Cell Cycle Specific Plasmid DNA Replication in the Nuclear Extract of *Saccharomyces cerevisiae*: Modulation by Replication Protein A and Proliferating Cell Nuclear Antigen†

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ABSTRACT: Plasmid DNA replication in nuclear extracts of *Saccharomyces cerevisiae* in vitro has been shown to be S-phase specific, similar to that observed in vivo. We report here a reconstituted in vitro system with partially purified replication proteins, purified replication protein A (RPA), and recombinant proliferating cell nuclear antigen (PCNA). Nuclear extracts from S-phase, G_1-phase, and unsynchronized yeast cells were fractionated by phosphocellulose chromatography. Protein fraction (polymerase fraction) enriched with replication proteins, including DNA polymerases (α, δ, etc.), was isolated, which was not capable of in vitro replication of supercoiled plasmid DNA. However, when purified yeast RPA and recombinant PCNA together were added to the polymerase fraction obtained from S-phase synchronized cells, in vitro plasmid DNA replication was restored. In vitro plasmid DNA replication with polymerase fractions from unsynchronized and G_1-phase cells could not be reconstituted upon addition of purified RPA and PCNA. RPA and PCNA isolated from various phases of the cell cycle complemented the S-phase polymerase pool to the same extent. Reconstituted systems with the S-phase polymerase pool, complemented with either the RPA- and PCNA-containing fraction or purified RPA and recombinant PCNA together, were able to produce replication intermediates (ranging in size from 50 to 1500 bp) similar to that observed with the S-phase nuclear extract. Results presented here demonstrate that both RPA and PCNA are cell cycle-independent in their ability to stimulate in vitro plasmid DNA replication, whereas replication factors in the polymerase fractions are strictly S-phase dependent.

Replication of the eukaryotic genome is limited to only once per cell cycle and proceeds with high fidelity and speed to avoid deleterious consequences for the cell's progeny (*1, 2*). The entire genome is duplicated during the S-phase of the cell cycle by initiating DNA synthesis from a large number of origins of DNA replication located within each chromosome (*2*).

Saccharomyces cerevisiae has provided a convenient genetic model for studies aimed at identifying factors and/or conditions that both mediate and regulate DNA synthesis. This organism undergoes normal mitotic division and exhibits conditional mutations in many genes that are essential for either the initiation or the elongation steps of replication (*1*−*4*). Although significant progress has been made in identifying factors such as DNA polymerases α, δ, etc. that catalyze eukaryotic DNA replication in vivo, the development of an in vitro eukaryotic DNA replication system using eukaryotic chromosomal origin of DNA replication has not been achieved (*4*−*6*). Systems based on frog oocytes, HeLa cells, and yeast nuclear extract have demonstrated the cell cycle dependence of genomic DNA replication using G_1-phase nuclei or chromatin as templates (*7, 8*). The introduction of a naked plasmid or phage DNA into the activated *Xenopus* egg extract leads to organization of the template into pseudo-nuclei, and initiation of DNA replication with no detectable sequence-specificity (*7, 9*).

Gasser and co-workers (*10*) have described a soluble system of prokaryotic plasmid DNA replication that is based on nuclear protein extract from yeast cells that are synchronized in S-phase. This system is able to support aphidicolin-sensitive semiconservative replication of primer-free, supercoiled plasmid DNA (*10*). Levels of replication in nuclear extracts of S-phase synchronized cells were shown to be significantly higher than in G_1-phase cells. Since most of the replication factors are present in equal amounts in both extracts (*10*), a question arises as to which component dictates S-phase specificity of DNA replication. It could be a cyclin-dependent kinase (*10*) or a replication factor. Despite the physiological dependence of the replication reaction on the specific cell cycle phase, the initiation event in vitro does not appear to require the presence of an ARS consensus sequence. Initiation may occur at DNA unwinding elements (DUE) that mimic origins of DNA replication (*10*), but it could also be initiated at all regions of helical instability in

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the supercoiled template that are melted in order to release the negative super-stress of the plasmid molecule. It has been shown that plasmid DNA replicates autonomously in *Caenorhabditis elegans* (*11*) and *Paramecium* (*12*). In many eukaryotic systems, systematic searches for ARS elements analogous to those in yeast have generally failed (*13*), and replication seems to initiate within any DNA sequence (*14*). Because of the unique S-phase dependence, we have explored the possibility that the system based on nuclear extract of *Saccharomyces cerevisiae*, uncomplicated by the initiation events, could be a good model for investigating the elongation step of DNA replication and identifying critical components of the "replisome" complex.

In this paper, we report partial reconstitution of this in vitro system and the requirement of some accessory factors for the S-phase dependent in vitro plasmid DNA replication. Our results suggest that in vitro replication of supercoiled plasmid DNA template requires both RPA[1] and PCNA. Both RPA and PCNA appear to be cell cycle-independent in carrying out their roles in S-phase specific DNA synthesis in vitro. The other replication factors in the nuclear extract are likely involved in maintaining the S-phase specificity.

MATERIALS AND METHODS

Yeast. The protease-deficient yeast strain of *Saccharomyces cerevisiae*, BJ 2168, was obtained from the Yeast Genetic Stock Center (Berkeley, CA).

Nucleic Acids, Enzymes, Plasmids, and Other Reagents. Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia (Piscataway, NJ) and were used without further purification. [α-^{32}P]dATP, [α-^{32}P]CTP, and [α-^{32}P]GTP were obtained from DuPont/NEN (Boston, MA). Yeast RPA and recombinant yeast PCNA, expressed in *E. coli* using a T7 expression system, were purified as described previously (*15*, *16*). Zymolase was purchased from Seikagaku America Inc. (Falmouth, MA). α-Factor was obtained from Research Genetics Inc. (Mobile, AL). The chromogenic substrates of the alkaline phosphatases NBT and BCIP were purchased from Invitrogen Inc. (Gaithersburg, MD). All chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Chemical Co. (Pittsburgh, PA). Protease inhibitors were obtained from Bachem Inc. (Los Angeles, CA). Plasmid *pSK*, known as *Bluescript SK+*, was obtained from Stratagene Inc. (La Jolla, CA). *pSKARS* was constructed by cloning the 837 bp *Hin*dIII/*Eco*RI fragment of the ARS1-Trp1 gene from plasmid *yRp7* into the *Hin*dIII/*Eco*RI site of *pSK*. Plasmids were purified by the Qiagen Plasmid Midi kit of Qiagen Inc. (Valencia, CA). Nicked and linear DNA were selectively removed from supercoiled DNA by acid−phenol extraction as described by Zasloff et al. (*17*). The purified plasmids were more than 95% pure in their supercoiled form. Antibodies against RPA p70, RPA p34, and PCNA were produced in mice using conventional immunological methods. Antisera were used without further purification.

Buffers. Buffer A contained 50 mM Tris/HCl (pH 7.5), 1 M sorbitol, 10 mM $MgCl_2$, and 30 mM DTT. Buffer B contained 50 mM Tris/HCl (pH 8), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and protease inhibitors. Buffer C contained 50 mM Tris/HCl (pH 7.5), 25% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and protease inhibitors. Buffer D-*x* contained 25 mM Tris/HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.005% NP-40, protease inhibitors, and *x* mM NaCl. Buffer E contained 20 mM HEPES−NaOH (pH 7.6), 5 mM $MgCl_2$, 0.3 mM EDTA−NaOH (pH 7.6), and 1 mM DTT. 2 × buffer F contained 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. 1 × TBE contained 89 mM Tris−borate (pH 8.3) and 2.5 mM EDTA.

Yeast Synchronization. Yeast cells were grown in a shaker incubator at 30 °C in YPD media to $OD_{600} = 0.25$. G_1- and S-phase synchronized yeast cells were obtained by α-factor synchronization as described (*10*, *18*). Synchronized cells were resuspended in buffer A and stored at −80 °C. Unsynchronized cells were grown in a shaker incubator at 30 °C in YPD media to $OD_{600} = 0.6$, harvested, and stored as described above.

Yeast Nuclear Extract Preparation. Synchronized or unsynchronized cells were washed twice with 3 volumes of buffer A. Spheroplasts were obtained following the procedure described by Verdier et al. (*19*). The presence of spheroplasts was confirmed microscopically after lysis with 1% SDS. All subsequent steps for the preparation of nuclear extract were performed at 4 °C. The spheroplasts were centrifuged for 5 min at 1500*g*, and washed twice with buffer B containing 15 mM KCl. The nuclei were released by Dounce homogenization, and the suspension was centrifuged for 30 min at 10000*g*. Nuclei were resuspended in buffer B, containing 100 mM KCl and 10% glycerol. After lysis of nuclei with 0.1 g/mL $(NH_4)_2SO_4$ for 20 min, the unlysed spheroplasts and cell debris were removed by centrifugation for 1 h at 25000*g*. The supernatant (Fraction I) was fractionated using 0.35 g/mL $(NH_4)_2SO_4$ (Fraction II). The pellet (Fraction II) was resuspended and dialyzed against buffer C for up to 4 h. The resulting nuclear extract was aliquoted and stored at −80 °C.

Fractionation of Yeast Nuclear Extract by Phosphocellulose Chromatography. The nuclear extract was dialyzed against buffer D-200 for 2.5 h and loaded onto a phosphocellulose column (1 × 2 cm), equilibrated with buffer D-200. The column was extensively washed with the same buffer, and all proteins bound to the matrix were eluted with buffer D-600. Both the flow through and eluted fractions were precipitated using 0.35 g/mL $(NH_4)_2SO_4$ for 1 h at 4 °C, and centrifuged for 30 min at 25000*g*. The pellets were resuspended and dialyzed as described above.

In Vitro Replication Assay. The replication reaction was carried out in a 25 μL reaction mixture that contained buffer E, 300 ng of supercoiled plasmid DNA, 40 mM creatine phosphate and 0.125 mg/mL creatine phosphokinase, 4 mM ATP, 70 μM of each rNTP, 100 μM of each dNTP, 0.125 μCi of [α-^{32}P]dATP, and protein as indicated. Standard assays were carried out for 60 min at 30 °C with 20−60 μg of nuclear extract. The optimal protein concentration of each

[1] Abbreviations: RPA, replication protein A; PCNA, proliferating cell nuclear antigen; ARS, autonomously replicating sequences; DUE, DNA unwinding elements; Aux fraction, fraction containing auxiliary factors derived from nuclear extract; Aux_S, Aux_{G1}, Aux_U fractions, fractions containing auxiliary factors derived from nuclear extract of S-phase, G_1-phase, and unsynchronized cells, respectively; Pol fraction, fraction containing polymerases derived from nuclear extract; Pol_S, Pol_{G1}, Pol_U fractions, fractions containing DNA polymerases derived from yeast nuclear extract of S-phase, G_1-phase, and unsynchronized cells, respectively; pol α, polymerase α; pol δ, polymerase δ; RFC, replication factor C.

extract was determined by titration. The reaction was stopped by adding 1 mL of 10% TCA/0.1 M sodium pyrophosphate, followed by incubation for 15 min on ice. DNA was immobilized on GFC glass microfiber filters (Whatman Inc.) and counted in a scintillation counter.

Polyacrylamide Gel Analysis of the Products of DNA Synthesis. Reactions were carried out as described above, except with 5 μCi of [α-^{32}P]dATP or 2.5 μCi of each [α-^{32}P]-CTP and [α-^{32}P]GTP. It was terminated by the addition of 2 μL of 500 mM EDTA/NaOH (pH 8). The DNA products were ethanol-precipitated and resuspended in 1 $\times$ formamide loading buffer F. The samples were heated at 95 °C for 2 min and loaded on a 7 M urea/6% polyacrylamide gel in 1 $\times$ TBE. The electrophoresis was carried out for 4 h in 1 $\times$ TBE buffer. Gels were dried and exposed to Fuji RX film at −80 °C for 12 h.

Immunological Analysis. Western transfer was performed using a Bio-Rad Trans-blot apparatus according to manufacturer's instructions. Antigen−antibody reaction was detected by utilizing alkaline phosphatase-conjugated second antibody with NBT and BCIP as its chromogenic substrates.

Other Methods. Protein concentrations were determined by the method of Bradford (*20*) using bovine serum albumin as a standard. SDS−PAGE was carried out as described by Laemmli (*21*).

RESULTS

In Vitro Replication of Supercoiled Plasmid DNA in Yeast Nuclear Extracts. In vitro DNA syntheses in nuclear extracts prepared from synchronized *Saccharomyces cerevisiae* cells in S-phase as well as from unsynchronized cells were carried out as described under Materials and Methods. The extent of DNA synthesis using supercoiled plasmid DNA templates with or without ARS is presented in Figure 1.

Two double stranded, supercoiled plasmids were used as templates: *pSK* (without ARS) and *pSKARS*, containing the ARS1 origin of replication of *Saccharomyces cerevisiae*. We observed a high level of replication of supercoiled plasmid DNA in nuclear extracts, prepared from cells that were synchronized in S-phase (Figure 1). The extent of DNA synthesis was similar for both plasmids, approximately 36 pmol for *pSKARS* and 33 pmol for *pSK*. When reactions were carried out using nuclear extracts from unsynchronized cells, a significantly reduced level of DNA synthesis was observed (Figure 1). The extent of DNA synthesis with *pSKARS* and *pSK* was approximately 15 pmol. These results are in good agreement with previous findings of in vitro supercoiled plasmid DNA replication in S-phase nuclear extracts (*10*, *22*).

To systematically examine the roles of various replication factors in this yeast nuclear extract system, nuclear extracts from S-phase, G_1-phase, and unsynchronized yeast cells were fractionated by phosphocellulose chromatography as described earlier (*23*). Columns were equilibrated with buffer, containing 200 mM NaCl. At this salt concentration, some DNA replication factors, along with other cellular proteins, do not bind to phosphocellulose and appear in the unbound fraction (auxiliary fraction − Aux fraction). Most replication factors, including DNA polymerases, which remain bound to the matrix were eluted using buffer containing 600 mM NaCl as described under Materials and Methods. The eluted

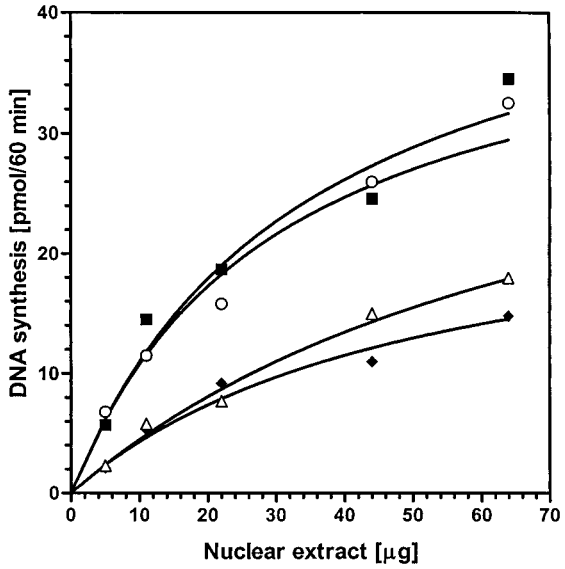

FIGURE 1: In vitro replication assay of supercoiled plasmid DNA in yeast nuclear extracts. Replication assays were carried out for 60 min at 30 °C, as described under Materials and Methods. Two double stranded plasmids were used as templates: *pSK*, which does not contain specific yeast origin of replication, and *pSKARS*, containing *ARS1*. Replication with nuclear extract obtained from S-phase cells using *pSK* (○) and *pSKARS* (■). Replication with nuclear extract obtained from unsynchronized cells using *pSK* (◆) and *pSKARS* (△). Representation of the data is a result of three different sets of experiments.

fraction facilitated high DNA polymerase activity on activated calf thymus DNA template, about ~150 pmol/30 min, and was termed polymerase fraction (Pol fraction). The total polymerase activities based on an activated calf thymus DNA assay were similar for the Pol fractions obtained from S-phase cells (Pol_S fraction), G_1-phase cells (Pol_{G1} fraction), and unsynchronized cells (Pol_U fraction) (data not shown). The Aux and Pol fractions were precipitated with 0.35 g/mL $(NH_4)_2SO_4$ and the protein pellets dialyzed and stored at −80 °C in small aliquots. These protein fractions were used for reconstitution of in vitro plasmid DNA replication.

Reconstitution of in Vitro Plasmid DNA Replication after Phosphocellulose Chromatography. After fractionation of nuclear extract from S-phase cells by phosphocellulose chromatography, the Pol_S fraction demonstrated very low levels of plasmid DNA replication (Figure 2A) in comparison to the total nuclear extract (Figure 1). The maximum DNA synthesis (13 pmol) observed for the Pol_S fraction was significantly lower than the maximum activity observed with nuclear extract from S-phase cells before fractionation (36 pmol). In contrast, polymerase fraction from unsynchronized cells possessed activity on supercoiled plasmid DNA template similar to that observed using nuclear extract from unsynchronized cells (Figure 2B).

We further examined the reconstitution of in vitro plasmid DNA replication by combining different fractions of nuclear extracts from S-phase and unsynchronized cells, obtained after phosphocellulose chromatography (Figure 2). When Aux_S or Aux_U fraction was added to the indicated amounts of Pol_S fraction, we observed stimulation of the DNA synthesis to 35 and 32 pmol, respectively (Figure 2A). The same experiment was carried out using Pol_U fraction (Figure 2B). Our attempts to reconstitute in vitro plasmid DNA replication using polymerase fraction from unsynchronized

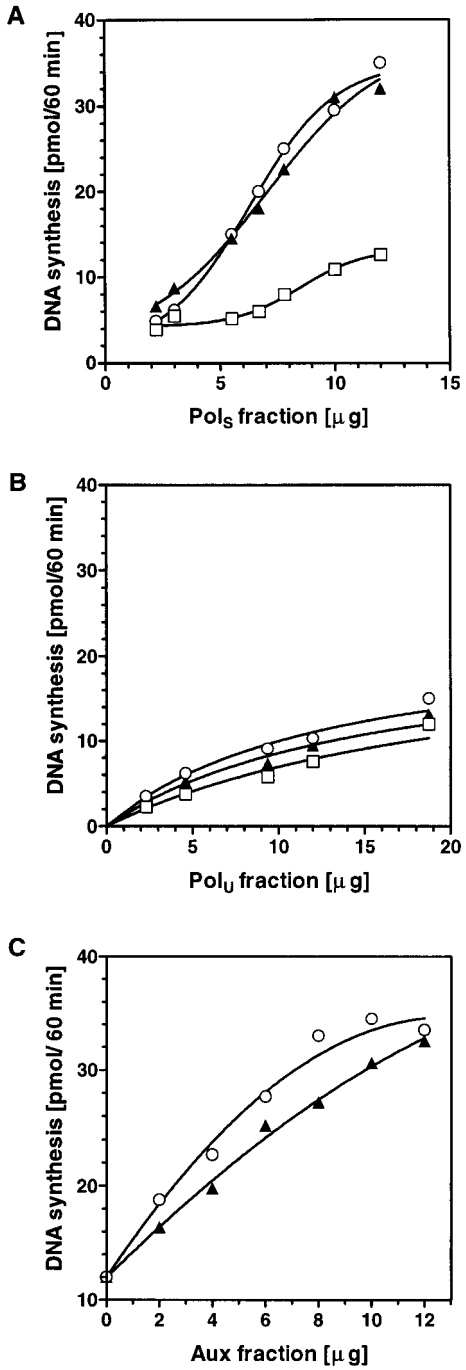

FIGURE 2: Reconstitution of in vitro supercoiled plasmid DNA replication with Pol fraction and Aux fraction. Replication assays were carried out as described in Figure 1 with 300 ng of supercoiled *pSKARS* as a template. (A) Reconstitution of the system combining either 10 μg of Aux$_S$ fraction or 12 μg of Aux$_U$ fraction with indicated amounts of polymerase fraction from S-phase cells (Pol$_S$ fraction): (□) Pol$_S$ fraction alone; (○) Pol$_S$ fraction + Aux$_S$ fraction; (▲) Pol$_S$ fraction + Aux$_U$ fraction. (B) Reconstitution of the system combining either 10 μg of Aux$_S$ fraction or 12 μg of Aux$_U$ fraction with indicated amounts of polymerase fraction from unsynchronized cells (Pol$_U$ fraction): (□) Pol$_U$ fraction alone; (○) Pol$_U$ fraction + Aux$_S$ fraction; (▲) Pol$_U$ fraction + Aux$_U$ fraction. (C) Titration of 12 μg of Pol$_S$ fraction with Aux$_S$ fraction (○) and Aux$_U$ fraction (▲). Representation of the data is a result of three different sets of experiments.

cells and Aux$_S$ or Aux$_U$ fraction were not successful. Neither Aux$_S$ nor Aux$_U$ fraction stimulated the activity of the Pol$_U$ fraction, and the level of replication remained essentially unchanged (Figure 2B). The maximum DNA synthesis

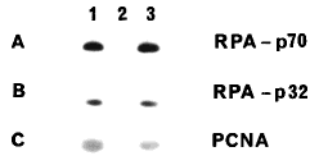

FIGURE 3: Western blotting with anti-RPA and anti-PCNA sera of S-phase nuclear extract and fractions after phosphocellulose chromatography. (A) Western blotting with anti-RPA p70 serum. (B) Western blotting with anti-RPA p32 serum. (C) Western blotting with anti-PCNA serum. Lane 1, Aux$_S$ fraction (10 μg); lane 2, Pol$_S$ fraction (12 μg); lane 3, S-phase nuclear extract (60 μg).

observed for Pol$_U$ fraction, Pol$_U$ fraction + Aux$_S$ fraction, or Pol$_U$ fraction + Aux$_U$ fraction was 12, 15, and 13 pmol, respectively, similar to the maximum activity possessed by the total nuclear extract from unsynchronized cells. The stimulation of plasmid replication by Aux fraction appears to be specific for the polymerase fraction obtained from S-phase cells.

The optimal concentrations of Aux$_S$ and Aux$_U$ fractions for reconstitution of the in vitro system were determined by titration of the Pol$_S$ fraction (12 μg). Our results indicated that maximum stimulation of DNA synthesis was observed with 8 μg of Aux$_S$ or 10 μg of Aux$_U$ fraction (Figure 2C). The extent of DNA synthesis was 34 and 33 pmol, respectively, which was similar to the maximum activity observed with nuclear extract from S-phase cells (36 pmol) (Figure 1). This result suggests that the maximum activity of the nuclear extract from S-phase cells can be completely recovered by addition of either Aux$_S$ or Aux$_U$ fraction to the Pol$_S$ fraction.

Reconstitution of in Vitro Plasmid DNA Replication with Pol Fraction, Purified RPA, and PCNA. Previous studies by us and others have demonstrated that at least two DNA replication factors, RPA and PCNA, do not bind to phosphocellulose matrix at 200 mM NaCl (*23*, *24*). Consequently, it is reasonable to assume that, after fractionation of nuclear extracts by phosphocellulose chromatography, at least these two replication factors should appear in the flow through. The low level of plasmid DNA replication of the Pol$_S$ fraction has raised the question of whether this fraction is completely depleted of RPA and PCNA, leading to diminished activity, or if there are other replication factors present in the auxiliary fraction which are essential for polymerases to complete DNA synthesis. Western blotting with anti-RPA p70, anti-RPA p32, and anti-PCNA sera showed that the Aux$_S$ fraction contained both RPA and PCNA (Figure 3A,B, lane 1), and that the Pol$_S$ fraction was completely devoid of these two replication factors (Figure 3A,B, lane 2). These results were also confirmed after fractionation of nuclear extracts obtained from unsynchronized and G_1-phase cells following phosphocellulose chromatography (data not shown).

To dissect the RPA and PCNA requirements for in vitro plasmid DNA replication, we reconstituted the system using purified yeast RPA, purified recombinant PCNA, produced in *E. coli*, and Pol fractions. Neither RPA nor PCNA alone was capable of stimulating the activity of the Pol$_S$ fraction to any significant extent (Figure 4A). The level of the plasmid replication remained relatively low, and the extent of DNA synthesis reached only 14 and 16 pmol, respectively, after addition of PCNA (100 ng) or RPA (200 ng) alone to the

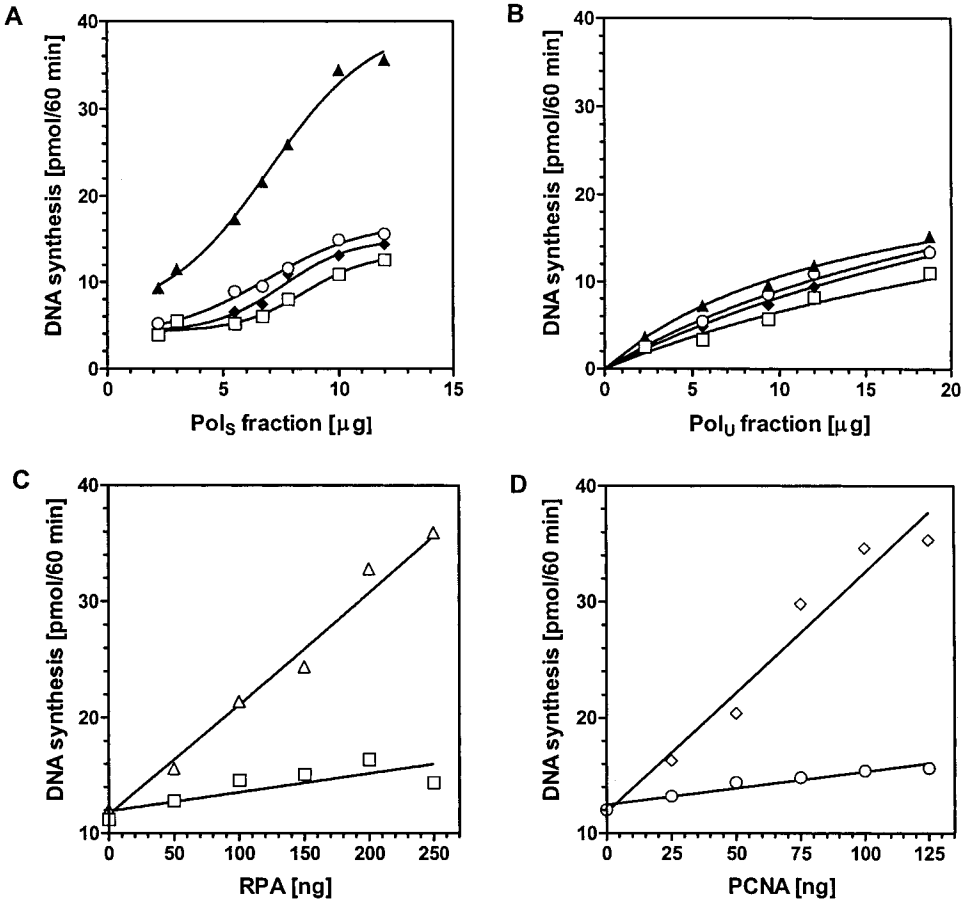

FIGURE 4: Reconstitution of in vitro supercoiled plasmid DNA replication with Pol fraction, purified RPA, and/or recombinant PCNA. The replication assays were carried out as described in Figure 1 with 300 ng of supercoiled *pSKARS* as a template. (A) Reconstitution of the system with Pol$_S$ fraction, purified RPA, and/or PCNA: (□) Pol$_S$ fraction alone; (◆) Pol$_S$ fraction + 100 ng of PCNA; (○) Pol$_S$ fraction + 200 ng of RPA; (▲) Pol$_S$ fraction + 100 ng of PCNA and 200 ng of RPA. (B) Reconstitution of the system with Pol$_U$ fraction, purified RPA, and/or PCNA: (□) Pol$_U$ fraction alone; (◆) Pol$_U$ fraction + 100 ng of PCNA; (○) Pol$_U$ fraction + 200 ng of RPA; (▲) Pol$_U$ fraction + 100 ng of PCNA and 200 ng of RPA. (C) The system was reconstituted with 12 μg of Pol$_S$ fraction in the presence of 100 ng of PCNA and subsequent titration with RPA: (□) Pol$_S$ fraction + PCNA; (△) Pol$_S$ fraction + PCNA, titrated with RPA. (D) The system was reconstituted with 12 μg of Pol$_S$ fraction in the presence of 200 ng of RPA and subsequent titration with PCNA: (○) Pol$_S$ fraction + RPA; (◇) Pol$_S$ fraction + RPA, titrated with PCNA. Representation of the data is a result of three different sets of experiments.

Pol$_S$ fraction (12 μg). However, a dramatic increase in DNA synthesis was observed with the addition of RPA and PCNA together (Figure 4A). When these two replication factors were added together to the Pol$_S$ fraction, DNA synthesis was increased 3-fold (Figure 4A). In contrast, reconstitution of in vitro plasmid DNA replication with the Pol$_U$ fraction, by the addition of RPA and PCNA, could not be restored (Figure 4B). The stimulation of DNA synthesis with the Pol fraction by RPA and PCNA appeared to be specific only for S-phase cells. As shown in Figure 4B, only a minor increase in DNA synthesis was observed upon addition of RPA and PCNA to the Pol$_U$ fraction, which was probably due to the presence of a small number of S-phase cells in the random population of unsynchronized cells.

The optimal concentrations of purified RPA and PCNA used for reconstitution of in vitro plasmid replication were determined by protein titration of the Pol$_S$ fraction. Titration of the Pol$_S$ fraction (12 μg) with RPA in the presence of 100 ng of PCNA was carried out, and the maximum activity was observed with 200 ng of RPA (Figure 4C). Titration of 12 μg of Pol$_S$ fraction in the presence of 200 ng of RPA with PCNA demonstrated that the maximum stimulation of plasmid DNA replication was observed after addition of 100 ng of PCNA (Figure 4D). The control titration with either RPA or PCNA individually did not change remarkably the level of replication of Pol$_S$ fraction (13 pmol) (Figure 4C,D). Consequently, the presence of RPA and PCNA together is required for in vitro reconstitution of plasmid DNA replication.

Analysis of the Products of in Vitro Reconstituted Plasmid DNA Replication by Denaturing Polyacrylamide Gel. Results presented above with nuclear extracts from synchronized and unsynchronized cells have demonstrated that there is a basal level of DNA synthesis in the polymerase fraction from S-phase cells that can be stimulated by RPA and PCNA. Consequently, we examined the products of in vitro reconstituted plasmid DNA replication. The products of in vitro plasmid DNA replication reconstituted from nuclear extract of cells that were synchronized in S-phase and Pol$_S$ fraction complemented with RPA and/or PCNA were analyzed on a 7 M urea/6% polyacrylamide gel. To determine if the products obtained were actually nascent DNA fragments (Okazaki), identical experiments were carried out using [α-^{32}P]dATP and [α-^{32}P]CTP/[α-^{32}P]GTP as labeled nucleotides. The Pol$_S$ fraction and Aux$_S$ fraction separately did not produce detectable replication intermediates (≥100 bp) (Figure 5A,B, lanes 3 and 4, respectively) even though we observed ~13 pmol of DNA synthesis with the Pol$_S$ fraction

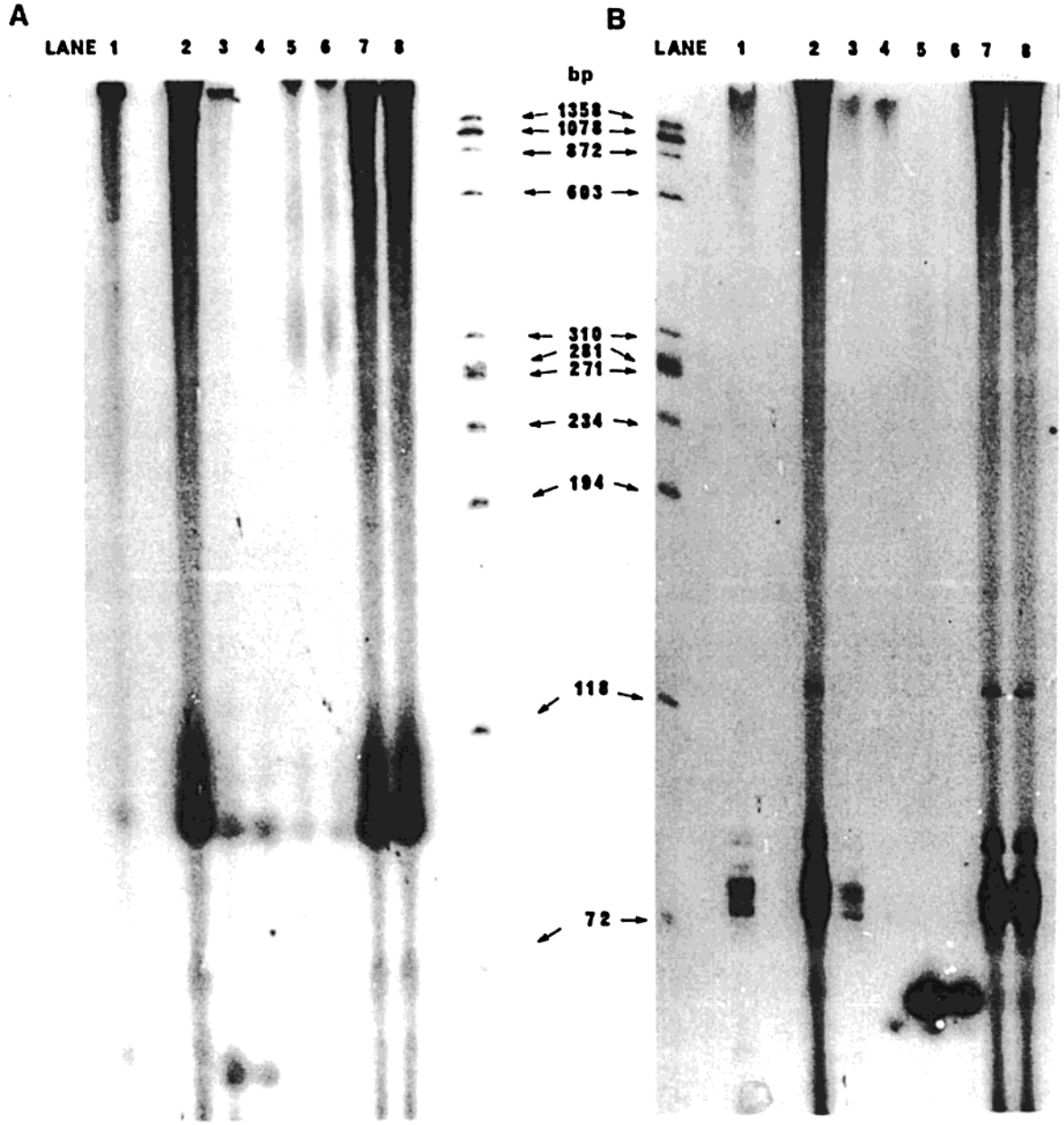

FIGURE 5: Denaturing polyacrylamide gel electrophoresis of the products of in vitro supercoiled plasmid DNA replication. The replication reactions were carried out as described in Figure 1 using 300 ng of *pSKARS*. The samples were ethanol precipitated and run on a 7 M urea/6% polyacrylamide gel. (A) Analysis of the replication intermediates after [α-^{32}P]dATP incorporation. (B) Analysis of the replication intermediates after [α-^{32}P]CTP/[α-^{32}P]GTP incorporation. The replication reactions were carried out with 60 μg of nuclear extract from unsynchronized cells (lane 1), 60 μg of nuclear extract from S-phase cells (lane 2), 10 μg of Aux_S fraction (lane 3), 12 μg of Pol_S fraction (lane 4), 12 μg of Pol_S fraction + 200 ng of RPA (lane 5), 12 μg of Pol_S fraction + 100 ng of PCNA (lane 6), 12 μg of Pol_S fraction + 10 μg of Aux_S fraction (lane 7), and 12 μg of Pol_S fraction + 200 ng of RPA and 100 ng of PCNA (lane 8).

in our filter assay (Figure 2A). However, a reconstituted system either with the Pol_S fraction and Aux_S fraction (Figure 5A, lane 7) or with the Pol_S fraction combined with purified RPA and PCNA (Figure 5A, lane 8) produced essentially identical patterns of [α-^{32}P]dATP-radiolabeled fragments of replication intermediates, ranging in size from 50 to 1500 bp. Similar results were observed with the total nuclear extract from S-phase cells (Figure 5A, lane 2). The appearance of a high molecular weight ladder of replication products, as a result of extension of ^{32}P-labeled RNA primers on the replication fork, was also observed after reconstitution of the system with Aux_S fraction (Figure 5B, lane 7) or RPA/PCNA added back to Pol_S fraction (Figure 5B, lane 8) in the presence of [α-^{32}P]CTP/[α-^{32}P]GTP nucleotides. The rNTP-labeled replication intermediates from a reconstituted system displayed the same electrophoretic profile as the products of the total nuclear extract from S-phase cells (Figure 5B, lane 2). In contrast, after incubation of *pSKARS* with the Pol_S fraction complemented with either RPA or PCNA alone, long replication products (>100 bp) were not detected (Figure 5A,B, lanes 5 and 6, respectively). With [α-^{32}P]CTP/[α-^{32}P]GTP incorporation, an accumulation of small fragments below 72 bp was observed (Figure 5B, lanes 5 and 6, respectively). These results demonstrate that both RPA and PCNA are required for processive extension of RNA primers leading to Okazaki fragments.

Nuclear extract from unsynchronized cells (Figure 5A,B, lane 1) and the reconstituted systems after fractionation by phosphocellulose chromatography (Pol_U fraction, Pol_U fraction + Aux_U fraction, and Pol_U pool + RPA and/or PCNA)

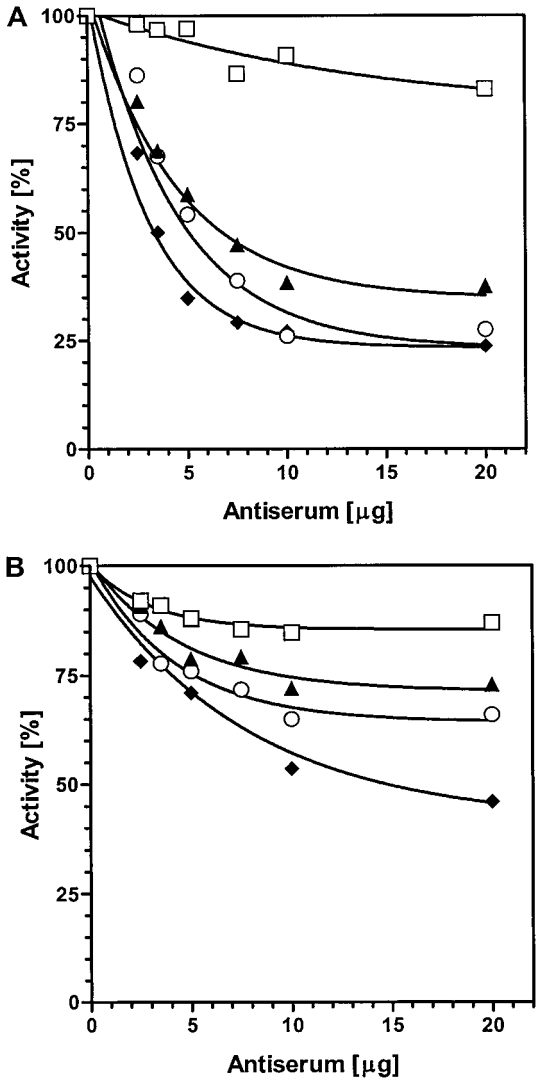

FIGURE 6: Inhibition of in vitro supercoiled plasmid DNA replication by specific mouse antisera. (A) Aliquots of 60 μg of nuclear extract from S-phase cells were preincubated at 30 °C with the indicated amounts of anti-pol α serum, anti-RPA serum, anti-PCNA serum, or nonimmune serum. After 15 min, 300 ng of *pSKARS* was added to each sample, and the replication reactions were continued for 60 min as described in Figure 1. (B) Preincubation of 60 μg of nuclear extract from unsynchronized cells with the same antisera was carried out as in (A), and the replication reactions were continued after addition of *pSKARS*. (◆) Anti-pol α serum; (○) anti-RPA serum; (▲) anti-PCNA serum; (□) nonimmune serum. Representation of the data is a result of three different sets of experiments.

were not capable of producing detectable replication intermediates (>50 bp) (data not shown).

Inhibition of the in Vitro Replication Reaction by Specific Antisera and Aphidicolin. We have analyzed the roles of RPA, PCNA, and pol α by inhibition of the in vitro supercoiled plasmid DNA replication with specific mouse antisera and aphidicolin. Nuclear extracts from S-phase and unsynchronized cells were preincubated with antiserum specific for pol α, RPA, or PCNA and used for in vitro plasmid DNA replication. Titration of nuclear extract from S-phase cells with different amounts of these antisera is shown in Figure 6A. The maximum inhibitions observed for anti-pol α, anti-RPA, and anti-PCNA sera were 78%, 73%, and 68%, respectively, suggesting that all of these replication factors are required for in vitro replication of plasmid DNA. When nuclear extract from unsynchronized cells was preincubated with the same antisera, only pol α was inhibited up to 53% (Figure 6B). Anti-RPA and anti-PCNA sera did not show significant inhibition of the replication reaction and were comparable to nonimmune control serum (Figure 6B). These findings suggest that the DNA syntheses observed in S-phase nuclear extract and the reconstituted systems are due to synthesis of Okazaki fragments or replication intermediates. The background synthesis (12−15 pmol) observed with the $Pol_{S/U}$ fraction or nuclear extract from unsynchronized cells is perhaps due to an aberration of DNA replication or DNA repair carried out by polymerases other than pol α and pol δ. Obviously, these processes are not true examples of DNA replication.

The products of plasmid DNA replication derived from S-phase nuclear extract in the presence or absence of anti-pol α, anti-RPA, anti-PCNA serum, or aphidicolin were analyzed on 7 M urea/6% polyacrylamide gels after labeling with [α-^{32}P]dATP or equal amounts of both [α-^{32}P]CTP and [α-^{32}P]GTP (Figure 7). The products in the absence of inhibitor could be observed as a ladder of replication intermediates (size range 50−1500 bp) after incorporation of [α-^{32}P]dATP into the newly synthesized DNA chains (Figure 7A, lane 1). The same size distribution of replication intermediates was detected as a result of [α-^{32}P]CTP/[α-^{32}P]-GTP incorporation into the RNA primers and their extension with dNTP on the replication fork (Figure 7B, lane 1). In contrast, preincubation of the extract with anti-pol α, anti-RPA, anti-PCNA sera, or aphidicolin inhibited the in vitro incorporation of both [α-^{32}P]dATP (Figure 7A, lanes 2−5) and [α-^{32}P]CTP/[α-^{32}P]GTP (Figure 7B, lanes 2−5) and did not produce a ladder of replication intermediates after 7 M urea/6% polyacrylamide gel analysis. The accumulation of small products (<72 bp) with [α-^{32}P]CTP/[α-^{32}P]GTP incorporation suggested that the elongation had been inhibited in an early stage of Okazaki fragment synthesis not only by anti-pol α but also by anti-RPA, anti-PCNA serum (5 μg), and aphidicolin (500 μg/mL) (Figure 7B, lanes 2−5). Surprisingly, all of these inhibitors appear to block the DNA chain elongation at the same stage in a very similar mechanism. No changes were observed in the products of replication after pretreatment of the extract with nonimmune serum as a control (Figure 7A,B, lane 6).

In Vitro Reconstitution of Plasmid DNA Replication with G_1-Phase Nuclear Extract. Results described above indicate that RPA, in conjunction with PCNA, stimulated the plasmid DNA replication activity of the Pol_S fraction, but was unable to stimulate the basal plasmid DNA replication activity of the Pol_U fraction. This invites the question of whether their participation in in vitro replication is specific only for the S-phase of the cell cycle. To answer this question, we prepared nuclear extract from cells that were synchronized in G_1-phase. Nuclear extract from G_1-phase cells was fractionated by phosphocellulose chromatography, as described above.

We have analyzed the replication activities of nuclear extracts from G_1-phase and S-phase cells. To compare these two in vitro systems, we chose the maximum DNA synthesis observed with nuclear extract from S-phase cells as 100% (Figure 8A). The total nuclear extract from G_1-phase cells (Figure 8B, bar 1) produced 15 pmol of DNA synthesis, which was significantly below the replication activity level

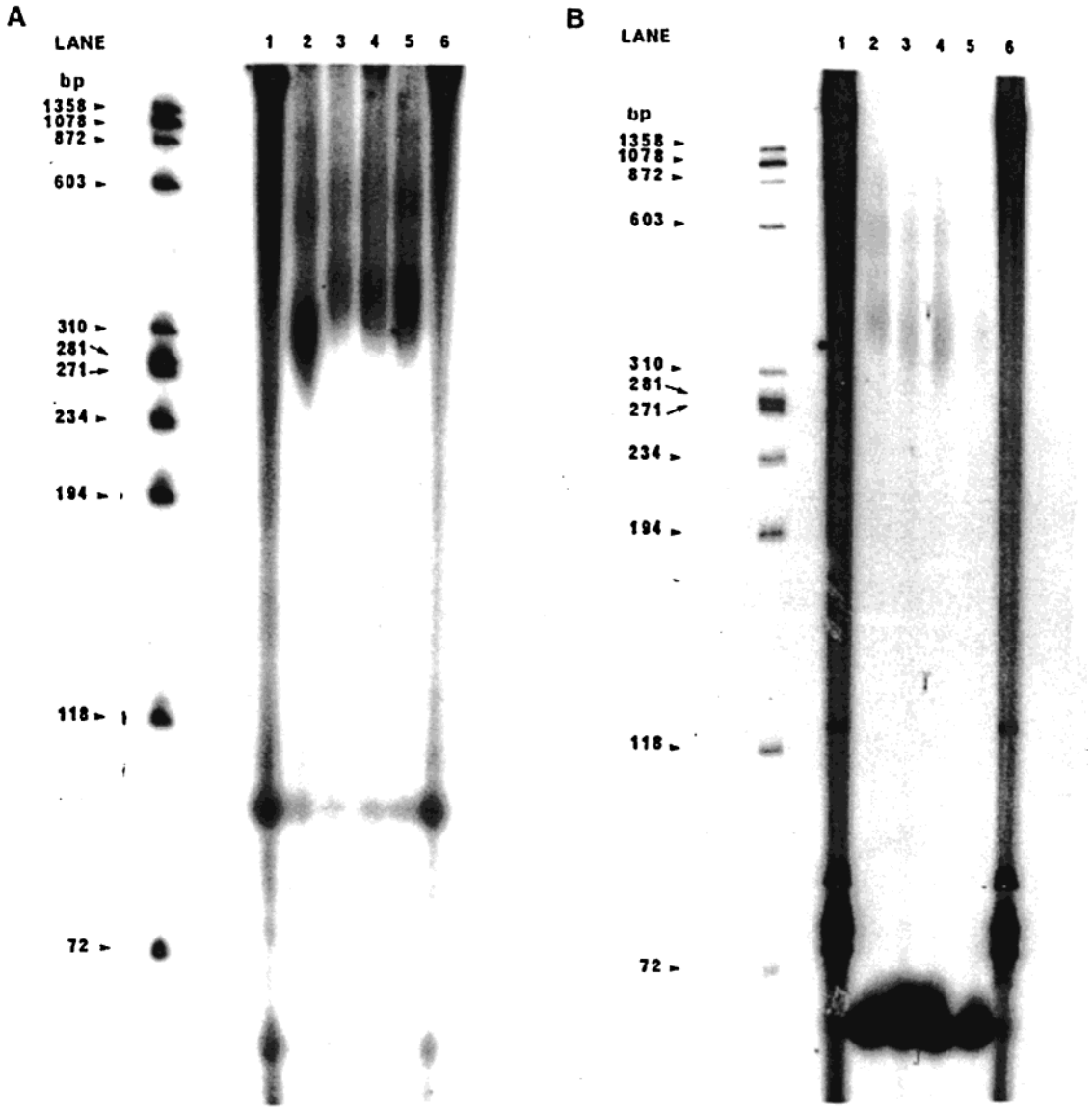

FIGURE 7: Denaturing polyacrylamide gel electrophoresis of the products of plasmid DNA replication in S-phase nuclear extract, inhibited with specific mouse antisera. 60 μg of S-phase nuclear extract was preincubated in 25 μL of complete reaction mixture with 5 μg of anti-pol α serum, anti-RPA serum, anti-PCNA serum, nonimmune serum, or 500 μg/mL aphidicolin, respectively. After 15 min, 300 ng of *pSKARS* was added to each sample, and the replication reactions were continued for 60 min. The samples were ethanol-precipitated and run on a 7 M urea/6% polyacrylamide gel. The products of replication were analyzed after subsequent autoradiography. (A) Analysis of the replication intermediates after [α-^{32}P]dATP incorporation. (B) Analysis of the replication intermediates after [α-^{32}P]CTP/[α-^{32}P]GTP incorporation. Lane 1, nuclear extract from S-phase cells; lane 2, nuclear extract from S-phase cells + anti-pol α serum; lane 3, nuclear extract from S-phase cells + anti-RPA serum; lane 4, nuclear extract from S-phase cells + anti-PCNA serum; lane 5, nuclear extract from S-phase cells + aphidicolin; lane 6, nuclear extract from S-phase cells + nonimmune serum.

of the nuclear extract derived from S-phase cells (Figure 8A, bar 1). However, there was no difference between the activities of Pol_{G1} fraction (Figure 8B, bar 3) and Pol_S fraction (Figure 8A, bar 3) in the absence of RPA and PCNA (12 pmol). The Aux_S and Aux_{G1} fractions did not appear to exhibit replication activity (Figure 8A,B, bars 2).

The remarkable differences between the two reconstituted systems were observed after combining the Pol fraction and $Aux_{S/G1}$ fractions or purified RPA and PCNA. As has been shown earlier, the replication activity of the Pol_S fraction was completely recovered after combining with either Aux_S fraction or purified RPA and purified recombinant PCNA together (Figure 8A, bars 4 and 8, respectively). These reconstituted systems possessed 95% and 99% of the maximum activity of nuclear extract from S-phase cells. When purified RPA or purified recombinant PCNA was added separately to the replication reaction, the DNA synthesis observed was only 48% and 40%, respectively, of the maximum activity of nuclear extract from cells that were synchronized in S-phase (Figure 8A, bars 6 and 7). Aux_{G1} fraction, added to the Pol_S fraction, also restored the activity of the Pol_S fraction to 94% or 34 pmol (Figure 8A, bar 5). In contrast, neither $Aux_{G1/S}$ fractions nor RPA and PCNA stimulated plasmid DNA replication, observed with Pol_{G1} fraction (Figure 8B, bars 4−8). The activities of these systems were similar, between 32% and 34%, respectively, of the maximum activity of S-phase nuclear extract.

Analysis of RPA and PCNA in Nuclear Extracts from S-Phase, G_1-Phase, and Unsynchronized Cells. The replication of supercoiled plasmid DNA in yeast nuclear extracts is completely dependent on S-phase, while extracts from cells arrested in G_1-phase are nearly inactive for replication (*10*).

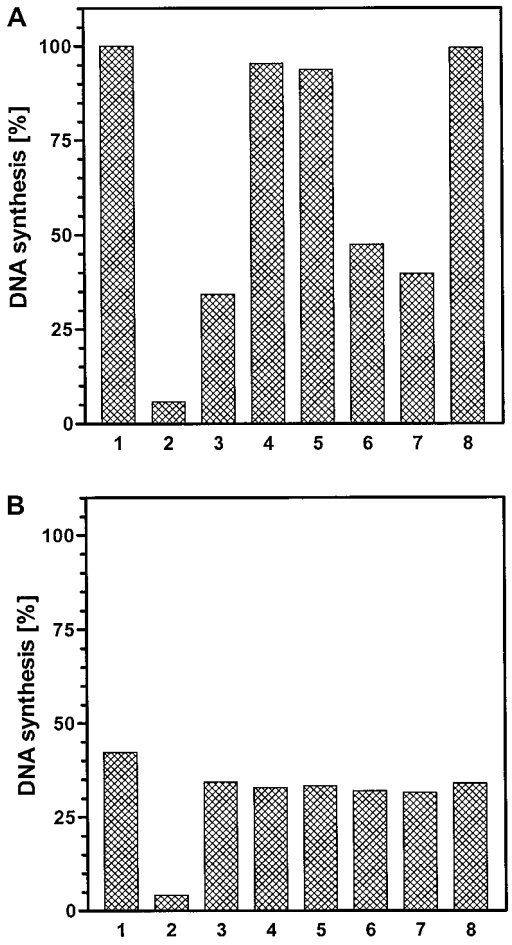

FIGURE 8: Comparison of in vitro plasmid DNA replication in S-phase, G_1-phase nuclear extracts, and reconstituted systems after their fractionation by phosphocellulose chromatography. The replication assays were carried out as described in Figure 1. The levels of replication of nuclear extracts from S-phase and G_1-phase cells, as well as of the reconstituted systems after phosphocellulose chromatography, were compared and the results summarized. The maximum activity, possessed by nuclear extract from S-phase cells, was expressed as 100% activity. (A) In vitro reconstitution of *pSKARS* replication using: 60 μg of S-phase nuclear extract (bar 1); 10 μg of Aux_S fraction (bar 2); 12 μg of Pol_S fraction (lane 3); 12 μg of Pol_S fraction + 10 μg of Aux_S fraction (bar 4); 12 μg of Pol_S fraction + 12 μg of Aux_{G1} fraction (bar 5); 12 μg of Pol_S fraction + 200 ng of RPA (bar 6); 12 μg of Pol_S fraction + 100 ng of PCNA (bar 7); 12 μg of Pol_S fraction + 200 ng of RPA and 100 ng of PCNA (bar 8). (B) In vitro reconstitution of *pSKARS* replication using: 60 μg of nuclear extract from G_1-phase cells (bar 1); 10 μg of Aux_{G1} fraction (bar 2); 12 μg of Pol_{G1} fraction (bar 3); 12 μg of Pol_{G1} fraction + 10 μg of Aux_{G1} fraction (bar 4); 12 μg of Pol_{G1} fraction + 12 μg of Aux_S fraction (bar 5); 12 μg of Pol_{G1} fraction + 200 ng of RPA (bar 6); 12 μg of Pol_{G1} fraction + 100 ng of PCNA (bar 7); 12 μg of Pol_{G1} fraction + 200 ng of RPA and 100 ng of PCNA (bar 8). Representation of the data is a result of three different sets of experiments.

Our results suggest that the requirement of RPA and PCNA for DNA synthesis in this system is also specific for S-phase. However, RPA and PCNA in Aux fractions after phosphocellulose chromatography of either unsynchronized cells (Aux_U fraction) or G_1-phase cells (Aux_{G1} fraction) can stimulate DNA synthesis as observed with the Pol_S fraction. Purified RPA, derived from unsynchronized yeast cells (*15*), and purified recombinant yeast PCNA (*16*) are capable of supporting the activity of DNA polymerases from the Pol_S fraction during in vitro plasmid DNA replication.

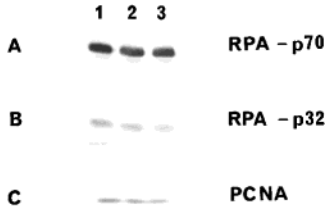

FIGURE 9: Western blotting with anti-RPA and anti-PCNA sera of nuclear extracts from S-phase, G_1-phase, and unsynchronized cells. (A) Western blotting with anti-RPA p70 serum. (B) Western blotting with anti-RPA p32 serum. (C) Western blotting with anti-PCNA serum. Lane 1, nuclear extract from S-phase cells (60 μg); lane 2, nuclear extract from G_1-phase cells (60 μg); lane 3, nuclear extract from unsynchronized cells (60 μg).

It is well-known that eukaryotic DNA replication is under specific checkpoint control and that most of the replication factors are phosphorylated in a cell cycle-dependent manner (*25*). Here we have analyzed RPA and PCNA in nuclear extracts from S-phase, G_1-phase, and unsynchronized cells using Western blotting with antisera specific for PCNA and two of the subunits of RPA (p70 and p32). As shown in Figure 9 there were no differences in the band intensities of RPA p70 (A), RPA p32 (B), and PCNA (C) from nuclear extracts of S-phase, G_1-phase, and unsynchronized cells (lanes 1, 2, and 3, respectively), indicating comparable levels of these polypeptides in different phases of the cell cycle.

DISCUSSION

Over the last 2 decades, there has been much progress in elucidating biochemical mechanisms underlying eukaryotic DNA replication. It started with the discovery of the origins of replication in the budding yeast *Saccharomyces cerevisiae*, named autonomously replicating sequences (ARS) (*26*). These replicators have been defined genetically and physically mapped and used for identification of many yeast replication proteins and regulators. Homologues of some yeast proteins involved in DNA replication have been identified in higher eukaryotes (*27, 28*).

Plasmid DNA Replication in the Nuclear Extract of Saccharomyces cerevisiae Is S-Phase-Specific and ARS-Independent. Two types of assays have been developed for studying the regulation and molecular mechanisms of eukaryotic DNA replication. In the first, template DNA for replication is provided in the form of intact nuclei, usually from cells arrested in G_1-phase (*7, 8, 29, 30*). In the second assay, supercoiled plasmid DNA is replicated in S-phase nuclear extract supplemented with nucleotides and an energy-regenerating system (*10, 22*).

A system has been described in which nuclei isolated from G_1-phase-arrested cells initiate specific DNA replication in nuclear extract derived from S-phase cells of S*accharomyces cerevisiae* (*30*). However, an origin-specific, cell-free system from budding yeast that initiates DNA replication on exogenously added DNA in vitro has never been discovered. Our results have confirmed that in vitro replication of supercoiled plasmid DNA in S-phase nuclear extract from the yeast *Saccharomyces cerevisiae* is ARS-independent, since the levels of DNA synthesis were similar for both *pSK* (without ARS) and *pSKARS* (with a 837 bp ARS1 origin of replication) (Figure 1). In contrast, even though it is origin-independent, the replication reaction in this in vitro system is specific for the S-phase of the cell cycle. We observed

successful DNA synthesis only in nuclear extracts obtained from cells that were synchronized in S-phase (Figures 1A and 8A) and not in nuclear extracts obtained from unsynchronized cells (Figure 1B) or cells that were arrested in late G_1-phase (Figure 8B). Our results also suggest that only nuclear extract from S-phase cells is capable of producing replication intermediates (>50 bp) (Figure 5A,B). In addition, nuclear extracts from unsynchronized cells (Figure 5A,B) and G_1-phase cells (data not shown) did not show detectable replication products.

In Vitro Supercoiled Plasmid DNA Replication Requires RPA and PCNA. Since initiation of the plasmid DNA replication in the cell-free in vitro system described here occurs without origin specificity, we focused our attention on elucidating the elongation step of this process and the most important components of the "replisome" that are required for this system. Two of the most important accessory factors for polymerases in DNA replication are RPA and PCNA. During the elongation step, RPA stimulates pol α/primase activity under certain conditions and is required for RFC- and PCNA-dependent DNA synthesis by DNA pol δ (*25*). PCNA acts as a processivity factor for pol δ during DNA replication (*31*−*34*).

The reconstitution of DNA replication with purified proteins has yielded great insight into the mechanism of DNA replication as well as other aspects of DNA metabolism such as DNA repair and recombination in prokaryotic and eukaryotic cells (*35*−*40*). We have described here reconstitution of in vitro plasmid DNA replication after partial fractionation of the replication factors of yeast nuclear extract (*23*). We separated the DNA polymerases from RPA and PCNA by phosphocellulose chromatography, which was confirmed by Western blotting using anti-RPA p70, anti-RPA p32, and anti-PCNA sera (Figure 3A,B).

Although the polymerase pools from the different nuclear extracts (Pol_S fraction, Pol_U fraction, and Pol_{G1} fraction) possessed high activity (∼150 pmol/30 min of DNA synthesis) on activated calf thymus DNA template, they showed very low levels of DNA synthesis during in vitro plasmid DNA replication (12−13 pmol) (Figure 2A,B; Figure 4A,B; Figure 8B). Neither the Pol_S fraction (Figure 5A,B) nor the Pol_U and Pol_{G1} fractions (not shown) produced detectable replication intermediates after analysis of the replication products by denaturing polyacrylamide gel electrophoresis. The system was successfully reconstituted after adding back Aux_S fraction to the Pol_S fraction (Figure 2A,C). In the second reconstitution of the in vitro plasmid replication, the Pol_S fraction was combined with purified yeast RPA and PCNA (Figure 4A,C,D). In both cases of reconstitution of the system, replication activity was similar to that obtained with total nuclear extract from S-phase cells. Thus, in the replication reaction, the accessory factors present in Aux_S fraction could be completely replaced with RPA and PCNA. In contrast, when RPA or PCNA was added separately to the Pol_S fraction, we did not observe stimulation of DNA synthesis (Figures 4A,C,D and 5). Our results suggest that in vitro plasmid DNA replication either with the Pol_S fraction plus Aux fraction or with the Pol_S fraction plus purified RPA and PCNA produces DNA replication intermediates as shown by denaturing polyacrylamide gel electrophoresis of [α-^{32}P]-dNTP- and [α-^{32}P]rNTP-labeled products (Figure 5A,B). Titration of S-phase nuclear extract with antisera specific for RPA and PCNA demonstrated a dose-dependent inhibition of the DNA synthesis comparable to anti-pol α serum (Figure 6A). In addition, analysis of the replication products after inhibition of in vitro plasmid DNA replication with anti-pol α, anti-RPA, anti-PCNA sera, or aphidicolin showed a remarkable reduction of the ladder of replication intermediates as visualized by denaturing polyacrylamide gel electrophoresis (Figure 7A,B). It was evident that all of these inhibitors blocked DNA chain elongation at the same stage in a very similar mechanism. We also observed an accumulation of small products below 72 bp in the case of [α-^{32}P]rNTP incorporation after inhibition of the replication reaction (Figure 7B) or after reconstitution of the system by adding back RPA and PCNA separately to the Pol_S fraction (Figure 5B). Without RPA and PCNA, the primase may still be able to carry out RNA primer synthesis, but without these important accessory factors, the elongation stops at an early stage of Okazaki fragment synthesis, and pol δ is not able to continue DNA synthesis on the replication fork. In conclusion, both RPA and PCNA are required for in vitro elongation of supercoiled plasmid DNA replication beyond initial RNA priming and extension.

The DNA Synthesis Observed in Nuclear Extracts from Unsynchronized and G_1-Phase Cells Does Not Require RPA and PCNA. Although in vitro replication of supercoiled plasmid DNA is S-phase specific, our results suggested that DNA synthesis also occurs in nuclear extracts prepared from unsynchronized cells (Figure 1B) and G_1-phase cells (Figure 8B). After separation of RPA and PCNA, the Pol_U and Pol_{G1} fractions produced low levels of DNA synthesis, similar to that observed with total nuclear extracts from unsynchronized and G_1-phase cells (Figures 2B, 4B, and 8B). The levels of DNA synthesis remained the same after adding back either Aux fraction or purified RPA plus PCNA to Pol_U and Pol_{G1} fractions (Figures 2B, 4B, and 8A). Anti-RPA and anti-PCNA sera did not inhibit the activity of nuclear extract from unsynchronized cells. On the other hand, the activity of pol α was inhibited up to 53% by anti-pol α serum (Figure 6B). Taken together, these data suggest that the low levels of DNA synthesis observed in nuclear extracts from unsynchronized and G_1-phase cells are probably due to some other form of DNA synthesis, such as DNA repair, rather than DNA replication, and this activity does not require RPA and PCNA. Some other polymerase, different from pol α and pol δ, could be responsible for this RPA- and PCNA-independent DNA synthesis (*40*).

RPA and PCNA from Nuclear Extracts of Unsynchronized or G_1-Phase Cells Are Able To Support in Vitro Supercoiled Plasmid DNA Replication of Pol_S Fraction. After partial fractionation of the replication factors of S-phase nuclear extract by phosphocellulose chromatography, in vitro supercoiled plasmid replication was successfully reconstituted by complementing the Pol_S fraction with either Aux_S fraction or purified yeast RPA and recombinant yeast PCNA. Our results also suggest that Aux fraction from unsynchronized (Aux_U fraction) or G_1-phase (Aux_{G1} fraction) nuclear extracts stimulates the replication activity of the Pol_S fraction at the same level of DNA synthesis observed with total S-phase nuclear extract (Figures 2A,C and 8A). It has been shown that both the large (p70) and the middle (p32) subunits of human and yeast RPA are phosphorylated in a cell-cycle-dependent manner (*41*). The phosphorylation of p32 subunit

has been characterized extensively. It is phosphorylated in the S- and G_2-phases of the cell cycle, and cyclin-dependent and DNA-dependent kinases have been identified as enzymes capable of phosphorylating p32 (*42*). However, the role of phosphorylation in the functions of RPA in DNA replication is still not clear, and even the link between the phosphorylation of RPA and S-phase checkpoint controls has been questioned (*25*). To date, phosphorylation of PCNA has not been reported. After Western blotting analysis, we found that the subunits of RPA, p70 and p32, as well as the 36 kDa monomer of PCNA were presented in similar amounts in S-phase, G_1-phase, and unsynchronized nuclear extracts of *Saccharomyces* cerevisiae (Figure 9). A possibility exists that the protein kinases in the S-phase nuclear extract could carry out phosphorylation of the accessory proteins such as RPA and PCNA, derived from cells synchronized in different phases of the cell cycle as well as of the recombinant ones. Further studies are necessary to address this issue.

Obviously, only replication carried out by cells synchronized in S-phase is sensitive to stimulation by RPA and PCNA. No inhibitors of in vitro plasmid DNA replication were also found in Aux_U, Aux_{G1}, Pol_U, and Pol_{G1} fractions, which could be a key to the understanding the S-phase specificity of this event (our unpublished data). This suggests that cell cycle-specific changes in protein−protein interactions between the replication proteins may be pivotal. It has been known that DNA replication is under the specific checkpoint control of the cell cycle and that cyclin-dependent Cdk kinases are responsible for activation of the replication factors by site-specific phosphorylation (*43*, *44*). Since RPA and PCNA from either unsynchronized or G_1-phase cells are capable of cooperating with the polymerases in the S-phase nuclear extracts, this control probably concerns the DNA polymerases or some other accessory factor(s), rather than RPA and PCNA.

In conclusion, we found the following: (i) Both RPA and PCNA together are required for stimulation of in vitro supercoiled plasmid DNA replication. (ii) The stimulation of polymerase activity by RPA and PCNA is confined to S-phase nuclear extract. (iii) RPA and PCNA appear to be cell cycle-independent in their ability to stimulate in vitro supercoiled plasmid DNA replication.

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REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) in *DNA Replication*, 2nd ed., W. H. Freeman and Co., San Francisco, CA.
2. DePamphilis, M. L. (2000) *J. Struct. Biol. 129*, 186−197.
3. Hartwell, L. H. (1971) *J. Mol. Biol. 59*, 183−194.
4. So, A. G., and Downey, K. M. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 129−155.
5. Coverley, D., and Laskey, R. A. (1994) *Cell 63*, 745−776.
6. Walter, J., and Newport, J. (2000) *Mol. Cells 5*, 617−627.
7. Gilbert, D. M., Miyazava, H., and DePamphilis, M. L. (1995) *Mol. Cell. Biol. 15*, 2942−2954.
8. Blow, J. J. (1996) in *Eukaryotic DNA replication* (Blow, J. J., Ed.) pp 143−165, IRL Press, New York.
9. Walter, J., Sun, L., and Newport, J. (1998) *Mol. Cells 1*, 519−529.
10. Braguglia, D., Heun, P., Pasero, P., Duncker, B. P., and Gasser, S. M. (1998) *J. Mol. Biol. 281*, 631−649.
11. Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991) *EMBO J. 10*, 3959−39678.
12. Kim, C. S., Preer, J. R., and Polisky, B. (1992) *Dev. Genet. 13*, 97−108.
13. Krysan, P. J., Smith, J. G., and Calos, M. P. (1993) *Mol. Cell. Biol. 13*, 2688−2696.
14. Gilbert, D. M. (2001) *Science 294*, 96−100.
15. Biswas, E. E., Chen, P. H., Gray, W., Li., Y. H., Ray, S., and Biswas, S. B (1993) *Biochemistry 32*, 3013−3019.
16. Biswas, E. E., Chen, P. H., and Biswas, S. B. (1995) *Protein Expression Purif. 6* 763−770.
17. Zasloff, M., Ginder, G. D., and Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 1139−1142.
18. Lue, N. F., and Kornberg, R. D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8839−8843.
19. Verdier, J. M., Stalder, R., Roberge, M., Amati, B., Sentenac, A., and Gasser, S. M. (1990) *Nucleic Acids Res. 18*, 7033−7039.
20. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
21. Laemmli, U. K. (1970) *Nature (London) 22*, 680−685.
22. Pasero, P., Duncker, B. P., and Gasser, S. M. (1999) *Methods: Compan. Methods Enzymol. 18*, 368−376.
23. Biswas, E. E., Chen, P., Gray, W., Li, Y., Ray, S., and Biswas, S. B. (1993) *Biochemistry 32*, 3020−3026.
24. Fien, K., and Stillman, B. (1992) *Mol. Cell. Biol. 12*, 155−163.
25. Waga, S., and Stillman, B. (1998) *Annu. Rev. Biochem. 67*, 721−751.
26. Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979) *Nature 282*, 39−43.
27. Chong, J. P., Thommes, P., and Blow, J. J. (1996) *Trends Biochem. Sci. 21*, 102−106.
28. Jallepalli, P. V., and Kelly, T. J. (1997) *Curr. Opin. Cell Biol. 9*, 358−363.
29. Krude, T., Jackman, M., Pines, J., and Laskey, R. A. (1997) *Cell 88*, 109−119.
30. Pasero, P., Braguglia, D., and Gasser, S. M. (1997) *Genes Dev. 11*, 1504−1518.
31. Tan, C. K., Castillo, C., So, A., and Downey, K. M. (1986) *J. Biol. Chem. 261*, 12310−12316.
32. Prelich, G., Tan, C. K., Kostura, M., Mathews, M. B., So, A., Downey, K. M., and Stillman, B. (1987) *Nature 326*, 517−520.
33. Tan, C. K., Sullivan, K., Li, X. Y., Tan, E. M., Downey, K. M., and So, A. (1987) *Nucleic Acids Res. 15*, 9299−9308.
34. Zhou, J.-Q., He, H., Tan, C.-K., Downey, K. M., and So, A. G. (1997) *Nucleic Acids Res. 25*, 1094−1099. Waga, S., and Stillman, B. (1994) *Nature 369*, 207−212.
35. Chalberg, M. D., and Kelly, T. J. (1989) *Annu. Rev. Biochem. 58*, 671−617.
36. Stillman, B. (1989) *Annu. Rev. Cell Biol. 5*, 197−245.
37. Xu, C. J., Grainge, I., Lee, J., Harshey, R. M., and Jayaram, M. (1998) *Mol. Cells 1*, 729−739.
38. Stephens, K. M., and McMacken, R. (1997) *J. Biol. Chem. 272*, 28800−28813.
39. Lee, J., Tribble, G., and Jayaram, M. (2000) *J. Mol. Biol. 296*, 403−419.
40. Prakash, S., and Prakash, L. (2000) *Mutat. Res. 30*, 13−24.
41. Din, S., Brill, S., Fairman, M. P., and Stillman, B. (1990) *Genes Dev. 4*, 968−977.
42. Brush, G. S., Anderson, C. W., and Kelly, T. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12520−12524.
43. Stillman, B. (1996) *Science 274*, 1659−1664.
44. Zeng, Y., Forbes, K. C., Wu, Z., Moreno, S., Piwnica-Worms, H., and Enoch, T. (1998) *Nature 395*, 507−510.

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