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The yeast analog of mammalian cyclin/proliferating-cell nuclear antigen interacts with mammalian DNA polymerase δ

(DNA replication/DNA polymerase III/processivity/cell cycle control/*Saccharomyces cerevisiae*)

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ABSTRACT **DNA polymerase III from *Saccharomyces cerevisiae* is analogous to the mammalian DNA polymerase δ by several criteria, including an increased synthetic activity on poly(dA)·oligo(dT) (40:1 nucleotide ratio) in the presence of calf thymus proliferating-cell nuclear antigen (PCNA), or cyclin. This stimulation assay has been used to purify the yeast analog of PCNA/cyclin (yPCNA) to homogeneity. yPCNA is a trimer or tetramer ($M_r \approx 82{,}000$) of identical subunits with a denatured M_r of 26,000. On a molar basis yPCNA and calf thymus PCNA/cyclin are equally active in stimulating DNA synthesis by DNA polymerase III. About 10 times more yPCNA than calf thymus PCNA/cyclin is needed, however, to stimulate calf thymus DNA polymerase δ, and the degree of stimulation obtained at saturating levels of yPCNA is a factor of 2–3 less than with calf thymus PCNA/cyclin. Both stimulatory proteins exert their effect in an identical fashion, i.e., by increasing the processivity of the DNA polymerase. Yeast DNA polymerases I and II and calf thymus DNA polymerase α are not stimulated by yPCNA. Treatment of logarithmic-phase cells with hydroxyurea blocks them in the S phase and produces a 4- to 5-fold increase in yPCNA.**

Proliferating-cell nuclear antigen (PCNA), also known as cyclin, is a cell cycle-regulated protein present at high levels in rapidly dividing cells (1–4). The intranuclear localization of PCNA/cyclin to regions of active DNA synthesis indicates that PCNA/cyclin plays a role in DNA replication (5). Recently, it has been shown that PCNA is required for *in vitro* simian virus 40 (SV40) DNA replication (6, 7). In its absence, early replicative intermediates accumulate and leading-strand DNA synthesis is impaired (7).

The additional identification of PCNA/cyclin as an accessory factor of DNA polymerase δ (8–10) suggests, in conjunction with the SV40 DNA replication data, a role for a complex of DNA polymerase δ and PCNA/cyclin in processive leading-strand DNA synthesis.

We have recently isolated from the yeast *Saccharomyces cerevisiae* a third nuclear DNA polymerase, which we call DNA polymerase III and which shows properties closely analogous to the mammalian DNA polymerase δ (11). These include a proofreading 3′ → 5′ exonuclease activity, sensitivity to the drug aphidicolin, and relative resistance to the nucleotide analog N^2-[*p*-(*n*-butyl)phenyl]dGTP (12). In addition, calf thymus PCNA/cyclin interacts with DNA polymerase III and promotes processive DNA synthesis by the enzyme (13). This finding prompted us to identify and purify a PCNA/cyclin-like activity in yeast by using stimulation of DNA polymerase III activity as an assay. The purification of this activity and its interaction with DNA polymerase III, as well as calf thymus DNA polymerase δ, is presented in this paper. Because the name cyclin implies that the expression of the gene and/or the activity of the protein is restricted to a part of the cell cycle, we prefer to use the more neutral term yPCNA (yeast proliferating-cell nuclear antigen) for the activity in yeast, in the absence of more comprehensive data on cell cycle control than presented here.

MATERIALS AND METHODS

Enzymes. *S. cerevisiae* DNA polymerases I and II were purified from the protease-deficient strain BJ405 (*MATα, trp1, pep4-3, prbl, prc1*) as described (13). DNA polymerase III fraction VIII (Fig. 1*A*) was used in all experiments. Calf thymus DNA polymerases α and δ were partially purified from fetal bovine thymus through the hydroxyapatite step as described (14). Homogeneous calf thymus PCNA purified from fetal bovine thymus was a generous gift of C. K. Tan (University of Miami) (8).

Purification of yPCNA. Yeast strain BJ405 was grown to late logarithmic phase, harvested, and broken with glass beads. The lysate was fractionated with polyethylenimine (Polymin P; BASF Wyandotte, Parsippany, NJ) to remove nucleic acids, with ammonium sulfate (0–51% saturation), and by phosphocellulose chromatography as described (11). The flowthrough and the wash of this column (fraction III) was the starting material for the purification of yPCNA. All steps were carried out at 0–4°C unless otherwise stated. Fraction III (1500 ml) was stirred with 400 ml of DEAE-Sephacel (Pharmacia) that had been equilibrated in buffer A [25 mM potassium phosphate, pH 7.0/25 mM KCl/10% (vol/vol) glycerol/2 mM EDTA/5 mM dithiothreitol/2 μM pepstatin A/2 μM leupeptin/2 mM benzamidine/0.5 mM phenylmethylsulfonyl fluoride]. After stirring for 2 hr, the resin was allowed to settle for 1 hr and the supernatant was decanted. The resin was resuspended in 500 ml of buffer A and allowed to settle, and the supernatant was decanted. The resin was then resuspended in 200 ml of buffer A and poured in a column containing 100 ml of resin. After packing, the column was washed with 1 liter of buffer A and bound protein was eluted with 5 liters of a linear gradient of 25 → 600 mM KCl in buffer A. yPCNA was eluted at 0.37–0.45 M KCl. Active fractions were pooled (500 ml), concentrated with a Millipore Minitan cassette system, and dialyzed twice against 10 volumes of buffer B [25 mM triethanolamine hydrochloride, pH 7.3/10% (vol/vol) glycerol/2 mM EDTA/5 mM dithiothreitol/2 μM pepstatin A/2 μM leupeptin/0.5 mM benzamidine) plus 25 mM NaCl (fraction IV, 80 ml).

Half of fraction IV was loaded on a 2-ml Mono Q column (Pharmacia). Protein was eluted with a 50-ml linear gradient of 25 → 500 mM NaCl in buffer B. The Mono Q column was attached to a Waters HPLC apparatus as described (11). Chromatography was at room temperature. The second half of fraction IV was chromatographed in an identical fashion.

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Abbreviations: PCNA, proliferating-cell nuclear antigen; yPCNA, yeast PCNA; SV40, simian virus 40.

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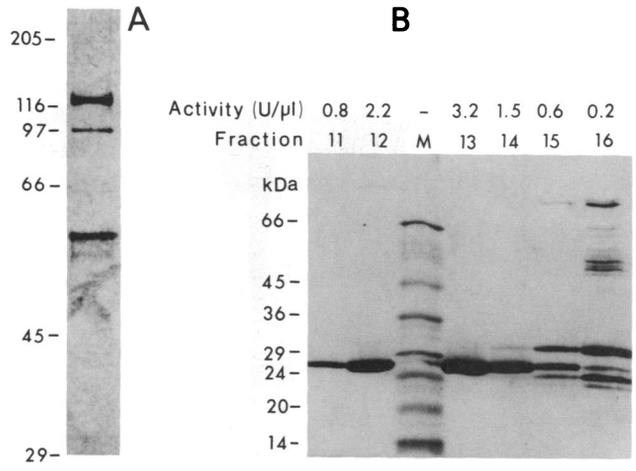

FIG. 1. NaDodSO$_4$/PAGE of yeast DNA polymerase III and yPCNA. (*A*) DNA polymerase III [fraction VII (11)] was further purified by sedimentation through a 10–30% (vol/vol) glycerol gradient in 20 mM potassium phosphate, pH 7.2/1 mM EDTA/5 mM dithiothreitol/1 mM benzamidine/2 μM pepstatin A/40 mM ammonium sulfate/50% (vol/vol) ethylene glycol/1.0% (vol/vol) ampholyte (pH 3.5–10; Pharmacia) for 44 hr at 0°C at 58,000 rpm (Beckman SW60 rotor). DNA polymerase III peak fraction was analyzed. Positions of molecular weight markers ($M_r \times 10^{-3}$) are indicated. (*B*) Glycerol gradient fractions (5 μl per lane) of yPCNA. See *Materials and Methods* for details. Fraction number and volume activity [units (U)/μl] are indicated above each lane. Lane M, molecular mass markers (kDa at left). Proteins were visualized by silver staining.

Active fractions were combined and dialyzed against buffer C [20 mM potassium phosphate, pH 7.0/10% (vol/vol) ethylene glycol/0.1 mM EDTA/10 μM $CaCl_2$/2 μM pepstatin A/2 μM leupeptin/5 mM dithiothreitol/0.5 mM benzamidine] (fraction V, 11 ml).

Fraction V was loaded on a 4.5-ml hydroxyapatite HPLC column (Bio-Rad) and eluted with a 25-ml gradient of 20 → 250 mM potassium phosphate (pH 7.0) in buffer C. Active fractions were dialyzed against buffer B (fraction VI, 7 ml).

Fraction VI was rechromatographed and concentrated over a 1-ml Mono Q column by elution with a 20-ml linear gradient of 25 → 500 mM NaCl in buffer B. Chromatography was at room temperature (fraction VII, 1.8 ml).

Seventy percent of fraction VII was chromatographed at room temperature over a 13-ml gel filtration column (Waters 300 SW) in buffer B plus 0.2 M NaCl by repeated injections of 250 μl (fraction VIII). To determine the Stokes radius of yPCNA, 50 μl of fraction VII was injected onto the column.

One hundred fifty microliters of fraction VII was sedimented through a 10–30% (vol/vol) glycerol gradient in buffer B plus 0.2 M NaCl at 58,000 rpm (Beckman SW60 rotor) for 20 hr at 4°C. Three-drop fractions were collected from the bottom of the tube (Fig. 1*B*). To determine the sedimentation coefficient, 75 μl of fraction VII was sedimented together with marker proteins. Two-drop fractions were collected.

Assays for yPCNA. The 25-μl assay mixtures contained 20 mM Tris·HCl (pH 7.8), 4% glycerol, 2.5 μg of bovine serum albumin, 5 mM dithiothreitol, 20 μM [^{3}H]dTTP (500 cpm/pmol), 9 mM $Mg(OAc)_2$, 0.25 μg of poly(dA)·$(dT)_{16}$ (40:1 nucleotide ratio), 1 unit of DNA polymerase III, and yPCNA or calf thymus PCNA. The mixtures were incubated at 37°C for 30 min and processed as described (11). One unit of yPCNA stimulates the synthetic activity of DNA polymerase III by 100%. When calf thymus DNA polymerase δ was used, the buffer in the assay was changed to 40 mM 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethyl-1,3-propanediol (Bistris)·HCl (pH 6.5) and the glycerol concentration to 10% (8).

In assays measuring processive DNA synthesis by the DNA polymerases, [α-^{32}P]dTTP (3000 cpm/pmol) replaced [^{3}H]dTTP and reaction volumes were lowered to 10 μl. Reaction times were adjusted so that <1 pmol of dTMP was incorporated per pmol of primer. The products were separated by electrophoresis in an 8% polyacrylamide gel in 80 mM Tris·HCl/80 mM borate/4 mM EDTA, pH 8.3, containing 7 M urea. The radioactive products were visualized by autoradiography on Kodak XAR film at −70°C with an intensifying screen.

RESULTS

Purification and Physical Characterization of yPCNA. The purification of yPCNA is summarized in Table 1. The initial steps of the purification scheme were based on the assumption that yPCNA had a chromatographic behavior similar to that of calf thymus PCNA—i.e., failure to bind to phosphocellulose and strong binding to a DEAE column (8). Initially, yeast DNA polymerase III was used to monitor column fractions of yPCNA. Because of the low but significant activity of DNA polymerase III on poly(dA)·oligo(dT) in the absence of yPCNA, this was often problematic when assaying for low levels of yPCNA. Later, calf thymus DNA polymerase δ was used in assays for yPCNA. Because this polymerase is stimulated by yPCNA but is completely inactive on poly(dA)·oligo(dT) (8), even very low levels of yPCNA could be easily and accurately measured. yPCNA purified through the gel filtration step was about 60% pure as judged by NaDodSO$_4$/PAGE. Glycerol gradient sedimentation purified yPCNA to homogeneity (Fig. 1*B*). Only small amounts of yPCNA could be isolated from logarithmic-phase

Table 1. Purification of yPCNA

Fraction	Step	Protein, mg	Activity, units	Specific activity, units/mg	Recovery, %
I	Cleared lysate*	55,000			
II	Dialyzed $(NH_4)_2SO_4$ precipitate	15,500			
III	Phosphocellulose flowthrough	12,700			
IV	DEAE-Sephacel	128	47,200	360	(100)†
V	Mono Q	9.4	29,400	3,100	62
VI	Hydroxyapatite	2.3	22,800	9,900	48
VII	Second Mono Q	1.03	16,400	15,900	35
VIII	Gel filtration‡	0.16	8,600	53,800	26

*From 2 kg (wet weight) of cells.
†Activity could not be measured prior to this step.
‡Seventy percent of fraction VII was taken through this step.

yeast cells. Assuming full recovery of yPCNA through fraction IV, 560 μg of yPCNA was present in 55 g of protein (cleared lysate) from 2 kg of yeast (Table 1). Varying the isolation conditions (e.g., increasing the ammonium sulfate in step 2 to 58% saturation) did not lead to an increased yield of yPCNA. In contrast, large amounts of PCNA can easily be isolated from mammalian cells. For instance, from 0.75 kg of fetal bovine thymus, 14 g of protein containing 11 mg of PCNA can be obtained in the cleared lysate (8).

The apparent molecular weight of the denatured protein is 26,000 (Fig. 1*B*). The native molecular weight of 82,400, calculated from gel filtration (Stokes radius 40 Å) and glycerol gradient centrifugation (5.0 S) (15), indicates that yPCNA is either a trimer or a tetramer of identical subunits. The isoelectric point of yPCNA is 5.3. *N*-Ethylmaleimide readily inactivates yPCNA [$t_{1/2}$ = 2.5 min with 2 mM *N*-ethylmaleimide at 0°C (12)].

yPCNA Stimulates DNA Polymerase δ. The effect of calf thymus PCNA and yPCNA on yeast DNA polymerase III or calf thymus DNA polymerase δ was measured by using poly(dA)·oligo(dT) (40:1 nucleotide ratio) as template primer (Fig. 2). DNA polymerase III was stimulated in an identical fashion by calf thymus and yeast PCNA (Fig. 2*A*). About 10 times more yPCNA than calf thymus PCNA was needed to obtain an equivalent degree of stimulation of DNA polymerase δ and, in addition, at saturating levels of PCNA, the calf thymus PCNA was 2–3 times more effective (Fig. 2*B*). Calf thymus DNA polymerase α or yeast DNA polymerase I or II were not stimulated by yPCNA (results not shown). The activity of yeast DNA polymerase III or calf thymus DNA polymerase δ on poly(dA)·oligo(dT) (40:1 nucleotide ratio) is low, primarily because of the low processivity of DNA synthesis by these enzymes (10, 13). Addition of either yPCNA or calf thymus PCNA increased processivity to full length of the available template for DNA polymerase III (Fig. 3, lanes 1–5) as well as for DNA polymerase δ (lanes 6–8). The processivity of yeast DNA polymerase I was not increased by addition of yPCNA (lanes 9–10). DNA polymerase II was fully processive on poly(dA)·$(dT)_{16}$ and addition of yeast or calf thymus PCNA had no effect (Fig. 3, lanes 11–12, and ref 13). At subsaturating levels of yPCNA, two size classes of products were synthesized by DNA polymerase III, one size class identical to that observed in the absence of yPCNA and one size class identical to that observed at saturating levels of yPCNA (Fig. 3, lanes 2 and 3).

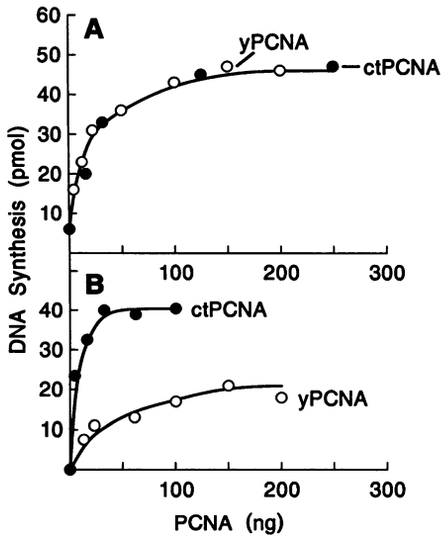

FIG. 2. Stimulation of DNA polymerase III (*A*) and DNA polymerase δ (*B*) by calf thymus PCNA (ctPCNA, ●) or yPCNA (○). DNA synthesis is expressed as pmol of dTMP incorporated.

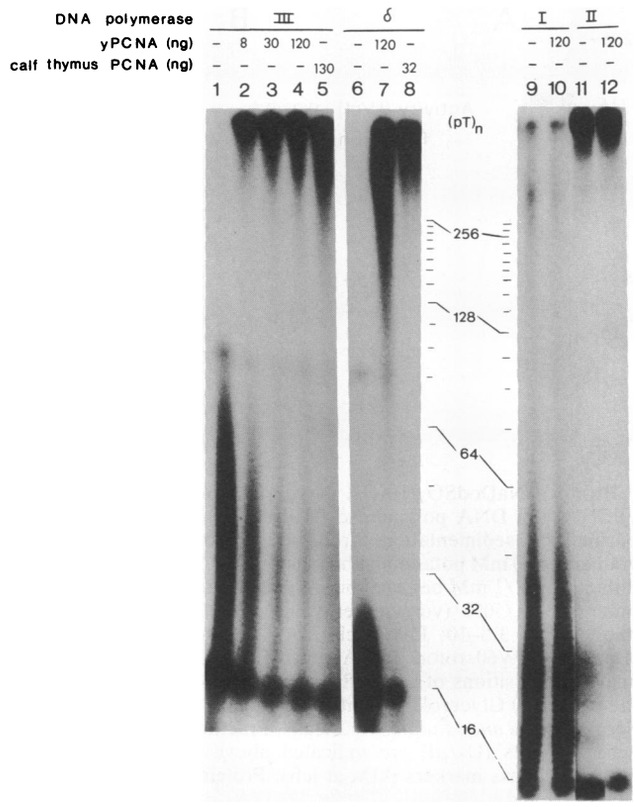

FIG. 3. Processivity of DNA polymerases. Approximately equal amounts of radioactivity were loaded in each lane. Lanes 1–8 and 9–12 are from different gels. The $(pT)_{16}$ ladder used as size markers was generated as described (13). Darker spots at $(pT)_n$ = 16 and $(pT)_n$ = 90 are autoradiographic artifacts due to the tracking dyes (Bromophenol blue and xylene cyanol FF, respectively).

yPCNA has no measurable enzymatic activity—i.e., no single- or double-stranded DNA-dependent exonuclease or endonuclease activity, no DNA polymerase activity, and no ATPase activity (data not shown). In addition, no binding of yPCNA to single-stranded or double-stranded DNA was detected in nitrocellulose filter binding experiments (data not shown).

Cell Cycle Regulation of yPCNA. Treatment of logarithmic-phase yeast cells with hydroxyurea causes arrest in the S phase (16, 17). Extracts from hydroxyurea-treated cells and control cells contain similar levels of DNA polymerase activity but the treated cells contain 4- to 5-fold higher levels of yPCNA (measured as specific activity; Table 2).

DISCUSSION

Since the first isolation of DNA polymerase δ from calf thymus (18), many different forms of this enzyme have been isolated (14, 19–23). The synthetic activity of some of these forms is not stimulated by PCNA (22, 24), and only one form of DNA polymerase δ isolated from fetal bovine thymus and the enzyme isolated from human placenta require PCNA for efficient synthesis on the model template–primer poly(dA)·oligo(dT) (8, 21). The form of DNA polymerase δ from calf thymus that interacts with PCNA has a subunit structure—i.e., two subunits of M_r 125,000 and 48,000 (14), very similar to that of yeast DNA polymerase III, which consists of two subunits of M_r 125,000 and 55,000 (Fig. 1*A*). The other forms of calf thymus DNA polymerase δ with different, more complex, subunit structures, are unaffected by PCNA (22, 24). DNA polymerase α of *Drosophila melanogaster* can be transformed into a polymerase with δ-like activities upon

Table 2. yPCNA in hydroxyurea-treated cells

Experiment	Hydroxyurea	Fraction II protein, mg	DNA polymerase, units	yPCNA, units
1	−	22.5	7500	54
	+	13.8	4400	158
2	−	25.3	8900	50
	+	20.8	6200	166

Strain BJ405 was grown with aeration in 3 liters of YPD medium at 30°C to OD_{660} = 1.0. Hydroxyurea was added (final concentration, 150 mM) to one-half of the culture, and the untreated and treated suspensions were aerated for another 75 min. Cells were then chilled, harvested, and lysed as described (11), except that protein was precipitated with 0.35 g of ammonium sulfate per ml. The precipitate was collected as usual, redissolved in buffer B and desalted by passage through a Sephadex G-25 column (Pharmacia) equilibrated in buffer B (fraction II). Total amount of protein and DNA polymerase activity was measured in fraction II. Fraction II was stirred for 2 hr with 3 ml of phosphocellulose, equilibrated in buffer B. The resin was pelleted at 1000 × *g* for 5 min and washed three times by resuspension and pelleting with 5 ml of buffer B. The combined supernatants were injected onto a 1-ml Mono Q column and protein was eluted as described for the second Mono Q column (see *Materials and Methods*). yPCNA activity was determined by measuring stimulation of DNA polymerase δ.

removal of a M_r 73,000 subunit (25). However, monoclonal antibodies to human DNA polymerase α do not crossreact with DNA polymerase δ, and a polyclonal antiserum to DNA polymerase δ fails to crossreact with DNA polymerase α (21). In yeast we found a similar lack of immunological crossreactivity between DNA polymerases I and III, the analogs of DNA polymerase α and δ, respectively (12).

Some lines of evidence indicate that PCNA stimulates DNA polymerase III or δ through a protein–protein interaction. Processivity assays with subsaturating levels of PCNA show two size classes of products, one class identical to that observed in the absence of PCNA and a second class identical to that obtained in the presence of saturating levels of PCNA (Fig. 3, lanes 1–4, and ref. 13). This argues against a mass-action effect exerted by PCNA. Neither calf thymus PCNA nor yPCNA has detectable DNA-binding activity (8). Calf thymus PCNA, however, increases the binding of calf thymus DNA polymerase δ to poly(dA)·oligo(dT) as assayed by nitrocellulose filter binding (8). Both calf thymus and yeast PCNA are acidic proteins with similar chromatographic properties. The PCNA from calf thymus and other mammalian species is a dimer of M_r 37,000 subunits, as determined by glycerol gradient centrifugation and $NaDodSO_4$/PAGE (8, 10). The calculated molecular weight of the polypeptide deduced from the highly homologous human or rat cDNA sequence, however, is only about 29,000 (26, 27), indicating an anomalous electrophoretic behavior of the mammalian PCNA. yPCNA is a trimer or tetramer of M_r 26,000 subunits. Unfortunately, crosslinking experiments with dimethyl suberimidate (25) did not allow an unequivocal determination of subunit structure (result not shown). In addition, yPCNA is immunologically distinct from the mammalian PCNAs. Two monoclonal antibodies to the rat PCNA, which crossreact with PCNAs from other mammalian species (28, 29), did not crossreact with yPCNA (data not shown). With this in mind it is remarkable that yeast DNA polymerase III interacts equally well with the calf thymus and the yeast PCNA (Fig. 2*A*). The converse is not true; about 10-fold more yPCNA than calf thymus PCNA is needed to attain a similar degree of stimulation of DNA polymerase δ (Fig. 2*B*). These experiments suggest that the PCNAs and the DNA polymerases with which they interact are highly conserved proteins. Both calf thymus and yeast PCNA likely share similar contact points for interaction with DNA polymerase III. Presumably, additional contact points for interaction with DNA polymerase δ are missing in yPCNA. Furthermore, levels of yPCNA sufficient to fully stimulate calf thymus DNA polymerase δ fail to restore leading-strand DNA synthesis in an *in vitro* SV40 DNA replication system lacking its endogenous PCNA (G. Prelich and B. Stillman, personal communication; see ref. 7). This may indicate that PCNA plays a larger role in chromosomal DNA replication beyond its interaction with DNA polymerase δ and that those requirements are not satisfied by yPCNA.

The regulation of mammalian PCNA in the cell cycle has been well studied, both by two-dimensional gel electrophoresis and by analysis of mRNA levels (3, 5, 26, 27). PCNA is synthesized at high levels in late G_1 and early S phase of the cycle. DNA synthesis, however, is not required for synthesis of PCNA. In the presence of hydroxyurea, an inhibitor of DNA synthesis, expression of PCNA continues undiminished (27, 30). In yeast, hydroxyurea causes cells to arrest in the S phase (16, 17). If in yeast, as in higher eukaryotes, PCNA is only synthesized at G_1/S and in S, and inhibition of DNA synthesis does not block synthesis of yPCNA, extracts isolated from cells blocked in the S phase should be enriched for yPCNA compared to extracts from nonsynchronous cells. This is, in fact, observed (Table 2), suggesting that regulation of yPCNA is similar to that of the mammalian PCNA. To study the regulation of yPCNA on a more defined scale as well as its precise function in DNA replication *in vivo*, its gene needs to be isolated. The study of the yPCNA gene and well-defined mutants in yPCNA should help enormously in shedding light not only on the control of DNA replication in yeast but also on similar events in mammalian cells.

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