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

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The B subunit of the DNA polymerase (pol) $\alpha$-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 $\alpha$-primase complex are assembled throughout the cell cycle, and physical association between newly synthesized pol $\alpha$ (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 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 substrates 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 $\alpha$-primase complex (pol1 $\alpha$-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 $\alpha$-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 yeasts pol $\alpha$-primase complex is indeed regulated during the cell cycle. The yeast pol $\alpha$-primase complex contains four polypeptides with apparent molecular masses of 180, 86, 58, and 48 kDa (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 $\alpha$ and p48 (Santocanale et al., 1993; Longhese et al., 1993). The p86 polypeptide (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 $\alpha$-primase complex at the origins of replication during the M/G1 transition of the cell cycle. This loading may involve 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 $\alpha$-primase subunits are transiently transcribed at the G1/S boundary (Johnson et al., 1987; Foiani et al., 1989; Johnson et al., 1990; Foiani et al., 1995), and this transcriptional program is mediated by cis- and trans-acting elements (Johnson and Lowndes, 1992; Koch and Nasmyth, 1994). We have recently shown that the pol $\alpha$-primase subunits are stable proteins, which are present in large excess within the cell, and their de novo synthesis, resulting in...
from the late G1 transcriptional burst, is not essential to enter S phase (Muzzi 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 EcoRI-Sall fragment from plasmid pol1-36, 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 EcoRI-Sall sites of plasmid YIp5 (Rose et al., 1987). Plasmids YEpL11 and pFE3 E3, respectively, the POL1 and POL2 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 MATa ade5-7 can1 leu2-3,112 trp1-289 ura3-52. Strain CG378pol1-36 (pMA2) has been already described (Muzzi Falconi et al., 1993). Strain CGMA1 is a CG378 derivative in which the chromosomal copy of POL1 has been replaced with the pol1-36 allele by the two-step procedure (Rothstein, 1991). Briefly, strain CG378 has been transformed with plasmid pMA1 linearized with Hpal, 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 35S protein labeling has been carried out as described previously (Foiani et al., 1994). After preparation of non-denaturing protein extracts, 5 × 106 35S 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 G1/S (Foiani et al., 1995; Johnstone et al., 1990; Foiani et al., 1989; Johnstone 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 POL1 gene, is phosphorylated in late S/early G2 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 dephosphorylated—
Phosphorylation of DNA pol α-Primase B Subunit

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
regulatory element (Pizzagalli et al., 1992), and tested the effect of this deletion on cell growth and B subunit phosphorylation. As shown in Fig. 4A, this mutation in the POL1 promoter did not affect growth rate, since the kinetic of growth of the pol1-Δ6 strain was indistinguishable 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 of S phase progression, as visualized by the accumulation of wild-type. FACSanalysisshowedthatdeletionofthe pol1-Δ6 did not affect growth rate, since the kinetic of growth of the 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. 4B). 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 G1 in the subsequent cell cycle (Johnston and Singer, 1983).

As shown in Fig. 5A, 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 the 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 G1/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. 5C), while both p86 and p91 were found in protein extracts prepared from wild-type cells (Fig. 5B).

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. 5C) or logarithmically growing pol1-Δ6 cells (Fig. 6A) 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. 6B). 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. 6B), 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
Phosphorylation of DNA pol \(\alpha\)-Primase B Subunit

**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 \(\alpha\)-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 G1/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 \(\alpha\)-primase complex in initiating DNA synthesis de novo (Wang, 1991). The successful production of a RNA-DNA chain by pol \(\alpha\)-primase represents the first polymerization event occurring at an origin of replication. Moreover, the switch of the pol \(\alpha\)-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 \(\alpha\)-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 \(\alpha\)-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 \(\alpha\)-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 \(\alpha\)-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 G1, 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 Cib5/Cib6 and of Cib1–4 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.

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