THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 15, Issue of April 12, pp. 8661–8666, 1996
Printed in U.S.A.

Phosphorylation of the DNA Polymerase α-Primase B Subunit Is Dependent on Its Association with the p180 Polypeptide*

(Received for publication, August 2, 1995, and in revised form, January 31, 1996)

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The B subunit of the DNA polymerase (pol) α-primase complex executes an essential role at the initial stage of DNA replication in *Saccharomyces cerevisiae* and is phosphorylated in a cell cycle-dependent manner. In this report, we show that the four subunits of the yeast DNA polymerase α-primase complex are assembled throughout the cell cycle, and physical association between newly synthesized pol α (p180) and unphosphorylated B subunit (p86) occurs very rapidly. Therefore, B subunit phosphorylation does not appear to modulate p180·p86 interaction. Conversely, by depletion experiments and by using a yeast mutant strain, which produces a low and constitutive level of the p180 polypeptide, we found that formation of the p180·p86 subcomplex is required for B subunit phosphorylation.

The development of cell-free systems capable to replicate viral DNA molecules has been essential in understanding the fundamental enzymology of eukaryotic DNA replication and in identifying most of the required cellular factors (Challberg and Kelly, 1989; Stillman, 1989; Hurwitz *et al.*, 1990; Waga and Stillman, 1994). The structure of these proteins appears to be conserved in all the eukaryotic organisms analyzed so far, and the genetic amenability of the yeast system has allowed testing of their function in DNA replication *in vivo* (Campbell, 1993).

Despite considerable advances in understanding the mechanistic properties of the replicative process, very little is known about the mechanisms that couple initiation of DNA replication to cell cycle progression (Coverley and Laskey, 1994; Heichman and Roberts, 1994). Entry into the S phase is likely to be controlled by the combined action of positive and negative regulators. In fact, current evidence is consistent with the notion that the onset of S phase requires the accumulation of cyclin-dependent kinase(s) and the concomitant destruction of cyclin-dependent kinase inhibitors (Hayles *et al.*, 1994; Schwob *et al.*, 1994). The substrates of the cyclin-dependent kinase promoting S phase entry are presently unknown, although it is legitimate to speculate that at least some proteins of the replicative apparatus itself might represent the physiological target. Indeed, specific subunits of critical replication factors are phosphorylated in a cell cycle-dependent manner (Din *et al.*, 1990; Dutta and Stillman, 1992; Foiani *et al.*, 1995; Nasheuer *et al.*, 1991).

Biochemical and genetic evidence have established that the DNA polymerase α-primase complex (pol[1] α-primase) plays an essential role in eukaryotic DNA replication, because of its unique ability to initiate DNA synthesis *de novo* (Wang, 1991); as a consequence, it is required for discontinuous lagging strand synthesis, as well as for initiation of DNA replication at an origin. This dual role of the pol α-primase complex makes it a potential target of the regulatory mechanisms controlling entry into S phase. By using the yeast *Saccharomyces cerevisiae* as a model system, we have combined *in vitro* and *in vivo* experimental approaches to test whether the function of the yeast pol α-primase complex is indeed regulated during the cell cycle. The yeast pol α-primase complex contains four polypeptides with apparent molecular masses of 180, 86, 58, and 48 kDa (Plevani *et al.*, 1985; Brooke *et al.*, 1991), and the structure and catalytic properties of this protein complex are conserved in a wide range of eukaryotic organisms (Wang, 1991). The p180 polypeptide is the pol α subunit, while DNA primase is a heterodimer of the 58- and 48- kDa polypeptides (Plevani *et al.*, 1985; Brooke and Dumas, 1991). The p48 subunit is sufficient for RNA primer synthesis *in vitro* (Santocanale *et al.*, 1993), and the p58 polypeptide stabilizes primase activity and mediates the interaction between pol α and p48 (Santocanale *et al.*, 1993; Longhese *et al.*, 1993). The p86 protein species (also called B subunit) directly interacts with the p180 polypeptide, but does not influence any of the enzymatic activity of the complex (Plevani *et al.*, 1985; Brooke *et al.*, 1991), suggesting that it may play a regulatory function. Accordingly, we have recently shown that the yeast B subunit specifically executes its essential function at the initial stage of DNA replication (Foiani *et al.*, 1994), and it is phosphorylated and dephosphorylated in a cell cycle-dependent manner (Foiani *et al.*, 1995). These observations led us to suggest that p86 might be involved in loading the pol α-primase complex at the origins of replication during the M/G_1 transition of the cell cycle. This loading may require specific protein-protein interactions with some initiation factors and may be modulated by post-translational modifications. The finding that the human B subunit physically interacts with the SV40 T-antigen and is phosphorylated in a cell cycle-dependent manner is in agreement with this model (Collins *et al.*, 1993).

The yeast genes encoding the pol α-primase subunits are transiently transcribed at the G_1/S boundary (Johnston *et al.*, 1987; Foiani *et al.*, 1989; Johnston *et al.*, 1990; Foiani *et al.*, 1995), and this transcriptional program is mediated by cis- and trans-acting elements (Johnston and Lowndes, 1992; Koch and Nasmyth, 1994). We have recently shown that the pol α-primase subunits are stable proteins, which are present in large excess within the cell, and their *de novo* synthesis, resulting

* This work was supported in part by grants from the Target Projects in Genetic Engineering and Biotechnology and Bioinstrumentation, by Progetto Bilaterale 95.00840.CT04 Centro Nazionale Ricerche Italy, by a grant from Associazione Italiana per la Ricerca sul Cancro, and by Contract CHRX-CT93-0248 from the European Union. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: pol, polymerase; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; HU, hydroxyurea.

from the late G_1 transcriptional burst, is not essential to enter S phase (Muzi Falconi *et al*., 1993; Foiani *et al*., 1995). Therefore, the significance of the transcriptional control of the corresponding genes remains obscure. By considering that the four subunits of the pol α-primase complex are always present during the different steps of the cell cycle and their level is not rate-limiting for entry into S phase, we wanted to test whether the assembly of the complex was regulated during the cell cycle and whether it was influenced by the phosphorylation state of the B subunit.

In this report we provide evidence that formation of the pol α-primase complex is not restricted to S phase, since the 4-subunit complex is assembled throughout the cell cycle. Phosphorylation of the B subunit of the complex (p86) is not required for productive interaction with the pol α polypeptide (p180), while previous assembly of the p86·p180 subcomplex is required for B subunit phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pMA1 has been constructed by inserting the 2,032-base pair *Eco*RI-*Sal*I fragment from plasmid pol1-Δ6, carrying an internal deletion of the *POL1* 5′ non-coding region from positions −237 to −156, which eliminates the cell cycle regulatory element from the *POL1* promoter (Pizzagalli *et al*., 1992), into the *Eco*RI-*Sal*I sites of plasmid YIp5 (Rose *et al*., 1987). Plasmids pGLΔ1 and pFE3 carrying, respectively, the *POL1* and *POL12* gene on the high copy number vector YEp24 have been already described (Pizzagalli *et al*., 1992; Foiani *et al*., 1994).

Yeast Strains—Strain CG378 is *MAT***a** *ade5–7 can1 leu2–3,112 trp1–289 ura3–52*. Strain CG378ΔPOL1 (pMA2) has been already described (Muzi Falconi *et al*., 1993). Strain CGMA1 is a CG378 derivative in which the chromosomal copy of *POL1* has been replaced with the *pol1-Δ6* allele by the two-step procedure (Rothstein, 1991). Briefly, strain CG378 has been transformed with plasmid pMA1 linearized with *Hpa*I, followed by 5-fluoroorotic acid treatment (Rothstein, 1991). The correct replacement has been verified by Southern blot analysis.

Preparation of Yeast Extracts, Western Blot Analysis, and Immunoprecipitation of the DNA Polymerase α-Primase Complex—Preparation of protein extracts from trichloroacetic acid-treated yeast cells and the procedure for Western blot analysis was described previously (Foiani *et al*., 1994), except that the ECL system (Amersham Corp.) has been used as the secondary antibody. Preparation of non-denaturing protein extracts and immunoprecipitation of the pol α-primase complex were performed as described (Foiani *et al*., 1995).

In Vivo Labeling of Yeast Proteins and Immunoprecipitation—In vivo ^{35}S protein labeling has been carried out as described previously (Foiani *et al*., 1995). After preparation of non-denaturing protein extracts, 5×10^7 ^{35}S total counts were adjusted to 1 ml with Tris-HCl, pH 8, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin and immunoprecipitated using the mouse monoclonal antibody Y48 (directed against the p180 pol α polypeptide) covalently linked to protein A-Sepharose CL-6B (Plevani *et al*., 1985) for 60 min at 4 °C. After being washed four times with the same buffer, the monoclonal antibody protein A-Sepharose was resuspended in 100 μl of 1% SDS, boiled for 4 min, and centrifuged. 900 μl of Tween-20 IP buffer (Foiani *et al*., 1995) was added to the supernatant. The pol α catalytic subunit and the B subunit of the pol α-primase complex were immunoprecipitated as described for immunoprecipitation of trichloroacetic acid-denatured proteins, using mouse monoclonal antibodies directed against the two polypeptides (Foiani *et al*., 1994, 1995). The final pellet was resuspended in 20 μl of Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Sambrook *et al*., 1989).

Fluorescence-activated Cell Sorter (FACS) Analysis and Cell Synchronization by α-Factor Treatment—Both FACS analysis and cell synchronization by α-factor treatment have been described previously (Foiani *et al*., 1994).

RNA Analysis—Extraction of total yeast RNA, Northern blotting analysis, and DNA probes have been already described (Pizzagalli *et al*., 1992).

RESULTS

The Four-subunit pol α-Primase Complex Is Assembled throughout the Cell Cycle—A network of specific protein-protein interactions is required for initiation of DNA replication at an origin. By considering that most eukaryotic replication proteins are present as multisubunit complexes, a possible mechanism to drive entry into S phase is to restrict the physical assembly of a functional complex to a specific stage of the cell cycle. Therefore, we tested whether the four subunit pol α-primase complex was physically assembled throughout the cell cycle, by immunoprecipitating the complex with anti-pol α monoclonal antibodies from protein extracts prepared from a synchronous cell culture. As shown in Fig. 1, Western blot analysis performed on the immunoprecipitates indicates that a fully assembled pol α-primase complex is not restricted to S phase, but is present at every stage of the cell cycle. The fully assembled pol α-primase complex found in α-factor arrested cells (*Time 0* in Fig. 1) has to be inherited from the previous cell cycle (maternal complex), since it is known that the pol α-primase polypeptides are periodically synthesized, following the transient transcription of their corresponding genes in G_1/S (Foiani *et al*., 1995; Johnston *et al*., 1990; Foiani *et al*., 1989; Johnston *et al*., 1987). Although the complex is assembled throughout the cell cycle, different isoforms of the associated B subunit can be found at different times (Fig. 1). We have previously shown that the B subunit exists in two forms of 86 and 91 kDa, and p91 arises by phosphorylation of p86 (Foiani *et al*., 1995). Moreover, maternal p86 is phosphorylated early in S phase, while the newly synthesized p86, which derives from the periodic transcription of the corresponding *POL12* gene, is phosphorylated in late S/early G_2 phase 70 min after its synthesis, and p91 is dephosphorylated to p86, while cells are exiting from mitosis (Foiani *et al*., 1995). Both forms are present in the pol α-primase complex immunoprecipitated from exponentially growing cells, before α-factor addition (−122-min sample in Fig. 1). In α-factor arrested cells, only dephosphoryl-

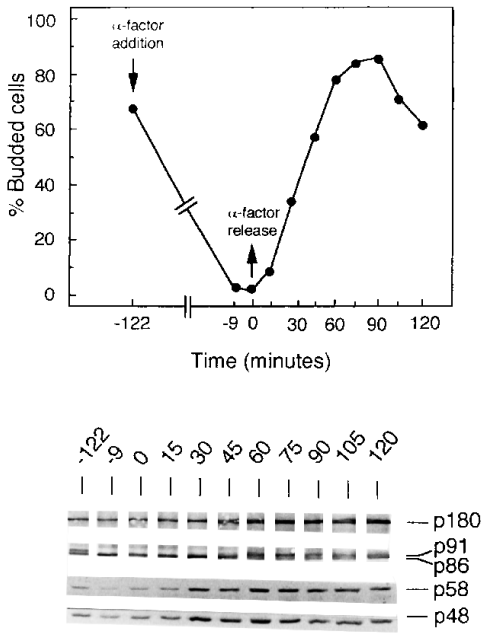

FIG. 1. **Immunoprecipitation of the pol α-primase complex from synchronously dividing cells.** *Top*, strain CG378 was synchronized by α-factor treatment and the degree of synchrony was measured by microscopic analysis of cell budding. *Bottom*, aliquots of the cell culture (50 ml) were taken at the indicated times and protein extracts were prepared using non-denaturing conditions (see "Experimental Procedures"). For each sample, 2 mg of total protein were immunoprecipitated and analyzed by SDS-PAGE electrophoresis and Western blotting with monoclonal or affinity-purified polyclonal antibodies against the individual pol α-primase subunits (Plevani *et al*., 1985; Santocanale *et al*., 1992; Foiani *et al*., 1994).

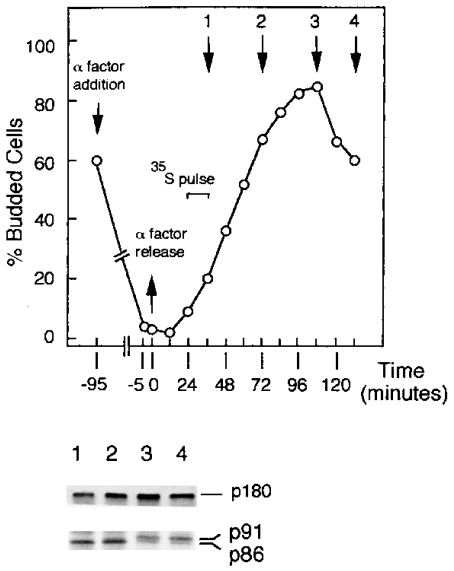

FIG. 2. **Kinetics of interaction between newly synthesized p180 and B subunit.** *Top*, budding profile of strain CG378 synchronized by α-factor treatment. After α-factor release, cells were labeled *in vivo* with a 12-min pulse of [^{35}S]methionine, performed at the time indicated by *bracket*, and chased with an excess of cold methionine. *Bottom*, at the times indicated by the *numbered arrows* on the top of the figure, aliquots of the culture (2 ml) were taken and protein extracts were prepared under non-denaturing conditions. The p180 and B subunit polypeptides were immunoprecipitated with the two-step method described under "Experimental Procedures."

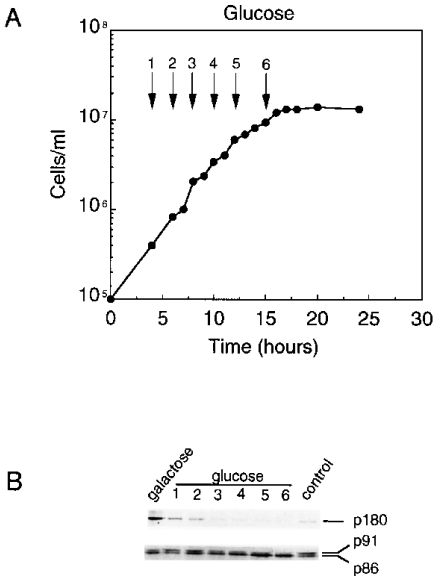

FIG. 3. **Effect of p180 depletion on B subunit phosphorylation.** *Panel A*, cells of strain CG378ΔPOL1 (pMA2) were grown at 28 °C under selective conditions in synthetic medium (Muzi Falconi *et al.*, 1993) containing 2% galactose to a concentration of 2 × 1O^6 cells/ml. Cells were filtered, washed, and resuspended in synthetic medium containing 2% glucose (time 0). Cell number was monitored by microscopic counting. Aliquots of 3 × 10^8 cells were taken at the indicated times (*numbered arrows*) for protein extract preparation and Western blot analysis. *Panel B*, 25 μg of total protein extracts were prepared by the trichloroacetic acid method (see "Experimental Procedures") and analyzed by SDS-PAGE electrophoresis followed by Western blotting with the appropriate antibodies. Control lane is a sample of protein extract (25 μg) prepared from exponentially growing CG378 isogenic cells.

ated p86 is present (maternal complex), while two complexes, containing either the p86 or p91 forms of B subunit, can be detected in S phase (45 min after α-factor release) and they persist until mitosis. Moreover, the appearance of phosphorylated p91 associated with the other subunits of the complex is concomitant with the timing of B subunit phosphorylation observed by Western blot analysis performed on crude extracts before immunoprecipitation (data not shown). These data show that assembly of the yeast pol α-primase complex is not restricted to a particular stage of the cell cycle, as previously observed in human cells (Nasheuer *et al.*, 1991) and two complexes, containing either p86 or p91, can coexist in S phase.

Phosphorylation of B Subunit Is Dependent on Its Previous Assembly with the pol α Polypeptide—As mentioned above, newly synthesized p86 remains unphosphorylated for 70 min after its synthesis. This lag could be due to the time required to assemble the newly synthesized pol α-primase complex, if B subunit phosphorylation can only occur after its physical interaction with pol α. To test directly this hypothesis, presynchronized yeast cells were pulse-labeled with [^{35}S]methionine at the time of maximal p86 synthesis (Foiani *et al.*, 1995) and, after chase, pol α·B subunit interaction was monitored by coimmunoprecipitation. As shown in Fig. 2, newly synthesized p86 was found to be associated with pol α immediately after the pulse, although it remained unphosphorylated for 70 min. Therefore, the establishment of a physical interaction between newly synthesized p86 and p180 is not the rate-limiting step preventing p86 phosphorylation.

We previously found that overexpression of the *POL12* gene, encoding the B subunit, resulted in accumulation of the p86 unphosphorylated form (Foiani *et al.*, 1995). To investigate whether a correct stoichiometry of the pol α-primase polypeptides was required for proper p86 modification, we analyzed its phosphorylation state in yeast strains carrying the *POL1*, *PRI1*, or *PRI2* genes, encoding, respectively, the p180, p48, and p58 pol α-primase polypeptides under the control of the repressible *GAL1* promoter.

Strain CG378ΔPOL1 (pMA2) carrying the *GAL1-POL1* fusion gene can grow for several generations under repressed conditions in glucose-containing media (Fig. 3, *panel A*) (Muzi Falconi *et al.*, 1993), even when the level of the p180 polypeptide drops far below its physiological level (Fig. 3, *panel B*) (Muzi Falconi *et al.*, 1993). Therefore, it was possible to test whether phosphorylation of B subunit was correlated to the progressive depletion of the pol α polypeptide. As shown in Fig. 3 (*panel B*), repression of *GAL1-POL1* expression did not change the ratio between the p86 and p91 forms of B subunit, until the level of p180 was lower than that of the wild-type control strain. Further decrease in the amount of p180 resulted in a proportional decrease of p91, indicating that the amount of p180 influences the extent of B subunit phosphorylation.

Analogous experiments performed on yeast strains carrying, respectively, the *PRI1* and *PRI2* genes under the control of the *GAL1* promoter have shown that progressive depletion of either the p48 or p58 polypeptides did not interfere with B subunit phosphorylation (data not shown). Therefore, the proper stoichiometry of all four subunits of the pol α-primase complex is not a prerequisite for B subunit phosphorylation, which is exclusively dependent on the level of p180. This finding is consistent with the long standing observation that the pol α-primase complex consists of two subcomplexes: heterodimeric DNA primase, containing the p48 and p58 polypeptides; and the p180·p86 subcomplex, which only shows DNA polymerase activity (Plevani *et al.*, 1985).

As an alternative approach to correlate the level of p180 with that of phosphorylated B subunit, we used a yeast strain constitutively producing a very low amount of the p180 polypeptide. Such a strain was constructed by replacing the chromosomal copy of the *POL1* gene with the constitutively repressed *pol1-Δ6* allele, which carries a deletion of the *POL1* cell cycle

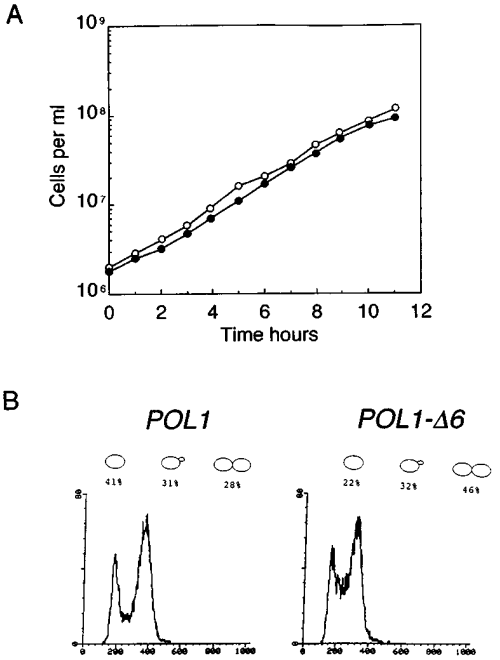

FIG. 4. **Growth rate and FACS analysis of the *pol1-Δ6* strain.** *Panel A*, growth rates of CG378 (○) and *pol1-Δ6* (●) isogenic strains were monitored by cell counting. *Panel B*, samples of exponentially growing CG378 (*POL1*) and *pol1-Δ6* cells were taken and the DNA content was measured by FACS analysis. Percentage of small budded, large budded, and unbudded cells (*inset*) was monitored by microscopic examination.

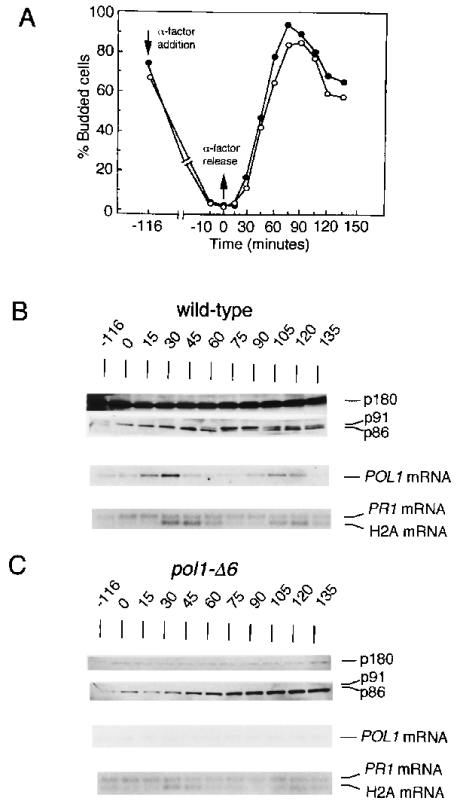

FIG. 5. **B subunit phosphorylation in synchronized *pol1-Δ6* cells.** *Panel A*, budding profile of CG378 (○) and *pol1-Δ6* (●) strains synchronized by α-factor treatment. *Panel B*, at the indicated times, samples of the CG378 synchronized culture were taken, protein extracts were prepared by the trichloroacetic acid method (Experimental Procedures), and 25 μg of total proteins were analyzed by SDS-PAGE electrophoresis and Western blotting with the appropriate antibodies. Total RNA was extracted at the same times, and 5 μg of RNA was loaded in each lane. The level of *POL1*, *PR1*, and H2A mRNAs was measured by Northern blot analysis as indicated under "Experimental Procedures." *Panel C*, samples taken from the synchronous *pol1-Δ6* culture were processed for Western and Northern blot analysis as described in *panel B*. The Northern blot performed to monitor the level of *POL1* mRNA in *pol1-Δ6* cells was developed after 3 weeks, while the other Northern blots shown in *panels B* and *C* were developed after 3 days.

regulatory element (Pizzagalli *et al.*, 1992), and tested the effect of this deletion on cell growth and B subunit phosphorylation. As shown in Fig. 4*A*, this mutation in the *POL1* promoter did not affect growth rate, since the kinetic of growth of the *pol1-Δ6* strain was undistinguishable from that of the isogenic wild-type. However, FACS analysis showed that deletion of the *POL1* cell cycle regulatory element resulted in a partial delay of S phase progression, as visualized by the accumulation of cells with an intermediate DNA content between 1C and 2C (Fig. 4*B*). Microscopic analysis of wild-type and *pol1-Δ6* cells indicated that the percentage of unbudded cells decreased in the mutant strain. This finding was expected, since it is known that a longer S phase leads to larger daughter cells at the time of cell separation, reducing the requirement for growth and time in G_1 in the subsequent cell cycle (Johnston and Singer, 1983).

As shown in Fig. 5*A*, after release from the α-factor block, *pol1-Δ6* cells were able to divide synchronously with a budding profile that was essentially undistinguishable from that of isogenic wild-type cells. Moreover, periodic synthesis of H2A mRNA in S phase was coincident in wild-type and mutant cells (Fig. 5, *B* and *C*). However, the steady-state level of *POL1* mRNA in the *pol1-Δ6* strain was barely detectable and periodic transcription of the *POL1* gene in G_1/S, which was evident in the isogenic wild-type strain, was completely abolished. Accordingly, the amount of the *POL1* gene product (p180 polypeptide) was strongly decreased, compared to that found in wild-type cells. These data support the notion that periodic transcription of the *POL1* gene is not required to drive entry into S phase, and that the p180 polypeptide is present in excess amount within the cell (Muzi Falconi *et al.*, 1993). When we tested the level of B subunit phosphorylation in synchronously dividing *pol1-Δ6* cells, only the p86 form was detectable on Western blots of total protein extracts (Fig. 5*C*), while both p86 and p91 were found in protein extracts prepared from wild-type cells (Fig. 5*B*).

The correlation between B subunit phosphorylation and the level of p180 can again be explained by assuming that physical interaction between p86 and p180 is required for p86 phosphorylation. In fact, the finding that only p86 is detectable on Western blots of protein extracts prepared from synchronously dividing (Fig. 5*C*) or logarithmically growing *pol1-Δ6* cells (Fig. 6*A*) can be ascribed to the low amount of B subunit that can associate with p180 due to the reduced level of the pol α polypeptide in the mutant strain. This assumption was corroborated by the finding that phosphorylated B subunit can instead be detected in *pol1-Δ6* cells if a sufficient amount of protein extract was immunoprecipitated with anti-p180 monoclonal antibodies (Fig. 6*B*). This result indicates that B subunit phosphorylation requires the physical interaction with p180 and, in turn, this association is modulated by the level of the pol α polypeptide.

Both p86 and p91 were present in the immunoprecipitate prepared from logarithmically growing *pol1-Δ6* cells (Fig. 6*B*), probably as a consequence of the cell cycle-dependent phosphorylation of B subunit. However, only the amount of p86 that is physically associated with p180 can be phosphorylated by the yet unidentified protein kinase responsible for such post-translational modification. In fact, phosphorylated p91 accumulated

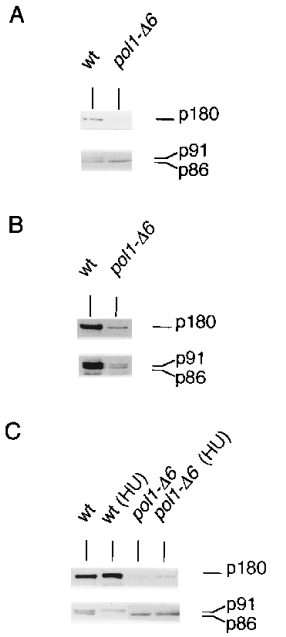

FIG. 6. **Phosphorylation state of pol α-associated B subunit in *pol1-Δ6* cells.** *Panel A*, Western blot analysis of 25 μg of total protein extracts prepared from exponentially growing CG378 (*wt*) and *pol1-Δ6* cells. *Panel B*, 5 mg of total protein from exponentially growing CG378 (*wt*) and *pol1-Δ6* cells were immunoprecipitated under non-denaturing conditions, followed by Western blot analysis with specific antibodies. *Panel C*, total protein extracts were prepared from exponentially growing CG378 (*wt*) and *pol1-Δ6* cells, or from cells of the same strains which have been arrested in S phase by treatment with 0.08 M HU for 3 h. 25 μg of total proteins for each sample were separated by SDS-PAGE electrophoresis, followed by Western blotting.

in protein extracts prepared from wild-type cells arrested by hydroxyurea (HU) treatment (Fig. 6*C*) (Foiani *et al*., 1995). Conversely, only p86 was detected in protein extracts prepared from *pol1-Δ6* cells arrested at the HU-dependent step, indicating that free p86 cannot be phosphorylated, even when cells are blocked for several hours in S phase, a stage of the cell cycle when a protein kinase capable to phosphorylate p86 is fully active.

The level of p86 phosphorylation could be exclusively dependent on the amount of the p86·p180 subcomplex or, alternatively, could be limited by one or more rate-limiting factors. If the first hypothesis is correct, we would predict that the amount of phosphorylated B subunit will increase in a yeast strain overproducing the p180 and p86 polypeptides. Indeed, as shown in Fig. 7, the total level of the p180·B subunit subcomplex was found to be higher in immunoprecipitates from protein extracts prepared from a yeast strain transformed with two high copy number plasmids carrying either the *POL1* or the *POL12* genes. This overproducing strain did not show any detectable cell cycle defect (data not shown), and the ratio between unphosphorylated p86 and phosphorylated p91 was similar to that found in the untransformed wild-type strain. When we analyzed the level of associated p48 and p58 in the immunoprecipitates shown in Fig. 7, we found that the amount of these polypeptides was slightly higher in the co-overproducing strain compared to the wild-type although their level, measured by Western blotting on total extracts, was identical in the two strains. We have shown previously that only 50% of DNA primase is associated in the four subunit pol α-primase complex (Santocanale *et al*., 1993), and an increase in the level of the p180·B subunit heterodimer might favor association of free primase subunits.

As expected, single overexpression of the *POL12* gene causes

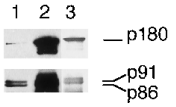

FIG. 7. **Phosphorylation state of B subunit in cells co-overexpressing the *POL12* and *POL1* genes.** 5 mg of total protein prepared from wild-type cells (*lane 1*) or from cells overexpressing the *POL12* and *POL1* genes (*lane 2*) were immunoprecipitated under non-denaturing conditions, followed by Western blot analysis with specific antibodies. *Lane 3* is the same sample analyzed in *lane 2*, except that the Western blot was developed for a few seconds instead than 5 min (*lanes 1* and *2*).

the accumulation of unphosphorylated p86 (Foiani *et al*., 1995), while *POL1* overexpression does not increase the level of p91 found in wild-type cells and the ratio between p86 and p91 (see Fig. 3). These findings indicate that p180·p86 association is necessary and sufficient for proper B subunit phosphorylation.

DISCUSSION

Replication of the eukaryotic genome is restricted to the S phase of the cell cycle, but preparation for S phase appears to occur while cells are exiting from mitosis. Recently it has been shown that the chromatin structure of yeast origins of replication changes from a post-replicative to a prereplicative state late in mitosis (Diffley *et al*., 1994). In this respect, it is quite intriguing that dephosphorylation of the yeast pol α-primase B subunit is coincident with the appearance of the prereplicative complexes late in mitosis (Foiani *et al*., 1995). By considering that the B subunit becomes phosphorylated in G_1/S and executes its function at the initial stage of DNA replication, before the HU-sensitive step (Foiani *et al*., 1994), it is reasonable to speculate that this protein might be the target of regulatory events controlling the onset of DNA replication. This hypothesis is strengthened by the unique ability of the pol α-primase complex in initiating DNA synthesis *de novo* (Wang, 1991). The successful production of a RNA-DNA chain by pol α-primase represents the first polymerization event occurring at an origin of replication. Moreover, the switch of the pol α-primase complex to the lagging strand template (Tsurimoto *et al*., 1990; Waga and Stillman, 1994) might signal the establishment of a productive multiprotein complex competent for further DNA replication.

We are interested in understanding: (i) the *in vivo* function of the four pol α-primase subunits, (ii) the physiological role of B subunit phosphorylation, and (iii) the rules controlling the physical interaction of the four subunits with each other and with other components of the replication apparatus. In yeast, the answer to these questions is further complicated by the finding that the genes encoding the pol α-primase polypeptides are periodically transcribed during the cell cycle, while the corresponding gene products are stable proteins (Muzi Falconi *et al*., 1993; Foiani *et al*., 1995). As a consequence, the cell contains two pools of pol α-primase polypeptides: the first one, which is inherited from the previous cell cycle (maternal pool); and the second one, which is *de novo* synthesized in S phase (newly synthesized pool). It is presently unclear whether these newly synthesized proteins play any role, since we have shown that proteins of maternal origins are sufficient to drive entry into S phase and to replicate chromosomal DNA (Muzi Falconi *et al*., 1993; Foiani *et al*., 1995).

A possible way to restrict the function of the pol α-primase to S phase would have been to modulate the assembly of the whole complex during the cell cycle. This question is particularly relevant in the yeast *S. cerevisiae,* because of the presence of maternal and newly synthesized proteins in normally cycling cells. The immunoprecipitation experiments described in this report indicate that the four subunits of the complex are firmly associated with each other throughout the cell cycle, similarly

to what it has been found in human cells (Nasheuer *et al*., 1991). Furthermore, association of newly synthesized subunits is extremely rapid. Therefore, transient association of one subunit does not appear to regulate the function of the whole complex.

Moreover, B subunit phosphorylation does not modulate the assembly of the complex. In fact, not only the B subunit is associated to the other polypeptides at every stage of the cell cycle but two complexes, containing either phosphorylated (p91) or unphosphorylated (p86) B subunit, can co-exist during most of the cell cycle starting from early S phase until mitosis. The presence of two complexes differing in the phosphorylation state of B subunit is likely due to its dual timing of phosphorylation. In fact, we have shown previously that maternal p86 is phosphorylated early in S phase, while newly synthesized p86, produced as a consequence of periodic transcription of the *POL12* gene in late G_1, becomes phosphorylated 70 min after its synthesis (Foiani *et al*., 1995).

Several mechanisms, which are not mutually exclusive, can be involved in preventing phosphorylation of newly synthesized p86 during this time interval. For example, it is possible that phosphorylation of p86 requires the previous assembly of the pol α-primase complex, and productive interaction among the four subunits may be a function of a threshold level of the *de novo* synthesized polypeptides. Alternatively, B subunit phosphorylation might occur only after this polypeptide, or the whole pol α-primase complex, are transported within the nucleus and, eventually, after they interact with DNA or with other protein components. Finally, B subunit phosphorylation might be controlled by a timing mechanism, and, under unperturbed conditions, two cyclin-dependent kinases might be responsible for phosphorylation of maternal or newly synthesized B subunit. According to this last hypothesis, it is interesting that the phosphorylation timings of maternal and newly synthesized p86 essentially parallel, respectively, the timings of Clb5/Clb6 and of Clb1–4 synthesis (Schwob and Nasmyth, 1993; Schwob *et al*., 1994).

Our finding that newly synthesized p86 rapidly associates with the p180/pol α polypeptide, while remaining unphosphorylated for 70 min, indicates that p86 phosphorylation is not a prerequisite for p180·p86 interaction and suggests that assembly of the complex occurs almost immediately after synthesis of its subunits. Therefore, assembly of the pol α-primase complex does not seem to be the rate-limiting step preventing B subunit phosphorylation under unperturbed conditions. However, p86 phosphorylation requires the formation of the p180·p86 subcomplex, while it does not appear to be influenced by a correct stoichiometry of the four pol α-primase subunits. In fact, progressive depletion of the p48 and p58 primase polypeptides does not change the ratio between phosphorylated and unphosphorylated B subunit (data not shown). Conversely, depletion of the p180 polypeptide causes an evident increase in the amount of unphosphorylated p86.

The correlation between the level of p180 and B subunit phosphorylation was also studied by using the *pol1-Δ6* strain, which carries an integrated copy of the *POL1* gene lacking its cell cycle regulatory element and producing a very low amount of p180 polypeptide. The finding that wild-type and *pol1-Δ6* cells have identical growth rates further confirms that periodic transcription of the *POL1* gene does not have an essential function under normal conditions (Muzi Falconi *et al*., 1993). In the *pol1-Δ6* mutant strain, the level of phosphorylated p91 is much lower than that found in the isogenic wild-type and correlates with the limited amount of p180 found in protein extracts prepared from the mutant strain. Moreover, three sets of evidence indicate that unassembled B subunit cannot be phosphorylated: first, overexpression of the *POL12* gene leads to accumulation of unphosphorylated p86 (Foiani *et al*., 1995); second, when *pol1-Δ6* cells are blocked in S phase by HU treatment, the phosphorylation of B subunit does not increase, while phosphorylated p91 accumulates in wild-type cells arrested by the same treatment; third, phosphorylated p91 in *pol1-Δ6* extracts can only be found in immunoprecipitates obtained with anti-pol α antibodies. Altogether, these data indicate that formation of the p180·p86 subcomplex is a prerequisite for B subunit phosphorylation.

The finding that co-overproduction of p180 and B subunit results in an increased level of p91, compared to that found in wild-type cells, indicates that the interaction between these polypeptides is sufficient for B subunit phosphorylation. Although other explanations can be envisaged, it is possible that p180·p86 association leads to a conformational change which allows proper B subunit phosphorylation. A major issue remains the discovery of the kinase(s) responsible for B-subunit phosphorylation and the definition of the biological significance of this event. Our finding that p180·p86 association is a prerequisite for B-subunit phosphorylation further complicates these studies. In fact, proper p180·p86 interaction will have to be considered in studying any mutant altered in B-subunit phosphorylation, and the possibility that specific protein-protein interaction will affect phosphorylation could be extended to other DNA replication proteins.

Acknowledgments—We thank M. Muzi Falconi for help in the construction of the pol1Δ6 strain, and we thank all the members of our laboratory for useful discussions and criticisms.

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