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Cell Cycle-Dependent Phosphorylation and Dephosphorylation of the Yeast DNA Polymerase α-Primase B Subunit

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The yeast DNA polymerase α-primase B subunit functions in initiation of DNA replication. This protein is present in two forms, of 86 and 91 kDa, and the p91 polypeptide results from cell cycle-regulated phosphorylation of p86. The B subunit present in G_1 arises by dephosphorylation of p91 while cells are exiting from mitosis, becomes phosphorylated in early S phase, and is competent and sufficient to initiate DNA replication. The B subunit transiently synthesized as a consequence of periodic transcription of the *POL12* gene is phosphorylated no earlier than G_2. Phosphorylation of the B subunit does not require execution of the *CDC7*-dependent step and ongoing DNA synthesis. We suggest that posttranslational modifications of the B subunit might modulate the role of DNA polymerase α-primase in DNA replication.

Current evidence indicates the existence of two major control points in the cell cycle: one at G_1/S, which regulates initiation of DNA replication, and one at G_2/M, which controls entrance into mitosis (reviewed in reference 54). Although great advances in understanding mitotic control have been made in the past few years, relatively little is known about the G_1 events that lead to the S phase (reviewed in reference 64).

S-phase nuclei contain a positive activator which can prematurely induce DNA synthesis in G_1 cells (62), although the nature of this S-phase-promoting factor remains elusive. Furthermore, all genomic DNA must be replicated once and only once during the S phase prior to entry into mitosis. The integrity of the nuclear envelope or selective transport into the nucleus of key regulatory factors at mitosis appears to play a relevant role in initiation of DNA replication and/or in preventing rereplication of the genome (3). Moreover, DNA replication is temporally and spatially regulated (16), and mechanisms exist which couple the S phase with correct progression through G_1 and entry into mitosis (14, 26, 50, 53).

To improve our knowledge of the regulatory processes controlling entrance into, execution of, and exit from S phase, it is essential to characterize the replication apparatus of a eukaryotic cell and to identify the factors which might be the ultimate targets of the regulatory circuits promoting G_1-to-S-phase transition. In principle, initiation of DNA replication may be controlled by the de novo synthesis of a subset of key replication proteins, by posttranslational modifications of DNA synthesis factors, or by changes in their intracellular localization.

The level of many replication proteins remains relatively constant going from the G_1 to the S phase, and this also appears to be true in *Saccharomyces cerevisiae*, although the amount of the corresponding mRNAs fluctuates during the yeast cell cycle, reaching a peak at the G_1/S-phase boundary (reviewed in references 37 and 48). Recently, we showed that active yeast DNA polymerase α (Pol α) can be inherited from the previous cell cycle and that de novo synthesis of the enzyme at G_1/S is not required for ongoing DNA synthesis (55). Therefore, the level of Pol α, as well as that of other stable replication proteins in yeast cells, is not rate limiting, although it is still possible that de novo synthesis of labile or limiting key protein factors is required to initiate DNA synthesis (38). Alternatively, the activity of critical replication proteins might be modulated by posttranslational modifications. For instance, it has been shown that the p34 subunit of replication protein A is phosphorylated at the G_1/S boundary in both yeast and metazoan cells (11, 13, 15, 23). Finally, cell cycle-regulated nuclear transport has been proposed to explain the role in initiation of DNA replication of the *CDC46*, *MCM2*, and *MCM3* gene products in *S. cerevisiae* (27, 76).

The four-subunit DNA Pol α-primase complex (Pol α-primase) is required during both the initiation and elongation steps of DNA replication (6, 32, 72, 75), and therefore its action is likely to be tightly regulated. The Pol α-primase complex in *S. cerevisiae* contains four polypeptides with apparent molecular masses of 180, 86, 58, and 48 kDa, which are encoded by single and essential genes called *POL1*, *POL12*, *PRI2*, and *PRI1*, respectively (20, 21, 35, 45, 59). The p180 polypeptide has been shown to be the catalytic Pol α subunit, while DNA primase is a heterodimer of the 58- and 48-kDa polypeptides (19, 60). The p48 subunit is sufficient for RNA primer synthesis in vitro, although an auxiliary role for the 58-kDa polypeptide in DNA primase activity in vivo cannot be excluded (42, 67). No enzymatic activity has been found associated with the 86-kDa protein species (B subunit), which is tightly bound to the p180 polypeptide (5). In vitro reconstitution studies with purified components indicate that p86 is not required for Pol α-primase interaction, and its presence does not change the catalytic properties of Pol α (4). It has been proposed that the human p86 homolog might serve as a molecular tether during DNA replication, because this subunit mediates the in vitro interaction between the human Pol α-primase complex and T antigen bound to the simian virus 40 origin of replication (9). Accordingly, we have shown that the B subunit of the yeast Pol α-primase complex executes an essential role at the initial stage of DNA synthesis before the hydroxyurea (HU)-sensitive step (20).

In this paper, we provide evidence that the B subunit is a stable protein which is phosphorylated and dephosphorylated in a cell cycle-dependent manner. The B subunit is transiently synthesized during the S phase as the unphosphorylated form (p86), which starts to be phosphorylated (p91) not earlier than G_2, while preexisting (maternal) p86 is phosphorylated in early S phase. Dephosphorylation of both maternal and newly syn-

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TABLE 1. Yeast strains used in this study

Strain	Description or genotype	Source or reference
Cy46	*ura3-52 leu2-3,112 trp1-63 pol12*::*LEU2*[pFE10 *POL12 TRP1 CEN6*]	21
Cy94	*ura3-52 leu2-3,112 trp1-63 pol12*::*LEU2*[pFE7 *POL12 URA3 CEN4*]	This study
Cy101	*ura3-52 leu2-3,112 trp1-63 pol12*::*LEU2*[pFE109 *pol12*Δ*T4,T5 URA3 CEN4*]	This study
K699	*MAT***a** *ura3-52 leu2-3,112 trp1-1 ade2-1 can1-100 his3-11,15 GAL psi*$^+$	K. Nasmyth
Cy345	*MAT***a** *ura3-52 leu2-3,112 trp1-1 ade2-1 can1-100 his3-11,15 GAL psi*$^+$ *pol12*::*LEU2*[pFE10 *POL12 TRP1 CEN6*]	This study
Cy348	*MAT***a** *ura3-52 leu2-3,112 trp1-1 ade2-1 can1-100 his3-11,15 GAL psi*$^+$ *pol12*::*LEU2*[pFE10 *POL12 TRP1 CEN6*][pFE122 *GAL1*::*POL12 URA3 CEN4*]	This study
Cy349	*MAT***a** *ura3-52 leu2-3,112 trp1-1 ade2-1 can1-100 his3-11,15 GAL psi*$^+$ *pol12*::*LEU2*[pFE122 *GAL1*::*POL12 URA3 CEN4*]	This study
CG378	*MAT***a** *ade5-7 can1 leu2-3,112 trp1-289 ura3-52*	L. H. Johnston
TP11B-4-1	*MAT***a** *ade1 leu2-3,112 ura3-52 prt1-1*	G. Johnston
1393	*MAT*α *trp1 ade1 leu2 his2 cdc28-13*	K. Nasmyth and S. Reed
H28CYB1	*MAT*α *his7 ura1 cdc28-1*	L. Hartwell
H4C1A1	*MAT***a** *his7 ura1 cdc4-1*	L. Hartwell
H7CYA1	*MAT***a** *his7 ura1 cdc7-4*	L. Hartwell
RM14-4B	*MAT***a** *bar1 his6 trp1 ura3 leu2 ade1 cdc7-1*	L. H. Johnston
SB646	*ura3 ade cdc7-3*	J. Campbell
H8C1A1	*MAT***a** *his7 ura1 cdc8-1*	L. Hartwell
17026	*MAT***a** *ade1 ade2 his7 lys2 tyr1 ura1 cdc21-1*	L. Hartwell
H2C2A1	*MAT***a** *his7 ura1 cdc2-2*	L. Hartwell
H6C1A1	*MAT***a** *his7 ura1 cdc6-1*	L. Hartwell
H9C1A1	*MAT***a** *his7 ura1 cdc9-1*	L. Hartwell
H5C1B1	*MAT***a** *his7 ura1 cdc5-1*	L. Hartwell
2593	*MAT***a** *ade1 ade2 gal1 his1 lys2 tyr1 ura1 cdc20-2*	K. Nasmyth and L. Hartwell
1082	*MAT*α *trp ade leu ura lys tyr cdc14-3*	K. Nasmyth
1081	*MAT***a** *ade1 ade2 ura1 tyr1 his7 lys2 gal1 cdc15-4*	K. Nasmyth
1941	*MAT***a** *ade1 ade2 gal1 his7 lys2 trp1 ura1 cdc18-1*	K. Nasmyth and A. Murray
1756	*MAT***a** *can1-100 ura3 ade2-1 met his3 trp1-1 ho-*β*gal swi5 SUP4-o cdc15-2*	K. Nasmyth
L128-20	*MAT***a** *ura3 trp1 ade5 dbf4-1*	L. H. Johnston
J2	*MAT***a**/α *arg4/ARG4 trp1-289/TRP1 ura3-52/URA3 his1/HIS1 trp2/TRP2 can1/CAN1 ade5/ADE5 leu2-3,112/LEU2 dbf2-2/dbf2-2*	L. H. Johnston

thesized B subunit occurs while cells are exiting mitosis. The essential function performed by the B subunit at the initial stage of DNA replication is likely to be carried out by the p86 polypeptide inherited from the previous cell cycle, since de novo synthesis of p86 is not required for ongoing DNA synthesis.

MATERIALS AND METHODS

Plasmids. Plasmid pAC27 (a generous gift from D. Hinkle, University of Rochester, Rochester, N.Y.) and plasmids pFE2, pFE3, pFE7, and pFE10 were described previously (20). Plasmid pFE109, containing an internal deletion of the *POL12* gene (*pol12*Δ*T4*, *T5*), was derived from plasmid pFE7 as described previously (20). Plasmid pFE122 was constructed by cloning the blunted 2.5-kb *Sac*I-*Kpn*I *POL12* fragment from plasmid pJN13 (5) into the *Bam*HI site of the centromeric plasmid pBM125 (55), downstream of the *GAL1* promoter.

Yeast strains. Plasmids pFE7 and pFE109 carrying the wild-type *POL12* gene and the *pol12*Δ*T4,T5* allele, respectively, were used to transform strain Cy46 (20). Strains Cy94 and Cy101 were derived from transformants cured from the pFE10 centromeric plasmid (*POL12 CEN6 TRP1*) by plasmid-shuffling procedures (20). Strain Cy345 was constructed as follows. Strain K699 was transformed with the centromeric plasmid pFE10, and the chromosomal copy of the *POL12* gene was disrupted by one-step gene replacement by using the 4.1-kb *Sac*I-*Kpn*I fragment from plasmid pAC27, as previously described (20). The replacement of the *POL12* chromosomal copy was verified by Southern analysis. Strain Cy345 was then transformed with plasmid pFE122 (*GAL1-POL12 CEN4 URA3*) to originate strain Cy348. Strain Cy349 was derived from strain Cy348 after curing from the pFE10 plasmid as described above. Other yeast strains and their genotypes are listed in Table 1.

Preparation of yeast protein extracts and Western blot analysis. Preparation of protein extracts from trichloroacetic acid-treated yeast cells was described previously (20). Ascitic fluid (1:2,000 dilution) of mouse monoclonal antibody 6D2 (20) has been used as primary antibody to detect the *POL12* gene product on Western immunoblots of protein extracts. Alkaline phosphatase-conjugated anti-mouse immunoglobulin G was used as the secondary antibody as described previously (18).

In vivo labelling of yeast proteins. For ^{35}S protein labelling, yeast cells were grown in synthetic medium (65) supplemented with the necessary amino acids and pulse-labelled with 250 μCi of [^{35}S]methionine (Amersham) per ml for 12 min, unless otherwise indicated in the figure legends. In some experiments, incorporation of [^{35}S]methionine was chased by addition of cold methionine at a final concentration of 2 mM. Then 100 μl of 50% cold trichloroacetic acid was added to 1 ml of cells, and the mixture was kept in ice for 10 min. Samples were spun in an Eppendorf microcentrifuge, and after two washes with cold acetone, the pellet was resuspended in 50 μl of boiling buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and an equal volume of glass beads was added. Cells were disrupted by vortexing for 3 min followed by boiling for 4 min. After addition of 1 ml of Tween 20 IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA), a crude protein extract was recovered by centrifugation for 2 min in a microcentrifuge. Protein labelling with $^{32}P_i$ (1 mCi/ml) was carried out for 1 h in exponentially growing cells in YEPD medium in which the P_i was removed by precipitation with Mg^{2+} at high pH (11). Protein extracts were prepared as described above. The level of the labelled precursors incorporated into proteins was monitored by spotting a 3-μl aliquot of the extract on GF/C fiberglass disks (Whatman), which were then kept for 3 min in 10% boiling trichloroacetic acid. The filters were washed three times with H_2O and twice with ethanol and then counted in a liquid scintillation apparatus.

Immunoprecipitation of the B subunit. Aliquots of the extract containing either 5×10^7 ^{35}S total counts or 10^9 ^{32}P total counts were adjusted to 1 ml and precleared overnight at 4°C by absorption with 60 μl of Gamma Bind Plus suspension (Pharmacia). A mixture of 6D2, 5E5, and 2H8 monoclonal antibodies (MAbs) recognizing the B subunit of the Pol α-primase complex (21) (5 μg each) was prebound to 60 μl of Gamma Bind Plus suspension and incubated with the precleared extract for 1 h at 4°C. After centrifugation, the immunoprecipitate was washed four times with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 8.0]) and the final pellet was resuspended in 100 μl of 1% SDS before being boiled for 4 min. The samples were then centrifuged for 2 min in a microcentrifuge, and 900 μl of Tween 20 IP buffer was added to the supernatant. The immunoprecipitation procedure was repeated, since we found that a second immunoprecipitation greatly reduced the background. Immunoprecipitates derived from ^{32}P-labelled cells were treated with 1 μg of RNase A for 15 min at 30°C. The final pellet was resuspended in 20 μl of Laemmli buffer, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (66).

Treatment of the B subunit with potato acid phosphatase. Protein extracts were prepared by resuspending yeast cells in an equal volume (wt/vol) of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–0.5 M NaCl–1 mM dithiothreitol–1 mM EDTA–20% glycerol–1 mM phenylmethylsulfonyl fluoride–1 μg of aprotinin per ml–1 μg of pepstatin per ml–1 μg of leupeptin per ml–20 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) per ml (all protease inhibitors were from Sigma) followed by homogenization in liquid nitrogen and centrifugation. A 1-ml sample of a 1:10 suspension (in the above buffer without dithiothreitol) of protein A–Sepharose CL-6B covalently linked to the anti-Pol α MAb y48 (60) was added to 100 μl of crude extract, and the mixture was incubated for 90 min at 4°C. After being washed with 1 ml of Tris-buffered saline, the MAb protein A-Sepharose was resuspended in 1 ml of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.0)–1 mM dithiothreitol, divided into two samples, and incubated at 30°C for 60 min with or without the addition of 20 μg of potato acid phosphatase. After being washed, the resin was resuspended in 20 μl of Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

Cell synchronization by α-factor treatment. Strain CG378 was synchronized by α-factor treatment as described previously (58). In all the synchronization experiments, time zero corresponds to the sample taken after release from the α-factor block by filtration and washing. At the indicated time intervals, aliquots of cells were sonicated for 10 s and immediately fixed by addition of an equal volume of 3.7% formaldehyde–0.15 M NaCl. Fixed cells were microscopically analyzed for the percentage of budded cells. Northern (RNA) analysis of total RNA from synchronous cells and the probes to detect histone H2A and *POL1* mRNA were described previously (58); the 4,360-bp *Xba*I-*Nco*I fragment spanning the *POL12* locus was used as probe to detect the *POL12* mRNA. Preparation of protein extracts, immunoprecipitation, and Western blot analysis are described above.

***POL12* expression driven by the *GAL1* promoter.** Strain Cy349 (Table 1) was used for analysis of *POL12* expression as previously described (55). Briefly, cells were grown at 28°C under selective conditions in synthetic medium containing 2% galactose to a concentration of 7×10^6 cells per ml. Glucose (final concentration, 2%) and α-factor were added to turn off *POL12* transcription driven by the *GAL1* promoter and to arrest cells in G_1. Cells were then filtered, washed, and resuspended in synthetic medium containing 2% glucose. Aliquots of the culture, which divided synchronously for at least two cell divisions, were taken every 15 min, and the level of the *GAL1-POL12* mRNA and that of the B subunit were monitored by Northern and Western blot analysis, respectively.

Cell cycle analysis with inhibitors and *cdc* strains. Cell cycle analysis with specific inhibitors or *cdc* strains was performed as described previously (11), unless otherwise indicated in figure legends. Briefly, α-factor was used at 2 μg/ml for 2 h, hydroxyurea was used at 80 mM for 3.5 h, and nocodazole was used at 20 μg/ml for 3.5 h. *cdc* strains were grown to 5×10^6 cells per ml at 23°C (permissive temperature) in YEPD medium (65) and then shifted to 38°C (restrictive temperature) in YEPD medium for 5 h. Effective cell cycle arrest was monitored by microscopy analysis.

Synchronization of *cdc15-2* mutant cells. The conditions used to synchronize *cdc15-2* mutant cells in late anaphase were exactly as previously described (71). Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining (20), and the percentage of anaphase spindles was measured by in situ immunofluorescence with anti-tubulin MAbs (39).

RESULTS

The B subunit of the yeast Pol α-primase complex is present in two forms which differ in their extent of phosphorylation. The molecular mass of the B subunit of the yeast Pol α-primase complex, deduced from the largest open reading frame found in the corresponding *POL12* gene, is 78.9 kDa (20, 68), but the apparent mass of the purified protein is 86 kDa (5, 20). Such a discrepancy is not uncommon; it might be due to the structure of the protein itself or to a variety of posttranslational modifications or both. We have produced a panel of MAbs which specifically recognize the yeast B subunit, and they have been useful in establishing the identity of the cloned *POL12* gene (20). Approximately equimolar amounts of two forms of the B subunit (86 and 91 kDa) were clearly distinguishable on Western blots of protein extracts prepared from logarithmically growing yeast cultures when cells were treated with trichloroacetic acid to inhibit any protease and phosphatase (Fig. 1A). To demonstrate that the two polypeptides were indeed different forms of the same gene product, we probed a Western blot of protein extract prepared from strain CY101, which contains a lethal chromosomal deletion of the *POL12* gene complemented by the *pol12*Δ*T4*, *T5* allele carried on a centromeric plasmid (20). The *pol12*Δ*T4*, *T5* allele has suffered a 12-codon deletion within the *POL12* open reading frame, causing synthesis of a smaller protein, which is still able to fully complement a lethal chromosomal disruption of the *POL12* gene. As shown in Fig. 1A, both immunoreactive polypeptides were smaller in the extract prepared from strain CY101 than in the wild-type extract, thus proving that they are translation products of the *POL12* gene.

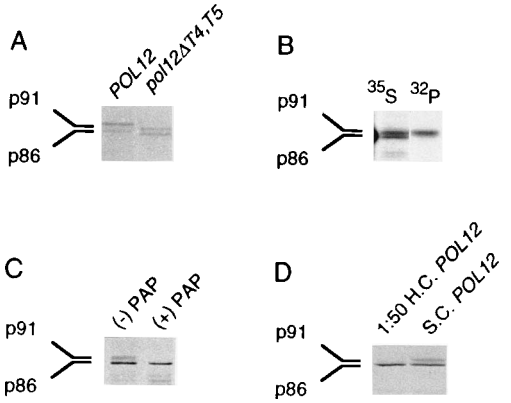

FIG. 1. The *POL12* gene product is present in two forms. (A) Western blots of protein extracts (40 μg) prepared from strain Cy94 (*POL12*) or its isogenic derivative Cy101 (*pol12ΔT4,T5*) were separated by SDS-PAGE, and the blots were probed with anti-B subunit MAb 6D2 as the primary antibody. (B) SDS-PAGE and autoradiography of immunoprecipitates obtained with anti-B-subunit MAbs from protein extracts prepared from CG378 cells metabolically labelled with [^{35}S]methionine (^{35}S) or $^{32}P_i$ (^{32}P). (C) Western blots of immunoprecipitates analyzed by SDS-PAGE after 60 min of incubation at 30°C in buffer with or without 20 μg of potato acid phosphatase (PAP). (D) Western blots of protein extracts prepared from CG378 cells (S.C.*POL12*) or from CG378 cells transformed with a high-copy-number plasmid (pFE3) carrying the *POL12* gene (1:50 H.C.*POL12*). The extract from cells overexpressing the *POL12* gene has been diluted 50-fold to identify the overexpressed polypeptide and its phosphorylation state. Since the amount of p91 form in the high-copy-number *POL12* strain is comparable to that in the untransformed *POL12* strain, the p91 polypeptide is no longer detectable in the 50-fold-diluted sample. S.C., single copy.

Both the 86- and 91-kDa polypeptides (called p86 and p91, respectively) were found in immunoprecipitates from ^{35}S-labelled cell extracts, while a single radioactive band comigrating with p91 was detected in immunoprecipitates from ^{32}P-labelled yeast extracts (Fig. 1B). Furthermore, the p91 polypeptide was converted to the p86 form by treatment with potato acid phosphatase (Fig. 1C). Therefore, p91 is a phosphorylated form of the *POL12* gene product. Several potential phosphorylation sites are present in the deduced amino acid sequence of the Pol α-primase B subunit, although at present we do not have any information on the amino acid residues phosphorylated in vivo. The availability of several *POL12* alleles carrying different internal deletions of the gene (20) should facilitate their mapping.

Surprisingly, only p86 was found to be overproduced in protein extracts prepared from cells transformed with a high-copy-number plasmid carrying the *POL12* gene under the control of its own promoter (Fig. 1D), while the level of p91 was maintained at the level found in cells transformed with the vector alone. It is worth noting that overexpression of the *POL12* gene does not cause any apparent phenotype and that the overproduced protein is correctly localized within the nucleus (20). Therefore, unbalanced stoichiometry of the complex subunits and failure to phosphorylate overproduced p86 do not affect nuclear transport, although p86 phosphorylation appears to be carried out by some rate-limiting mechanism.

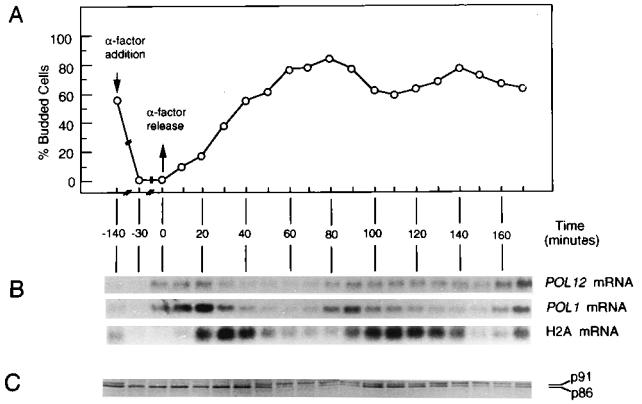

FIG. 2. Cell cycle-regulated phosphorylation of the B subunit. A logarithmically growing culture (7×10^6 cells per ml) of strain CG378 was synchronized by α-factor treatment. Times of addition and removal of α-factor are indicated by arrows, and samples were taken at the indicated times. (A) Budding profile. (B) Total RNA (5 μg) was used to monitor the fluctuation of the *POL12*, *POL1*, and H2A histone gene transcripts during the cell cycle by Northern blot analysis. (C) Total protein (25 μg) present in extracts prepared at the indicated times was separated by SDS-PAGE and analyzed on a Western blot probed with the anti-B-subunit 6D2 MAb.

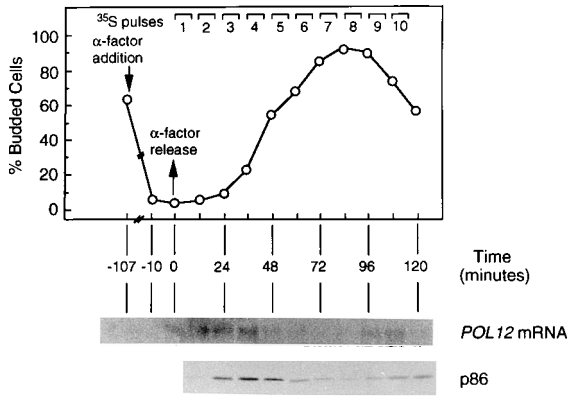

FIG. 3. Transient synthesis of the B subunit during the cell cycle. A logarithmically growing culture of strain CG378 was synchronized by α-factor treatment; the times of addition and removal of α-factor are indicated by arrows. Pulses (12 min in duration; indicated by brackets) with [^{35}S]methionine were performed after release from the α-factor block. The budding profile was monitored by microscopic analysis, and total RNA and protein extracts were prepared from samples of synchronously dividing cells taken at the indicated times. Total RNA (5 μg per lane) was used to monitor the fluctuation of the *POL12* mRNA on a Northern blot, and aliquots of the protein extracts each containing 5×10^7 ^{35}S total counts were immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

The *POL12* gene is periodically transcribed, and the reciprocal level of the two B-subunit forms changes during the cell cycle. In *S. cerevisiae*, the transcript level of most DNA synthesis genes peaks at the G_1/S boundary, and it has been demonstrated that the sequence 5′-ACGCGT-3′ (also called *Mlu*I cell cycle box [MCB]) is the essential *cis*-acting element required for the regulated expression of these genes (reviewed in reference 37). Since the 5′ noncoding region of the *POL12* gene contains two MCBs at positions −221 and −198 from the first ATG, we tested whether the level of *POL12* mRNA fluctuated during the cell cycle with the same kinetics as for other DNA synthesis genes. Moreover, since the B subunit of the Pol α-primase complex performs an essential function at an early stage of DNA replication before the HU-sensitive step and is present in two forms, we also tested the phosphorylation state of the protein during the cell cycle. Yeast cells were synchronized by α-factor treatment (Fig. 2A) (55), and the levels of *POL12* mRNA and the B-subunit polypeptide were measured in cells which divided synchronously for two generations. As shown in Fig. 2B, the level of the *POL12* transcript fluctuated, reaching a peak before the accumulation of histone H2A mRNA. Identical kinetics was observed for the *POL1* mRNA in the same synchronous culture analyzed in this experiment (55). Therefore, the *POL12* gene belongs to the class of periodically expressed DNA synthesis genes which are all likely to be coregulated through the MCB and common transcription factors (12, 41, 43, 48, 73). Western blot analysis of protein extracts (Fig. 2C) indicated that the B subunit is present at every stage of the cell cycle but that the levels of the two forms of B subunit are not constant. Only the p86 polypeptide was present in cells blocked by α-factor treatment, whereas phosphorylated p91 became clearly detectable when ~60% of cells had budded after the accumulation of the H2A mRNA, which is considered a marker of the middle of the S phase (28). A limited level of p86 was present during the G_2 and M phases of the first cell cycle; however, p91 was certainly the most abundant at both stages. A fluctuation in the relative level of p86 and p91 was still visible in the second cell cycle, although cell division synchrony was decreasing. These data show that the B subunit is a stable protein and that its steady-state level at most doubled following the peak of *POL12* transcription, similar to what we found for the p180 Pol α polypeptide (55).

The B subunit is periodically synthesized during the cell cycle. Because of the periodic transcription of the *POL12* gene at G_1/S, the p86 polypeptide present in α-factor-arrested cells (Fig. 2) must have been inherited from the previous cell cycle. To correlate the time of *POL12* transcription with the synthesis of the corresponding polypeptide, the rate of B-subunit synthesis in a synchronous culture was monitored by performing several pulses with [^{35}S]methionine during the cell cycle. The level of the labelled protein was analyzed by gel electrophoresis of immunoprecipitates derived from protein extracts prepared at the end of the pulses. As shown in Fig. 3, the B subunit was transiently synthesized as the p86 unphosphorylated form. The maximal rate of synthesis occurred when 10 to 50% of cells had budded (early S phase), although residual synthesis was observed at later times.

This observation raises the question whether the de novo synthesized protein plays any role in the initiation of DNA synthesis within the same cell cycle. Although a direct answer to this question is not yet available, we observed that yeast cells can enter the S phase in the absence of *POL12* transcription. In fact, CY349 cells, containing a lethal *POL12* chromosomal disruption complemented by a *GAL1-POL12* fusion (Table 1; see Materials and Methods) were able to enter the S phase when they were synchronized by α-factor treatment and released in glucose-containing media (data not shown). Similar to what we found for the Pol α polypeptide (55), cells were able to divide for several generations in glucose, although *POL12* transcription was no longer detectable and the level of B subunit dropped far below the physiological level measurable in isogenic wild-type cells. Therefore, de novo synthesis of the B subunit does not appear to be required to support ongoing DNA synthesis.

Newly synthesized p86 and maternal p86 are phosphorylated at different times during the cell cycle. The above-described data show that the B subunit is transiently synthesized in the S phase as the p86 form and that the newly synthesized protein does not seem to be necessary to drive G_1/S-phase transition. The p86 B subunit present at START and inherited

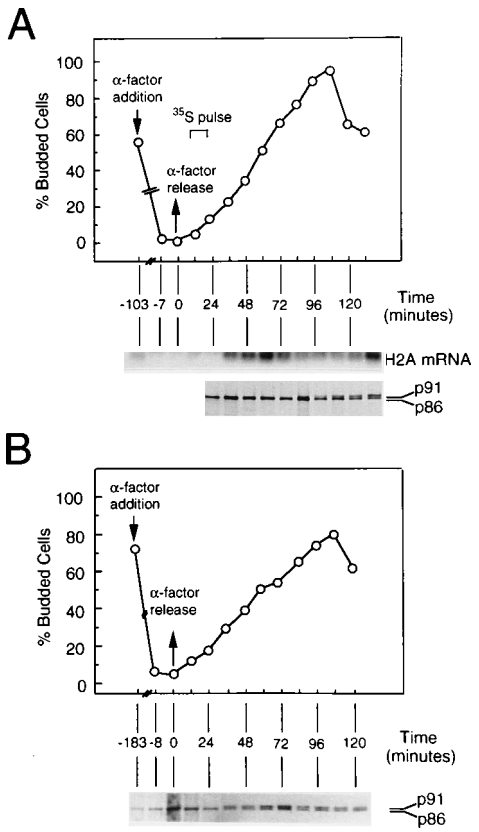

FIG. 4. Dual phosphorylation timing of newly synthesized and maternal B subunit. (A) A logarithmically growing culture of strain CG378 was synchronized by α-factor treatment. The times of addition and removal of α-factor are indicated by arrows, and the budding profile was monitored by microscopic analysis. After a 12-min pulse with [^{35}S]methionine (indicated by the bracket), cellular labelling was chased by addition of an excess of cold methionine. Samples of cells were taken at the indicated times, and the levels of the H2A histone transcript and B subunit were analyzed, respectively, by Northern blotting and immunoprecipitation followed by SDS-PAGE and autoradiography. Aliquots containing 5 μg of total RNA or 5×10^7 ^{35}S total counts were analyzed in each lane. (B) A logarithmically growing culture of strain CG378 was pulse-labelled for 12 min with [^{35}S]methionine before concomitant addition of an excess of cold methionine and α-factor. The budding profile was monitored microscopically, and aliquots of the protein extracts containing 5×10^7 ^{35}S total counts were immunoprecipitated and analyzed in each lane.

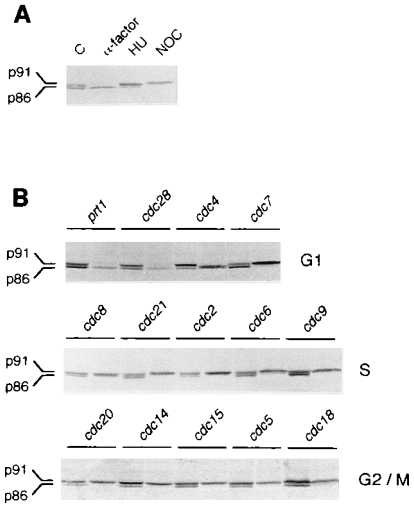

FIG. 5. B subunit in cells arrested at different stages of the cell cycle. (A) Western blot of protein extracts prepared from exponentially growing CG378 cells (C) or after treatment of cultures of the same strain with α-factor, HU, or nocodazole (NOC). (B) Western blots of protein extracts prepared from different *cdc* mutant strains (Table 1; also see the text). For each of the mutant strains tested, the extract analyzed in the left lane was prepared from cells grown at the permissive temperature (25°C), while the extract analyzed in the right lane was prepared from cells shifted to the restrictive temperature (38°C) for 5 h.

from the previous cell cycle appears to be sufficient to perform this task. Since it was reasonable to hypothesize that phosphorylation of p86 could play a critical role in modulating B-subunit function, we decided to test the phosphorylation timing of the two pools of protein (maternal and newly synthesized) by carrying out pulse-chase labelling experiments. De novo synthesized p86 was followed by pulse-labelling of synchronously dividing cells at the time of maximal B-subunit synthesis and then by a chase with an excess of cold methionine. The time of phosphorylation of B subunit inherited from the previous cell cycle was monitored by pulse-labelling of logarithmically growing cells followed by a chase with cold methionine and concomitant α-factor addition. When more than 95% of cells were found to be arrested at START, α-factor was removed and samples of synchronously dividing cells were collected. In both experiments, the time of appearance of phosphorylated p91 was analyzed by SDS-PAGE of immunoprecipitates obtained from protein extracts prepared at different times after the chase.

As shown in Fig. 4A, phosphorylation of newly synthesized p86 starts 96 min after release from the α-factor block, when ~90% of cells have formed large buds. This time follows the periodic accumulation of histone H2A mRNA, indicating that phosphorylation of newly synthesized B subunit starts not earlier than G_2. Only approximately 50% of p86 seems to be converted into p91. Either phosphorylation of de novo synthesized p86 is rate limiting or dephosphorylation of p91 is also cell cycle controlled and cell synchrony late in the experiment is not sufficient to identify the stage in which all p86 is phosphorylated.

As shown in Fig. 4B, the B subunit already present in α-factor-arrested cells migrates as a single polypeptide of 86 kDa, in agreement with the Western blot analysis presented in Fig. 2C. This pool of protein of maternal origin starts to be phosphorylated 36 min after release from the α-factor block, when 30% of cells are budded. Its phosphorylation timing is clearly anticipated relative to that of the newly synthesized B subunit and corresponds approximately to early S phase. This dual timing of phosphorylation may explain the pattern of overall p86 phosphorylation during the cell cycle observed by Western blotting and shown in Fig. 2C. Moreover, the pulse-chase technique, which allows the distinction between the two pools of protein, is by far more sensitive than Western blotting to monitor the time of transition from p86 to p91.

When exponentially growing cells were pulse-labelled with [^{35}S]methionine and samples were taken and analyzed at different times after the chase, the B subunit began to be phosphorylated ~75 min after the pulse (data not shown), in agreement with results from synchronous cultures (Fig. 4A). This finding indicates that α-factor treatment does not influence the timing of the phosphorylation process, and from this experiment, we could calculate that the half-life of the protein is >6 h.

Unphosphorylated p86 accumulates only in cells arrested in G_1. The above-described pulse-chase experiments have been useful in determining the time of phosphorylation of the ma-

ternal and newly synthesized B subunit in synchronously dividing cells. Moreover, as shown in Fig. 5, specific inhibitors and cell division cycle (*cdc*) mutants (25, 61) were used to test which form of the B subunit accumulated when the execution of essential functions required for correct cell cycle progression was prevented. As already pointed out, only the p86 form was found in cells arrested at START by α-factor treatment. Conversely, the p91 form of the B subunit drastically accumulated when cell growth was blocked with HU or nocodazole (Fig. 5A), which arrest cell cycle progression in S or G_2/M by interfering with deoxynucleotide biosynthesis or tubulin polymerization, respectively (34, 69). The small amount of p86 still detectable in HU-arrested cells is probably due to incomplete cell cycle block, since morphological examination and DAPI staining of these cells indicated that only 80 to 90% were blocked with a typical dumbbell-shaped morphology (data not shown).

As shown in Fig. 5B, equimolar amounts of p86 and p91 were present in extracts prepared from cells grown at the permissive temperature (25°C) for all the *cdc* mutant strains tested. Conversely, after a shift to 38°C, p86 was the only form present in cells carrying temperature-sensitive mutations in the *CDC28* and *PRT1* genes. The *CDC28* gene encodes the budding yeast p34 protein kinase, and execution of the *CDC28*-dependent step is required for passage through START and commitment to complete the cell division cycle (reviewed in references 57 and 63). The *PRT1* gene, also known as *CDC63*, encodes a subunit of the eukaryotic initiation factor 3 (eIF3) translational factor (7), and mutations in this gene block cells in pre-START (24). The *CDC4* gene encodes a protein homologous to β-transducins and is required to execute an event following the *CDC28*-dependent step in G_1 (22, 30). We consistently found that *cdc4-1* mutant cells shifted to 38°C contained, besides the p86 form, an additional protein band with an intermediate molecular mass between 86 and 91 kDa, possibly representing an intermediate product of the phosphorylation reaction. The *CDC7* gene product is a protein kinase required for G_1/S transition and for initiation but not elongation of DNA replication (30, 31, 77). Moreover, cells aligned at the *CDC7*-dependent step can complete DNA synthesis in the presence of cycloheximide, indicating that protein synthesis is not required to complete DNA replication after this step (29). The p91 form clearly accumulated in *cdc7-4* mutant cells after the shift to 38°C (Fig. 5B). Analogous results (not shown) were obtained with cells containing the *cdc7-1* or *cdc7-3* allele and with the *dbf4-1* strain carrying a mutation which affects a regulatory subunit of the *CDC7* gene product (33, 40).

Only the p91 form was found in protein extracts prepared after the shift to the restrictive temperature (Fig. 5B) of all the other *cdc* mutants tested, which lacked functions required for execution of cell cycle events necessary for progression through S, G_2, and mitosis.

From our overall analysis of the *cdc* mutants, we conclude that phosphorylated p91 is the form of the B subunit which accumulates when correct progression of the cell cycle from early S to late mitosis is prevented. Conversely, p86 is the only form of the B subunit found in cells arrested in G_1 before the *CDC7*-dependent step. The results obtained with *cdc* mutants must be interpreted cautiously, because it is known that some biochemical pathways continue normally, even in the presence of a cell cycle block caused by a temperature-sensitive *cdc* mutation. However, the conclusion based on the analysis of the tested *cdc* mutants is essentially in agreement with the timing of B-subunit phosphorylation observed in synchronously dividing cells. Furthermore, consistent results were obtained with a variety of mutations and treatments that cause cell cycle arrest at a specific stage.

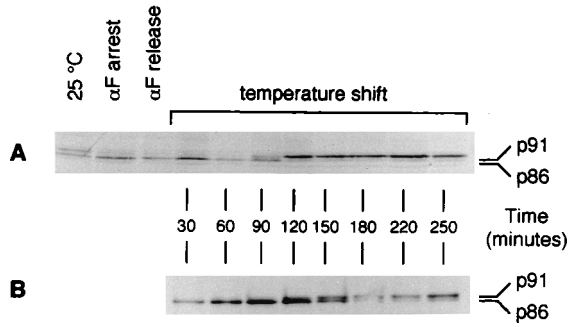

FIG. 6. B-subunit phosphorylation does not require execution of the *CDC7*-dependent step. Cells carrying the *cdc7-4* allele were grown in synthetic medium at 25°C to a concentration of 5×10^6 cells per ml before addition of α-factor (αF). When more than 95% of the cells were unbudded, the α-factor block was released by washing and resuspending the cells with prewarmed medium (38°C). [^{35}S]methionine (250 μCi/ml) was added to part of the culture. Samples of cells, kept at 38°C, were taken at the indicated times and processed for Western blot analysis (A) or immunoprecipitation, SDS-PAGE, and autoradiography (B). Aliquots of protein extracts containing 5×10^7 ^{35}S total counts were immunoprecipitated and analyzed in each lane of panel B.

Synthesis and phosphorylation of the B subunit do not require execution of the *CDC7*-dependent step. It is well known that execution of the *CDC7*-dependent step is required for initiation of DNA synthesis in *S. cerevisiae* (30, 31). To test whether B-subunit synthesis and phosphorylation require the execution of the *CDC7*-dependent step, *cdc7-4* cells were synchronized by α-factor treatment and released from the α-factor block at the restrictive temperature in the presence of [^{35}S]methionine. Samples were taken at 30-min intervals and analyzed by immunoprecipitation and Western blot to correlate the time of synthesis and phosphorylation of p86 with the steady-state level of the B subunit. As shown in Fig. 6, de novo synthesis of the B subunit can occur even when the *CDC7* gene product is inactivated by the temperature shift. Both maternal and newly synthesized p86 become phosphorylated, indicating that the protein kinase responsible for p86 phosphorylation is unlikely to be the *CDC7* gene product and that its activity is not dependent on *CDC7* function. Phosphorylation of de novo synthesized p86 clearly takes place later than phosphorylation of the maternal protein, in agreement with the results of the pulse-chase experiments (Fig. 4). In fact, as shown in Fig. 6A, the appearance of the p91 form in the Western blot starts 90 min after the temperature shift, while the labelled p91 form is detectable after 120 min (Fig. 6B).

It is not yet known whether *cdc7* cells may initiate the replicative process but arrest at a very early stage (before DNA synthesis). Our finding that phosphorylation of p86 takes place in *cdc7-4*-arrested cells suggests that this modification does not require DNA synthesis. Since the Cdc28 protein kinase remains active in *cdc7*-arrested cells (2), B-subunit phosphorylation might be mediated either directly or indirectly by the *CDC28* gene product. Alternatively, a kinase that acts downstream by a timing mechanism might be produced between the *CDC4*- and *CDC7*-dependent steps. In any case, while new protein synthesis is not required for DNA synthesis after the *CDC7*-dependent step (30), protein synthesis after the *CDC28*-dependent step is necessary for B-subunit phosphorylation. In fact, such modification did not occur in *cdc28-13* mutant cells after release from the temperature block in the presence of cycloheximide (our unpublished observation), although peri-

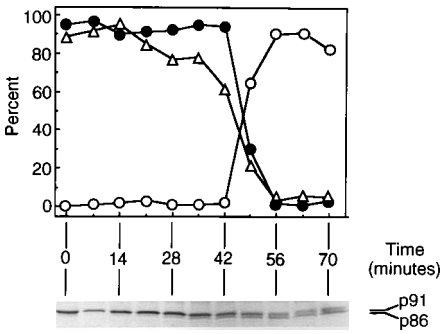

FIG. 7. Dephosphorylation of the p91 form of the B subunit occurs while cells exit mitosis. A *cdc15-2* mutant culture was blocked by a shift to 36°C for 3 h. At time zero, the arrested culture was released into fresh medium at the permissive temperature (25°C) and aliquots of cells were taken at 7-min intervals. At the indicated times, protein extracts were prepared and analyzed by SDS-PAGE and Western blotting with the anti-B subunit 6D2 MAb as the primary antibody. Cell morphology and nuclei were visualized microscopically after DAPI staining to calculate the percentage of cells with one (○) or two (●) nuclei. The percentage of cells containing elongated anaphase spindles was measured with anti-tubulin antibodies by indirect immunofluorescence (△).

odic transcription of DNA synthesis genes is not prevented under the same conditions (46).

Dephosphorylation of p91 occurs while cells are exiting mitosis. From the analysis of the B subunit in *cdc*-arrested cells, it appears that phosphorylated p91 is present in cells blocked late in mitosis (*cdc15*, *cdc5*, *cdc18*, and *dbf2* [Fig. 5 and data not shown]) whereas p86 accumulates in cells arrested early in G_1 (*prt1*). To examine the appearance of the p86 form more closely, *cdc15-2* cells were synchronously blocked at the restrictive temperature in mitosis and then released into fresh medium at the permissive temperature (70, 71). Samples were taken at intervals after release and analyzed by Western blotting and microscopy (Fig. 7). In this experiment, unphosphorylated p86 starts to be detectable in *cdc15-2* cells 35 min after release from the temperature block. At this time, ~90% of cells in the population have large buds and contain two separate nuclei. Cells with one single nucleus abruptly appear 49 min after the release. To define further when the p91-to-p86 conversion is starting, the state of the mitotic spindle was used as a cytological marker. At 35 min, when p86 becomes clearly detectable, nearly 80% of cells are at a late stage in mitosis, when the daughter nuclei are still connected by elongated spindles (71).

It is known that perturbation of mitotic events might cause G_1 cell cycle regulatory events, such as execution of START, to occur earlier (36). However, by considering that *prt1*-arrested cells, which are blocked in the pre-START interval, accumulate the p86 form and that p91 dephosphorylation begins before the breakdown of the mitotic spindle, we suggest that the p91-to-p86 transition occurs while cells are exiting mitosis and before execution of the *PRT1*-dependent step.

DISCUSSION

An experimental strategy to try to understand the mechanisms controlling entry into, progression of, and exit from the S phase consists of identifying which replication proteins are targets of the cell cycle circuits and then working back to the identification of the regulatory pathway(s). Of the known replication proteins, the replication protein A single-stranded DNA-binding protein and the Pol α-primase complex are likely to be involved in controlling initiation of DNA synthesis. In fact, studies with the simian virus 40 in vitro replication system indicate that these two protein complexes are required for initiation of both the leading and lagging strands (8, 32, 72, 74). Unwinding at origins is probably the first step in DNA synthesis, and in the simian virus 40 system, this reaction is stabilized by the functional interaction between T antigen, replication protein A, and Pol α-primase (52). The Pol α-primase complex is unique in its ability to start DNA synthesis de novo and must be loaded at replication origins to initiate leading-strand DNA synthesis. Subsequently, through a complex mechanism requiring other replication factors, Pol α-primase will switch to synthesize the Okazaki fragments on the lagging strand (72, 74, 75).

Increasing evidence indicates that the in vivo role of proteins involved in initiation of DNA replication is modulated by phosphorylation and dephosphorylation events carried out by a variety of protein kinases and phosphatases. In fact, the role of T antigen in initiation of DNA replication is positively and negatively regulated by its state of phosphorylation (47, 49). Similarly, the p34 subunit of replication protein A is phosphorylated at the G_1/S boundary in both yeast and mammalian cells (11, 13, 15, 23), and we have found that the yeast Pol α-primase complex B subunit undergoes cell cycle-dependent phosphorylation and dephosphorylation events. It has also been shown that the human B subunit is hyperphosphorylated in mitosis (56), and the phosphorylation state of this polypeptide might account for the finding of two populations of murine Pol α-primase complexes, only one of which interacts with polyomavirus T antigen (51).

Transcriptional and posttranscriptional regulatory mechanisms controlling entry into the S phase might be superimposed in budding and fission yeasts. In fact, the large majority of DNA synthesis genes in *S. cerevisiae* and at least two genes in *Schizosaccharomyces pombe* are periodically transcribed at the G_1/S boundary (38, 44, 48). However, it has been demonstrated that mutations in the regulatory genes (41, 43) or deletion of the MCB (our unpublished observations) do not fatally perturb cell cycle progression in *S. cerevisiae*. The B subunit of the Pol α-primase complex is periodically synthesized following the peak of the *POL12* mRNA. However, de novo synthesis of the protein does not seem to be required for entry into the S phase, similar to what was found for the catalytic Pol α polypeptide (55), indicating that the level of at least these stable replication proteins is not rate limiting. In fact, cells can grow and replicate their DNA even when the amount of B subunit is smaller than that found under physiological conditions. Although we cannot exclude the possibility that the newly synthesized protein, which arises from periodic transcription of the *POL12* gene, can be used during the same cell cycle, the in vivo function of the B subunit is likely to be regulated by the cell cycle-dependent phosphorylation and dephosphorylation events described in this work.

The B subunit of the Pol α-primase complex is quite unusual. In fact, a temperature-sensitive *pol12* allele fails to execute a function required before the HU-sensitive step (20), while the Pol α-primase complex is required during all of S phase to carry out discontinuous DNA synthesis of the lagging strand (74). It appears that yeast cells contain two pools of B subunit, one inherited from the previous cell cycle and the second resulting from periodic translation of the *POL12* mRNA early in S phase. As discussed above, the maternal B subunit appears to be competent and sufficient to support DNA synthesis.

The following circumstantial evidence indicates that the B subunit might play a regulatory role. (i) The biochemical prop-

erties of the Pol α-primase complex in vitro are not influenced by the presence of the B subunit (4). (ii) The yeast B subunit is required for the initial stage of DNA synthesis before the HU-sensitive step (20), and the human homolog mediates the in vitro interaction between Pol α-primase and T antigen (9). (iii) The B subunit is phosphorylated and dephosphorylated in a cell cycle-dependent manner (see above). At present, of the replication proteins, only the p34 subunit of replication protein A and the B subunit of Pol α-primase complex are known to be periodically phosphorylated during the cell cycle. We suggest that the unphosphorylated form of the B subunit is active before undergoing DNA synthesis. In fact, we found that the B subunit is dephosphorylated late in mitosis and that the execution point of the *pol12-T9* mutant precedes the HU-sensitive step (20), whereas phosphorylated B subunit accumulates in HU-arrested cells. Since recent data support the possibility that prereplicative complexes are already assembled late in mitosis (1, 10), we suggest that the unphosphorylated B subunit might mediate the loading of the Pol α-primase complex at origins of replication at that stage.

When the *POL12* gene is present on a high-copy-number plasmid, the overproduced B subunit is mostly present as the unphosphorylated form (see above) and the overproduced protein is localized within the nucleus (20). Therefore, we suggest that B-subunit phosphorylation does not influence its nuclear transport, although it might require the stoichiometric assembly of the Pol α-primase subunits. In fact, preliminary experiments indicate that p86 phosphorylation is dependent on assembly with the p180 Pol α polypeptide. However, it is unlikely that assembly and disassembly of the Pol α-primase polypeptides is modulated by B-subunit phosphorylation, since both the p86 and p91 forms are present in immunopurified Pol α-primase preparations (17).

At present we do not know whether phosphorylation of the B subunit is a consequence of some steps in replication or whether it is causally associated with the regulation of the process. Neither do we know whether phosphorylation of maternal B subunit has the same functional significance as phosphorylation of the newly synthesized protein. Intriguing possibilities, which are not mutually exclusive, might correlate phosphorylation of the B subunit (and/or other replicative proteins) to mechanisms which might trigger some steps in DNA replication or prevent unscheduled reinitiation events. Identification of the amino acid residues in the B subunit which are phosphorylated in vivo, their mutagenesis, and the identification of the kinase(s) and phosphatase(s) involved in such a process will be required to clarify the physiological significance of B-subunit phosphorylation.

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