained enzymatic activity, and this activity was sensitive to DNA polymerase inhibitors, butylphenyl-dGTP and aphidicolin (Wang, 1991). Thus, we concluded that the B18 antibodies specifically recognized the *S. pombe* DNA polymerase α.

To test the immunoreactivity of DNH2, affinity-purified DNH2 antibodies were used for immunoblotting GST-polδ fusion protein, GST protein, and proteins from *S. pombe* whole cell lysates. The DNH2 antibodies recognized a protein of 125 kDa in *S. pombe* whole cell lysates whose mass was in good agreement with the predicted molecular mass of *S. pombe* DNA polymerase δ protein. Further purification of *S. pombe* DNA polymerase δ by DEAE-Sephacel chromatography resulted in a significant enhancement of the signal of the 125-kDa protein by immunoblotting (Figure 3D, lane 2). This 125-kDa protein was not detected by preimmune IgYs in either whole cell ly-

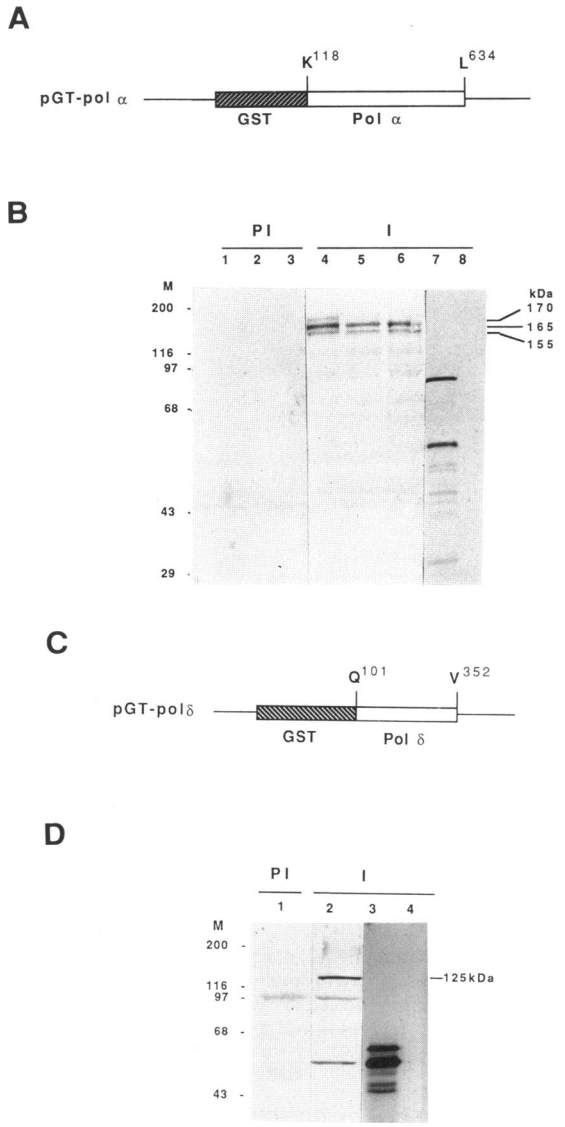

Figure 3. Analysis of B18 and DNH2 antibodies. (A) Construct for GST-polα fusion protein. The pGT-polα is an expression construct for GST-polα fusion protein in *E. coli*. (B) Analysis of B18 antibodies. B18 antibody was generated against GST-polα fusion protein as described under MATERIALS AND METHODS. One hundred micrograms of protein from whole cell lysates of *S. pombe* 972h$^-$ (lanes 1 and 4), *cdc10* (lanes 2 and 5), and *cdc25* strains (lanes 3 and 6), 1 μg of GST-polα (lane 7), and 1 μg of GST protein (lane 8) were separated by 8% SDS-PAGE and analyzed by immunoblotting. Protein blot was incubated with either preimmune (PI) or affinity-purified B18 antibodies (I). Molecular weight marker is depicted under M, and molecular mass of *S. pombe* DNA polymerase α proteins is indicated. (C) Construct for GST-polδ fusion protein. The pGT-polδ is an expression construct for GST-polδ fusion protein in *E. coli*. (D) Analysis of DNH2 antibodies. DNH2 antibodies were generated against GST-polδ fusion protein as described under MATERIALS AND METHODS. The whole cell lysates from *S. pombe* 972h$^-$ strains were partially purified by DEAE-Sephacel chromatography as described under MATERIALS AND METHODS. The eluted protein fractions from the DEAE-Sephacel columns (lanes 1 and 2), 1 μg of GST-polδ (lane 3), and 1 μg of GST protein (lane 4) were separated by 8% SDS-PAGE and analyzed by immunoblotting. The protein blot was incubated with either preimmune (PI) or affinity-purified DNH2 antibodies (I). Molecular weight marker is depicted under M and a 125-kDa *S. pombe* DNA polymerase δ is indicated.

sates or in the partially purified protein fractions (Figure 3D, lane 1). Affinity-purified DNH2 antibodies were readily reactive to GST-polδ fusion protein but not able to recognize GST proteins (Figure 3D, lanes 3 and 4). By immunoblot analysis, the affinity-purified DNH2 antibodies were also shown to be cross-reactive with a 140-kDa protein in *S. cerevisiae* whole cell lysates, which is identical in mass to *S. cerevisiae* DNA polymerase δ protein. Although the DNH2 antibodies recognized the *S. pombe* DNA polymerase δ protein by immunoblotting, they were unable to immunoprecipitate native DNA polymerase δ protein from *S. pombe* whole cell lysates.

Expression of S. pombe DNA Polymerases α and δ Proteins During the Cell Cycle

We investigated the steady-state protein levels of DNA polymerases α and δ in *S. pombe* for two cell cycles. To obtain a large number of synchronized cells, a conditional-lethal *cdc25* strain was used for block and release synchronization. A culture of *cdc25* strain was arrested at G2 at nonpermissive temperature and released back to the actively cycling state for two cell cycles at permissive temperature. Cell cycle synchrony was monitored by the percentage of septated cells in the population (Figure 4A). Affinity-purified B18 antibodies were used to investigate the level of DNA polymerase α proteins in *S. pombe* whole cell lysates for two cell cycles by immunoblotting. As a control of constant expression during the cell cycle, the level of *S. pombe* p34^{cdc2} protein was also monitored by immunoblotting (Simanis and Nurse, 1986). Anti-PSTAIR antibody was used to detect the p34^{cdc2} protein on the same immunoblot used for identifying *S. pombe* DNA polymerase α. The results from immunoblotting analysis indicated that three protein bands of *S. pombe* DNA polymerase α—170, 165, and 155 kDa—were present throughout two cell cycles (Figure 4B). The p34^{cdc2} protein was also detected at a constant level in whole cell lysates throughout two cell cycles. Even though the protein cluster of *S. pombe* DNA polymerase α was present throughout the cell cycle, a slightly weaker signal of 170-kDa protein band was observed around S phase of the cell cycle. To further investigate this observation, the whole cell lysates of each fraction were partially purified by phosphocellulose chromatography as described under MATERIALS AND METHODS. *S. pombe* DNA polymerase α proteins in the partially purified fractions were detected by immunoblotting with affinity-purified B18 antibodies for

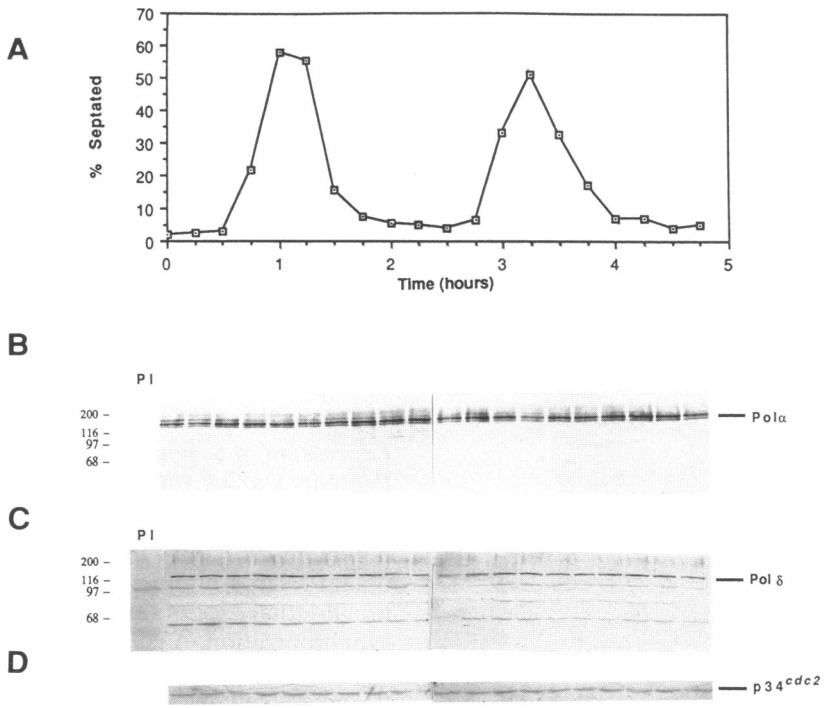

Figure 4. Steady-state protein levels of *S. pombe* DNA polymerases α and δ during the cell cycle. The *cdc25* cell culture was synchronized by block and release as described under MATERIALS AND METHODS. (A) Percentage of septated cells in synchronized culture for two cell cycles. The percentage of septated cells was calculated by counting ≥200 cells at each time point after release from the cell cycle arrest. Time (in hours) indicates the time points when the cell fractions were collected. (B) Steady-state protein level of *S. pombe* DNA polymerase α during the cell cycle. One hundred micrograms of protein from whole cell lysates of each cell fraction were analyzed by immunoblotting with either preimmune (PI) or with affinity-purified B18 antibodies. Whole cell lysates from the cell fraction collected 1 h after release were used for immunoblotting with preimmune antibodies. The *S. pombe* DNA polymerase α protein is indicated. (C) Steady-state protein level of *S. pombe* DNA polymerase δ during the cell cycle. Equal amounts of whole cell lysates from each cell fraction were partially purified by DEAE-Sephacel chromatography as described under MATERIALS AND METHODS. Entire eluted protein fractions from DEAE-Sephacel columns were separated by 8% SDS-PAGE and analyzed by immunoblotting. Protein blot was incubated either with preimmune (PI) or affinity-purified DNH2 antibodies. Partially purified fraction from cell sample collected 1 h after release was used for immunoblotting with preimmune antibodies. The 125-kDa *S. pombe* DNA polymerase δ protein is indicated. (D) Level of steady-state $p34^{cdc2}$ protein during the cell cycle. The same immunoblot used to detect *S. pombe* DNA polymerase δ in C was probed with anti-PSTAIR antibody. The $p34^{cdc2}$ protein is indicated.

two cell cycles. The results indicated that the three protein bands of *S. pombe* DNA polymerase α were present constantly throughout the cell cycle without any significant variation.

Affinity-purified DNH2 antibodies were used to investigate the steady-state level of *S. pombe* DNA polymerase δ protein during the cell cycle by immunoblotting. The DNH2 antibodies detected a weak protein band of 125 kDa together with smaller proteins in whole cell lysates. To enhance the signal of the DNA polymerase δ protein, the whole cell lysates from each cell fraction were partially purified by DEAE-Sephacel chromatography as described under MATERIALS AND METHODS. *S. pombe* DNA polymerase δ proteins in the partially purified fractions were detected by immunoblotting with affinity-purified DNH2 antibodies for two cell cycles. The same immunoblot used to detect *S. pombe* DNA polymerase δ was also incubated with anti-PSTAIR antibodies to monitor the level of $p34^{cdc2}$ protein in the partially purified fractions during the cell cycle. Results indicated that the level of *S. pombe* DNA polymerase δ protein was constant throughout two cell cycles (Figure 4C). The $p34^{cdc2}$ proteins were also present throughout two cell cycles at a constant level (Figure 4D). This verified that the same amounts of protein from each cell fraction were isolated by DEAE-Sephacel purification and loaded on the gel. The results from these immunoblot analyses indicate that *S. pombe* DNA polymerases α and δ proteins are constantly expressed during the cell cycle.

Enzymatic Activity of S. pombe DNA Polymerases During the Cell Cycle

We further investigated the enzymatic activities of *S. pombe* DNA polymerases during the cell cycle. Total DNA polymerase activities in *S. pombe* whole cell lysates were assayed in vitro for two cell cycles. The *S. pombe*

wild-type strain 972h$^-$ was synchronized by centrifugal elutriation. Equal amounts of protein from whole cell lysates of each cell fraction were assayed for total DNA polymerase activities using gapped calf thymus DNA as primer-template (Wang *et al.*, 1984). Results indicated that total DNA polymerase activity was present throughout two cell cycles. Because B18 antibody was able to immunoprecipitate enzymatically active *S. pombe* DNA polymerase α, the B18 antibody was used to investigate the enzymatic activity of *S. pombe* DNA polymerase α during the cell cycle. Four independent isogenic *S. pombe* strains containing conditional *cdc* mutations (*cdc10, cdc22, cdc25,* and *cdc2*) were used to block cells at discrete stages of the cell cycle (G_1, S, G_2, and G_2/M). Affinity-purified B18 antibodies were then used to immunoprecipitate *S. pombe* DNA polymerase α protein from whole cell lysates prepared from each arrested cell culture. DNA polymerase α enzymatic activities in the immunoprecipitates were assayed in vitro. The *S. pombe* DNA polymerase α proteins bound to B18 antibodies in each cell lysate were also analyzed by immunoblotting. The results indicated that comparable amounts of DNA polymerase α protein were immunoprecipitated from each cell lysate by B18 antibodies (Figure 5A). Under the same conditions, preimmune IgYs were unable to isolate any *S. pombe* DNA polymerase α proteins from whole cell lysates (Figure 5A). Activity assays of the immunoprecipitates showed that a less than two- to threefold increase of *S. pombe* DNA polymerase α enzymatic activity was detected in the cell lysates isolated from the S phase-arrested *cdc22* strains. This may be due to a slight increase in the amount of DNA polymerase α protein immunoprecipitated from the arrested *cdc22* strain. However, significant amounts of *S. pombe* DNA polymerase α activities were also present in the cell lysates arrested at G_1, G_2, and G_2/M phases of the cell cycle (Figure 5B).

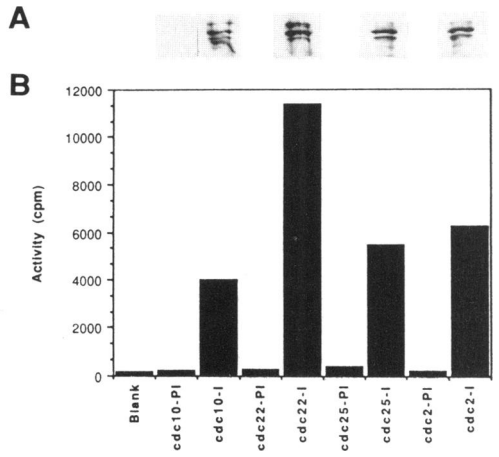

Figure 5. *S. pombe* DNA polymerase α enzymatic activities during the cell cycle. *S. pombe cdc10, cdc22, cdc25,* and *cdc2* strains were synchronized at discrete stages of the cell cycle. Affinity-purified B18 antibodies were used to immunoprecipitate *S. pombe* DNA polymerase α proteins from the arrested cell lysates as described under MATERIALS AND METHODS. (A) The *S. pombe* DNA polymerase α proteins immunoprecipitated by B18 antibodies at discrete stages of the cell cycle. Proteins immunoprecipitated by preimmune and B18 antibodies from arrested cell lysates were analyzed by immunoblotting. Preimmune antibodies were incubated with arrested *cdc10* cell lysates. Protein blot of the immunoprecipitates was incubated with B18 antibodies. Results are shown above the enzymatic activities from the corresponding arrested cells. (B) *S. pombe* DNA polymerase α enzymatic activities at discrete stages of the cell cycle. Immunoprecipitates by preimmune (PI) and affinity-purified B18 antibodies (I) from the arrested cell lysates were assayed for DNA polymerase enzymatic activity. Relative acid precipitable radioactivities (cpm) from the assay are shown.

DISCUSSION

Progress has been made in understanding the regulation of the expression of genes involved in DNA replication or DNA precursor synthesis in different organisms. In mammalian cells, most genes involved in DNA synthesis or metabolism are expressed constitutively during the cell cycle. In some cases, the proteins are modified post-translationally in a cell cycle-dependent manner (Sherley and Kelly, 1988; Wahl *et al.*, 1988; Morris and Mathews, 1989; Tseng *et al.*, 1989; Nasheuer *et al.*, 1991). In *S. cerevisiae,* the transcriptional expression of many DNA synthesis genes are coordinately induced at the G1/S boundary. The cell cycle-dependent transcription of these budding yeast DNA synthesis genes appears to be mediated by the common promoter element, *Mlu* I motif (MCB), and MCB binding complexes (Andrews and Herskowitz, 1990; Dirick *et al.*, 1992; Lowndes *et al.*, 1991, 1992a,b). In *S. pombe,* studies of the expression of DNA synthesis genes thus far are only limited to two genes. The transcriptional expression of *cdc22*$^+$ gene is periodically induced at the G1/S boundary (Gordon and Fantes, 1986). The promoter of the *cdc22*$^+$ gene has two *Mlu* I motifs that were shown to be involved in the cell cycle-dependent transcriptional expression of this gene. In contrast, *S. pombe cdc17*$^+$ gene is constitutively expressed at the transcriptional level throughout the cell cycle (White *et al.*, 1986).

In this report, we have shown that the steady-state transcripts of *S. pombe pol*α^+ and *pol*δ^+ genes are expressed throughout the cell cycle with a slight increase of the *pol*α^+ transcript during S phase (Figure 2B). However, the *pol*α^+ transcript was expressed at a constant level in the G_1, S, G_2, and G_2/M arrested *cdc* strains, showing no apparent increase of the transcript in S phase arrested *cdc22* strain. Because *cdc22* strain is arrested at a specific phase of the cell cycle at nonpermissive temperature, it is possible that the *cdc22* strain was arrested just before the induction of *pol*α^+ transcript in S phase. The slight difference in the results from two experiments also may be originated from different nature of *S. pombe* strains under different experimental

conditions. These results, nevertheless, indicated that the transcriptional expression of *S. pombe* $pol\alpha^+$ and $pol\delta^+$ genes is different from that of *S. cerevisiae* homologs that exhibit sharp induction of transcription at G1/S junction. Neither *S. pombe* $pol\alpha^+$ nor $pol\delta^+$ genes contain any canonical *Mlu* I motif in their upstream sequences. The $pol\alpha^+$ gene, however, has five near-match *Mlu* I motifs in the upstream sequences (Figure 1B). Studies of budding yeast *CDC9* and *TMP1* genes suggest that the *Mlu* I hexamer sequence is an integral component for the cell cycle-dependent transcriptional expression of these genes (Lowndes *et al.*, 1991; McIntosh *et al.*, 1991). However, the integrity of the *Mlu* I sequences within the promoter does not seem to be absolutely necessary for transcription of the *TMP1* gene. The transcription of *TMP1* was not impaired by replacing *Mlu* I motifs with near-match *Mlu* I sequences within the native *TMP1* promoter (McIntosh *et al.*, 1991). We have not investigated whether any of the five near-match *Mlu* I elements in *S. pombe* $pol\alpha^+$ gene is involved in the transcription of this gene during the cell cycle. Data presented in this report has confirmed that *S. pombe* has at least two different modes of regulating the expression of DNA synthesis genes. One regulatory mode is the cell cycle-dependent transcriptional activation mediated by MCB and MCB-binding activity that is exemplified by $cdc22^+$ gene expression. The second mode of regulation confers transcriptional expression throughout the cell cycle, which is shown in the expression of the $cdc17^+$, $pol\alpha^+$, and $pol\delta^+$ genes.

Thus far, at least three *S. pombe* genes involved in DNA synthesis ($cdc17^+$, $pol\alpha^+$, and $pol\delta^+$) were found to be expressed differently from their budding yeast homolog genes. The difference in the regulation of the DNA synthesis genes may reflect the differences in the cell cycle regulation of these two yeasts. The *S. cerevisiae* cell cycle is mainly regulated at the G_1/S (Pringle and Hartwell, 1981), whereas the *S. pombe* cell cycle is mainly regulated at the G_2/M (Fantes and Nurse, 1977). The coordinated expression of the DNA synthesis genes at the G_1/S boundary may be a part of an elaborate network of regulation imposed on the entry into the S phase in budding yeast. Although *S. cerevisiae* exhibits the distinct periodic transcriptional expression of DNA synthesis genes, it has not been demonstrated how most of the proteins encoded by these genes and their enzymatic activities are regulated during the cell cycle. In one well-studied case, the transcriptional expression of the *RFA2* gene encoding the middle subunit of *S. cerevisiae* replication protein-A, $p34^{RP\text{-}A}$, was reported to be cell cycle-dependent (Brill and Stillman, 1991). However, the $p34^{RP\text{-}A}$ protein was present constantly during the cell cycle and was shown to be phosphorylated during S phase (Din *et al.*, 1990). Thus, the significance of the periodic and coordinated transcriptional expression of the DNA synthesis genes at the G_1/S boundary remains to be elucidated in budding yeast.

In this report, two polyclonal antibodies, B18 and DNH2, against *S. pombe* DNA polymerases α and δ were used to investigate the level of these proteins during the cell cycle. The DNA polymerase α specific antibodies, B18, reproducibly recognized three protein bands of 170, 165, and 155 kDa with the 165-kDa protein as a predominant species (Figure 3B). The predicted molecular mass deduced from the coding sequence of *S. pombe* $pol\alpha^+$ gene is 159 kDa. The difference between the predicted and the observed 170-kDa protein is reminiscent of that observed for human DNA polymerase α protein. The human DNA polymerase α has a predicted molecular mass of 165 kDa (Wong *et al.*, 1988) but displays a protein cluster of 180-, 165-, and 155-kDa proteins by an SDS gel analysis (Hsi *et al.*, 1990). It is demonstrated that the observed larger 180-kDa human DNA polymerase α protein is due to both phosphorylation and glycosylation (Hsi *et al.*, 1990; Nasheuer *et al.*, 1991). The 165- and 155-kDa proteins are shown to be the proteolytically degraded products of the modified 180-kDa protein (Hsi *et al.*, 1990). Our preliminary data indicated that *S. pombe* DNA polymerase α is a phosphoprotein. All three *S. pombe* DNA polymerase α protein bands are phosphorylated, and the degree of phosphorylation is proportional to the amount of each protein analyzed on a gel. We do not know if the *S. pombe* DNA polymerase α is glycosylated or modified in other manners. It is possible that like human DNA polymerase α, the 170-kDa *S. pombe* DNA polymerase α protein has additional protein modifications and the 165- and 155-kDa proteins are the proteolytically degraded products of the modified 170-kDa protein.

We also showed that the *S. pombe* DNA polymerases α and δ proteins were present throughout the cell cycle. To investigate whether the slight variation of 170-kDa *S. pombe* DNA polymerase α protein (Figure 4B) is due to a cell cycle-dependent protein modification or an experimental variation, each crude cell lysate was partially purified by phosphocellulose chromatography to enhance the signal of 170-kDa protein. Results from the immunoblotting analysis on the partially purified fractions indicated that the DNA polymerase α protein cluster was present throughout the cell cycle without any increase or decrease of any protein species.

Our data indicated that the *S. pombe* DNA polymerase α enzymatic activities were present at all stages of the cell cycle. It should be noted that the DNA polymerase enzymatic activities were measured with artificially gapped DNA as primer-template, not representing the in vivo chromosomal scenario. These findings suggest that there must be regulatory mechanisms in vivo that modulate the DNA polymerase activities at initiation and termination of DNA replication.

The expression of *S. pombe* DNA polymerases α and δ resembles that of human DNA polymerase α, which is constantly expressed at the levels of transcript, protein, and enzymatic activity during the cell cycle (Wahl *et al.*, 1988). These findings raise the possibility that *S. pombe* DNA polymerases α and δ might be phosphorylated in a cell cycle-dependent manner like human DNA polymerase α (Nasheuer *et al.*, 1991). We have found that *S. pombe* DNA polymerase α is a phosphoprotein. We are currently investigating whether *S. pombe* DNA polymerases α and δ exhibit cell cycle-dependent phosphorylation.

Genetic studies from both budding and fission yeasts suggest that protein kinases are involved in the regulation of the G_1/S transition. In budding yeast, *CDC28* and *CDC7* protein kinases are essential for initiation of DNA replication (Hartwell, 1974). In addition, budding yeast *DBF2* protein has been implicated to be a protein kinase involved in the regulation of initiation of DNA replication (Johnston *et al.*, 1990a). In fission yeast, the *cdc2* protein kinase plays a role in the G_1/S transition as well as in the G_2/M transition (Nurse and Bissett, 1981). These findings suggest that progression from start to DNA initiation may be regulated by protein phosphorylation. The fact that the essential replication proteins including DNA polymerases are constantly expressed during the cell cycle suggests that the replicative function of these proteins might be modulated by protein modification, possibly by protein phosphorylation, during the cell cycle. It is attractive to speculate that these proteins involved in DNA replication may be targets of the protein kinases that regulate cell cycle progression. This might be an efficient way to ensure that DNA replication occurs only once during the cell cycle.

ACKNOWLEDGMENTS

We thank Dr. J.M. Mitchison for insightful and intellectual advice on *S. pombe* cell cycle, Dr. M. Yanagida for generously providing us with the *S. pombe* histone *H2A1* gene, and Dr. R. Booher for *ura4*$^+$ and *cdc2*$^+$ genes and *cdc* strains, for many helpful discussions during the course of this study, and for critical review of the manuscript. We also thank Teresa Biegel for excellent technical assistance. This study was supported by a National Institutes of Health grant CA-54415 from the National Cancer Institute.

REFERENCES

Andrews, B.J., and Herskowitz, I. (1990). Regulation of cell cycle-dependent gene expression in yeast. J. Biol. Chem. *265*, 14057–14060.

Araki, H., Ropp, P.A., Johnson, A.L., Johnston, L.H., Morrison, A., and Sugino, A. (1992). DNA polymerase II, the probable homolog of mammalian DNA polymerase ϵ, replicates chromosomal DNA in the yeast *Saccharomyces cerevisiae*. EMBO J. *11*, 733–740.

Bauer, G.A., and Burgers, P.M.J. (1990). Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene. Nucleic Acids Res. *18*, 261–265.

Boulet, A., Simon, M., Faye, G., Bauer, G.A., and Burgers, P.M.J. (1989). Structure and function of the *Saccharomyces cerevisiae CDC2* gene encoding the large subunit of DNA polymerase III. EMBO J. *8*, 1849–1854.

Breeden, L., and Nasmyth, K. (1987). Similarity between cell-cycle genes of budding yeast and fission yeast and the *Notch* gene of *Drosophila*. Nature *329*, 651–654.

Brill, S.J., and Stillman, B. (1991). Replication factor-A from *Saccharomyces cerevisiae* is encoded by three essential genes coordinately expressed at S phase. Genes Dev. *5*, 1589–1600.

Budd, M.E., Wittrup, K.D., Bailey, J.E., and Campbell, J.L. (1989). DNA polymerase I is required for premeiotic DNA replication and sporulation but not for X-ray repair in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9*, 365–376.

Challberg, M.D., and Kelly, T.J. (1989). Animal virus DNA replication. Annu. Rev. Biochem. *58*, 671–717.

Damagnez, V., Tillit, J., Recondo, A.-M., and Baldacci, G. (1991). The *POL1* gene from fission yeast, *Schizosaccharomyces pombe*, shows conserved amino acid blocks specific for eukaryotic DNA polymerase alpha. Mol. Gen. Genet. *226*, 182–189.

Din, S.-U., Brill, S.J., Fairman, M.P., and Stillman, B. (1990). Cell cycle-regulated phosphorylation of DNA replication factor-A from human and yeast cells. Genes Dev. *4*, 968–977.

Dirick, L., Moll, T., Auer, H., and Nasmyth, K. (1992). A central role for *SWI6* in modulating cell cycle start-specific transcription in yeast. Nature *357*, 508–513.

Elledge, S.J., and Davis, R.W. (1990). Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. *4*, 740–751.

Fantes, P.A. (1979). Epistatic gene interactions in the control of division in fission yeast. Nature *279*, 428–430.

Fantes, P.A., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth modulated size control over nuclear division. Exp. Cell. Res. *107*, 377–386.

Foiani, M., Santocanale, C., Plevani, P., and Lucchini, G. (1989). A single essential gene, *PRI2*, encodes the large subunit of DNA primase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9*, 3081–3087.

Forsburg, S.L., and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Annu. Rev. Cell Biol. *7*, 227–256.

Gassmann, M., Thommes, P., Weiser, T., and Hubscher, U. (1990). Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB J. *4*, 2528–2532.

Gordon, C.B., and Campbell, J.L. (1991). A cell cycle-responsive transcriptional control element and a negative control element in the gene encoding DNA polymerase α in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *88*, 6058–6062.

Gordon, C.B., and Fantes, P.A. (1986). The *cdc22* gene of *Schizosaccharomyces pombe* encodes a cell cycle regulated transcript. EMBO J. *5*, 2981–2985.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe:* a system for gene disruption and replacement using the *ura4* gene as a selective marker. Mol. Gen. Genet. *215*, 81–86.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974). *Schizosaccharomyces pombe*. In: Handbook of Genetics 1. ed. R.C. King, New York: Plenum Press, 395–446.

Hartwell, L.H. (1974). *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. *38*, 164–198.

Hsi, K.-L., Copeland, W.C., and Wang, T.S.-F. (1990). Human DNA polymerase α catalytic polypeptide binds ConA and RCA and contains a specific labile site in the N-terminus. Nucleic Acids Res. *18*, 6231–6237.

Hurwitz, J., Dean, F.B., Kwong, A.D., and Lee, S.-H. (1990). The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. *265*, 18043–18046.

Johnson, L.M., Snyder, M., Chang, L.M.-S., Davis, R.W., and Campbell, J.L. (1985). Isolation of the gene encoding yeast DNA polymerase I. Cell *43*, 369–377.

Johnston, L.H., Eberly, S.L., Chapman, J.W., Araki, H., and Sugino, A. (1990a). The product of the *Saccharomyces cerevisiae* cell cycle gene *DBF2* has homology with protein kinases and is periodically expressed in the cell cycle. Mol. Cell. Biol. *10*, 1358–1366.

Johnston, L.H., White, J.H.M., Johnson, A.L., Lucchini, G., and Plevani, P. (1987). The yeast DNA polymerase I transcript is regulated in both the mitotic cell cycle and in meiosis and is also induced after DNA damage. Nucleic Acids Res. *15*, 5017–5030.

Johnston, L.H., White, J.H.M., Johnson, A.L., Lucchini, G., and Plevani, P. (1990b). Expression of the yeast DNA primase gene, *PRI1*, is regulated within the mitotic cell cycle and meiosis. Mol. Gen. Genet. *221*, 44–48.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature *227*, 680–685.

Lane, D., and Harlow, E. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Lowndes, N.F., Johnson, A.L., Breeden, L., and Johnston, L.H. (1992a). *SWI6* protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast. Nature *357*, 505–508.

Lowndes, N.F., Johnson, A.L., and Johnston, L.H. (1991). Coordination of expression of DNA synthesis genes in budding yeast by a cell-cycle regulated *trans* factor. Nature *350*, 247–250.

Lowndes, N.F., McInerny, C.J., Johnson, A.L., Fantes, P.A., and Johnston, L.H. (1992b). Control of DNA synthesis genes in fission yeast by the cell cycle gene *cdc10*$^+$. Nature *355*, 449–453.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Matsumoto, S., and Yanagida, M. (1985). Histone gene organization of fission yeast: a common upstream sequence. EMBO J. *4*, 3531–3538.

McIntosh, E.M., Atkinson, T., Storms, R.K., and Smith, M. (1991). Characterization of a short, *cis*-acting DNA sequence which conveys cell cycle stage-dependent transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *11*, 329–337.

Mitchison, J.M. (1988). Synchronous culture and age fractionation. In: Yeast: A Practical Approach. ed. I. Campbell and J.H. Duffus, Oxford & Washington, DC: IRL Press, 51–63.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. In: Guide to Yeast Genetics and Molecular Biology. ed. C. Guthrie and G.R. Fink, San Diego, CA: Academic Press, 795–823.

Morris, G.F., and Mathews, M.B. (1989). Regulation of proliferating cell nuclear antigen during the cell cycle. J. Biol. Chem. *264*, 13856–13864.

Nasheuer, H.-P., Moore, A., Wahl, A.F., and Wang, T.S.-F. (1991). Cell cycle-dependent phosphorylation of human DNA polymerase α. J. Biol. Chem. *266*, 7893–7903.

Nasmyth, K., and Nurse, P. (1981). Cell division cycle mutants altered in DNA replication and mitosis in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. *182*, 119–124.

Nurse, P., and Bissett, Y. (1981). Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. Nature *292*, 558–560.

Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. *146*, 167–178.

Pearson, B.E., Nasheuer, H.-P., and Wang, T.S.-F. (1991). Human DNA polymerase α gene: sequence controlling expression in cycling and serum stimulated cells. Mol. Cell. Biol. *11*, 2081–2095.

Pignede, G., Bouvier, D., Recondo, A.-M., and Baldacci, G. (1991). Characterization of the *POL3* gene product from *S. pombe* indicates interspecies conservation of the catalytic subunit of DNA polymerase δ. J. Mol. Biol. *222*, 209–218.

Pizzagalli, A., Valsasnini, P., Plevani, P., and Lucchini, G. (1988). DNA polymerase I gene of *Saccharomyces cerevisiae:* nucleotide sequence, mapping of a temperature-sensitive mutation, and protein homology with other DNA polymerases. Proc. Natl. Acad. Sci. USA *85*, 3772–3776.

Pringle, J.R., Adams, A.E.M., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yeast. In: Guide to Yeast Genetics and Molecular Biology. ed. C. Guthrie and G.R. Fink, San Diego, CA: Academic Press, 565–602.

Pringle, J.R., and Hartwell, L.H. (1981). The *Saccharomyces cerevisiae* cell cycle. In: The Molecular Biology of the Yeast *Saccharomyces:* Life Cycle and Inheritance. ed. J.N. Strathern, E.W. Jones, and J.R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 97–142.

Sherley, J.L., and Kelly, T.J. (1988). Regulation of human thymidine kinase during the cell cycle. J. Biol. Chem. *263*, 8350–8358.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene *cdc2*$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell *45*, 261–268.

Sitney, K.C., Budd, M.E., and Campbell, J.L. (1989). DNA polymerase III, a second essential DNA polymerase, is encoded by the *S. cerevisiae CDC2* gene. Cell *56*, 599–605.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene *67*, 31–40.

Solomon, M.J., Glotzer, M., Lee, T.H., Phillippe, M., and Kirschner, M.W. (1990). Cyclin activation of p34^{cdc2}. Cell *63*, 1013–1024.

Stillman, B. (1989). Initiation of eukaryotic DNA replication *in vitro*. Annu. Rev. Cell Biol. *5*, 197–245.

Storm, R.K., Ord, R.W., Greenwood, M.T., Miradammadi, B., Chu, F.K., and Belfort, M. (1984). Cell cycle-dependent expression of thymidylate synthase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *4*, 2858–2864.

Tseng, B.Y., Prussak, C.E., and Almazan, M.T. (1989). Primase p49 mRNA expression is serum stimulated but does not vary with the cell cycle. Mol. Cell. Biol. *9*, 1940–1945.

Tsurimoto, T., and Stillman, B. (1991). Replication factors required for SV40 DNA replication *in vitro*. DNA structure specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. J. Biol. Chem. *266*, 1950–1960.

Wahl, A.F., Geis, A.M., Spain, B.H., Wong, S.W., Korn, D., and Wang, T.S.-F. (1988). Gene expression of human DNA polymerase α during cell proliferation and the cell cycle. Mol. Cell. Biol. *8*, 5016–5025.

Wang, T.S.-F. (1991). Eukaryotic DNA polymerases. Annu. Rev. Biochem. *60*, 513–552.

Wang, T.S.-F., Hu, S.-Z., and Korn, D. (1984). DNA primase from KB cells. Characterization of a primase activity tightly associated with immunoaffinity-purified DNA polymerase α. J. Biol. Chem. *259*, 1854–1865.

White, J.H.M., Barker, D.G., Nurse, P., and Johnston, L.H. (1986). Periodic transcription as a means of regulating gene expression during the cell cycle: contrasting modes of expression of DNA ligase genes in budding and fission yeast. EMBO J. *5*, 1705–1709.

White, J.H.M., Green, S.R., Barker, D.G., Dumas, L.M., and Johnston, L.H. (1987). The *CDC8* transcript is cell cycle regulated in yeast and is expressed coordinately with *CDC9* and *CDC21* at a point preceding histone transcription. Exp. Cell. Res. *171*, 223–231.

Wong, S.W., Wahl, A.F., Yuan, P.M., Arai, N., Pearson, B.E., Arai, K.-I., Korn, D., Hunkapiller, M.W., and Wang, T.S.-F. (1988). Human DNA polymerase α gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. *7*, 37–47.

Wright, A., Maundrell, K., Heyer, W.-D., Beach, D., and Nurse, P. (1986). Vectors for the construction of gene banks and the integration of cloned genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Plasmid *15*, 156–158.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene *33*, 103–119.

Zhang, J., Chung, D.W., Tan, C.-K., Downey, K.M., Davie, E.W., and So, A.G. (1991). Primary structure of the catalytic subunit of calf thymus DNA polymerase δ: sequence similarities with other DNA polymerases. Biochemistry *30*, 11742–11750.