Early Events in DNA Replication Require Cyclin E and Are Blocked by p21^{CIP1}

Peter K. Jackson,* Stephane Chevalier,‡ Michel Philippe,‡ and Marc W. Kirschner*

*Harvard Medical School, Department of Cell Biology, Boston, Massachusetts 02115; ‡Departement de Biologie et Génétique du Developpement, CNRS Unité Associée 256, Universite of Rennes I, Campus de Beaulieu, 35042 Rennes, France

Abstract. Using immunodepletion of cyclin E and the inhibitor protein p21$^{WAF/CIP1}$, we demonstrate that the cyclin E protein, in association with Cdk2, is required for chromosomal replication in Xenopus extracts. The requirement for cyclin E occurs early in the replication process and cyclin E/Cdk2 is not required for the elongation phase of replication on single-stranded substrates. Although cyclin E/Cdk2 is likely to be the major target by which p21 inhibits the initiation of sperm DNA replication, p21 can inhibit single-stranded replication through a mechanism dependent on PCNA. While the cyclin E/Cdk2 complex appears to have a role in the initiation of DNA replication, another Cdk kinase, possibly cyclin A/Cdk, may be involved in a later step controlling the switch from initiation to elongation. The provision of a large maternal pool of cyclin E protein shows that regulators of replication are constitutively present, which explains the lack of a protein synthesis requirement for replication in the early embryonic cell cycle.

THE cell cycle is now known to be driven by complexes of stable kinases of the Cdc2 class and unstable regulatory subunits, called cyclins. In the best studied example, cyclin B and Cdc2 initiate mitosis. It is thought that cyclin B either phosphorylates specific substrates or that it activates other kinases that promote mitosis (for a recent review see King et al., 1994). The discovery of a set of cyclins and kinases expressed in G1 in eukaryotic cells has suggested that the G1-S transition also depends on similar reactions. Among the cyclins expressed at G1 and S, cyclins A, D, and E, and the Cdk2 kinase have been shown to be required for some aspect of G1 progression or the G1/S transition (for recent reviews see Draetta, 1994; Heichman and Roberts, 1994; Hunter and Pines, 1994). While these cyclins may have a role in progression through G1, cyclins A and E can associate with the Cdk2 kinase and have been suggested to control initiation or continuation of DNA replication itself (see Heichman and Roberts, 1994).

Cyclin E associated kinase activity peaks late in G1, when the retinoblastoma gene product, p105^{Rb}, becomes phosphorylated (Dulic et al., 1992; Koff et al., 1992; Lew et al., 1991). The Rb related protein p107 may be a direct target of cyclin E/Cdk2 because complexes containing these proteins together with the transcription factor E2F, which is thought to regulate S phase–specific gene expression, have been seen (Lees et al., 1992). Overexpression of cyclin E in an osteosarcoma cell line induces p105^{Rb} hyperphosphorylation and inhibits its growth-suppressive function, while overexpression in human fibroblasts leads to a shortened G1 phase (Hinds et al., 1992; Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). Treatment of mink lung cells with growth inhibitory factor TGF-β prevents the activation of cyclin E-Cdk2 and Rb hyperphosphorylation and inhibits cell cycle progression before the G1-S transition (Koff et al., 1993). These results suggest that cyclin E may play a role upstream of a transcriptional cascade, controlling the synthesis of components for S phase. Other perturbations of cyclin E or Cdk2 function, such as microinjection of antibodies against Cdk2 or cyclin E, or transfection of a dominant–negative cdk2 gene, give further support for some role of these proteins in G1-S progression (Pagano et al., 1993; van den Heuvel and Harlow, 1993; Ohtsubo et al., 1995). A more direct assay of a role for cyclin E in S phase progression was the effect of ectopic expression in Drosophila, which induced S phase in cells that had exited the cell cycle; mutants lacking cyclin E were blocked at the G1-S boundary (Knoblich et al., 1994).

Perhaps the most puzzling cyclin requirement for S phase function is that for human cyclin A (Pines and Hunter, 1989). While this cyclin has hallmarks of a mitotic cyclin including a destruction box, its levels and associated kinase activity apparently peak in S phase, and microinjection studies support that it is required in S phase (Girard et al., 1991; Pagano et al., 1992). Its association with p105^{Rb}, Cdk2, and E2F transcription factors suggest a role in S phase–specific transcription (Lees et al., 1992).

Xenopus egg extracts are useful for studying the direct effects of cell cycle regulators on biochemical targets in the absence of transcriptional control circuits. These ex-

Address all correspondence to P. K. Jackson, Harvard Medical School, 25 Shattuck St., Boston, MA 02115. Tel.: (617) 432-2294. Fax: (617) 432-0420.

tracts recapitulate major events of the cell cycle (Blow and Laskey, 1986; Lohka and Masui, 1984; Lohka and Maller, 1985; Murray and Kirschner, 1989). Replication of permeabilized sperm nuclei in interphase extracts from Xenopus eggs faithfully reproduces several important aspects of cell cycle control seen in vivo: the initiation and completion of semi-conservative chromosomal DNA replication (Blow and Nurse, 1990; Blow and Laskey, 1986); subsequent rounds of replication and a requirement that the nucleus must pass through mitosis in order to be relicensed for replication (Blow and Watson, 1987; Blow and Laskey, 1988; Hutchison et al., 1987); and a dependence of replication on the reformation of the interphase nucleus, on nuclear transport, and on the formation of replication foci (Cox, 1992; Leno et al., 1992; Mills et al., 1989; Newport and Spann, 1987; Newport and Kirschner, 1984; Sheehan et al., 1988). In addition, replication proteins RP-A, PCNA, DNA polymerase α, and RF-C have been demonstrated to be present and in some cases required in this in vitro system (Adachi and Laemmli, 1992, 1994; Fang and Newport, 1991; Hutchison et al., 1989; Zuber et al., 1989; Jackson, unpublished data).

We have begun to examine the linkage between control of the cell cycle and control of DNA replication by asking whether cyclin E is required for DNA replication in vitro and by determining its immediate targets. Previous work by Fang and Newport (1991, 1993) had identified a role for Cdk2 in DNA replication and demonstrated the association of forms of a 54-kD Cdk2-associated protein, potentially a cyclin. We demonstrate that cyclin E is the major partner of Cdk2 in Xenopus extract and is likely the Cdk2-associated protein. Using immunodepletion and the p21 protein, an inhibitor of cyclin-dependent kinases, we show that cyclin E is required for a step preceding the initiation of DNA replication and the formation of replication foci. We identify that a later transition from initiation to elongation is controlled by a cyclin/Cdk kinase. Cyclin E or Cdk2 do not seem to be required for subsequent elongation. Thus, cyclin E is essential for and participates in the earliest steps of chromosomal initiation, perhaps by either promoting the recruitment of replication proteins to origins or by controlling the step that unwinds the DNA.

Materials and Methods

Preparation of Extracts

Xenopus laevis egg extracts were prepared from laid eggs as described (Murray and Kirschner, 1989), except that dejellied eggs were activated in 1× MMR with calcium ionophore A23187 (Calbiochem-Novabiochem, La Jolla, CA) at 1 μg/ml until cortical contraction was observed, typically <4 min. Interphase extracts were made by addition of cycloheximide to 100 μg/ml to prevent synthesis of mitotic cyclins. Typically, experiments were performed with multiple fresh extracts, but for some analyses extracts were supplemented to 200 mM sucrose, flash frozen, and stored at −80°C. In some experiments, dejellied eggs were presoaked in 200 μg/ml cycloheximide for 15–30 min before activation with A23187. This treatment resulted in an inhibition of measurable cyclin A associated H1 kinase activity in interphase egg extracts, but had little effect on the cyclin E–associated H1 kinase activity.

Preparation of Demembranated Sperm Nuclei

Sperm nuclei were isolated from *X. laevis* testes as described (Sawin and Mitchison, 1991), except that the testes were macerated between two sintered glass microscope slides. The sperm was stored in 250 mM sucrose, 15 mM Hepes (pH 7.4), 1 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.1% β-mercaptoethanol, 10 μg/ml each of leupeptin, chymostatin, and pepstatin with 0.3% BSA and 30% glycerol at −80°C. Sperm stocks were 10^5 sperm/μl or ~300 ng DNA/μl.

Replication Assays

Reactions were typically carried out by mixing 10 μl of cycloheximide-stabilized interphase extract with 25 or 50 ng sperm DNA equivalents (~8,000 sperm) or 25 or 50 ng single-stranded M13 DNA (Pharmacia Fine Chemicals, Piscataway, NJ). Reactions were incubated at 23°C for the indicated times. For end-point assays of replication, incubation was typically for 180 min. For efficient extracts (30–100% of input DNA replicated), kinetic studies revealed that little additional incorporation into sperm DNA was seen after this time. M13 replication typically ran to completion within 60–90 min. Reactions were quenched by addition of 100 μl of SDS–proteinase K stop buffer (0.5% SDS), 20 mM EDTA, 20 mM Tris-HCl, pH 8.0, 1 mg/ml proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and incubated at 37°C for 30 min. Digested samples were extracted with phenol-chloroform and aliquots were spotted onto GF-C glass fiber filters (Whatman Paper, Clifton, NJ), precipitated in ice-cold 10% TCA/5% sodium pyrophosphate for 30 min, washed three times in ice-cold 1 M HCl, 2 times in 95% ethanol, and dried filters were counted by liquid scintillation and normalized to equivalent samples that were not TCA precipitated.

The efficiency of replication was measured by two methods. In the first, the number of sperm added was set such at ~25 or 50% of the maximum capacity of extract to replicate sperm DNA (~10 ng/μl or 3,200 sperm/μl). The specific activity of dATP in extract was based on the published concentration of (50 μM) dATP in extract (Blow and Laskey, 1986). The amount of DNA replicated was then calculated from the ratio of TCA precipitable counts to the counts added and the efficiency of replication from the ratio of DNA replicated to DNA input. In the second method, the percentage of sperm nuclei brightly stained by rhodamine dUTP after replication was compared with the percentage of nuclei stained with Hoechst 33258.

H1 Kinase Assays, Western Blotting

Typically, 5–10 μl of extract was diluted 1:10 in immunoprecipitation buffer (IPB: 100 mM NaCl, 50 mM β-glycerophosphate, 5 mM EDTA 0.1% Triton X-100, 1 mM DTT + protease inhibitors) to which was added a fixed amount of antisera specific to Xenopus Cdc2, Cdk2, or cyclins A1, B1, B2, or E. Typically, 1–2 μl crude antisera was demonstrated to be in antibody excess for 10 μl interphase extract. After 1–2 h incubation on ice, ~15 μl of protein A–Sepharose Cl4B was added and tumbled for ~30 min. Immunoprecipitates were washed four times in IPB, three times in kinase buffer (KB: 50 mM NaCl, 20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 0.02% Triton X-100), aspirated, mixed with 30 μl kinase cocktail (1× KB, 250 μg/ml histone H1, 0.4 mM NaATP, 0.25 μCi/μl [^{32}P]-g-ATP), incubated at 25°C for 15 min, quenched in sample buffer, resolved by SDS-PAGE, dried onto paper, and the assay quantitated on a Molecular Dynamics PhosphorImager (Eugene, OR).

Western blotting was performed by blotting SDS–polyacrylamide gels onto nitrocellulose, blocked in Tris-buffered saline with 0.5% Tween 20 and 5% Carnation non-fat dry milk. Affinity purified antibodies were used at 0.5–1 μg/ml; crude sera were used as indicated: PCNA COOH-terminal sera (1:300), RP-A COOH-terminal sera (1:500). Blocking experiments were performed by preincubating antibodies with a 10-fold molar excess of antigen or peptide. Secondary reagents were horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch, Inc.) with detection by chemiluminescence (Renaissance, New England Nuclear, Boston, MA).

Production of Bacterially Expressed GST-Cyclins, GST-p21, GST-Cdk2

Various GST-cyclin or MalE cyclin fusion proteins were prepared. Due to the insolubility of many cyclins, batch binding of GST-cyclins to beads proved inefficient. The following protocol evolved. Cyclin fusion proteins were expressed in BL21 pLys or NB42. Overnight cultures were inoculated into 1 liter pre-warmed LB and grown to OD_{600} ~0.5 in 2.8 liters Fernbach flasks. Flasks were plunged into ice-water and chilled to 18°C. IPTG was added to 100 μM and flasks were shaken at room temperature

for 2 h. Bacteria were pelleted, washed 1× in NTE (100 mM NaCL, 10 mM Tris, pH 8.0, 1 mM EDTA) + 1 mM PMSF/10 μg/ml each of leupeptin, pepstatin, and chymostatin (lpc[1] inhibitors), and frozen in liquid nitrogen. Extracts were suspended in PBS with PMSF and lpc inhibitors, sonicated, supplemented to 250 mM KCL/15 mM DTT, and centrifuged at 18,000 rpm in an SS34 rotor. From 6 liters of bacteria, approximately 100 mls of supernatant were produced and passed at 0.5 ml/min over a 5–15 ml GSH-agarose column (G4510; Sigma Chem. Co., St. Louis, MO) fitted with a flow adapter. The column was washed (~20 column volumes) with 0.1% Tween 20/PBS/1 mM DTT and then with 20 column volumes of PBS/1 mM DTT. The column was eluted in 1 ml bumps with glutathione buffer (5 mM glutathione/50 mM Tris, pH 7.7, 250 mM KCl, 2 mM DTT), and peak fractions pooled. The protein was dialyzed against XB^- (100 mM KCL/50 mM Hepes, pH 7.7), concentrated using an Amicon 10 concentrator, made 40% in glycerol, and stored at ~10–20 mg/ml total protein at −80°C. For the less soluble cyclins like cyclin A1, preparations were sometimes contaminated with bacterial heat shock proteins and the actual concentration of cyclin was estimated from Coomassie-stained gels.

Antibody Production, Purification, and Production of Antibody Beads

GST-Xenopus cyclin A1 and GST-Xenopus cyclin B1 constructs were kindly provided by Doug Kellogg and Andrew Murray (Univ. of California, San Francisco, CA). Inclusion body preparation of these GST-cyclins fusion proteins were gel purified, electroeluted, and used to raise antisera in rabbits (Babco Inc., Emeryville, CA). Xenopus cyclin E (Chevalier, S., and M. Philippe, unpublished results) was cloned in pGEX2 and a GST-cyclin E fusion protein produced. This protein was considerably more soluble than GST-cyclin A or B. Affinity purification employed the use of a MalE-cyclin E fusion generated by cloning cyclin E into the pMALc vector (New England Biolabs, Beverly, MA). This soluble protein was purified on an amylose resin column (NEB), following the manufacturer's protocol. The protein was dialyzed against 100 mM $NaHCO_3$ pH 8.5 and coupled to Affigel 10 (Biorad) following the manufacturer's specifications. These MalE-cyclin E beads were used for affinity purification of anti-cyclin E serum essentially as described (Harlow and Lane, 1988). The majority of the cyclin E reactivity was present in the base eluate. Pooled antibody fractions were dialyzed against XB^- (100 mM KCl/50 mM Hepes, pH 7.7/1 mM DTT) and concentration on a pre-washed Centriprep 30 concentrator. These antibodies were used for antibody blocking experiments. Anti-cyclin A2 antibodies were generously provided by Mike Howell and Tim Hunt (ICRF Clare Hall Labs). Anti-Cdc2 antibodies were raised against a COOH-terminal peptide as described (Solomon et al., 1990). Anti-Cdk2 antibodies were raised against a COOH-terminal peptide (sequence n-CPFFRDVSRPTPHLI) coupled to keyhole limpet hemocyanin (KLH) and affinity purified on a column of peptide coupled to Sulfolink beads (Pierce, Rockford, IL) following the manufacturer's instructions. Additional affinity-purified anti-Cdk2 antibodies were generously provided by John Newport (Univ. of California, San Diego, CA). Anti-RPA sera were raised against COOH-terminal peptides (n-CNIRKMATQGV) and used crude or affinity-purified on a peptide-Sulfolink bead column. Additional anti-Xenopus cyclin E sera were raised against NH_2- and COOH-terminal peptides (n-CPVIRNPAAEK and n-CDKKQKSDPAD). Anti-PCNA sera were raised against a COOH-terminal peptide of Xenopus PCNA (n-CMEHVKYYLAPKIEDEEAS-c).

Immunofluorescence, Nuclear Assembly, and Nuclear Transport Assays

Interphase extracts were mixed with sperm nuclei and either digoxygenin-11-dUTP (Boehringer Mannheim Biochemicals), biotin-21-dUTP (Clontech, Palo Alto, CA), fluoroscein-11-dUTP, or rhodamine-11-dUTP (Amersham Corp., Arlington Heights, IL) at 40 μM. Reactions were incubated at 23°C for indicated times, diluted 1:10 in XB/0.05% Triton X-100, overlaid on a sucrose cushion (XB/30% sucrose/0.05% Triton X-100) and spun for 5 min at 1,300 *g* onto polylysine-coated coverslips. Coverslips were fixed in methanol at −20°C for 5 min, briefly dried, rehydrated in PBS/3% BSA/0.1% Triton X-100. Some experiments were performed with EGS fixation as described (Mills et al., 1989) and similar results obtained. Anti-RP-A staining was performed with crude anti-RP-A (p70) COOH-terminal peptide sera at 1:1,000 or with affinity-purified antibody with blocking by the original peptide. Anti-cyclin E staining was performed with affinity-purified antibodies at 0.5 μg/ml. Secondary reagents were either rhodamine-conjugated anti-digoxygenin Fab fragments (Boehringer-Mannheim Biochemicals), FITC-streptavidin, TRITC-streptavidin, or Texas red– or TRITC-conjugated donkey anti–rabbit antibody (Jackson ImmunoResearch Labs, Inc., West Grove, PA). DNA was stained with Hoechst 33258 at 0.5 μg/ml for 5 min. Coverslips were mounted in Fluoromount G (Fisher Scientific, Pittsburgh, PA) with 1 mg/ml phenylene diamine as an anti-quenching agent, visualized on a 63×/1.4 NA oil-immersion lens on a Zeiss Axiophot, and photographed with Kodak TMAX 400 film. Nuclear transport was assayed with rhodamine-histone (Minden et al., 1989) or a synthetic peptide nuclear transport substrate coupled to fluorescent BSA (Newmeyer and Forbes, 1990).

For the quantitation experiment in Fig. 8 *C*, standard replication reactions were incubated with biotin- or digoxygenin-dUTP for 1 or 3 h and with dATP for 3 h. Quantitation of the fluorescent dUTP staining was accomplished using a Photometrics CH250 digital camera with a TK1024AB cooled CCD and the Universal Imaging Corporation Metamorph imaging system.

For the time course experiments in Fig. 9, parallel samples were incubated and removed at various times, diluted in XB-/0.05% Triton X-100 with or without denatured salmon sperm DNA (50 μg/ml) and kept on ice for 30 min before pelleting onto coverslips. Cross-over from the fluorescein and Texas red channels was minimal as shown by comparing samples stained with either secondary to those stained with both.

suc1-Bead Production and Depletion Protocol

Suc1 protein was expressed in the T7 expression system as described (Brizuela et al., 1987) with the following modifications. After expression of suc1 protein in bacterial strain BL21 pLysS and a cycle of freeze-thaw, lysates were prepared in 50 mM Hepes, pH 8.0, 5 mM EDTA with protease inhibitors, sonicated, and centrifuged at 100,000 *g* for 30 min. A 30–50% ammonium sulfate salt cut was taken, heat treated at 60°C for 3 min, and recentrifuged. This supernatant was run on a 120 ml Superdex 75 column and peak fractions pooled. Protein was dialyzed against 100 mM $NaHCO_3$/0.5 M NaCl and coupled to CNBr-Sepharose (Pharmacia Fine Chemical Co.) at 30 mg/ml following the manufacturer's instructions. The high density of coupling of Suc1 protein was important for efficient depletion with small amounts of beads. Control beads were either coupled without protein or with ~20 mg/ml BSA. Two important effects of using smaller amounts of beads may be reducing the dilution of extracts and reducing non-specific adsorption of activities to the beads themselves. Using less than 0.25 vol beads per volume of extract reduced non-specific inhibition by control beads.

Immunodepletion

Immunodepletions with crude sera were performed by binding of clarified, crude rabbit sera to protein A-Sepharose Cl4B beads in XB^- for 45 min, washing beads four times in XB^-, resuspending the final wash as a 50% slurry. Typically, 40 μl of this slurry was added to a 0.5 ml Eppendorf tube for each immunodepletion, spun down in a Eppendorf microfuge for 30 seconds, and aspirated nearly dry with a 25 gauge needle. 100 μl of interphase extract was added, the tube tapped gently to mix, and the bead-extract mix rocked very slowly on ice for 60 min. Beads were spun out in an Eppendorf microfuge (Brinkman Instruments, Westbury, NY) for 10 min at 13,000 rpm at 4°C. Control depletions used preimmune serum from the same rabbit. At an intermediate concentration of sera that substantially inhibited replication (30 μl sera versus ~20 μl beads), we would expect the maximum binding capacity of the beads used to be ~50 μg and the amount of IgG to be maximally ~300 μg (sixfold excess). The purified cyclin E antibodies described above were bound and coupled to protein A-Sepharose Cl4B beads using dimethyl pimilimidate as described (Simanis and Lane, 1985). These were washed in XB, and varying volumes of beads mixed with interphase extracts before assay for replication or H1 kinase activity. Minimal leaching of antibody was demonstrated by Western blotting of the depleted extracts for Ig heavy chain. Measurements of cyclin or Cdk H1 kinase activity after immunodepletion were performed by diluting extract 1:10 in IP buffer and clearing these diluted extracts with protein A-Sepharose Cl4B to remove any residual antibody that might in-

1. *Abbreviations used in this paper*: GST, glutathione S-transferase; IVT, in vitro translation; KLH, keyhole limpet hemocyanin; lpc, leupeptin, pepstatin, and chymostatin; MBP, maltose-binding protein.

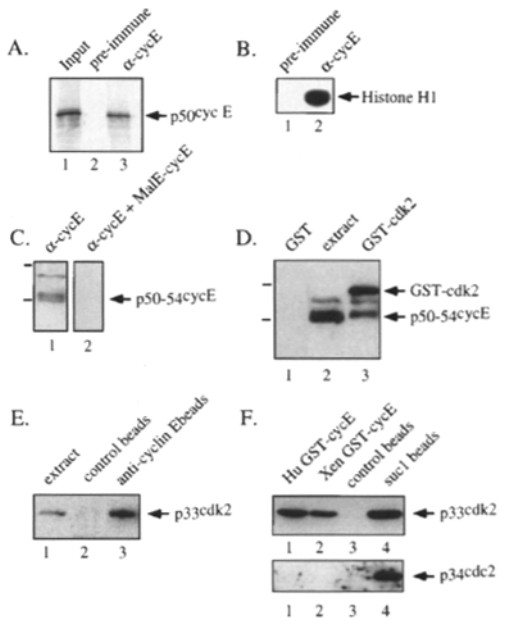

Figure 1. Cyclin E associates with Cdk2 to generate H1 kinase activity in interphase extract. (*A*) Anti-Xenopus cyclin E sera. Antisera generated against a GST-Xenopus cyclin E fusion protein (lane *3*) or pre-immune sera (lane *2*) were incubated with [^{35}S]-labeled in vitro translated Xenopus cyclin E (unprecipitated input, lane *1*), precipitated with protein A–Sepharose beads, resolved by SDS-PAGE, and visualized by autoradiography. (*B*) Cyclin E–dependent kinase activity is present in interphase extracts from Xenopus eggs. Antisera against cyclin E (lane *2*) or preimmune sera (lane *1*) were added to interphase extracts of Xenopus eggs and precipitated on protein A–Sepharose beads. Precipitates were washed and assayed in an immune complex kinase assay with histone H1 as a substrate. (*C*) Cyclin E protein has multiple 50–54-kD species. Interphase extracts were resolved on 11% SDS–polyacrylamide gels, blotted to nitrocellulose filters, and analyzed by immunoblotting with affinity purified anti-cyclin E antibodies (0.5 μg/ml) with (lane *2*) or without (lane *1*) competition by a MalE-cyclin E fusion protein. Secondary antibody was a HRP-conjugated donkey anti–rabbit and visualization was by enhanced chemiluminescence. Molecular mass markers are 49 and 70 kD. (*D*) A GST-Cdk2 protein precipitates the Cyclin E protein.A GST-human CDK2 protein or GST protein (~1 μg) was incubated with 10 μl of interphase extract, bound to glutathione agarose, washed, fractionated by SDS-PAGE, and blotted to nitrocellulose. Blots were incubated with affinity-purified anti-cyclin E antibodies as above. Lanes: *1*, GST protein; *2*, extract; *3*, GST-Cdk2. The GST-Cdk2 protein can be visualized because of its abundance and a small amount of anti-GST activity in the antibody preparation. Reblotting this filter with antibodies against GST or a C-terminal peptide from Cdk2 establish the indicated band as the unique GST-Cdk2 species. Molecular mass markers are 49 and 70 kD. (*E*) Anti-cyclin E antibodies coprecipitate the Cdk2 protein. Interphase extract was incubated with anti-GST-Xenopus cyclin E polyclonal antisera coupled to protein A-Sepharose or control rabbit IgG beads, washed, resolved by SDS-PAGE, and prepared for immunoblots with antisera raised against a Cdk2 C-terminal peptide. Lanes: *1*, Interphase extract; *2*, control beads; *3*, Anti-cyclin E beads. (*F*) A GST-cyclin E protein precipitates Cdk2, but not Cdc2. Human or Xenopus GST-cyclin E fusions (lanes *1* and *2*) were incubated with interphase extract, bound to glutathione agarose, washed, fractionated by SDS-PAGE, and blotted to nitrocellulose. Blots were incubated with either anti-Cdk2 sera (*upper panel*) or anti-Cdc2 (*lower panel*). For comparison, control (lane *3*) or suc1 beads (lane *4*) were incubated with interphase extracts and analyzed similarly.

terfere with the assay. Immunoprecipitations and H1 kinase assays were then performed as described above.

Inhibition with Purified Cyclin E Antibodies

Antibody preparations were polyclonal rabbit antisera raised against a GST-Xenopus cyclin E fusion protein and were affinity purified on a column with an immobilized Mal E-Xenopus cyclin E fusion. Antibodies were concentrated and dialyzed against 100 mM KCl, 20 mM Hepes, pH 7.4. Control antibodies were purified rabbit IgG.

p21 Inhibition of Replication

GST-p21 was prepared as above and stored at 10–20 mg/ml in XB$^-$. Various dilutions of GST-p21 were prepared in XB$^-$ + 1 mg/ml acetylated BSA as carrier. Typically, 1 μl of this dilution was added to a 10 μl replication reaction and the reaction run for 180 min. In the kinetic experiments, multiple aliquots of the replication reaction were set up and at the indicated times, 1 μl of XB containing 300 ng of p21 inhibitor was added, mixed gently with a pipet, and their incubation continued. At the end of the time course, all reactions were quenched and analyzed as above.

PCNA Binding

Approximately 2 μg of various GST fusions were added to 20 μl of interphase extract, incubated at room temperatures for 45 min, diluted in IPB, washed four times, and analyzed for PCNA binding by SDS-PAGE and Western blotting.

Gel Filtration Analysis

Gel filtration to size the cyclin E complex was performed using high-speed supernatants (HSS) from interphase egg extract. HSS was prepared by diluting LSS 1:4 in XB$^-$ and centrifuging in a TLS-55 for 1 h at 4°C. HSS was filtered through a 0.45 μ filter and 200 μl chromatographed on either a Superose 6 (24 ml) or a Superdex 200 column (120 ml) on a Pharmacia FPLC. A 10 ml TSK3000 column run on a Hewlett-Packard 1040 HPLC was also employed. For the Superdex 200 column, 4 ml fractions were collected, each containing substantially different proteins, and marker resolution was superb.

Results

Generation of Cyclin E Antisera and Characterization of the Cyclin E-Cdk2 Kinase Complex

In order to study the activities of cyclin E, we raised a polyclonal rabbit antiserum against a bacterially expressed glutathione S-transferase (GST)-Xenopus cyclin E fusion protein. This serum specifically immunoprecipitated the single radiolabeled 50-kD cyclin E protein species generated by in vitro translation (IVT) in rabbit reticulocyte lysates (Fig. 1 *A*). The serum also specifically immunoprecipitated cyclin E-associated histone H1 kinase activity from interphase extracts of Xenopus eggs (Fig. 1 *B*).

The antiserum was affinity purified on beads coupled to a Mal E-Xenopus cyclin E fusion protein. These affinity purified antibodies also precipitated the IVT cyclin E protein and histone H1 kinase activity as above (not shown). Immunoblots with these antibodies specifically recognized an apparent triplet of cyclin E species between 50 and 54 kD in interphase extracts, although a lower mobility species (~57 kD) is typically observed (Fig. 1 *C*). When the 50-kD species of in vitro translated cyclin E was added to

interphase extract, it generated multiple forms similar to those seen for endogenous cyclin E (not shown).

The specificity of the cyclin E antiserum was demonstrated by three criteria. First, this serum recognizes in vitro translated Xenopus cyclin E protein, but not Xenopus cyclins A1, A2, B1, or B2. Second, the recognition of endogenous cyclin E by this serum is blocked by a Mal E-cyclin E fusion protein (Fig. 1 *C*) and not by bacterially expressed GST-fusions of cyclins A1, A2, or B1 (not shown). Finally, the Mal E-cyclin E fusion protein blocks immunoprecipitation of H1 kinase activity by the cyclin E antiserum.

It is likely that the multiple cyclin E species are similar to the previously reported 54-kD "doublet" species that coprecipitated with Cdk2 (Fang and Newport, 1991). In support of this idea, a GST-Cdk2 protein precipitated several species of cyclin E protein (Fig. 1 *D*). Apparently, the lower mobility forms, which are more highly phosphorylated (not shown), preferentially associate with Cdk2.

Immunoprecipitation with anti-cyclin E antibodies efficiently coprecipitated Cdk2, while control antibodies did not (Fig. 1 *E*), consistent with studies in mammalian cells (Dulic et al., 1992; Koff et al., 1991, 1992). Human or Xenopus GST–cyclin E protein bound specifically to Cdk2 and not Cdc2 when incubated with interphase extracts (Fig. 1 *F*), indicating that the cyclin E-associated kinase activity was Cdk2, not Cdc2 dependent. Further, addition of the GST-cyclin E protein generated an active kinase complex as assayed by precipitation with anti-GST antibodies or with glutathione-agarose (not shown). This ability of added GST-cyclin E protein to generate an active kinase complex with Cdk2 was important for the rescue experiments that follow.

Analysis of extracts fractionated by high speed centrifugation revealed that Cdk2 or cyclin E proteins are largely soluble. Fractionation of this high speed supernatant by gel filtration and analysis of these fractions by immunoprecipitation and H1 kinase assay showed that >75% of both cyclin E– and Cdk2-associated H1 kinase activities are present in an apparent complex of approximately 440 kD. Immunoblotting showed that the majority of the cyclin E protein appeared in this complex and that Cdk2 coprecipitated with cyclin E in those fractions containing this apparent complex. Thus, cyclin E and Cdk2 each appear to be a major partner of the other in interphase extract.

A Requirement for Cyclin E in DNA Replication

Cdk2 was previously shown to be required for DNA replication in interphase extracts (Fang and Newport, 1991). Because cyclin E is a major partner of Cdk2, we expected that cyclin E would also be required for replication in these extracts. To test this idea, we used the anti-cyclin E sera to show that either immunodepletion of cyclin E or addition of concentrated, affinity-purified anti-cyclin E antibodies inhibited replication and that this inhibition was overcome by addition of cyclin E protein.

Anti-GST-Xenopus cyclin E antisera bound to protein A–Sepharose beads depleted replication activity from interphase extracts by as much as 75% (Fig. 2 *A*). Replication activity could be restored by the addition of GST-Xenopus cyclin E protein (Fig. 2 *B*), whereas the GST moiety alone

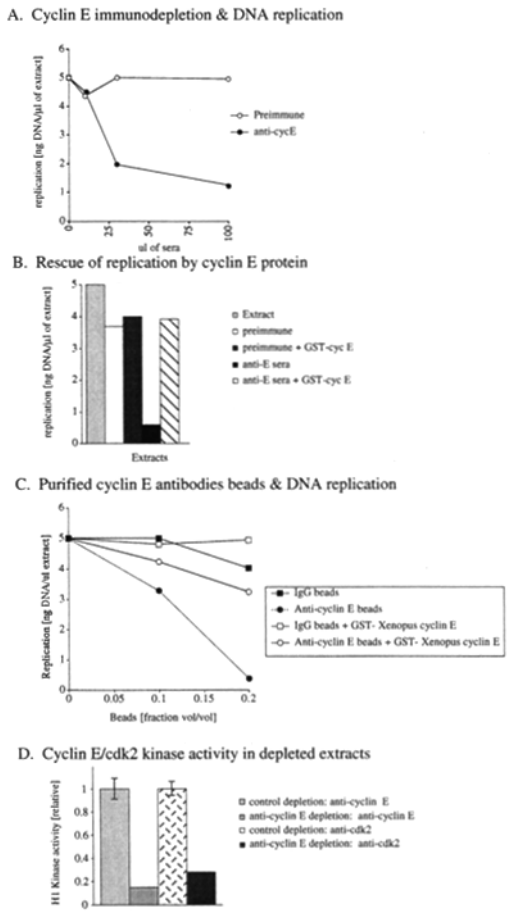

Figure 2. Immunodepletion of replication activity from interphase extract with anti-cyclin E antibodies and rescue by cyclin E protein. (*A*) Immunodepletion of replication activity by anti-cyclin E sera is specific and dose dependent. Increasing amounts of either preimmune or immune rabbit sera generated against Xenopus cyclin E were prebound to protein A–Sepharose beads, washed in XB, and rocked gently with interphase extract (0.2 volumes beads/volume extract) for 45 min. Beads were spun out at 13,000 rpm for 5 min and supernatants assayed for sperm replication activity (described in Materials and Methods). Briefly, permeabilized sperm were mixed with depleted or control extract (5 ng sperm DNA/μl extract) containing an energy-regenerating system, and [^{32}P]-dATP, and incubated at 23°C for 180 min. Reactions were quenched and quantitatively assayed for acid-insoluble counts (described in Materials and Methods). (*B*) Immunodepletion is rescued by bacterially expressed cyclin E. Immunodepletions were performed as in *A*. Sperm replication reactions were performed as above except that reactions were supplemented with GST-cyclin E (~50 ng/μl). (*C*) Immunodepletion with anti-cyclin E antibody beads. Affinity purified cyclin E antibodies were coupled to protein A–Sepharose beads and used to immunodeplete extracts of cyclin E. Addition of cyclin E protein (~0.5 μM) was used for rescue. (*D*) Measurement of residual cyclin E- or Cdk2-dependent H1 kinase activity after immunodepletion with cyclin E antibody beads. After immunodepletion with anti-cyclin E antibody beads (~90% inhibition of sperm replication), extracts were subsequently immunoprecipitated with anti-Xenopus cyclin E polyclonal rabbit antiserum or anti-Cdk2 COOH-terminal peptide antiserum and H1 kinase activity measured. Duplicate assays were performed and average results and representative standard deviations shown.

had no effect (not shown). Further, addition of a GST-Xenopus cyclin B1 did not rescue replication suggesting a specific role for cyclin E. However, addition for GST-Xenopus cyclin A2 did stimulate DNA replication. Thus, although the endogenous cyclin E is supporting DNA replication in egg extracts, cyclin A, but not cyclin B, can substitute for this requirement.

Cyclin E depletion of extracts actually caused slight stimulation of replication of single-stranded DNA (not shown). This is consistent with what we have observed with depletions of Cdk2 kinase with anti-Cdk2 antibodies or suc1 beads. Thus, cyclin E depletion appears to inhibit double stranded, but not single stranded replication, consistent with a role for cyclin E in initiation of replication.

To improve our efficiency of depletion, we covalently coupled affinity-purified anti-cyclin E antibodies directly to beads. Immunodepletion with these beads gave a dose-dependent reduction in replication activity (up to >90% inhibition) that could be substantially restored by addition of GST-Xenopus cyclin E protein (Fig. 2 *C*). After depletion with 0.2 vol of these beads, approximately 11% of cyclin E–associated H1 kinase activity and 24% of Cdk2-associated H1 kinase activity remained (Fig. 2 *D*); immunoblots showed similar percentages of cyclin E (~10%) or Cdk2 protein (~30%) remained (not shown). Thus, most of the Cdk2-associated kinase activity is depletable by cyclin E antibodies, consistent with cyclin E being the major cyclin associated with Cdk2.

Addition of a baculovirus-expressed human Cdk2 protein or a GST-Cdk2 fusion protein alone did not stimulate replication. These Cdk2 proteins were functional and capable of being activated in extracts as assayed by their associated H1 kinase activity upon reprecipitation from interphase extract. In contrast, addition of anti-Cdk2 immunoprecipitates to cyclin E–depleted extracts stimulated replication very strongly, suggesting that the activated Cdk2 kinase, presumably in complex with cyclin E and any associated proteins, was effective in promoting replication. The inability of Cdk2 alone to rescue replication suggests that cyclin E depletion blocks replication because cyclin E is required rather than because Cdk2 is depleted during cyclin E depletion.

The Cyclin E–Cdk2 Kinase Does Not Fully Complement Suc1-Depletion

Previous studies had suggested that depletion of interphase extracts with beads coupled to the cyclin-Cdk associated protein, Suc1, was capable of completely depleting activities required for DNA replication (Blow and Nurse, 1990). Since Cdk2 is required for DNA replication (Fang and Newport, 1991), and Suc1 beads can precipitate the cyclin complexes including cyclin E-Cdk2, we expected that Suc1 depletion would deplete the cyclin E–Cdk2 complex. Suc1 depletion removed >70% of Cdk2 protein and >90% of the cyclin E protein (Fig. 3 *A*) and >70% of Cdk2- and >85% of cyclin E associated H1 kinase activity. This depletion resulted in a loss of >95% of the ability of these extracts to replicate sperm DNA. Addition of a cocktail of cyclin E and Cdk2, but not Cdk2 protein alone, was able to partially rescue these Suc1–depleted extracts (Fig. 3 *B*). Screening a panel of bacterially expressed cyclins for their ability to rescue replication in Suc1-depleted extracts, we have found that most cyclins did not rescue replication including Xenopus cyclins B1, B2, mouse cyclin D1, and yeast CLN2 and CLB2 (not shown). However, Xenopus cyclins A1, A2, or human cyclin A strongly stimulated replication in these extracts. Thus, while the cyclin E/Cdk2 complex is an important Suc1-depletable factor, other factors depleted by Suc1 beads, including other cyclins, may be important for replication (see Discussion). When we adjusted the amount of GST-cyclin A2, -cyclin B1, or -cyclin E to normalize the amount of histone H1 kinase activity generated after reprecipitation of the GST cyclins with anti-GST antibodies, we found that only cyclin A2 or E, but not cyclin B1, would stimulate DNA replication in extracts depleted with Suc1 beads.

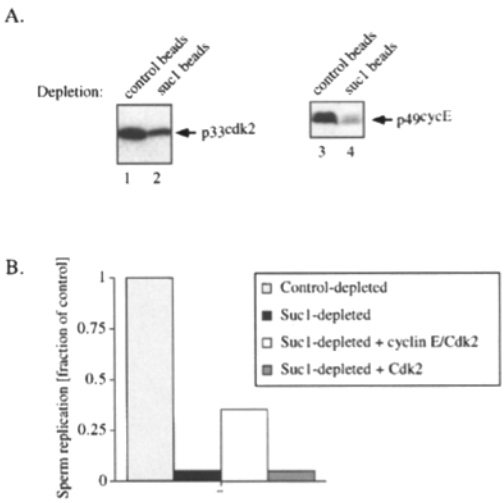

Figure 3. Depletion of replication activity by Suc1-affinity removes Cyclin E, but probably other factors important for replication. (*A*) Suc1 beads deplete cyclin E and Cdk2 protein. Western blot analysis of residual Cdk2 (lanes *1* and *2*) and cyclin E protein (lanes *3* and *4*) after depletion with control (lanes *1* and *3*) or Suc1 beads (lanes *2* and *4*). (*B*) Cyclin E partially rescues replication in Suc1-depleted extract. Extracts depleted with Suc1 or control beads were assayed for their ability to replicate sperm DNA as described above. Reactions were supplemented with Cdk2 alone or with cyclin E plus Cdk2 (each at 100 ng/μl).

Cyclin E Is Required for an Early Step in DNA Replication

In order to understand which steps in DNA replication require cyclin E, we used affinity-purified antibodies against cyclin E to block replication. Direct addition of these antibodies efficiently inhibited sperm replication; this inhibition could be blocked by a maltose-binding protein (MBP)-Xenopus cyclin E fusion protein (Fig. 4 *A*), but not by GST-cyclin B1 (not shown).

Proper nuclear assembly and nuclear envelope formation are essential for chromosomal replication and appeared to be unaffected by addition of anti-cyclin E antibodies (Fig. 4 *B*). Nuclear transport is also essential for replication (Cox, 1992) and was unaffected in extracts blocked with added cyclin E antibody as judged by the nuclear transport of a fluorescently labeled carrier protein

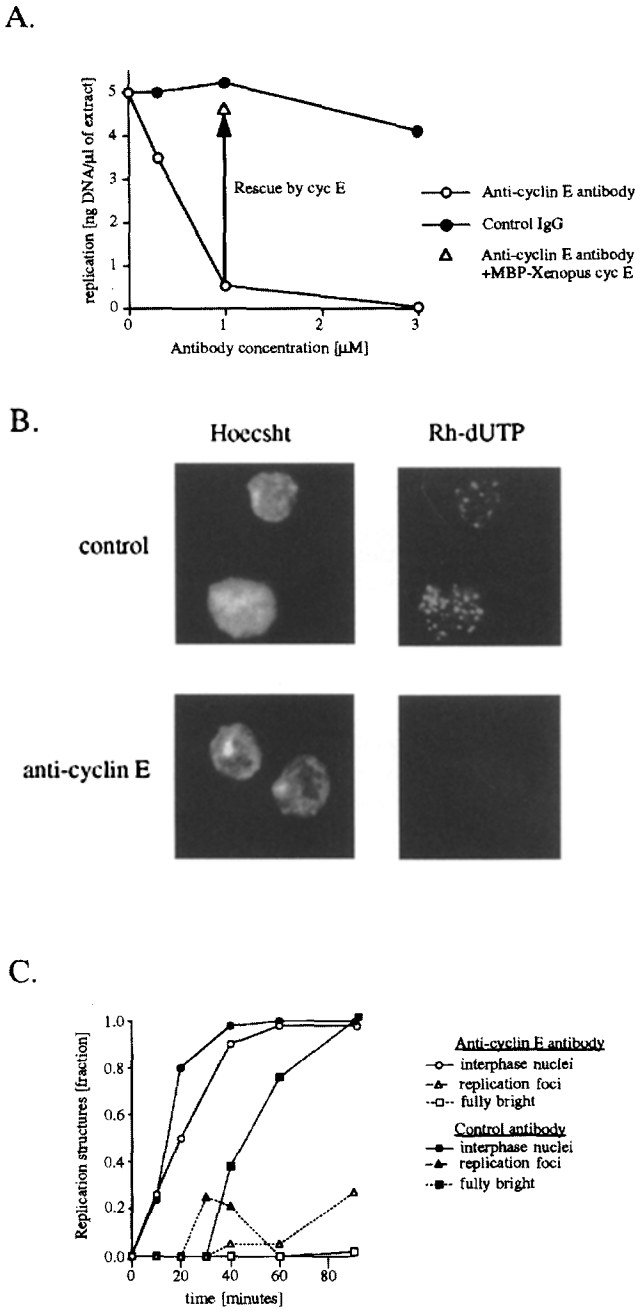

Figure 4. Anti-cyclin E antibodies block at an early step in DNA replication. (*A*) Antibody inhibition of replication in interphase extracts. Interphase extracts were mixed with sperm DNA and varying amounts of affinity purified anti-Xenopus cyclin E antisera and assayed for sperm DNA replication (see Materials and Methods). The approximate level of cyclin E in Xenopus interphase extract is ~0.1 μM, so addition of anti-cyclin E antibodies to an apparent 5–10-fold molar excess was sufficient to block >90% of replication. (*B*) Addition of cyclin E antibodies blocks formation of replication foci. Anti-cyclin E antibodies (1 μM) or control rabbit IgG was added to interphase extract containing rhodamine-dUTP (40 μM) and sperm templates. Replication reactions were run for 60 min, assembled nuclei spun onto coverslips, fixed, and counterstained with Hoechst 33258. (*C*) Cyclin E antibodies block replication before replication focus formation. Replication reactions were set up with anti-cyclin E or control antibodies as in Fig. 3 *A* with rhodamine-dUTP included to visualize replication foci. At the indicated times, aliquots were diluted and fixed, spun onto coverslips, counterstained with Hoechst 33258 for DNA, and visualized by indirect immunofluorescence and phase contrast microscopy. Quantitation is described in Materials and Methods (n = 100). Replication foci were structures similar to those in *B* and "fully bright" refers to fully replicated nuclei with intense overall rhodamine-dUTP staining.

coupled to a nuclear localization signal (see Materials and Methods). At the highest doses, addition of anti-cyclin E antibodies had a subtle effect on nuclear morphology, with the highest doses causing some condensation of the DNA. It is unclear whether the block to DNA replication induces the apparent condensation or whether cyclin E has some role in controlling chromosome condensation or dynamics. Given the abundance of cyclin E (~100 nM), a role in chromosome scaffolding seems less likely.

To test whether cyclin E was required at an early step in replication, we examined the effect of anti-cyclin E antibody addition on the formation of replication foci. These foci of replication, which can be visualized by incubation with fluorescently labeled dUTP, have been shown to coincide with replication proteins associated with the initiation complex such as PCNA, RP-A, and DNA polymerase-α (Adachi and Laemmli, 1992; Nakayusa and Berezney, 1989; Raska et al., 1989). Addition of anti-cyclin E antibodies caused a profound decrease and a delay in the appearance of replication foci, suggesting that replication was blocked at an early step (Fig. 4 *B*). A time course of replication showed that the anti-cyclin E antibodies blocked the formation of replication foci and of later forming fully replicated nuclei (Fig. 4 *C*). We achieved a similar block by depletion with Suc1-beads or anti-cyclin E antibody beads. Thus, cyclin E appears to be required for an early event in sperm replication.

p21$^{CIP/WAF}$ Inhibits Cyclin E/Cdk2 Kinase and Replication of Sperm DNA

Recent studies have shown that p21$^{CIP1/WAF1}$ is a specific inhibitor of cyclin-dependent kinases (Dulic et al., 1992; El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). We found that a bacterially expressed GST-human p21 fusion protein potently inhibited double-stranded sperm DNA replication at lower concentration (~100–200 nM), but required higher levels to block single-stranded replication (~10 μM; Fig. 5 *A*). We have found that another inhibitor of cyclin E/Cdk2, called p27^{KIP1} (Polyak et al., 1994*a*,*b*; Toyoshima and Hunter, 1994) also blocked replication of sperm DNA (not shown). Recent studies have also seen that p21 can block sperm replication (Strausfeld et al., 1994; Adachi and Laemmli, 1994; Yan and Newport, 1995) and that this inhibition is mediated by the NH_2-terminal cyclin/Cdk2 binding domain of p21, which binds to cyclin/Cdk2 (Chen et al., 1995). The ability of p21 to block single-stranded DNA replication in interphase extracts was also shown to function through the COOH-terminal domain of p21, which binds the replication protein PCNA (Chen et al., 1995).

As shown in Fig. 5 *B*, p21 inhibited the endogenous Cdk2-, cyclin E-, and cyclin A1-dependent kinases when added to interphase extract, but was less effective in inhibiting Cdc2 or cyclin B1–dependent kinases. The increased

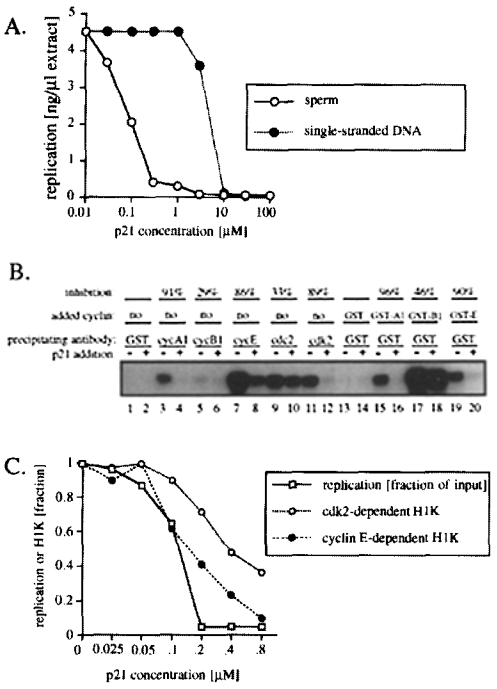

Figure 5. p21 coordinately inhibits replication and cyclin-E/Cdk2-dependent kinase activity. (*A*) p21 inhibits sperm replication at a much lower dose than single stranded DNA replication in interphase extracts. 10 μl of a cycloheximide-stabilized interphase extract was mixed with either permeabilized Xenopus sperm or purified single-stranded (M13) DNA (5 ng/μl) and varying amounts of GST-p21 protein. DNA replication was assayed as described. (*B*) p21 strongly inhibits cyclin E/Cdk2 complexes, but not cyclin B/Cdc2 complexes. 10 μl of interphase extract were mixed with various combinations of p21 (300 ng/μl) and GST cyclins (300 ng/μl). Extracts were incubated at 23°C for 45 min, diluted in 400 μl immunoprecipitation buffer, and immunoprecipitated with antisera specific for Xenopus cyclin A1, B1, E, Cdc2, or Cdk2. Immune complex kinase assays for H1 kinase activity were performed, visualized on SDS-polyacrylamide gels, and quantitated (described in Materials and Methods). (*C*) Dose response of p21 inhibition of DNA replication and cyclin E- or Cdk2-dependent kinase activity. Interphase extracts were prepared for parallel replication and immune complex kinase reactions. Varying amounts of GST-p21 protein were added at the beginning of replication reaction (180 min at 23°C). For analysis of cyclin-dependent kinase activity, GST-p21 was added to extracts and these were incubated for 45 min at 23°C. Extracts were then diluted into immunoprecipitation buffer and antisera specific for Xenopus cyclin E or Cdk2 was added in excess (see Materials and Methods). Average values are presented ($n = 3$), standard deviations were <10%. The approximate level of cyclin E protein in interphase extract is 5 ng/μl or 0.1 μM.

H1 kinase activity on addition of GST-cyclin A1 or E showed that the extracts have an excess capacity to activate these kinases, and that this activity is inhibitable by GST-p21 (Fig. 4 *B*).

The dose responses of p21 for inhibiting replication, cyclin E-, and Cdk2-dependent H1 kinase activity were fairly similar (Fig. 5 *C*). The amount of p21 required to half-maximally inhibit replication (~100 nM) and cyclin E-dependent kinase was roughly equivalent to the in vivo levels of cyclin E (100 nM) or Cdk2 (60 nM) proteins (Kobayashi et al., 1991*a*,*b*; Chevalier, S., and M. Philippe, unpublished data), and was consistent with the idea that p21 would stoichiometrically inhibit the cyclin E–Cdk2 complex. However, the inhibition of replication more closely paralleled the inhibition of cyclin E-dependent kinase activity than the Cdk2-dependent kinase activity. Thus, there may be additional Cdk2-dependent kinase complexes that do not contain cyclin E, as also suggested by our gel filtration analysis of Cdk2-dependent kinase activity (not shown). Direct addition of p21 to the kinase reaction after immunoprecipitation inhibited Cdk2- or cyclin E–dependent H1 kinase activity with a similar dose dependence (data not shown).

Addition of MBP-cyclin E protein rescued the p21 inhibition of sperm replication (Fig. 6 *A*). Thus, p21 and cyclin E appear to have antagonistic functions on a similar pathway leading to DNA replication.

p21 Inhibits Single-stranded DNA Replication via a PCNA-Dependent Mechanism

Recently, it has been demonstrated that p21 inhibits SV-40 replication via its interaction with PCNA (Waga et al., 1994*b*; Flores-Rozas et al., 1994). If this were the mode of inhibiting chromosomal replication, we would predict that added PCNA should suppress the inhibitory effects of p21. We did not observe any rescue of p21 inhibition of sperm replication when bacterially expressed human PCNA was added to egg extracts (Fig. 6 *A*). The bacterially expressed PCNA was functional for in vitro replication in the SV-40 system and associated directly with human p21 (Waga et al., 1994*b*). PCNA also did not block the inhibition of the cyclin E–associated H1 kinase by GST-p21 (data not shown). This is consistent with published work showing that PCNA does not affect p21 inhibition of the H1 kinase activity of cyclin/Cdk2 complexes, nor association of p21 with cyclin/Cdk complexes (Zhang et al., 1994). Further, it is consistent with cyclin/Cdk and PCNA binding to independent sites on p21 and the cyclin/Cdk2 binding domain blocking double-stranded DNA replication (Chen et al., 1995).

Inhibition of single-stranded DNA replication at higher concentrations of p21 was rescued by PCNA, but not the cyclin E protein (Fig. 6 *B*). Thus, apparently p21 can antagonize single-stranded replication by a PCNA-dependent mechanism in Xenopus extract, but it requires concentrations of p21 roughly stoichiometric with those of PCNA in extract (~8 μM). Presumably we cannot observe this mode of inhibiting chromosomal replication in the presence of a cyclin E/Cdk2-dependent step that is inhibited at ~100-fold lower concentration. We have previously shown that the COOH-terminal PCNA binding domain of p21 will inhibit single-stranded DNA replication in Xenopus extract (Chen et al., 1995).

Since we were using mammalian p21 and complexes of cyclin-Cdk2-PCNA and p21 have not been demonstrated in Xenopus, we asked whether any of these associations could be detected in Xenopus extract. Endogenous Xenopus PCNA from interphase extract associated strongly with GST-p21 (Fig. 6 *C*). In the extract, endogenous PCNA bound at low levels to GST fusions of Xenopus cyclins E, A2, and B, and at higher levels with GST-human

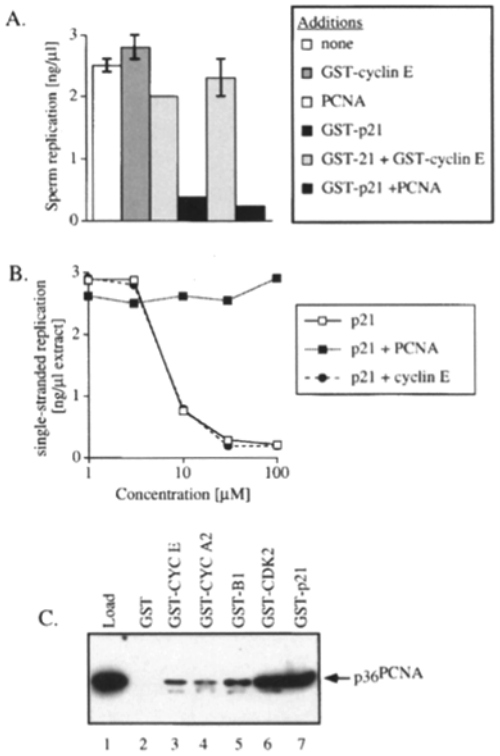

Figure 6. p21 inhibits sperm DNA and single-stranded DNA replication through cyclin E- and PCNA-dependent mechanisms, respectively. (*A*) Cyclin E, but not PCNA, rescues p21 inhibition of sperm replication. A sperm DNA replication assay was set up as described with [^{32}P]-dATP and 2.5 ng/µl of sperm DNA and combinations of various bacterially expressed proteins: GST-21 (7 ng/µl), GST-Xenopus cyclin E (30 ng/µl), or human PCNA (100 ng/µl). Quantitation was based on two independent assays from one extract, but results were consistent in independent extracts. (*B*) PCNA, but not cyclin E, rescues p21 inhibition of single-stranded DNA replication. Similar extracts with single stranded DNA substrate (M13) were performed. Additions of p21 with or without a two to three-fold molar excess of cyclin E or PCNA were added as indicated. (*C*) Association of PCNA with cyclins, Cdk2, and p21. The following fusions were added to interphase egg extracts: GST (lane 2), GST-Xenopus cyclin E (lane 3), GST-Xenopus cyclin A2 (lane 4), GST-Xenopus cyclin B1 (lane 5), GST-human Cdk2 (lane 6), or GST-human p21 (lane 7). Extracts were incubated, washed, resolved by SDS-PAGE, blotted, and stained with antiserum raised against the COOH-terminal peptide from Xenopus PCNA.

Cdk2 (Fig. 6 *C*); in addition, PCNA co-immunoprecipitates with cyclin E (not shown). These associations suggest an endogenous p21-like protein to couple PCNA to Cdk2. Thus, endogenous Xenopus PCNA is competent to associate with complexes of these proteins.

p21 Inhibits prior to the Aphidicolin-sensitive Step in DNA Replication

To ascertain whether cyclin E is required only for an early step in replication or whether it acts throughout replication, we assayed kinetically the exact time at which p21 inhibits replication. As shown in Fig. 7 *A*, sperm replication, as measured by a bulk assay of [^{32}P-α]-dATP incorporation, begins about 30 min after sperm addition; by 90 min >80% of the DNA is replicated ($t_{1/2} \sim 60$ min). Added single-stranded DNA has a much shorter lag to replication (<10 min; $t_{1/2} \sim 30$ min). Addition of p21 (~200 nM) early in the sperm replication reaction completely inhibited subsequent replication (Fig. 7 *B*). However, after 30 min there was little effect of added p21, despite the fact that less than 5% of the sperm DNA had been replicated. This suggests that the effect of p21 is confined to an early step in replication. In contrast, addition of the polymerase inhibitor aphidicolin blocked replication well after the p21 sensitive period is completed ($t_{1/2} \sim 60$ min). The difference in kinetics was not affected by increasing the concentration of inhibitors.

Indicated on the axis of the same plot (Fig. 7 *A*) are the approximate times of nuclear formation and rhodamine-dUTP replication foci. Although the period of p21 sensitivity begins before the completion of nuclear formation, nuclear formation was not affected by p21 (see below; Fig. 8 *B*). Thus, a step in DNA replication after nuclear formation and before the appearance of dUTP foci is blocked by the addition of p21.

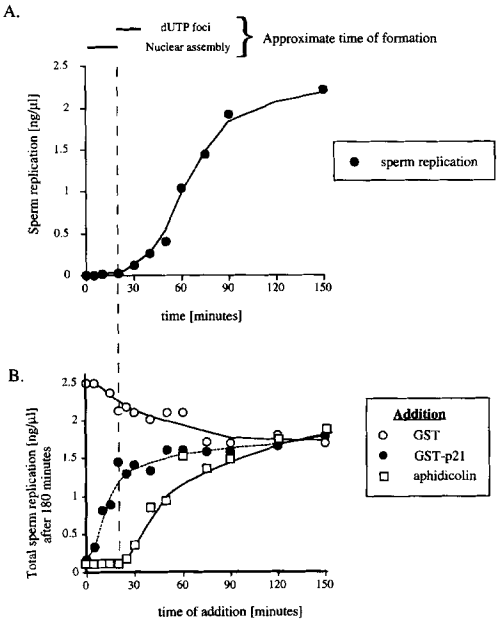

Figure 7. p21 inhibits sperm replication only before initiation. (*A*) A time course of sperm DNA replication. An interphase replication assay was set up as described with [^{32}P]-dATP and 2.5 ng/µl of sperm DNA. At the indicated times, aliquots were removed and assayed for cumulative DNA replication. In a parallel set of reactions, aliquots were removed at various times and assayed microscopically for nuclear assembly by Hoechst staining or dUTP focus formation. Approximate times for nuclear assembly (5–20 min) and the formation of rhodamine-dUTP replication foci (25–35 min) are indicated. (*B*) A window of sensitivity for p21 inhibition. In a parallel time course to part A, individual 10 µl aliquots were incubated for 180 min. At various times indicated, either GST-p21 (350 ng: ●), GST (350 ng: ○), or aphidicolin (20 µg/ml: □) was added to aliquots and gently mixed. Averages ($n = 2$) of normalized data are shown. Standard deviations were approximately ±5% for all points. The dotted line approximates the end of nuclear assembly.

p21 Blocks the Appearance of Replication Foci

Because the period of sensitivity to p21 occurs before the appearance of replication foci, we wished to know if p21 blocked their formation. A dose response for p21 inhibition of replication foci (Fig. 8 *A*) showed that the sensitivity of these structures to p21 is intermediate (~1–3 μM) between the sensitivity of sperm and single-stranded DNA replication (~100–300 nM and ~10 μM, respectively). Using the visual assay, p21 blocked the appearance of replication foci at ~1 μM, while 100–300 nM p21 did not inhibit replication foci (Fig. 8 *B*). The p21 block of replication foci at 1 μM was fully rescued by cyclin E protein, but not by PCNA. Further, the p21 NH_2-terminal cyclin/Cdk2 binding domain (Chen et al., 1995) was able to block the appearance of replication foci at similar doses to the full length p21 (not shown). This suggested that the appearance of these foci is dependent on the Cdk2 kinase. The COOH-terminal PCNA binding domain blocked the appearance of replication foci at approximately 10 μM (not shown). This is consistent with a role for PCNA for the visualization of these replication structures. Thus, p21 has three modes of inhibition. First, the 100–200 nM block to the bulk of dATP incorporation, which we interpret as inhibiting the ability of cyclin/Cdk2 to promote switching to the elongation phase. Second, the 1–2 μM block to formation of replication foci, which apparently targets the ability of cyclin/Cdk2 to promote the initiation of DNA replication. Third, the 10 μM block to single-stranded DNA replication, with PCNA as its target, which in the context of chromosomal replication, may be important for the visualization of replication foci.

To show that the inhibition of replication was not simply due to different thresholds for detection by different assays, we measured the ability of the polymerase inhibitor aphidicolin to block dATP incorporation (measuring acid-insoluble counts) and the formation of replication foci (by quantitative fluorescence microscopy). We found that inhibition of replication by aphidicolin gives similar decreases in signal in the two assays (Fig. 8 *C*). Thus, the different doses of p21 required to block in the two assays suggests distinct mechanisms rather than different thresholds for detection. This also suggests that the residual <5% of replication seen with 0.3–1 μM p21 represents the nucleotide incorporated into replication foci. This result is consistent with a recent study demonstrating that the amount of nucleotide incorporation into replication foci is a small percentage of overall replication (Yan and Newport, 1995).

p21 Blocks the Formation of Initiation Complexes from Pre-replication Foci

While the function of replication foci as assayed by biotin-dUTP incorporation can be blocked by p21, we were inter-

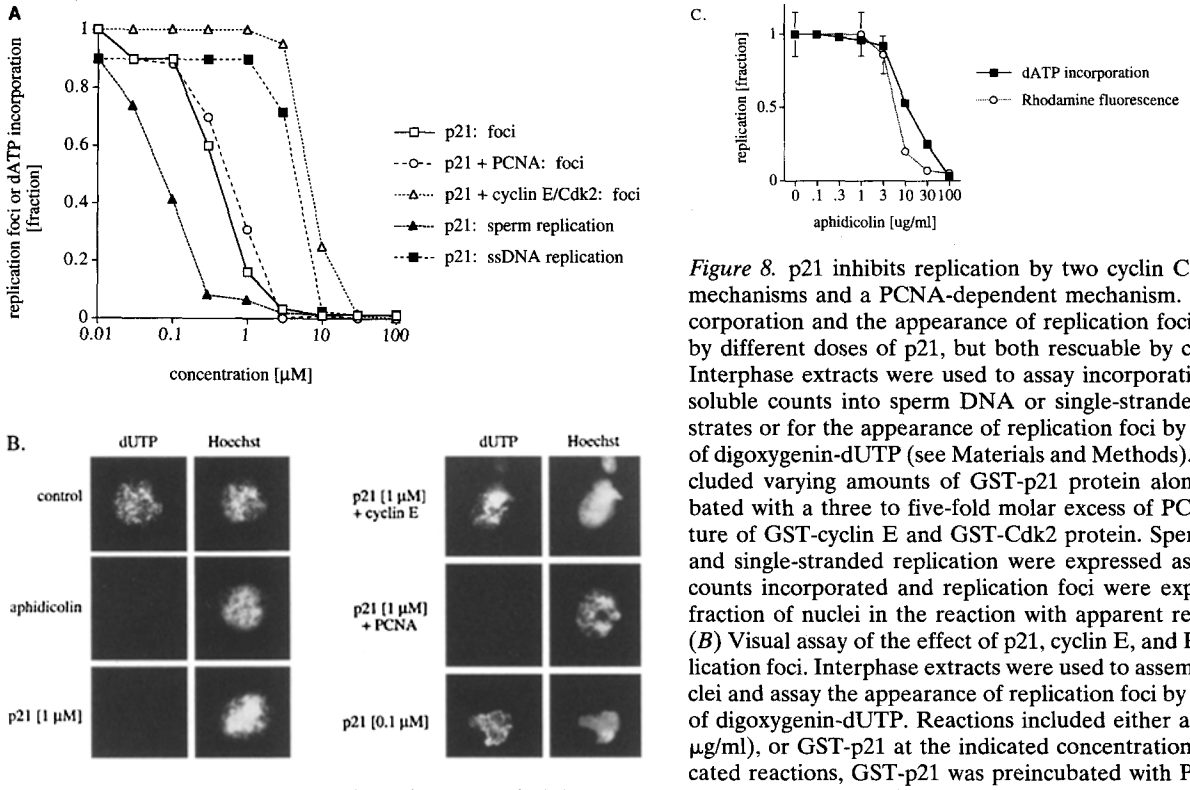

Figure 8. p21 inhibits replication by two cyclin Cdk dependent mechanisms and a PCNA-dependent mechanism. (*A*) dATP incorporation and the appearance of replication foci are inhibited by different doses of p21, but both rescuable by cyclin E/Cdk2. Interphase extracts were used to assay incorporation of acid insoluble counts into sperm DNA or single-stranded (M13) substrates or for the appearance of replication foci by incorporation of digoxygenin-dUTP (see Materials and Methods). Reactions included varying amounts of GST-p21 protein alone or preincubated with a three to five-fold molar excess of PCNA or a mixture of GST-cyclin E and GST-Cdk2 protein. Sperm replication and single-stranded replication were expressed as a fraction of counts incorporated and replication foci were expressed as the fraction of nuclei in the reaction with apparent replication foci. (*B*) Visual assay of the effect of p21, cyclin E, and PCNA on replication foci. Interphase extracts were used to assemble sperm nuclei and assay the appearance of replication foci by incorporation of digoxygenin-dUTP. Reactions included either aphidicolin (20 μg/ml), or GST-p21 at the indicated concentrations. In the indicated reactions, GST-p21 was preincubated with PCNA (5 μM) or a cocktail of GST-cyclin E/GST-Cdk2 (each 5 μM). (*C*) A comparison of the effect of aphidicolin on the visual and dATP incorporation assays. Varying amounts of aphidicolin were used to block DNA replication in Xenopus interphase extracts. The amount of replication by measuring acid insoluble counts was compared with the amount of fluorescence measured by quantitative digital microscopy (see Materials and Methods). Similar results were obtained using biotin- or digoxygenin-dUTP incorporation. Mean values for the acid insoluble counts (n = 2) and mean and representative standard deviations for arbitrary fluorescence intensity (n = 10) are shown as a fraction of replication in untreated extracts.

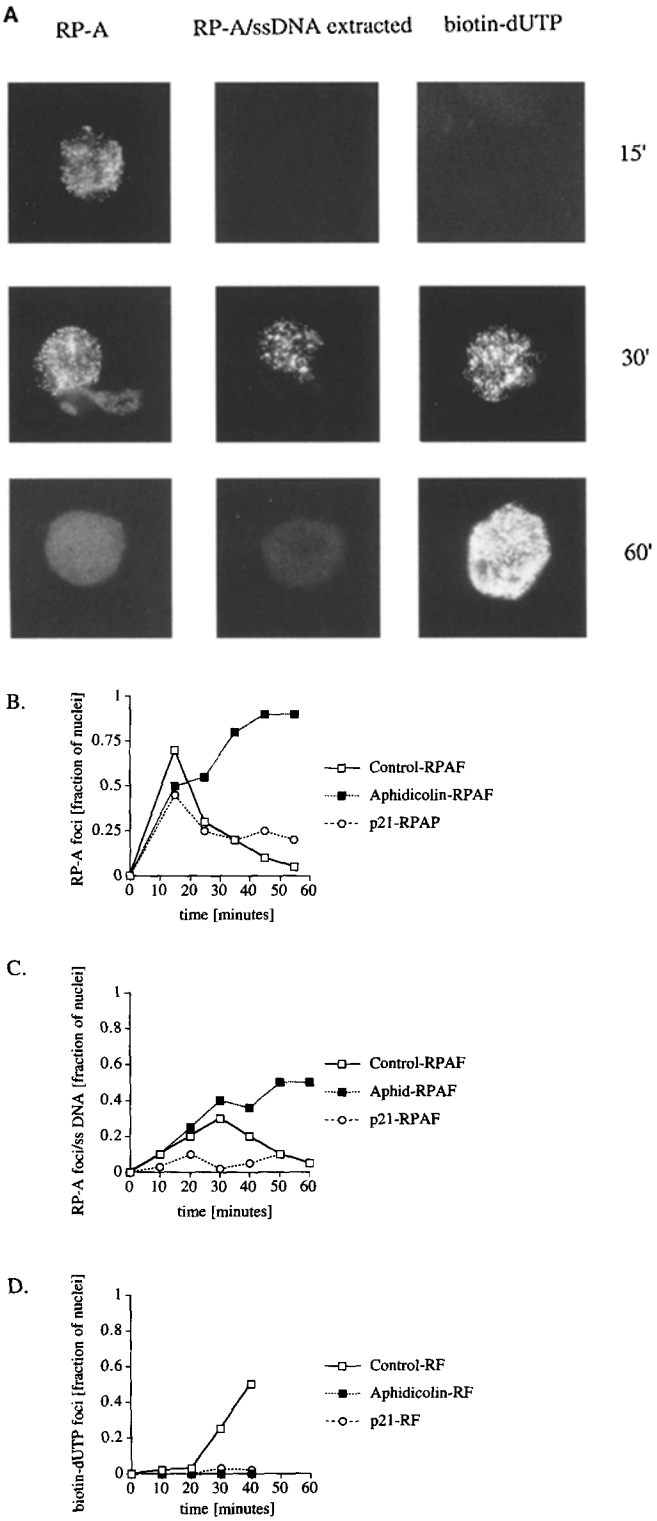

Figure 9. p21 blocks the formation of initiation complexes from pre-replication foci. (*A*) Appearance of RP-A and biotin dUTP staining in nuclei assembled in Xenopus interphase extract. Interphase extracts were used to synchronously assemble sperm nuclei, which were either extracted with single-stranded salmon sperm DNA or not, and spun onto coverslips and fixed in methanol at various times (see Materials and Methods). Replication was assayed by including biotin-dUTP in the reaction and counterstaining with FITC-streptavidin. RP-A staining was performed by using a rabbit anti-RP-A (p70) COOH-terminal peptide antiserum with Texas red donkey anti-rabbit secondary antibody. The appearance of representative RP-A staining (*left hand column*), RP-A staining after single-stranded DNA extraction (*central column*), or biotin-dUTP replication foci (*right hand column*) at the indicated times is shown. The RP-A foci resistant to single-stranded DNA extraction are thought to be associated with unwound DNA. (*B*) A time course of the appearance of RP-A staining. In the synchronous replication reaction described above, the appearance of RP-A staining was assayed at various times. Parallel time courses in the presence of GST-p21 (3 μM), aphidicolin (50 μg/ml), or without addition are shown. (*C*) A time course of single-stranded DNA extraction resistant RP-A staining. In an experiment parallel to that in *B*, samples were removed, diluted, and extracted on ice with single-stranded DNA for 30 min before spinning onto coverslips and fixing. (*D*) A time course of biotin dUTP incorporation. In the same reaction in *B*, the incorporation of biotin-dUTP was visualized.

ested in the effect of p21 on the formation of the replication initiation complexes themselves. These initiation complexes contain replication proteins including the single-stranded DNA binding protein RP-A. As seen by indirect immunofluorescence, RP-A appears as a set of punctate spots soon after sperm decondensation and at least 15 min before the appearance of dUTP replication foci (Fig. 9 *A*). Recently, Adachi and Laemmli (1994) have shown that the appearance of these RP-A "pre-replication foci" appear as organized domains on chromatin even without the presence of the nuclear membrane or when initiation is blocked by the addition of p21. However, these RP-A foci were only loosely associated with chromatin since they could be extracted with single-stranded DNA (Fig. 9 *A*). Yan and Newport (1995) further show that even after depletion of Cdk2, pre-replication foci appear. The ability of p21 addition or Cdk2 depletion to block the initiation step was inferred by Adachi and Laemmli (1994) by RP-A becoming resistant to single-stranded DNA extraction, coincident with RP-A becoming bound to unwound DNA, and by Yan and Newport (1995) on the basis of a quantitative increase in RP-A associated fluorescence and an eventual shift of RP-A staining from focal to diffuse.

We have made use of highly efficient interphase extracts to examine the timing of replication events. In these extracts, the appearance of RP-A foci occurs by 10–15 min and becomes diffuse by 60 min (Fig. 9 *A* and see time course in *B*). The appearance of replication foci visualized by the incorporation of biotin dUTP becomes apparent at ~30 min (Fig. 9 *A* and see time course in *D*). At later times, the pattern of dUTP incorporation becomes more complicated. Thus, there is a brief window of overlap around 30 min during which RP-A and nucleotide incorporation are both focal and some nuclei show colocalization by double immunofluorescence (Yan and Newport, 1995; Jackson, unpublished data). We examined a similar time course of RP-A staining where we incubated the nuclei for various times and then extracted the nuclei with single-stranded DNA to distinguish RP-A bound to unwound DNA. In this time course, the percentage of remaining RP-A foci is reduced to less than 30% and peaks slightly later than the unextracted foci (~30 min), at a time where replication foci as seen by biotin-dUTP incorporation is beginning. This would be consistent with a transi-

tion from "pre-replication" foci to focal RP-A associated with unwound DNA in initiation structures (see Adachi and Laemmli, 1994). The final transition to diffuse RP-A staining occurs later and we also saw the reappearance of RP-A focal structures following the completion of replication, as observed by Yan and Newport (1995).

We examined whether the appearance of RP-A staining in pre-replication foci and initiation complexes was blocked by GST-p21 at concentrations sufficient to block replication foci (3 μM). The early appearance of RP-A pre-replication foci was not appreciably affected by p21, confirming previous reports (Fig. 9 *B*; Adachi and Laemmli, 1994; Yan and Newport, 1995). In contrast, the later appearance of RP-A initiation structures was substantially diminished by the addition of p21 (Fig. 9 *C*). The addition of aphidicolin, an inhibitor of DNA polymerase α, did not block the appearance of either pre-replication foci or initiation complexes containing RP-A (Fig. 9, *B* and *C*), but rather blocked the disappearance of these foci. This would be consistent with a requirement for DNA polymerase activity to disassemble an RP-A containing initiation complex before later steps in replication. Both p21 and aphidicolin blocked the formation of biotin-dUTP containing replication foci (Fig. 9 *D*). Thus, p21 apparently blocks the transition from pre-replication foci to initiation complexes and aphidicolin blocks the transition from initiation to subsequent polymerase activity. This is similar to what was argued by Yan and Newport (1995), but they used a quantitative increase in diffuse RP-A staining as a marker for the transition from initiation to later replication.

Discussion

The Cyclin E/Cdk2 Complex Is Necessary for DNA Replication

Several experiments presented here show that in Xenopus interphase extracts, cyclin E is specifically required for early steps of chromosomal DNA replication. These steps may include initiation or the switch to elongation, but not later steps of replication, such as elongation itself. The specificity of the step in replication that is affected is suggested because treatments that block cyclin E inhibited replication of sperm, but not single-stranded, DNA. These blocking treatments include immunodepletion of cyclin E protein, the addition of purified anti-cyclin E antibodies, and the addition of the cyclin E/Cdk2 inhibitor $p21^{CIP1}$. These blocks could be rescued by adding Xenopus cyclin E protein, but not generally by other cyclins.

A complex of activated Cdk2 with cyclin E and possibly some associated proteins is the form important for replication. The cyclin E complex is ~440 kD and contains ~75% of the Cdk2-associated H1 kinase activity in the activated egg. The Xenopus cyclin E protein appears in interphase extracts as multiple species of 50–54 kD. Therefore, the previously identified, Cdk2-associated 54–kD protein "doublet" (Fang and Newport, 1991) is likely to be composed of forms of cyclin E and the requirement for the cyclin E/Cdk2 complex can be shown by depletion of either component. Immunoprecipitated Cdk2 kinase was a potent activator of replication in cyclin E–depleted extracts in the sperm replication assay, whereas Cdk2 protein did not stimulate replication. The inability of Cdk2 alone to rescue replication demonstrates that cyclin E–depletion blocks replication because of a cyclin E requirement rather than because the associated Cdk2 is missing after cyclin E depletion.

Recent reports of low molecular weight inhibitors of cyclin-dependent kinases suggest that the in vivo activity of these kinases may be highly dependent on inhibitors (Dulic et al., 1992; El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Polyak et al., 1994*a*,*b*; Toyoshima and Hunter, 1994). Since added p21 or p27 will affect sperm replication and the cyclin E/Cdk2 kinase activity, this type of negative regulation could be functional in egg extracts. We are presently limited by the lack of direct probes for these proteins in Xenopus. However, in interphase extracts, we have noticed consistently much higher levels of cyclin E dependent H1 kinase activity by immunoprecipitation than by direct measurements of activity in crude extracts. Thus, during immunoprecipitation, we may be gaining kinase activity by losing inhibitory activities. Recent evidence has suggested the presence of CDK inhibitors in egg extracts (Lee, T., A. Philpott, and M. Kirschner, unpublished results). Thus, the activity of cyclin E or Cdk2 kinases may be effectively buffered by these inhibitors.

p21 Can Work through Multiple Mechanisms

The inhibition of cyclin/Cdk2 kinases is not the unique mode by which p21 inhibits DNA replication. Recently, p21 was shown to inhibit the reconstituted SV-40 replication reaction by association with PCNA (Flores-Rozas et al., 1994; Waga et al., 1994*a*,*b*). We have shown here that p21 will inhibit sperm replication at a dose similar to the concentration of cyclin E/Cdk2 and can be rescued by cyclin E protein, but not by PCNA. The failure of PCNA to rescue p21 inhibition of sperm replication and the inability of PCNA to block p21 inhibition of cyclin/Cdk-associated H1 kinase activity (Zhang et al., 1994) suggested that PCNA and cyclin E/Cdk2 bind to independent sites on p21. Recent experiments demonstrate that separate domains of p21 bind to cyclin/Cdk2 versus PCNA and that the cyclin/Cdk2 binding domain inhibits the cyclin E kinase (Chen et al., 1995; Jackson, P. K., unpublished results). The cyclin/Cdk2 binding domain mimicked the properties of full length p21 in Xenopus extract: it inhibited sperm replication activity at similar concentrations and was rescuable by cyclin E, but not PCNA. These experiments strongly support the model that p21 inhibits sperm replication by a cyclin/Cdk2-dependent mechanism. However, it has recently been demonstrated that cyclin A or E will rescue p21 inhibition of sperm replication (Strausfeld et al., 1994). Although the p21 inhibition of replication does not clearly distinguish between a requirement for cyclin A or cyclin E, both p21 and depletion of cyclin E block at initiation. Thus, we cannot rule out an additional requirement for cyclin A at initiation, but the requirement for cyclin E is established by the immunodepletion (see below).

The abundance of PCNA in interphase extracts is much greater (~8 μM; Leibovici et al., 1990), so p21 inhibition of sperm replication was unlikely to work by simple stoichiometric association with PCNA in the egg. However, at much higher doses, similar to those of endogenous PCNA, p21 can inhibit single-stranded DNA replication in

Xenopus extract and that inhibition is rescuable by PCNA. This is consistent with a mechanism where PCNA is required for single-stranded replication and where PCNA is blocked by stoichiometric amounts of p21, as recently suggested (Flores-Rozas et al., 1994). Further, the COOH-terminal PCNA binding domain of p21 will inhibit either sperm or single-stranded DNA replication at ~10 μM (Chen et al., 1995) and these inhibitions are rescued by PCNA and not cyclin E. Thus, the PCNA binding function of full-length p21 can inhibit sperm DNA replication in Xenopus extracts, but this activity was masked by the more sensitive cyclin E/Cdk2-dependent mode of inhibition. In general, the mode by which p21 inhibits replication appears to depend on the concentrations of its two targets, but the importance of these targets for the function of p21 is not yet fully understood.

Cyclin Requirements for DNA Replication

Depletion of interphase extract by Suc1 beads substantially depleted cyclin E and inhibited replication. This depletion could only be partially rescued by a GST-cyclin E fusion protein, suggesting that cyclin E is an important, but not unique target of Suc1-depletion. What might the other Suc1-depleted factors be? While we found that most cyclins did not rescue replication in Suc1-depleted extracts, cyclin A almost completely rescued replication. This result raises the question of what are the respective roles of cyclin A and E?

Several studies suggest that cyclin A is required for S phase in cultured somatic cells (Girard et al., 1991; Pagano et al., 1992). Although cyclin A could function at several steps in replication, recent studies have colocalized cyclin A with replication proteins RP-A and PCNA and with replication origins, suggesting that it may play a role at the replication complex (Cardoso et al., 1993; Sobczak-Thepot et al., 1993). However, we have yet to directly identify a cyclin A requirement in interphase extracts. Xenopus cyclin A1 is expressed and accumulates in the first cell cycle almost immediately after fertilization or activation (Minshull et al., 1990), although its function remains unclear. Accumulation of cyclin A1 can be blocked by pre-soaking eggs in cycloheximide before activation (Shibuya et al., 1992) and this treatment does not suppress the ability of interphase extracts to efficiently replicate their DNA, even though there is no measurable cyclin A1–associated H1 kinase activity (Jackson, P. K., and M. N. Kirschner, unpublished data). Moreover, immunodepletion experiments by Fang and Newport (1991) and antisense experiments by Walker and Maller (1991) indicated that cyclin A1 is not required for DNA replication in the first or second cell cycles. Our experiments have failed to show an effect of cyclin A1 immunodepletion on DNA replication (Jackson, P. K., unpublished results). Thus, there is no apparent requirement for cyclin A1 in DNA replication. Perhaps this is not surprising given that Xenopus cyclin A1 has homology to and expression consistent with mitotic cyclins (Minshull et al., 1990) in contrast to the human cyclin A, which is expressed during S phase (Pines, 1993). More recently, a second Xenopus A-type cyclin, called cyclin A2, which has a higher degree of homology to human cyclin A, has been identified (Howe, J. A., et al., 1995). The Xenopus cyclin A2 protein can stimulate replication strongly, and thus would be an appealing candidate for another cyclin required for replication (Jackson, P. K., and M. W. Kirschner, unpublished results). Would cyclin E and cyclin A function similarly or at different steps in DNA replication?

What Are the Cyclin-dependent Steps for DNA Replication?

The block to appearance of replication foci with cyclin E antibodies or addition of 1 μM p21 suggests that the initiation of replication is affected. Further, sperm replication reaction is sensitive to p21 for a window in the first 15–30 min, during which initiation complexes form. Thus, cyclin E might control any of a series of steps in initiation such as origin recognition, DNA unwinding, stabilization of unwound DNA by the single-stranded DNA binding protein RP-A, or priming activity by polymerase α.

The earliest known step in chromosomal replication is the association of RP-A with DNA. Adachi and Laemmli (1994) recently showed that p21 did not block the formation of RP-A "pre-replication" foci. These structures are loosely associated with chromatin and can be extracted with single-stranded DNA. However, p21 did block the ability of RP-A to become tightly associated with regions of unwound DNA. We have obtained a similar block of the ability of RP-A to tightly associate with DNA with either the full-length p21 or the domain of p21 that associates with cyclin E/Cdk2 (Jackson, P. K., and M. W. Kirschner, unpublished data). It appears that cyclin E or a close relative is important for replication upstream of the step where RP-A binds unwound DNA.

At lower concentrations of p21 (0.2 μM), replication foci appear even though overall DNA replication as measured by dATP incorporation is reduced to less than 5%. This result could suggest that replication foci are irrelevant for DNA replication, or, more likely, that p21 has a second, more sensitive mode of inhibition. Thus, in this concentration range, p21 blocks the transition from the formation of replication foci to the elongation phase of DNA replication. We do not know what this p21 sensitive transition might be. One clue may be the experiment showing that the PCNA binding domain of p21 will inhibit replication foci at 10 μM concentration (Chen et al., 1995). This block is rescuable by PCNA. Thus, the visualization of replication foci apparently requires PCNA. An interesting possibility is that p21 can block the switching from polymerase α-dependent primer formation to processive replication using polymerase δ and PCNA, possibly via its dual association with PCNA and a cyclin/Cdk2 complex. Recent results by Hurwitz and colleagues suggested that loading of PCNA onto DNA may be blocked by p21 (Flores-Rozas et al., 1994).

Cyclins may thus take two roles in replication, as suggested by the two classes of events blocked by p21. Cyclin E appears to have a role in initiation, perhaps controlling origin firing or unwinding events. Its role in this step is supported by an overall requirement for cyclin E in replication, the ability of cyclin E to promote the function of replication foci as assayed by biotin dUTP incorporation, and the earlier timing of cyclin E expression seen in mam-

malian cells. The association of Cdk2 with RP-A foci in the nucleus suggests that cyclin/Cdk2 complexes may be directly assembled with the replication machinery and possibly have a structural role in addition to its kinase activity (Cardoso et al., 1993; Sobczak-Thepot et al., 1993). We have found that cyclin E is predominantly nuclear in tissue culture cells and is present in nuclei assembled in extracts (Jackson, P. K., and M. W. Kirschner, unpublished data). We have observed cyclin E protein in punctate, subnuclear domains. Although this localization is provocative, additional studies will be required to address its localization relative to cyclin A, Cdk2, and replication proteins like PCNA or RP-A.

A second role for cyclins may promote switching from priming to processive replication, and this could be a role for cyclin A. The colocalization of cyclin A and Cdk2 with PCNA (Cardoso et al., 1993; Sobczak-Thepot et al., 1993) and the later timing of cyclin A expression relative to cyclin E would be consistent with a cyclin A role at this step. Further, in rescuing replication in Suc1-depleted extracts, cyclin A appears more effective than cyclin E at promoting dATP incorporation. Since the bulk of dATP incorporation is limited by the switch to elongation, cyclin A appears the more likely candidate to control this step. Quantitation of the relative ability of cyclin A and E to promote initiation versus switching and a closer examination of when they become localized to specific replication structures may help establish their respective roles.

We thank Doug Kellogg and Andrew Murray for Xenopus cyclin A1, B1, and B2 GST fusion proteins, David Morgan for baculovirus CDC2, CDK2, and cyclins, James Roberts and Andy Koff for human cyclin E and communicating unpublished results, Joan Massague for p27, Mike Howell and Tim Hunt for Xenopus cyclin A2, sera, and advice, David Beach for p21 clones, Zvi Kelman and Mike O'Donnell for PCNA protein, Douglas Forbes for the synthetic nuclear transport substrate, and John Newport for affinity-purified Cdk2 sera and advice. We thank members of the Kirschner lab, Brian Burke, Ellen Shibuya, and Anindya Dutta for advice, and Randy King and Tim Bestor for critical reading of the manuscript.

We acknowledge the support of the Ligue Departementale d'Ile et Vilaine de la Recherche sur le Cancer (to M. Philippe) and a grant from the National Institute of Health (to M. W. Kirschner: GM39023-05). P. K. Jackson is a Merck Fellow of the Life Sciences Research Foundation.

Received for publication 3 January 1995 and in revised form 2 April 1995.

References

Adachi, Y., and U. K. Laemmli. 1992. Identification of nuclear pre-replication centers: immunolocalization study of replication protein A. *J. Cell Biol.* 119: 1–15.

Adachi, Y., and U. K. Laemmli. 1994. Study of the cell cycle-dependent assembly of the DNA pre-replication centres in Xenopus egg extracts. *EMBO J.* 13:4153–4164.

Amon, A., S. Irniger, and K. Nasmyth. 1994. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of g1 cyclins in the next cycle. *Cell.* 77:1037–1050.

Blow, J., and P. Nurse. 1990. A cdc2-like protein is involved in the initiation of DNA replication in Xenopus egg extracts. *Cell.* 62:855–862.

Blow, J., and J. V. Watson. 1987. Nuclei act as independent and integrated units of replication in a Xenopus cell-free DNA replication system. *EMBO J.* 6: 1997–2002.

Blow, J. J., and R. A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell.* 47:577–587.

Blow, J. J., and R. A. Laskey. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature (Lond.).* 332:546–548.

Brizuela, L., G. Draetta, and D. Beach. 1987. $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. *EMBO J.* 6:3507–3514.

Cardoso, M. C., H. Leonhardt, and B. Nadal-Ginard. 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and cdk2 specifically localize at subnuclear sites of DNA replication. *Cell.* 74:979–992.

Chen, J., P. K. Jackson, M. W. Kirschner, and A. Dutta. 1995. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature (Lond.).* 374:386–388.

Cox, L. 1992. DNA replication in cell-free extracts from Xenopus eggs is prevented by disrupting nuclear envelope function. *J. Cell Sci.* 101:43–53.

Draetta, G. F. 1994. Mammalian G1 Cyclins. *Curr. Opin. Cell Biol.* 6:842–846.

Dulic, V., E. Lees, and S. Reed. 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science (Wash. DC).* 257:1958–1961.

El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75:817–825.

Fang, F., and J. Newport. 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell.* 66:731–742.

Fang, F., and J. W. Newport. 1993. Distinct roles of cdk2 and cdc2 in RP-A phosphorylation during the cell cycle. *J. Cell Sci.* 106:983–994.

Flores-Rozas, H., Z. Kelman, F. B. Dean, Z. Pan, J. W. Harper, S. J. Elledge, M. O'Donnell, and J. Hurwitz. 1994. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase d holoenzyme. *Proc. Natl. Acad. Sci. USA.* 91: 8655–8659.

Girard, F., U. Strausfeld, A. Fernandez, and N. J. Lamb. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell.* 67: 1169–1179.

Gu, Y., C. W. Turck, and D. O. Morgan. 1993. Inhibition of CDK2 activity in vivo by an associated 20k regulatory subunit. *Nature (Lond.).* 366:707–710.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 cdk-interacting protein CIP1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell.* 75:806–816.

Heichman, K. A., and J. M. Roberts. 1994. Rules to replicate by. *Cell.* 79:557–562.

Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell.* 70:993–1006.

Howe, J. A., M. Howell, T. Hunt, and J. W. Newport. 1995. Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation. *Genes & Dev.* 9:1164–1176.

Hunter, T., and J. Pines. 1994. Cyclins and cancer II: cyclin D and Cdk inhibitors come of age. *Cell.* 79:573–582.

Hutchison, C. J., D. Brill, R. Cox, J. Gilbert, I. Kill, and C. C. Ford. 1989. DNA replication and cell cycle control in Xenopus egg extracts. *J. Cell Sci. (Suppl.)* 12:197–212.

Hutchison, C. J., R. Cox, R. S. Drepaul, M. Gomperts, and C. C. Ford. 1987. Periodic DNA synthesis in cell-free extracts of Xenopus eggs. *EMBO J.* 6: 2003–2010.

King, R. W., P. K. Jackson, and M. W. Kirschner. 1994. Mitosis in Transition. *Cell.* 79:563–571.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C. F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. *Cell.* 77:107–120.

Kobayashi, H., R. Golsteyn, R. Poon, E. Stewart, J. Gannon, J. Minshull, R. Smith, and T. Hunt. 1991*a*. Cyclins and their partners during Xenopus oocyte maturation. *Cold Spring Harb. Symp. Quant. Biol.* 56:437–447.

Kobayashi, H., J. Minshull, C. Ford, R. Golsteyn, R. Poon, and T. Hunt. 1991*b*. On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in Xenopus laevis. *J. Cell Biol.* 114:755–765.

Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. Roberts. 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell.* 66:1217–1228.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J. Harper, S. Elledge, T. Nishimoto, D. Morgan, B. Franza, and J. Roberts. 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science (Wash. DC).* 257:1689–1694.

Koff, A., M. Ohtsuki, K. Polyak, J. M. Roberts, and J. Massague. 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. *Science (Wash. DC).* 260:536–539.

Lees, E., B. Faha, V. Dulic, S. Reed, and E. Harlow. 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes & Dev.* 6:1874–1885.

Leibovici, M., M. Gusse, R. Bravo, and M. Mechali. 1990. Characterization and developmental expression of Xenopus proliferating cell nuclear antigen (PCNA). *Dev. Biol.* 141:183–192.

Leno, G., C. Downes, and R. Laskey. 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in Xenopus egg extract. *Cell.* 69:151–158.

Lew, D. J., V. Dulic, and S. I. Reed. 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell.* 66:1197–1206.

Lohka, M., and Y. Masui. 1984. Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations from amphibian eggs. *J. Cell Biol.* 98:1222–1230.

Lohka, M. J., and J. L. Maller. 1985. Induction of nuclear-envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J.*

Cell Biol. 101:518–523.

Mills, A., J. Blow, J. White, W. Amos, D. Wilcock, and R. Laskey. 1989. Replication occurs at discrete foci spaced throughout nuclei replicating in vitro. *J. Cell Sci.* 94:471–477.

Minden, J. S., D. A. Agard, J. W. Sedat, and B. M. Alberts. 1989. Direct cell lineage analysis in Drosophila melanogaster by time-lapse, three-dimensional optical microscopy of living embryos. *J. Cell Biol.* 109:505–516.

Minshull, J., R. Golsteyn, C. S. Hill, and T. Hunt. 1990. The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. *EMBO J.* 9:2865–2875.

Murray, A. W., and M. W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature (Lond.).* 339:275–280.

Nakayusa, H., and R. Berezney. 1989. Mapping replicational sites in the eucaryotic nucleus. *J. Cell Biol.* 108:1–11.

Newmeyer, D. D., and D. J. Forbes. 1990. A N-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding to the nuclear pore. *J. Cell Biol.* 110:547–557.

Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processsses. *Cell.* 48:219–230.

Newport, J. W., and M. W. Kirschner. 1984. Regulation of the cell cycle during early Xenopus development. *Cell.* 37:731–742.

Ohtsubo, M., and J. M. Roberts. 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science (Wash. DC).* 259:1908–1912.

Ohtsubo, M., A. M. Theodoras, J. Schumacher, and J. M. Roberts. 1995. Human cycline, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* 15:2612–2624.

Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek, and G. Draetta. 1993. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J. Cell Biol.* 121:101–111.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11:961–971.

Pines, J. 1993. Cyclins and cyclin-dependent kinases: take your partners. *Trends Biosci.* 18:195–197.

Pines, J., and T. Hunter. 1989. Isolation of human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. *Cell.* 58:833–846.

Polyak, K., J. Kato, M. Solomon, C. J. Sherr, J. Massague, J. M. Roberts, and A. Koff. 1994*a*. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & Dev.* 8:9–22.

Polyak, K., M. Lee, H. Erdjument-Bromage, A. Koff, J. M. Roberts, P. Tempst, and J. Massague. 1994*b*. Cloning of p27KIP1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell.* 78:59–66.

Raska, I., K. Koberna, M. Jarnik, V. Petrasovicova, J. Bednar, K. Raska, and R. Bravo. 1989. Ultrastructural immunolocalization of cyclin/PCNA in synchronized 3T3 cells. *Exp Cell Res.* 184:81–89.

Resnitzky, D., M. Gossen, H. Bujard, and S. Reed. 1994. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell Biol.* 14:1669–1679.

Sawin, K. E., and T. J. Mitchison. 1991. Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* 112:925–940.

Sheehan, M., A. D. Mills, A. M. Sleeman, R. Laskey, and J. Blow. 1988. Steps in the assembly of replication-competent nuclei in a cell-free system from Xenopus eggs. *J. Cell Biol.* 106:1–12.

Sherr, C. J. 1994. G1 phase progression: Cyclin on cue. *Cell.* 79:551–555.

Shibuya, E. K., A. J. Polverino, E. Chang, M. Wigler, and J. V. Ruderman. 1992. Oncogenic Ras triggers the activation of a 42 kDa mitogen activated protein kinase in extracts of Xenopus oocytes. *Proc. Natl. Acad. Sci. USA.* 89:495–501.

Simanis, V., and D. P. Lane. 1985. An immunoaffinity purification procedure for SV40 large T antigen. *Virology.* 144:88–100.

Sobczak-Thepot, J., F. Harper, Y. Florentin, F. Zindy, C. Brechot, and E. Puvion. 1993. Localization of cyclin A at the sites of cellular DNA replication. *Exp. Cell Res.* 206:43–48.

Solomon, M. J., M. Glotzer, T. Lee, M. Philippe, and M. W. Kirschner. 1990. Cyclin Activation of $p34^{cdc2}$. *Cell.* 63:1013–1024.

Strausfeld, U. P., M. Howell, R. Rempel, J. L. Maller, T. Hunt, and J. J. Blow. 1994. Cyclin-dependent kinase inhibitor blocks the initiation of DNA replication in Xenopus extracts. *Curr. Biol.* 4:1–9.

Toyoshima, H., and T. Hunter. 1994. p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell.* 78:67–74.

van den Heuvel, S., and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science (Wash. DC).* 262:2050–2054.

Waga, S., G. Bauer, and B. Stillman. 1994*a*. Reconstitution of complete SV40 DNA replication with purified replication factors. *J. Biol. Chem.* 8:10923–10934.

Waga, S., G. J. Hannon, D. Beach, and B. Stillman. 1994*b*. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature (Lond.).* 369:574–578.

Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature (Lond.).* 366:701–704.

Yan, H., and J. Newport. 1995. An analysis of the regulation of DNA synthesis by cdk2, Cip1, and Licensing Factor. *J. Cell Biol.* 129:1–15.

Zhang, H., G. J. Hannon, and D. Beach. 1994. p21-containing cyclin kinases exist in both active and inactive states. *Genes & Dev.* 8:1750–1758.

Zuber, M., W. Yasui, E. M. Tan, and M. Ryoji. 1989. Quantitation and subcellular localization of proliferating cell nuclear antigen (PCNA/cyclin) in oocytes and eggs of Xenopus laevis. *Exp. Cell Res.* 182:384–393.