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The B Subunit of the DNA Polymerase α-Primase Complex in *Saccharomyces cerevisiae* Executes an Essential Function at the Initial Stage of DNA Replication

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The four-subunit DNA polymerase α-primase complex is unique in its ability to synthesize DNA chains de novo, and some in vitro data suggest its involvement in initiation and elongation of chromosomal DNA replication, although direct in vivo evidence for a role in the initiation reaction is still lacking. The function of the B subunit of the complex is unknown, but the *Saccharomyces cerevisiae POL12* gene, which encodes this protein, is essential for cell viability. We have produced different *pol12* alleles by in vitro mutagenesis of the cloned gene. The in vivo analysis of our 18 *pol12* alleles indicates that the conserved carboxy-terminal two-thirds of the protein contains regions that are essential for cell viability, while the more divergent NH_2-terminal portion is partially dispensable. The characterization of the temperature-sensitive *pol12-T9* mutant allele demonstrates that the B subunit is required for in vivo DNA synthesis and correct progression through S phase. Moreover, reciprocal shift experiments indicate that the *POL12* gene product plays an essential role at the early stage of chromosomal DNA replication, before the hydroxyurea-sensitive step. A model for the role of the B subunit in initiation of DNA replication at an origin is presented.

Biochemical and genetic evidence indicate that the DNA polymerase α-DNA primase complex (Pol α-primase) is involved in initiation of DNA replication at an origin and in the synthesis of Okazaki fragments on the lagging strand of the replication fork (reviewed in references 12, 37, and 67). Studies of simian virus 40 (SV40) DNA replication in vitro have demonstrated that Pol α-primase, in conjunction with replication protein A and T antigen, is required for the initiation of DNA synthesis on duplex DNA molecules containing the SV40 origin of replication (13, 38, 66). As the replication fork progresses, Pol α-primase primes and extends new DNA chains on the lagging-strand template, as a result of its unique ability to start DNA synthesis de novo.

An in vitro yeast DNA replication system, capable of initiating DNA replication specifically and efficiently at yeast origin sequences (ARS), is still lacking. Therefore, the definition of the role of the yeast Pol α-primase polypeptides depends on the analysis of their biochemical properties and on the production and characterization of mutations in the corresponding genes (for a review, see reference 12).

The structure and catalytic properties of the Pol α-primase complex subunits are highly conserved in a wide range of eukaryotic organisms (67). The protein complex of the yeast *Saccharomyces cerevisiae* contains four polypeptides with apparent molecular masses of 180, 86, 58, and 48 kDa (7, 53). The p180 polypeptide has been shown to be the catalytic Pol α subunit, while the DNA primase is a heterodimer of the 58 and 48 kDa polypeptides (6, 7, 52, 53). We have shown that 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 (62). In fact, p58 mediates and/or stabilizes the binding of the catalytic p48 primase subunit to the p180 Pol α polypeptide (41).

Cloning of the *S. cerevisiae POL1*, *PRI1*, and *PRI2* genes, which encode the p180, p48, and p58 subunits of the yeast complex, respectively, and the study of *pol1*, *pri1*, and *pri2* lethal and conditional alleles have been essential in establishing the roles of the corresponding gene products in mitotic DNA replication and in identifying functional domains in the polymerase and primase polypeptides (8, 26, 29, 42, 51, 54). Moreover, *pol1*, *pri1*, and *pri2* conditional mutants are defective in premeiotic DNA synthesis and show an enhanced rate of intrachromosomal recombination and spontaneous mutation, which is generally correlated with the severity of their defects in cell growth and DNA synthesis (9, 41, 42). Since these mutant strains fail to accumulate high-molecular-weight DNA products (9, 29), the formation of nicked and gapped replicated DNA molecules might be responsible for the hyperrecombination phenotype usually found in yeast DNA synthesis mutants (33).

No enzymatic activity has been found to be associated with the 86-kDa protein species (B subunit), which is tightly bound to the p180 polypeptide, and the physiological role of p86 is presently unknown (7). In vitro reconstitution studies with purified components indicate that p86 is not required for polymerase-primase interaction, and its presence does not change the catalytic properties of Pol α (6, 7). Recently, 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 SV40 origin of replication (14).

As a first step in identifying the physiological role of the B subunit of the yeast Pol α-primase complex in vivo, we have mutagenized by the two-codon insertion mutagenesis method (3) the essential *POL12* gene, which was recently found to encode the p86 polypeptide (7, 36a). The analysis of

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the resulting mutations provides a map of essential domains of the B subunit. Furthermore, the characterization of a temperature-sensitive *pol12* allele demonstrates that wild-type p86 is required for in vivo DNA synthesis and correct progression through S phase. Finally, by mapping the *POL12*-dependent step with respect to the hydroxyurea (HU)-sensitive step, we found that the 86-kDa B subunit plays an essential role at the early stage of chromosomal DNA replication.

MATERIALS AND METHODS

Plasmids. Plasmid pJN7 contains an 8.3-kb *Kpn*I yeast genomic fragment carrying the *POL12* gene in the pBluescript SK+ plasmid vector (Stratagene). Plasmid pAC27 was constructed as follows: the 3.1-kb *LEU2 Bgl*II fragment was used to replace the 1,458-bp *Bgl*II region internal to the *POL12* open reading frame (ORF), and the generated 4.9-kb *Sac*I-*Kpn*I fragment, with the disrupted *POL12* gene, was then cloned in the pBluescript SK+ vector. Plasmids pJN7 and pAC27 were generous gifts from D. Hinkle (University of Rochester, Rochester, N.Y.). Plasmid pFE2 was produced by inserting the blunted 4.3-kb *Xba*I-*Nco*I fragment, containing the entire *POL12* gene, into the *Nru*I site of YCp50 (59). Plasmid pFE7 is a pFE2 derivative in which the genomic *Sac*I site at position −143 from the first ATG of the *POL12* ORF has been destroyed by T4 DNA polymerase treatment (61). This mutation does not cause any phenotypic change compared with pFE2. Plasmid pFE10 is a derivative of the *ARS1 TRP1 CEN6* pLA411 plasmid (50) containing the blunted 4.3-kb *Xba*I-*Nco*I *POL12* fragment inserted into the *Nru*I site. Plasmid pFE3 contains the blunted 4.3-kb *Xba*I-*Nco*I *POL12* fragment inserted into the blunted *Sal*I site of YEp24 (59). Plasmid pFE118 is a pFE2 derivative containing a *Sac*I site created in the 3′ noncoding region at position +2191 from the first *POL12* ATG. Plasmid pFE132 contains the 2.8-kb *Eco*RI-*Sac*I *POL12* fragment from plasmid pFE118 inserted into plasmid pRS316 (64). Plasmid pFE135 is a pFE132 derivative in which the *Bam*HI site at position +1946 from the first *POL12* ATG has been filled in by *Escherichia coli* Klenow fragment treatment (61). Plasmid pFE130 carries the 2.9-kb *Sph*I fragment from a pFE7 derivative plasmid, containing the *pol12-T9* allele inserted between the *Eco*RI and *Nru*I sites of YIp5, after filling in with Klenow fragment and exonucleolytic treatment with T4 DNA polymerase (61).

Yeast strains. Strain TD-28 (*MAT***a** *ino1 ura3-52 can1*) has been previously described (29). Strains H1514 (*MAT*α *ura3-52 trp1-63 leu2-3,112*) and H1515 (*MAT***a** *ura3-52 trp1-63 leu2-3,112*) were constructed by A. M. Cigan in A. G. Hinnebusch's laboratory (National Institutes of Health, Bethesda, Md.). CYd6 is a diploid strain obtained by crossing strains H1514 and H1515. One of the two copies of the *POL12* gene in CYd6 has been disrupted by one-step gene replacement (59), by using the 4.1-kb *Sac*I-*Kpn*I fragment from plasmid pAC27, to generate strain CYd24. Strain CY46 (*ura3-52 trp1-63 leu2-3,112 pol12*::*LEU2*[pFE10 *POL12 TRP1 CEN6*]) is a meiotic segregant of a CYd24 transformant containing the pFE10 plasmid. Strain CY196 (*MAT*α *ura3-52 trp1-63 leu2-3,112 pol12-T9*) is isogenic to strain H1514 and was obtained by transformation of H1514 with plasmid pFE130 linearized with *Bam*HI and subsequent 5-fluoroorotic acid treatment (59) of the obtained transformants; the replacement of the *POL12* wild-type copy has been verified by Southern analysis. Strain CY211 was obtained by replacing the wild-type *POL12* gene with the *pol12-T9* allele in strain CG378 (*MAT*α *ade5 can1 leu2-3 leu2-112 trp1-289 ura3-52*) as described above for the construction of strain CY196.

Mutagenesis of the *POL12* gene. To obtain two-codon insertions in the *POL12* gene (3), plasmid pFE7 was linearized by partial digestion with *Taq*I or *Mae*II followed by ligation in the presence of the oligonucleotide 5′-CGAGCT-3′, which generates a single *Sac*I site. The ligation mixture was cut with *Sac*I, and the 1.3-kb *Sac*I fragment, containing the kanamycin resistance (Kan^r) gene isolated from plasmid pUC4-KISS (Pharmacia), was added before ligation and transformation of the *E. coli* kanamycin-sensitive strain DH5α (Bethesda Research Laboratories). To obtain two-codon insertions, Kan^r insertion plasmids were digested with *Sac*I and religated, leaving a *Sac*I site between the cytosine and guanine nucleotides of each original *Taq*I and *Mae*II site. Plasmids were used to transform yeast strain CY46 either before or after excision of the Kan^r cassette. Transformants were selected on SC plates without uracil and tryptophan (59) at 25°C and tested for their ability to grow on the same medium at different temperatures. No dominant phenotype was observed for any of the tested mutations. Transformants were then assayed for their ability to loose the *TRP1 POL12* pFE10 centromeric plasmid after growth under nonselective conditions at 25°C by the plasmid-shuffling procedure (29). Trp$^-$ Ura$^+$ clones, containing the different *pol12* alleles on the pFE7 derivative plasmids, were then assayed for their ability to grow when streaked on YPD or SC plates (59) incubated at different temperatures. In-frame deletions of the *POL12* gene (see Fig. 4) were constructed by digestion with *Sac*I of two plasmids containing different two-codon insertions followed by ligation on the same plasmid of the NH_2- and COOH-terminal portions of *POL12* derived from the two original mutant plasmids. The effect of the generated deletions was assayed in vivo as described above. The nature and extent of the insertion and deletion mutations were controlled by direct nucleotide sequencing.

Preparation of yeast extracts. Total protein extracts were prepared from 4×10^8 cells collected from logarithmically growing yeast cultures. Cells were washed with 20% trichloracetic acid in order to prevent proteolysis and were resuspended in 200 μl of 20% trichloroacetic acid at room temperature. After addition of the same volume of glass beads, cells were disrupted by vortexing for 2 min. Glass beads were washed twice with 200 μl of 5% trichloroacetic acid, and the resulting extract was spun for 10 min at 3,000 rpm in an Eppendorf microcentrifuge at room temperature. The pellet was resuspended in 200 μl of Laemmli buffer (61), neutralized by adding 100 μl of 1 M Tris base, boiled for 3 min, and finally clarified by centrifugation as described above. Twenty-five-microgram aliquots of the extract, as determined by a Bio-Rad protein assay (61), were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

Production of monoclonal antibodies and Western blot (immunoblot) analysis. BALB/c mice were immunized subcutaneously with 30 μg of Pol α-primase complex immunopurified as previously described (53). The immunization protocol, fusion of spleen cells to NSO myeloma cells, and screening of hybridoma supernatants by enzyme-linked immunosorbent assay were performed according to published procedures (30). Positive hybridomas were tested for their ability to positively react with the B subunit of the Pol α-primase complex on Western blots of the purified complex, as previously described (25). Ascites fluids were pro-

duced as previously described (62), and antibodies were isolated by protein A-Sepharose adsorption and elution.

Fluorescence-activated cell sorter (FACS) analysis. Cells were grown in YPD medium, sonicated for 15 s, collected by centrifugation, and suspended in 70% ethanol for 16 h. Cells were then washed in 0.25 M Tris-HCl (pH 7.5) and suspended in the same buffer containing 2 mg of RNase A per ml. Samples were incubated for 12 h at 37°C and collected by centrifugation, and the pellet was resuspended in 0.5 M pepsin freshly dissolved in 55 mM HCl. Cells were then washed in 180 mM Tris-HCl (pH 7.5)–190 mM NaCl–70 mM $MgCl_2$ and stained in the same buffer containing 50 μg of propidium iodide per ml. Samples were then diluted 10-fold in 50 mM Tris-HCl (pH 7.8) and analyzed by using a Becton Dickinson FACScan.

Mapping the *POL12*-dependent step within the cell cycle by reciprocal shift experiments. The rationale of reciprocal shifts to map the order of events during the yeast cell cycle has been described previously (32, 55) and is discussed further in Results. Strain CY196 was used in experiments IA to IH of Table 2. In these experiments, the HU concentrations were 0.1 and 0.3 M, respectively, when used in the first and second incubations. In the first incubation it was necessary to keep the yeast cultures in the presence of 0.1 M HU for 3.5 h to obtain >90% arrested cells. In the second incubation HU was used at 0.3 M to prevent cells from bypassing the HU block. The shift to the restrictive temperature was performed for 4.5 h at 37°C to obtain >90% arrested large-budded cells during the first incubation and to allow their efficient recovery. In the second incubation, the temperature was raised to 38°C to increase the tightness of the temperature-sensitive block. Exponentially growing CY196 cultures (about 90% budded cells) underwent the first incubation in YPD or YPD plus HU liquid medium, and, after mild sonication, cells were spotted on YPD or YPD plus HU agar plates for the second incubation. The percentage of budded cells present in the first incubation and producing adjacent pairs of large-budded cells in the second incubation was scored microscopically at different times. The values reported in Table 2 refer to the 6-h time point, when >90% of cells in the control experiments IC and IG gave raise to microcolonies in the second incubation. Strain CY211 was used in experiments IIA to IIE. In experiment IIA, an exponentially growing culture was shifted directly to 38°C. In experiments IIB to IIE, cells were synchronized by addition of 2 μg of α-factor per ml (final concentration) and incubation at 25°C for 2.5 h. Approximately 80 and 20% of the cells were, respectively, unbudded or large budded after this treatment. α-factor was removed by filtration, and cells were either directly plated after sonication on YPD (experiments IIB and IIC) or resuspended in YPD liquid medium containing 0.1 M HU and incubated for an additional 2.5 h at 25°C before being plated on YPD (experiments IID and IIE). A 2.5-h incubation in the presence of HU was sufficient to obtain >90% large-budded cells. The percentage of adjacent pairs of large-budded cells in the second incubation was scored as described above, and the values reported in Table 2 again refer to the 6-h time point.

RESULTS

Six independent mouse monoclonal antibodies recognize the 86-kDa polypeptide encoded by the *POL12* gene. The second-largest polypeptide (B subunit) of the yeast Pol α-primase complex is encoded by the *POL12* gene (7, 36a). This gene is essential for cell viability, since tetrads heterozygous for the

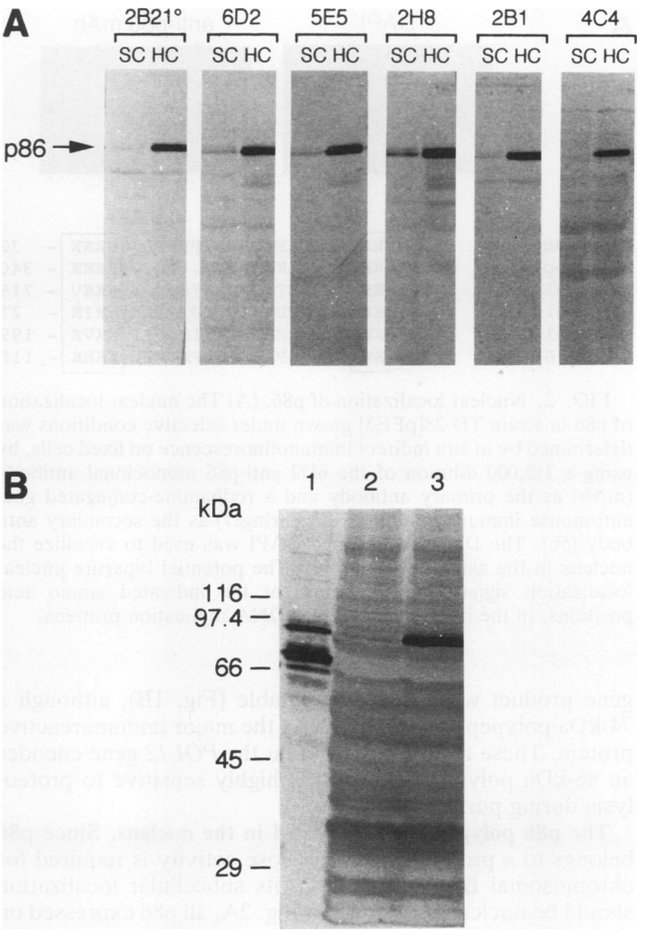

FIG. 1. Western blot analysis of crude extracts from yeast cells overexpressing the *POL12* gene. (A) Hybridoma cell lines producing the 2B2, 6D2, 5E5, 2H8, 2B1, and 4C4 monoclonal antibodies were raised as described in Materials and Methods, and ascites fluids at a dilution of 1:2,000 were used to probe by immunoblotting 30 μg of crude protein extract from strains TD-28[YEp24] (SC) and TD-28[pFE3] (HC). The position of the *POL12* gene product (86 kDa) is indicated by the arrow. (B) A mixture of the six monoclonal antibodies (1:2,000 dilution) was used to probe a Western blot containing an aliquot of immunopurified Pol α-primase complex (lane 1) or 30 μg of crude protein extract from strain TD-28[YEp24] (lane 2) or TD-28[pFE3] (lane 3).

POL12 disruption contain only two viable spores (data not shown). The predicted molecular mass of the *POL12* gene product is 78.9 kDa, while the purified protein migrates as an 86-kDa polypeptide when analyzed by SDS-PAGE (7). Since most of our immunopurified Pol α-primase complex preparations contain a major 74-kDa protein band (53, 62) and a panel of monoclonal antibodies was produced against this complex, it was of interest to verify whether some of these antibodies recognized the 86-kDa polypeptide in crude extracts when proteolysis was prevented.

As shown in Fig. 1A, six independent antibodies specifically identified an 86-kDa protein species which is enriched in extracts from cells containing the *POL12* gene on a high-copy-number plasmid. When an immunopurified Pol α-primase complex was probed on a Western blot with a mixture of the six monoclonal antibodies, a band migrating with the same electrophoretic mobility as that of the *POL12*

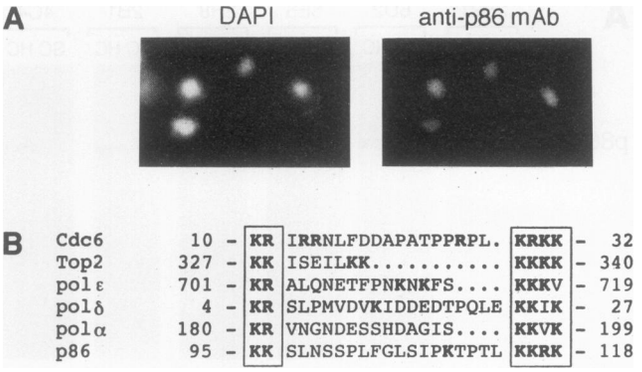

FIG. 2. Nuclear localization of p86. (A) The nuclear localization of p86 in strain TD-28[pFE3] grown under selective conditions was determined by in situ indirect immunofluorescence on fixed cells, by using a 1:2,000 dilution of the 6D2 anti-p86 monoclonal antibody (mAb) as the primary antibody and a rodhamine-conjugated goat anti-mouse immunoglobulin G (Boehringer) as the secondary antibody (56). The DNA-binding dye DAPI was used to visualize the nucleus in the same cells (56). (B) The potential bipartite nuclear localization signal (boxed) found, at the indicated amino acid positions, in the listed *S. cerevisiae* DNA replication proteins.

gene product was clearly detectable (Fig. 1B), although a 74-kDa polypeptide was found as the major immunoreactive protein. These results confirm that the *POL12* gene encodes an 86-kDa polypeptide which is highly sensitive to proteolysis during purification.

The p86 polypeptide is localized in the nucleus. Since p86 belongs to a protein complex whose activity is required for chromosomal DNA replication, its subcellular localization should be nuclear. As shown in Fig. 2A, all p86 expressed on a high-copy-number plasmid under the control of its own promoter is detected in the nucleus by indirect immunofluorescence with monoclonal antibodies against the *POL12* gene product. When the *POL12* gene was present in single copy, no immunofluorescent cells were found under the same experimental conditions (data not shown). These data confirm the specificity of the immunological reagents used and suggest that p86 is present at a low level in a wild-type background.

Consistent with the finding that p86 is a nuclear protein, a survey of the primary amino acid sequence of the *POL12* gene product identifies a region, spanning 23 residues from position 95 to position 118, containing two clusters of basic amino acids separated by an 18-amino-acid spacer (Fig. 2B). This bipartite motif functions as a nuclear localization signal (21) and is present in several yeast DNA replication proteins (Fig. 2B).

Two-codon insertion mutagenesis and deletion analysis of the *POL12* gene. The amino acid sequence derived from the largest *POL12* ORF shows significant homology with those of the corresponding subunits of the Pol α-primase complex from human, mouse, and *Drosophila melanogaster* cells (14, 15, 43). Clusters of conserved amino acid residues are mostly confined within the COOH-terminal two-thirds of these proteins (14). It is worth noting that the *POL12* gene product exhibits weak homology (20% identity and 41% similarity) also with the second-largest polypeptide (B subunit) of the yeast DNA Pol II (ε) complex, encoded by the *DPB2* gene (1). The most conserved region among the p86 homologs is proximal to the carboxyl terminus and contains the VINPG motif, which is found also in the *DPB2* gene product (Fig. 3). The NH_2-terminal half of p86 is more divergent, although, as in the human homolog (14), several putative $p34^{Cdc28/cdc2}$ phosphorylation sites can be found (data not shown).

In order to evaluate the physiological role of p86 and to define its putative functional domains, we mutagenized the *POL12* gene on a centromeric plasmid by the two-codon insertion method (3). As described in more detail in Materials and Methods, an oligonucleotide that creates a new and unique *Sac*I restriction site was inserted at random into various *Taq*I and *Mae*II sites in the *POL12* gene (Fig. 4A), and linker insertions were selected by ligation of the bacterial Kan^r gene in the *Sac*I site. The mutated plasmids were tested for their abilities to complement the lethal effect of a chromosomal *pol12::LEU2* disruption, either before or after the excision of the Kan^r cassette, by assaying yeast cell viability and growth rates at 19, 25, 37, and 39°C after plasmid shuffling. No complementation was found with plasmids carrying the Kan^r cassette in the *POL12* gene at all the positions indicated in Fig. 4B (data not shown). Therefore, the largest *POL12* ORF is coextensive with the genetic complementation unit.

As summarized in Table 1, the two-amino-acid insertion Leu-261–Glu–Leu–Asp-262 (*pol12-T7*) resulted in cell lethality, while insertions Ile-417–Glu–Leu–Asp-418 (*pol12-T9*) and Asn-535–Glu–Leu–Val-536 (*pol12-M2*) caused temperature sensitivity for growth at 37°C. All of the other two-amino-acid insertions (*pol12-T2*, *pol12-T3*, *pol12-T5*, *pol12-T6*, and *pol12-T8*) had no effect. In-frame deletions (Fig. 4B) were also generated as described in Materials and Methods and tested as described above. From this analysis (Table 1) we found that neither the 30-amino-acid region spanning from Asp-6 to Val-35 (*pol12-ΔT2,T3*) nor the 45-amino-acid region spanning from Ser-181 to Leu-224 (*pol12-ΔT4,T6*) was essential for p86 function. However, both a large in-frame deletion spanning from Glu-36 to Leu-261 (*pol12-ΔT3,T7*) and a small deletion spanning from Glu-411 to Ile-417 (*pol12-ΔT8,T9*) were lethal to the cell. Filling in of the *Bam*HI site at position +1946 from the first ATG of the *POL12* ORF generates a frameshift mutation leading to the synthesis of a protein lacking the last COOH-terminal 41 amino acids of

Sc p86	628	P D I M I I P S E L Q H F A R V V Q N V V V I N P G R F I R	657
Dm p73	591	- N V L - L - - D Q R - - I - L - N D C L - - - - - - V A D	620
Mouse p68	535	- - V F - V - - - - R Y - V K D I F G C - C V - - - - L T K	564
Human p67	535	- - V L - - - - - - R Y - V K D - L G C - C V - - - - L T K	564
Sc Dpb2	646	S T M V L C D I T S A Q - D L T Y N G C K - - - - - S - - H	675

FIG. 3. A stretch of amino acids is conserved in the B subunits of DNA Pol α and Pol ε. Numbers indicate the borders of the compared regions. Only nonidentical amino acid residues are shown for polypeptides other than yeast p86. Sc, *S. cerevisiae*; Dm, *D. melanogaster*.

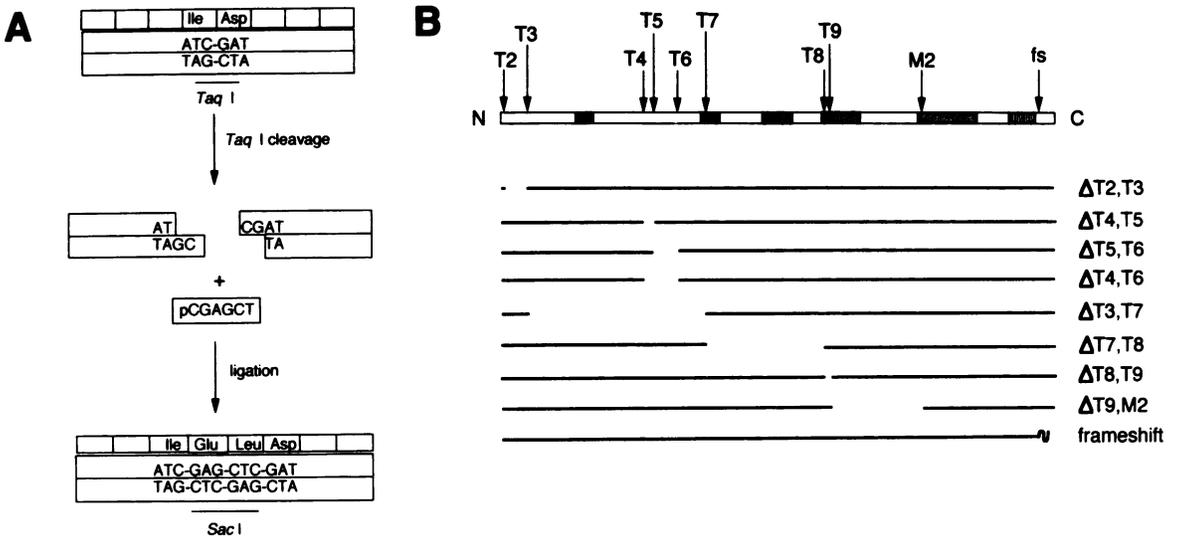

FIG. 4. Linker insertion mutagenesis of the *POL12* gene. (A) Relevant steps of the two-amino-acid linker insertion mutagenesis method (see text for details). (B) The bar represents schematically the p86 protein, with arrows indicating the positions of the two-amino-acid insertions at *Taq*I (T2 to T9) and *Mae*II (M2) restriction sites, as well as the frameshift mutation at position +1946 (fs) of the *POL12* ORF. The black box represents the putative nuclear localization signal, while the gray boxes indicate the amino acid regions conserved in the p86 homologs from other eukaryotes. In the bottom part of the figure, the in-frame deletions and the frameshift mutation described in the text are diagrammatically represented.

wild-type p86 and carrying an altered amino acid sequence from position 664 to 674 (Fig. 4B). This mutation is lethal, and therefore the p86 region from Ser-664 to Ser-705 is likely to be essential for p86 function.

TABLE 1. Growth phenotypes of the *pol12* mutants[a]

Allele	Growth level[b] at:		Insertion-deletion[c]
	25°C	37°C	
Wild type	++++++	+++++++	
T2	++++++	+++++++	I-5–**EL**–D-6
T3	++++++	+++++++	V-35–**EL**–E-36
T5	++++++	+++++++	T-193–**SS**–S-194
T6	++++++	+++++++	L-224–**SS**–S-225
T7	–	–	L-261–**EL**–D-262
T8	++++++	+++++++	L-410–**EL**–E-411
T9	+++	–/+	I-417–**EL**–D-418
M2	+++++	+	N-535–**EL**–V-536
ΔT2,T3	++++++	+++++++	E-6–L-36
ΔT3,T7	–	–	E-36–L-262
ΔT4,T5	++++++	+++++++	S-181–S-194
ΔT4,T6	++++++	+++++++	S-181–S-225
ΔT5,T6	++++++	+++++++	S-194–S-225
ΔT7,T8	–	–	E-262–L-411
ΔT8,T9	–	–	E-411–L-418
ΔT9,M2	–	–	E-418–L-536
Frameshift	–	–	S-664–**ILCTNYCPMPZ**

[a] Transformants of strain CY46 containing YCp50 derivatives carrying the indicated *pol12* mutations and cured of the *TRP1* plasmid carrying the *POL12* wild-type gene were streaked on YPD plates, which were incubated at different temperatures.

[b] The level of growth was evaluated after 3 days. A minus indicates no growth, while the number of plus signs indicates the extent of growth compared with that of strains carrying the wild-type *POL12* gene on plasmid pFE2, pFE7, or pFE132. No growth at both 25 and 37°C indicates the inability of the corresponding transformant to lose the *POL12* copy on the *TRP1* plasmid.

[c] The positions of the two-amino-acid insertions (boldface) and the boundaries of the in-frame deletions are indicated by numbers. The position of the frameshift mutation and the modified amino acid sequence (boldface) of the derived truncated protein are also shown. Amino acid residues are indicated in the one-letter code.

All of the two-amino-acid insertions causing either a lethal (*pol12-T7*) or a temperature-sensitive (*pol12-T9* and *pol12-M2*) phenotype are located within amino acid regions conserved in the p86 homologs from other species (Fig. 4B). All of the in-frame deletions without any detectable effect remove amino acid residues in nonconserved regions within the NH_2-terminal third of the protein. Conversely, *pol12-ΔT8,T9*, which removes only seven amino acids in a conserved region, is lethal, as are larger deletions in both the NH_2- and COOH-terminal halves of p86.

The *POL12* gene product is required for in vivo DNA synthesis and for progression through S phase. The production of a conditional mutation in the *POL12* gene (*pol12-T9*) allowed us to investigate the physiological role of the corresponding gene product. Since the plasmid carrying the original *pol12-T9* mutation was lost at high frequency, resulting in a slow-growth phenotype even at 25°C (Table 1), the wild-type chromosomal copy in strain H1514 was replaced with the *pol12-T9* mutant allele (see Materials and Methods). The growth rates, cell morphologies, DNA contents, and kinetics of DNA synthesis of the wild-type and isogenic mutant strains were compared at the permissive and nonpermissive temperatures.

As shown in Fig. 5A, the growth rate of the *pol12-T9* mutant strain at 25°C was only slightly different from that of the isogenic wild-type strain. Conversely, after the shift to the restrictive temperature (37°C), the *pol12-T9* strain exhibited a clear growth defect and mutant cells arrested as dumbbell-shaped cells with a single nucleus, as observed by 4',6-diamidino-2-phenylindole (DAPI) staining (data not shown).

Even at 25°C, *pol12-T9* cells were somewhat larger than normal and more likely than wild-type cells to be large budded, although the doubling times were similar in the two strains (110 and 100 min, respectively). Furthermore, the percentage of unbudded G_1 cells at 25°C was significantly lower in the pol12-T9 strain than in the wild-type strain (Fig. 6A). This reduced proportion of G_1 cells could result from a delay during progression through S phase. It has been shown

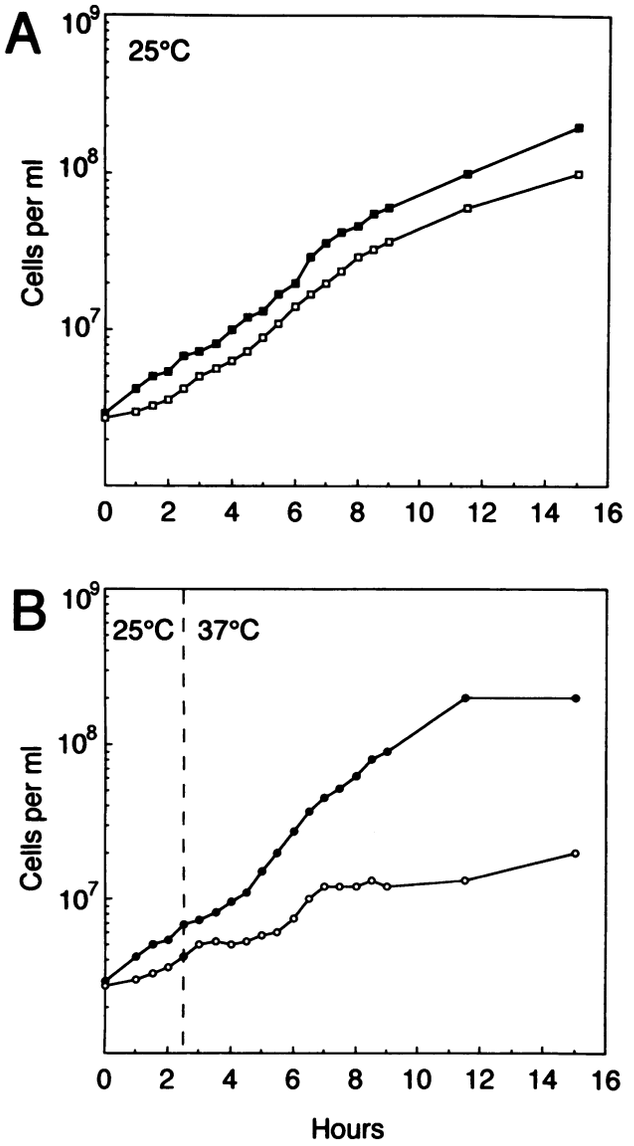

FIG. 5. Growth rate of the temperature-sensitive *pol12-T9* mutant strain. Cultures of wild-type (closed symbols) and *pol12-T9* (open symbols) cells, logarithmically growing at 25°C, were divided into two aliquots, which were incubated at 25°C (A) or shifted to 37°C at the indicated time (B). Cell numbers were monitored by direct cell counting with a light microscope.

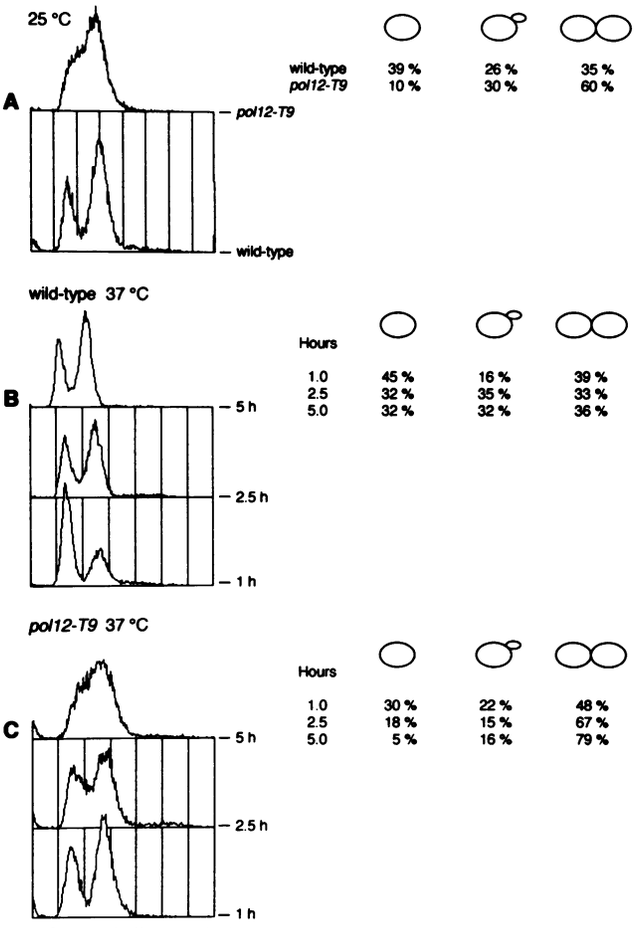

FIG. 6. Cell morphology and FACS analysis of wild-type and *pol12-T9* cells. Samples from the cultures grown at 25 or 37°C shown in Fig. 5 were collected at the times indicated and analyzed morphologically and for DNA content by FACS. The percentages of unbudded, small-budded, and large-budded cells were determined after sonication for 15 s and fixation in an equal volume of 3.7% formaldehyde–0.15 M NaCl followed by counting of 10 independent fields for a total of at least 200 cell units.

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 (23, 39). The decrease in the G_1 population in the *pol12-T9* strain is likely to be due to this indirect mechanism rather than to direct acceleration of the G_1-S transition, because *pol12-T9* cells are larger than wild-type cells. In fact, mutations that reduce the length of G_1 by accelerating the G_1-S transition are usually associated with a smaller size (16). When the DNA contents of the mutant and wild-type cells were analyzed by FACS at both the permissive and nonpermissive temperatures, the *pol12-T9* mutant strain was found to exhibit an abnormal DNA content even at 25°C (Fig. 6A). Consistent with the morphological observation, a very low proportion of mutant cells had a normal G_1 1C DNA content. Also, a higher percentage of mutant cells than of wild-type cells were traversing S phase. After 1 h at 37°C, the proportion of cells in G_1 increased in both the wild-type and mutant strains, as a result of the temperature shock (60) (Fig. 6B and C). However, the wild-type population exhibited a normal distribution of G_1 and G_2 cells after either 2.5 or 5 h at 37°C, as determined by cell morphology and FACS analysis (Fig. 6B). Conversely, after 2.5 h at 37°C the *pol12-T9* population accumulated an abnormal amount of S-phase cells, which were still present after 5 h, at which time the majority had arrested as dumbbell-shaped cells with a nearly 2C DNA content (Fig. 6C). Therefore, the *pol12-T9* mutation must affect cell cycle progression by increasing the length of S phase. All of the defective phenotypes resulting from the *pol12-T9* mutation, together with the biochemical evidence that p86 is tightly bound to the replicative DNA Pol α, strongly suggest a direct role for the *POL12* gene product in some aspects of chromosomal replication. To address this question more directly, we analyzed the kinetics of DNA synthesis in the wild-type and *pol12-T9* strains at permissive and nonpermissive temperatures. As shown in Fig. 7A, no

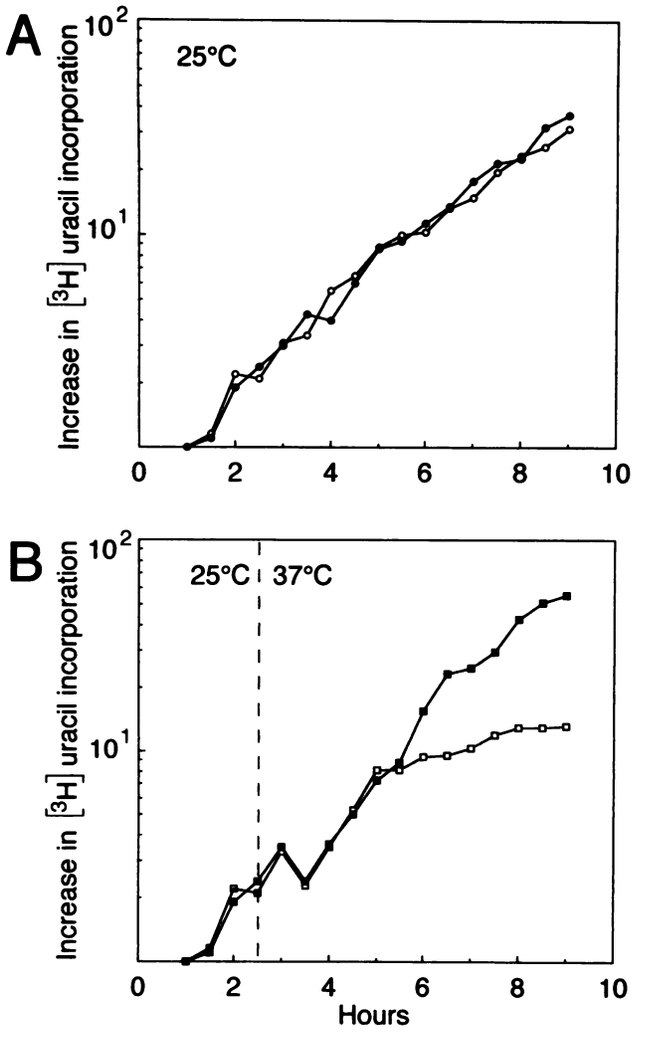

FIG. 7. Kinetics of DNA synthesis for wild-type and *pol12-T9* mutant cells. Exponentially growing cultures at 25°C were supplemented with [5,6-^{3}H]uracil at 7 μCi/ml and were divided into two aliquots, which were incubated at 25°C (A) or shifted to 37°C at the indicated time (B). [^{3}H]uracil incorporation into DNA or RNA was measured as previously described (29). The increase in [^{3}H]uracil incorporation is given as the ratio between the amount of radioactivity measured at the indicated times and that found after 1 h of incubation at the permissive temperature in the presence of the labeled precursor.

substantial difference in the incorporation of the labeled precursor into DNA was observed between the two strains at 25°C. However, after the shift to the restrictive temperature, *pol12-T9* cells exhibited a clear defect in DNA synthesis (Fig. 7B). A comparable defect in RNA synthesis was not observed (data not shown), indicating that the primary defect of this mutant is an impairment in DNA replication.

Mapping the *POL12*-dependent step within the yeast cell cycle by reciprocal shift experiments. The process of DNA replication can be divided into initiation, elongation, and termination events, which require different functions executed in a temporal order that can be established, in yeast cells, by using cell division cycle mutants (*cdc* mutants) and/or inhibitory drugs (31, 32, 36, 55). Among the different replication factors, the Pol α-primase complex is peculiar, because it appears to play a bifunctional role during the initiation reaction, at an origin and in the elongation step (67, 68). However, it is unknown whether all components of the complex are involved in both reactions. Therefore, we decided to map the *POL12*-dependent step with respect to the HU-sensitive step, since it is known that HU inhibits the elongation reaction and causes cells to arrest as large-budded cells (32, 65). If the *POL12* gene product was primarily involved in initiation of DNA replication, *pol12* temperature-sensitive mutant cells would complete the cell cycle at the restrictive temperature after HU removal and arrest as adjacent pairs of large-budded cells (see Materials and Methods). Alternatively, if p86 played a role in DNA elongation and, therefore, the *POL12*-dependent step overlapped the HU-sensitive step, the shift from HU to the *pol12* restrictive conditions would not allow the cells to proceed further in the cell cycle and they would arrest as single large-budded cells. Reciprocal shift experiments were carried out with *pol12-T9* strains, and the results are summarized in Table 2. When exponentially growing *pol12-T9* cultures blocked with HU at the permissive temperature were shifted to 38°C (Table 2, experiment IA), over 80% of the cells could recover from the HU block, since they arrested as adjacent pairs of large-budded cells. A shift of exponentially growing *pol12-T9* cultures (10% unbudded and 90% budded cells) from 25 to 38°C (experiment IB) caused the cells to arrest either as single large-budded cells (14%) or as pairs of adjacent large-budded cells (86%), as expected for a tight mutation leading to arrest in the first cell cycle. The HU treatment was reversible and not lethal (experiment IC), since 89% of cells were able to form colonies after HU removal, although the HU block was complete throughout the experiment (experiment ID). These data suggest that the *POL12* gene product is required before the HU-sensitive step. The results of experiments IE to IH in Table 2 are consistent with this interpretation. In fact, transfer of *pol12-T9* cells from 37°C to HU at 25°C should cause growth arrest with cells as single large-budded cells if the HU-sensitive step followed the step requiring the *POL12* function. Indeed, the proportion of budded cells able to execute the HU-sensitive step (pairs of adjacent large-budded cells in HU) was markedly reduced when the first incubation was carried out at 37°C instead of 25°C (experiments IE and IF). The *pol12-T9* arrest at 37°C was reversible (experiment IG), while the temperature-sensitive block was complete throughout the experiment (experiment IH).

To further confirm the ordering of the temperature-sensitive step with respect to the HU-sensitive step during the cell cycle, we performed the reciprocal shift experiment on α-factor-synchronized *pol12-T9* cultures (Table 2, experiments IIB to IIE). For this purpose we used strain CY211, which carries the *pol12-T9* mutation in a genetic background different from that of strain CY196, which was used in the previous experiments. As shown by experiment IIA, strain CY211 behaves like strain CY196 when shifted from 25 to 38°C. When cells were synchronized in G_1 with α-factor and then released from the pheromone treatment at 38°C (experiment IIB), they arrested in the first cell cycle as single large-budded cells, as expected for a tight mutation affecting a function required after start. Conversely, cells completed the first cell cycle and arrested as pairs of adjacent large-budded cells when α-factor-presynchronized cultures were incubated in the presence of HU and released from HU at 38°C (experiment IID). These data demonstrate that the *POL12* gene product plays an essential role at the initial stage of the DNA replication, before the HU-sensitive step.

TABLE 2. Mapping of the *POL12*-dependent step with respect to the HU-sensitive step

Strain	Expt	Conditions[a] of first incubation	Second incubation			
			Conditions[a]	Total no. of cells examined	% of budded cells that produce:	
					One large-budded cell	Two adjacent large-budded cells[b]
CY196	IA	HU	38°C	115	16	84
	IB	25°C	38°C	114	14	86
	IC	HU	25°C	120	11	
	ID	HU	HU	100	91	
	IE	37°C	HU	104	81	19
	IF	25°C	HU	117	10	90
	IG	37°C	25°C	107	15	
	IH	37°C	38°C	103	97	
CY211	IIA	25°C	38°C	112	20	80
	IIB	α-Factor	38°C	134	85	15
	IIC	α-Factor	25°C	123	12	
	IID	α-Factor→HU	38°C	125	16	84
	IIE	α-Factor→HU	25°C	142	10	

[a] See Materials and Methods for details.
[b] Cells gave rise to microcolonies in cases for which no number is given.

DISCUSSION

Chromosome replication in S phase is a central event in all eukaryotic cell cycles, and the timing of entry into S phase is tightly regulated (reviewed in references 18, 24, 40, and 57). Further, DNA replication is limited to once per cell cycle, and control mechanisms exist to prevent rereplication and entrance into mitosis when replication is not properly completed (reviewed in references 17, 22, 27, 33, 40, 46, and 49). However, the molecular details of turning replication on and off are still largely unknown (58). In fact, while a number of genes have been shown to be necessary for the linkage between the START commitment control and entry into S phase (reviewed in references 48 and 57), the molecular mechanisms which regulate the activity of the replication machinery are not yet understood. There is some evidence that essential replication proteins are modified in a cell cycle-dependent manner (20, 28, 47), suggesting that entry and exit into S phase might be modulated by posttranslational modifications that activate or repress the activity of replication factors.

Among the three DNA polymerases involved in chromosomal DNA replication (67), DNA Pol α, with the tightly bound DNA primase, plays a peculiar function since it is involved both in initiation of DNA replication at an origin and in the elongation reaction. This dual role has been suggested by analysis of the biochemical and catalytic properties of the Pol α-primase complex and by reconstitution studies with purified components, mostly performed with the SV40 in vitro DNA replication system (reviewed in references 37 and 67). However, direct in vivo evidence of the dual role of the Pol α-primase complex is still lacking.

The four-subunit structure of the Pol α-primase complex has been highly conserved in eukaryotic organisms as phylogenetically distant as yeasts and mammals (67). A protein, ranging in size from 67 to 86 kDa (B subunit), has been found tightly associated with the p180 Pol α polypeptide. In vitro reconstitution experiments indicate that yeast p86 is not required for the formation of a physical complex between Pol α and the primase polypeptides (6). Furthermore, p86 is not necessary for RNA primer formation and does not affect any of the parameters of Pol α catalysis, such as processivity or the K_m for deoxynucleoside triphosphates or for the primer terminus (7). Nonetheless, a functional *POL12* gene, encoding the 86-kDa B subunit, is required for cell viability, suggesting that this polypeptide performs an essential function. Recently, it has been shown that the human Pol α-primase complex stimulates the ATP-dependent binding of SV40 T antigen to the viral origin of replication (44), and the B subunit appears to play a crucial role in mediating the T antigen–Pol α-primase interaction (14).

The physiological characterization of the 18 different *pol12* alleles described in this work, together with the finding that the B subunit of Pol α-primase in all eukaryotic organisms analyzed so far shows conserved regions within the COOH-terminal two-thirds of the proteins (14), suggests that this region might play an essential role for the interaction between p86 and the p180 Pol α polypeptide. In fact, all but one of the mutations in this region lead either to a temperature-sensitive phenotype or to cell lethality. Conversely, most of the two-amino-acid insertions in the more divergent NH_2-terminal region, and even some significant amino acid deletions in this portion of the protein, do not cause any altered phenotype, although larger deletions in the same region, which include the nuclear localization signal, are lethal. Proteins likely to be involved in the initial stages of DNA replication, such as the Pol α-primase complex and replication protein A, are involved in species-specific interactions (5, 45, 63). The NH_2-terminal 230 amino acids of the human Pol α-primase B subunit are sufficient for in vitro interaction with T antigen (14), and it is known that this interaction is species specific (45). Therefore, the more divergent NH_2-terminal region of the Pol α-primase B subunit might have evolved to interact with species-specific key factors, such as origin recognition proteins, and therefore would lack the tight evolutionary constraints expected for protein domains involved in a catalytic function.

Among strains carrying the different mutations produced in the *POL12* gene, the characterization of the *pol12-T9* temperature-sensitive mutant strain allowed us to gain relevant information about the role of the Pol α-primase B subunit in DNA replication in vivo. We have shown that the *pol12-T9* mutant strain is impaired in DNA synthesis in vivo after shift to the nonpermissive temperature. Under restrictive conditions, cells arrest with a dumbbell shape, with an

undivided nucleus at the neck between mother and daughter cells. This terminal phenotype is typical of budding-yeast DNA synthesis mutants (12).

When the cell morphology and DNA content of *pol12-T9* cells were analyzed at the permissive and nonpermissive temperatures, cells showed a defect in cell cycle progression, probably because of an S-phase delay, and arrested with a nearly 2C DNA content. This finding is not surprising, because even cells with mutations in essential DNA polymerases often arrest with a greater-than-normal 1C DNA content (2, 10, 68), and this behavior is interpreted to be a consequence of mutations which only partially affect the core replication machinery at the nonpermissive temperature. The observation that *pol12-T9* cells can complete one round of cell division and DNA replication at the restrictive temperature before arresting with a nearly 2C DNA content could be the result of residual and abnormal DNA synthesis that may activate the *RAD9* checkpoint (33, 68). It will be of interest to verify the *RAD9* dependency of different *pol12* alleles, since this dependency appears to be allele specific and is influenced by the experimental conditions (68).

Because eukaryotic DNA synthesis is initiated at multiple origins, it is impossible to distinguish, solely on the basis of DNA content, between a defect in initiation and one in elongation. For the same reason, a lag in S phase does not distinguish incomplete initiation from an elongation defect. Because the Pol α-primase complex appears to be involved in both the initiation and elongation steps of DNA replication, we used the *pol12-T9* mutant to determine the time of action of the *POL12* gene product with respect to the HU-sensitive step. We found that the B subunit of the Pol α-primase complex is able to perform its function even when DNA elongation is inhibited by HU, as has been observed for the *CDC46* (35) and *CDC6* (11) gene products, which also appear to have essential roles in initiation of DNA replication. The analysis of the *pol12-T9* allele does not rule out the possibility that the B subunit might also play a role in the elongation step of DNA replication, and more alleles will be produced and complementary approaches will be taken to clarify this point. Nonetheless, our results showing that p86 is required at a stage preceding the HU-sensitive step clearly imply that this protein is essential for initiation of DNA replication in vivo.

Defective interactions among proteins required for initiation of DNA replication would possibly result in a less efficient activation of replication origins, leading to an increase in the length of S phase, as we detected in the *pol12-T9* mutant. Since one of the initial stages of DNA replication requires the loading of the Pol α-primase complex at an origin of replication, we suggest that the *POL12* gene product mediates the interaction between the Pol α-primase complex and proteins involved in modulating origin function (Fig. 8), such as the origin recognition complex (4), replication protein A (13), or the *CDC6* and *CDC46* gene products, whose biochemical functions are still unknown. The finding that the human B subunit mediates Pol α-T antigen interaction in vitro (14) is in agreement with this model. Since the origin recognition complex appears to be bound at yeast origins of replication during most or all of the cell cycle (19), it is reasonable to suggest that origin firing may be regulated by interactions with other critical protein factors (18). In this view, the p86 B subunit may have a regulatory function in activating DNA synthesis by loading the Pol α-primase complex at the origin, either by direct interaction with the origin recognition complex or by mediating the assembly of the initiation complex through the interaction with replication protein A or other, yet-unidentified key protein factors (indicated by the question mark in Fig. 8). We have recently found that the yeast Pol α-primase B subunit is a stable protein which is posttranslationally modified in a cell cycle-dependent manner (unpublished data). Therefore, we propose that changes in the phosphorylation state of p86 might modulate the regulatory role of the B subunit in origin firing.

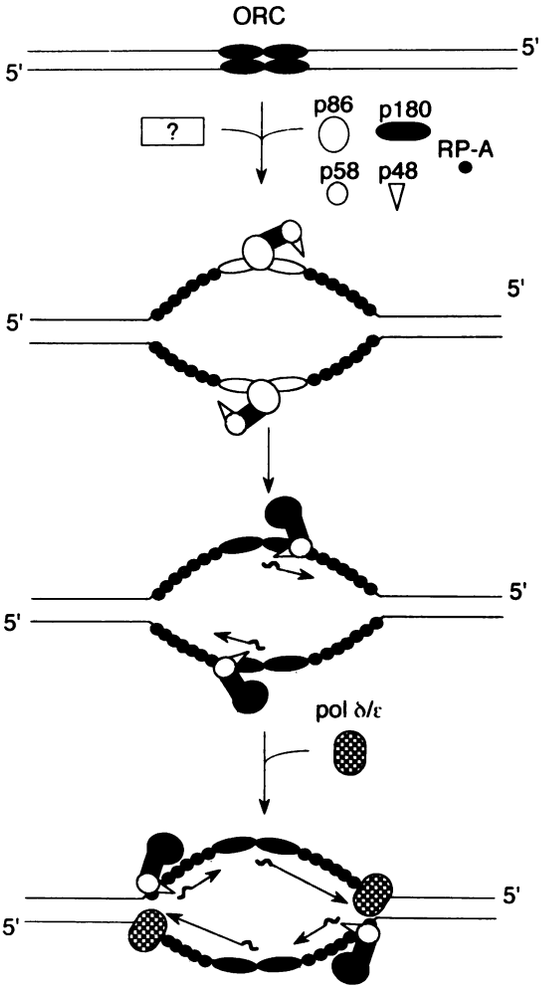

FIG. 8. Model for a regulatory role of the Pol α-primase B subunit in initiation of DNA replication at an origin. Relevant proteins are indicated with different symbols. A change from black to white for the origin recognition complex (ORC) and p86 symbols indicates a change in the activity of these proteins (see text for details).

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