

# Nuclear Proteins of Quiescent *Xenopus laevis* Cells Inhibit DNA Replication in Intact and Permeabilized Nuclei

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**Abstract.** Quiescent cells from adult vertebrate liver and contact-inhibited or serum-deprived tissue cultures are active metabolically but do not carry out nuclear DNA replication and cell division. Replication of intact nuclei isolated from either quiescent *Xenopus* liver or cultured *Xenopus* A6 cells in quiescence was barely detectable in interphase extracts of *Xenopus laevis* eggs, although *Xenopus* sperm chromatin was replicated with ~100% efficiency in the same extracts. Permeabilization of nuclei from quiescent *Xenopus* liver or cultured *Xenopus* epithelial A6 cells did not facilitate efficient replication in egg extracts. Moreover, replication of *Xenopus* sperm chromatin in egg extracts was strongly inhibited by a soluble extract of isolated *Xenopus* liver nuclei; in contrast, complementary-strand synthesis on single-stranded DNA templates in egg extracts was not affected. Inhibition was specific to endogenous molecules localized preferentially in quiescent as opposed to

proliferating cell nuclei, and was not due to suppression of cdk2 kinase activity. Extracts of *Xenopus* liver nuclei also inhibited growth of sperm nuclei formed in egg extracts. However, the rate and extent of decondensation of sperm chromatin in egg extracts were not affected. The formation of prereplication centers detected by anti-RP-A antibody was not affected by extracts of liver nuclei, but formation of active replication foci was blocked by the same extracts. Inhibition of DNA replication was alleviated when liver nuclear extracts were added to metaphase egg extracts before or immediately after  $Ca^{++}$  ion-induced transition to interphase. A plausible interpretation of our data is that endogenous inhibitors of DNA replication play an important role in establishing and maintaining a quiescent state in *Xenopus* cells, both in vivo and in cultured cells, perhaps by negatively regulating positive modulators of the replication machinery.

VIRTUALLY all liver cells in adult vertebrates, and contact-inhibited and/or serum-deprived cultured cells have exited from the cell cycle and entered a quiescent state, G0. Quiescence is a unique phase of cell cycle during which no DNA replication takes place (<0.2% of cells in intact mammalian livers incorporate [<sup>3</sup>H]thymidine), yet the cells maintain other metabolic and physiological functions (Baserga, 1985; Pardee, 1989). Entry into, and maintenance of, quiescence are postulated to be regulated by the coordination of many quiescence-specific factors (Rabinovitch and Norwood, 1980; Stein and Yanishevsky, 1981; Stein and Atkins, 1986; Bedard et al., 1987; Padmanabhan et al., 1987; Nuell et al., 1991; Del Sal et al., 1992).

The possible role of negative regulatory molecules in quiescence was first raised by studies of cell fusion. Fusion of quiescent human fibroblasts with proliferating fibro-

blasts results in blockage of entry into S phase of the proliferating cells if fusion occurs at least 3 h before the G1/S boundary (Rabinovitch and Norwood, 1980; Stein and Yanishevsky, 1981). However, ongoing DNA synthesis is not affected in heterodikaryons (Stein and Yanishevsky, 1981), suggesting that steps leading to initiation of DNA replication may be targets of the negative control molecules. It has been suggested that inhibitory genomic DNA sequences are preferentially modified (rearrangement, amplification) in quiescent cells (Padmanabhan et al., 1987): HeLa cells transfected with genomic DNA from quiescent WI-38 human embryo fibroblasts become blocked in DNA synthesis (Padmanabhan et al., 1987). Comparison of proteins expressed in quiescent vs proliferating chicken heart mesenchymal cells identified a 20-kD protein expressed specifically in the quiescent cells (Bedard et al., 1987), but no evidence was presented that directly links this protein to negative regulation of cell proliferation. A 34-kD homeobox protein, Gax, has been cloned from rat vascular smooth muscle cells (Gorski et al., 1993). Gax mRNA is downregulated in a dose-dependent manner up to 15-fold by mitogen stimulation, and downregulation is

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correlated with upregulation of DNA synthesis: Gorski et al. (1993) suggest that Gax has a negative regulatory function in G0 to G1 transition. Similarly, a series of growth arrest-specific genes (*gas*)<sup>1</sup> have been identified that are expressed only in quiescent but not proliferating NIH 3T3 cells (Schneider et al., 1988). Ectopic expression of *gas* 1, which encodes a transmembrane protein, leads to inhibition of DNA synthesis in normal or transformed NIH 3T3 cells (Del Sal et al., 1992). Another seemingly similar negative regulatory protein, GADD 153 (or CHOP), is a 29-kD nuclear protein that also blocks cell growth and DNA replication (Ron and Habener, 1992), but GADD 153 is believed to function at the G1 to S transition point (Barone et al., 1994).

Cell-free egg extracts from *Xenopus laevis* can be used not only to study the mechanism of eukaryotic DNA replication, but also the molecules that regulate replication during the cell cycle (Benbow and Ford, 1975; Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison et al., 1987; Newport, 1987). Egg extracts effectively support both complementary-strand synthesis on single-stranded DNA templates and replication of *Xenopus* sperm chromatin or double-stranded DNA templates (Mechali and Harland, 1982; Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison et al., 1987; Newport, 1987). Replication of sperm chromatin or double-stranded DNA in egg extracts has been successfully used as an assay for endogenous inhibitors of DNA replication found in *Xenopus* oocytes (Zhao and Benbow, 1994); a high molecular protein, p245, that is a cell cycle-dependent inhibitor of chromosomal DNA replication, has been purified to electrophoretic homogeneity (Zhao, J., and R.M. Benbow, manuscript submitted for publication).

In this study, we show that both intact and permeabilized nuclei from quiescent *Xenopus* cells failed to replicate effectively in egg extracts. Replication of *Xenopus* sperm chromatin in *Xenopus* egg extracts was used as an assay to identify endogenous inhibitors of DNA replication found in nuclei of quiescent adult *Xenopus* liver cells. Negative regulation by endogenous inhibitor molecule(s) from quiescent cells appeared to control initiation of DNA replication, since these inhibitors did not block chain elongation on single-stranded templates. Moreover, these inhibitors appear to be specific to quiescent cells, since similar nuclear extracts prepared from asynchronous *Xenopus* cultured A6 cells do not block replication of *Xenopus* sperm chromatin. We speculate that the quiescent cell inhibitors may abrogate DNA replication in egg extracts by "annulling" positive regulatory molecules.

## Materials and Methods

### Preparation of Liver Nuclei

Liver nuclei were prepared by a modification of a previous procedure (Blobel and Potter, 1966). Livers were removed from decapitated mature male or female frogs and put on ice immediately. All subsequent procedures were carried out at 4°C. Livers were minced and washed several times

in phosphate buffered saline, pH 7.5 (PBS; 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>·7 H<sub>2</sub>O, 2.68 mM KCl, 137 mM NaCl). 2 ml of 0.25 M sucrose in nuclear isolation buffer A (NIBA: 5 mM Tris HCl, pH 7.5, 2 mM MgCl<sub>2</sub>, 3.3 mM CaCl<sub>2</sub>, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM PMSF, 1 mM EDTA, 2 mM β-mercaptoethanol, 10 μg/ml of leupeptin, pepstatin A, and aprotinin) were added per gram of liver. The minced tissues were homogenized gently in chilled Dounce homogenizer with 4–6 strokes until ~30–50% of the cells were broken. The homogenate was filtered through four layers of cheesecloth. 2 ml of 2.3 M sucrose in NIBA buffer were added for each ml of homogenate. 1 ml of sucrose-homogenate was layered onto a step gradient containing 1 ml of 2.5 M sucrose in NIBA buffer overlaid with 2.5 ml of 2.3 M sucrose in NIBA buffer, and centrifuged at 29,000 rpm (100,000 g) in an SW50.1 rotor (Beckman Instrs., Fullerton, CA) for 2 h at 4°C. Nuclei at the interphase between 2.3 M and 2.5 M sucrose buffer were removed, washed three times with nuclear isolation buffer B (NIBB: 50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM spermidine, 0.15 mM spermine, 2 mM β-mercaptoethanol, 10 μg/ml of leupeptin, pepstatin A, and aprotinin), and centrifuged at 2,500 rpm (1,000 g) in an HB-4 rotor (Sorvall Instruments, Wilmington, DE) for 10 min at 4°C. Nuclear pellets were suspended in a small volume of NIBB buffer with 5% (vol/vol) glycerol and used immediately. Permeabilized nuclei were obtained by treatment with lysolecithin (Sigma Chem. Co., St. Louis, MO) as described previously (Blow and Laskey, 1988). To verify permeabilization, nuclei were incubated with FITC-dextran (mol wt 150,000) for 5–10 min (Newmeyer and Wilson, 1991) and examined with an Olympus BHS microscope.

To isolate nuclei for nuclear extracts, a slightly harsher method with greater yields was used: 10–14 strokes of the homogenizer until ~80% of the cells were broken. Nuclear pellets prepared as above were suspended in a small volume of NIBB buffer containing 5% (vol/vol) glycerol and stored at –70°C until preparation of extracts.

### Preparation of *Xenopus* Egg Extracts

Metaphase egg extracts were prepared as described previously (Blow, 1993) with modifications. Female *Xenopus* were injected with 500–600 IU human chorionic gonadotropin (Sigma). Eggs were collected in high salt Barth's solution (15 mM Tris.HCl, pH 7.4, 110 mM NaCl, 2 mM KCl, 2 mM NaHCO<sub>3</sub>, 1 mM MgSO<sub>4</sub>, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>), and dejellied with 2% cysteine HCl, pH 7.8, 2 mM EGTA in high salt Barth's solution. Dejellied eggs were washed twice in high salt Barth's solution with 2 mM EGTA, and then twice with prechilled unactivating extraction buffer (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 2 mM β-mercaptoethanol). Eggs were packed by centrifuging for 1 min at 3,000 rpm (1,500 g) in an HB-4 rotor. Excess buffer was removed, and eggs were crushed by centrifuging at 12,000 rpm (24,000 g) for 10 min at 4°C in an HB-4 rotor. The cytoplasmic layer was removed slowly using a 3-ml syringe with an 18-gauge needle. Cytochalasin B, leupeptin, aprotinin, and pepstatin A were added to the extracts to final concentrations of 10 μg/ml. The extracts were centrifuged at 30,000 rpm (100,000 g) for 15 min at 4°C in an SW50.1 rotor. The clear golden layer was collected, made 2% glycerol (vol/vol) and frozen in 15-μl aliquots in liquid nitrogen.

For preparation of interphase egg extracts, dejellied eggs were washed three times in Barth's solution (15 mM Tris.HCl, pH 7.4, 88 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>), activated for 5 min with 0.5 μg/ml calcium ionophore at room temperature and washed four times in extraction buffer (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM β-mercaptoethanol) at 4°C. All further steps were the same as for metaphase extracts except the second high speed centrifugation was in an SW50.1 rotor at 15,000 rpm (10,000 g) for 15 min.

### In Vitro DNA Replication Assay

After thawing at room temperature, interphase egg extracts were supplemented with extract dilution buffer (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM β-mercaptoethanol, 10 μg/ml of leupeptin, pepstatin A and aprotinin, 270 mM creatine phosphate, 13.5 mM ATP, 1 mg/ml creatine phosphokinase) to 15% by volume (Blow, 1993). 250 μg/ml cycloheximide was added to ensure only one round of DNA replication during incubation (Blow, 1993). Unless otherwise specified, each reaction contains 30 μl of egg extracts, 0.15 μg *Xenopus* sperm chromatin or other DNA templates, 5 μl liver nuclear extracts or NIBB buffer as control, 2 μCi [ $\alpha$ -<sup>32</sup>P]dATP (New England Nuclear/Du Pont Company, Boston, MA). Assays were carried out at room temperature for the indicated time and terminated with stop solution (Zhao and Benbow, 1994). Samples were di-

1. Abbreviations used in this paper: CSF, cytostatic factor; *gas*, growth arrest-specific gene; MPF, maturation promoting factor; NIBA, nuclear isolation buffer A; NIBB, nuclear isolation buffer B; RLF, replication licensing factor; RP-A, replication protein A; SLO, streptolysin-O; ss, single-stranded.

gested with proteinase K (0.5 mg/ml) for 1 h at 37°C and electrophoresed on 0.8% agarose gels. After electrophoresis, the gels were dried and radioactivity was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Two bands were usually observed for replicated nuclear chromatin on an agarose gel; the upper band corresponds to DNA presumably complexed with proteins that remains at the origin of the gel; the lower band represents DNA that migrated into the gel. DNA replication was measured as incorporation of [<sup>32</sup>P]dAMP into high molecular weight species (Zhao and Benbow, 1994).

Inhibition of DNA replication was calculated as a percentage of control DNA synthesis, which is [<sup>32</sup>P]dAMP incorporation in the presence of *Xenopus* liver nuclear extracts divided by that with NIBB buffer containing 5% (vol/vol) glycerol (DNA synthesis [% control]). Synthesized DNA and percentage of input DNA replicated were quantitated by the method described previously assuming that the dATP pool in egg extracts was 50 μM (Blow and Laskey, 1986).

### Preparation of Extracts of *Xenopus* Liver Nuclei

To make extracts, nuclei were thawed, sonicated for 5 s, and chilled on ice for 10 s. The sonication-chilling cycle was repeated eight times and the sonicate was centrifuged at 16,000 g for 20 min. The protein concentration of the supernatant was adjusted to 1 mg/ml measured by the Bradford assay using bovine plasma γ-globulin (Bio-Rad Laboratories, Hercules, CA) as standard, and stored in aliquots at -70°C.

Nuclear extracts of liver cells were also prepared by the method of Gorski et al. (1986). In this method, extracts were made by nuclear lysis and ammonium sulfate precipitation of nuclear proteins. Extracts made by this method also inhibited replication of sperm chromatin, but less than those prepared by sonication. The data in this study were all obtained using sonicated extracts.

### Cell Culture and Preparation of Nuclei and Nuclear Extracts from *Xenopus* A6 Cells

*Xenopus* epithelial A6 cells (kindly provided by Dr. K. Harkin, Cell and Hybridoma Facility, Iowa State University, Ames, IA) were grown in modified L-15 medium supplemented with 10% FBS (Smith and Tata, 1991). A quiescent state was induced by growing cells in modified L-15 medium supplemented with 0.5% FBS for the indicated times after the cells had become confluent.

Intact nuclei of *Xenopus* A6 cells were prepared according to the method described by Leno and colleagues (Leno et al., 1992). Cells were suspended at a concentration of  $5 \times 10^5$  cells/ml in prechilled Pipes buffer (50 mM Pipes, pH 7.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM EGTA) with 1 μg/ml of leupeptin, pepstatin, aprotinin and 1 mM dithiothreitol, and mixed with an equal volume of Pipes buffer containing 1.5 IU/ml streptolysin-O (SLO, Sigma Chem. Co.). After incubating 30 min on ice, cells were centrifuged at 0°C and washed twice with Pipes buffer. Cell pellets were resuspended in 1 ml of Pipes buffer and incubated for 5 min at 37°C. Permeabilized nuclei were obtained by treatment with lysolecithin as described previously (Blow and Laskey, 1988), and verified as above.

To make extracts of nuclei of cycling or quiescent *Xenopus* A6 cultures, cells were removed by scraping, and washed twice with PBS buffer. Cell pellets were suspended in prechilled hypotonic buffer (10 mM Tris.HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>) and incubated on ice for 15 min. The cell suspension was homogenized with 8–10 strokes in a Dounce homogenizer, and made 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 2 mM β-mercaptoethanol, 0.5 mM PMSF, 1 mM EDTA, 10 μg/ml each of leupeptin, pepstatin A, and aprotinin. Nuclei from *Xenopus* A6 cells were then isolated as described above, and extracts were prepared by sonication and stored in aliquots at -70°C.

### Flow Cytometry

*Xenopus* A6 cells were trypsinized and washed three times in PBS. 1 ml of cold ethanol was added to each 10<sup>6</sup> cells/ml and samples were left at 4°C overnight. The fixed cells were centrifuged and stained by hypotonic lysis in 1 ml of staining solution (50 μg/ml of propidium iodide, 0.1 mg/ml RNase A in 0.1% NP-40 and 0.1% trisodium citrate) for 20 min at 4°C. Fluorescence was measured using an EPICS XL flow cytometer (Coulter, Middleton, WI) and DNA histograms were produced from listmode data using Elite software and analyzed using Multi-Cycle software (Phoenix Flow System, San Diego, CA).

### [<sup>3</sup>H]Thymidine Uptake in *Xenopus* A6 Cells

*Xenopus* A6 cells in one 100-mm petri dish were incubated with 20 μCi [methyl, 1, 2, [<sup>3</sup>H]thymidine (3,000 Ci/mmol; New England Nuclear/Du Pont Company) for 1 h. After rinsing with PBS, cells were centrifuged and frozen. Cell pellets were vortexed in 0.5 ml of 0.3 M NaOH. After incubating on ice for 15 min, cells were added with an equal volume of 20% TCA and incubated on ice for another 15 min. The extracts were filtered through Whatman GF/A glass filters. Dried filters were quantitated in Scintiverse (Fisher Scientific, Pittsburgh, PA) using an LKB 1218 Rackbeta liquid scintillation counter.

### Proteolytic Treatment of Nuclei

Permeabilized nuclei (~10<sup>8</sup>) were incubated with 2.5 ml of 10 μg/ml trypsin in PBS for 5 min at room temperature. Reactions were stopped by adding an equal volume of PBS with 20% newborn bovine serum. The resulting nuclei were centrifuged at 1,000 g at 0°C for 10 min and washed twice with PBS containing 10 μg/ml leupeptin, pepstatin and aprotinin, and 1 mM PMSF. Nuclear pellets were resuspended in NIBB buffer to  $2 \times 10^7$ /ml.

### Preparation of *Xenopus* Sperm Nuclei and <sup>3</sup>H-labeled Bacteriophage λ DNA

*Xenopus* sperm chromatin was prepared by a previous method (Blow and Laskey, 1986). Single-stranded (ss) M13 DNA was purchased from GIBCO BRL (Grand Island, NY). Bacteriophage λ DNA was prepared from strain λ CI857R am5h (a gift from Dr. P.A. Pattee, Department of Microbiology, Iowa State University) according to the method described by Sambrook et al. (1989) with the addition of [<sup>3</sup>H]thymidine (New England Nuclear/Du Pont Company) to 5 mCi/ml during thermal induction.

### Trichloroacetic Acid Precipitation of <sup>3</sup>H-labeled λ Phage DNA

After incubation of the <sup>3</sup>H-labeled λ phage DNA in egg extracts with *Xenopus* liver nuclear extracts or NIBB buffer, the samples were treated with 0.5 mg/ml proteinase K (Hutchison et al., 1987; Zhao and Benbow, 1994). Acid-insoluble <sup>3</sup>H-labeled DNA was precipitated with 5% TCA collected on Whatman 934-AH filters, dried, and radioactivity on the filters quantitated in Scintiverse using an LKB 1218 Rackbeta liquid scintillation counter.

### Glycerol Gradient Sedimentation

Duplicate 4.8-ml 15–30% (vol/vol) linear glycerol gradients were made with buffer containing 50 mM Tris.HCl, pH 7.5, 200 mM KCl, 2 mM β-mercaptoethanol, 1 mM EDTA, 10 μg/ml of leupeptin, pepstatin A, and aprotinin. After being equilibrated at 4°C for 10 h, gradients were overlaid with 200 μl of *Xenopus* liver nuclear extract or standard proteins, respectively. Bovine liver catalase (11.3 S), rabbit muscle aldolase (7.4 S), bovine serum albumin (BSA, 4.22 S), and cytochrome c from horse heart (1.9 S) were used as standard proteins. Gradients were centrifuged in an SW50.1 rotor at 40,000 rpm (200,000 g) for 24 h at 3°C. Gradients were fractionated from bottom to top and 35 fractions were collected.

### Immunoprecipitation and Cdk2 Kinase Assay

*Xenopus* egg extracts were mixed with either liver nuclear extracts or NIBB buffer as described above, incubated at room temperature for 30 min and then precleared with nonspecific rabbit polyclonal IgG for 1 h at 4°C. 1 μg of anti-cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 15 μl of suspended agarose-protein A solution (Santa Cruz Biotechnology) were added to the precleared samples. Immunoprecipitation was carried out at 4°C overnight with constant shaking. Agarose beads were washed four times with RIPA buffer (50 mM Tris.HCl, pH 7.5, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM sodium pyrophosphate) and four times with reaction buffer (20 mM Tris.HCl, pH 7.5, 4 mM MgCl<sub>2</sub>) (Dulic et al., 1992). The beads were then mixed with 30 μl of reaction buffer containing 5 μg histone H1 (GIBCO BRL) and 5 μCi [γ-<sup>32</sup>P]ATP (New England Nuclear/DuPont Company). After incubation at 30°C for 30 min, the reaction was stopped by adding twofold concentrated Laemmli gel sample buffer (Laemmli, 1970). Proteins were separated by 12% SDS-PAGE electrophoresis. Histone H1 was visualized by Coomassie

blue staining and the gel was fixed (10% acetic acid, 20% methanol), dried, and analyzed with a Molecular Dynamics PhosphorImager.

### Phase-Contrast Microscopy of Nuclei

Nuclei formed in egg extracts or high speed supernatant prepared as previously described (Zhao and Benbow, 1994) were examined and photographed under an Olympus BHS microscope.

### Chromatin Decondensation

Interphase egg extracts were centrifuged at 30,000 rpm (100,000 g) for 1 h at 4°C in an SW50.1 rotor and the transparent layer (cytosol) was collected, mixed with 7% glycerol (vol/vol), and frozen in 15- $\mu$ l aliquots in liquid nitrogen. *Xenopus* sperm chromatin was incubated with the cytosol supplemented with an energy-regenerating system (40 mM creatine phosphate, 2 mM ATP, 150  $\mu$ g/ml creatine phosphokinase) at room temperature for the indicated times.

### Indirect Immunofluorescence of RP-A and Fluorescence Detection of Replicating DNA In Vitro

For immunofluorescent staining of replication protein A (RP-A), 40,000 demembrated *Xenopus* sperm nuclei were incubated with 30  $\mu$ l cytosol of interphase *Xenopus* egg extracts for the indicated time. Decondensed sperm chromatin was fixed with 1 mM EGS (ethylene glycobis-[succinimidylsuccinate]) (Pierce, Rockford, IL) for 30 min at 37°C. After fixation, sperm nuclei were centrifuged onto coverslips and treated with 0.25% Triton X-100 for 5 min at room temperature. The coverslips were washed, stained with 1:200 diluted rabbit anti-RP-A-antibody (a generous gift from Dr. J. Newport), washed, and stained with fluorescein-conjugated goat anti-rabbit antibody. Staining was for 1 h, followed by 3  $\times$  5 min washes with PBS.

To study DNA synthesis in situ in nuclei in egg extracts, 0.15  $\mu$ g sperm chromatin was incubated with 30  $\mu$ l interphase egg extracts which had been supplemented with extract dilution buffer. 30  $\mu$ M biotin-11-dUTP (Sigma) was added to egg extracts after 45 min incubation and pulse-labeled for 3 min. The nuclei were fixed with EGS, centrifuged onto coverslips, and treated with Triton X-100 as described above. Coverslips were stained with 200  $\mu$ l staining solution containing 10  $\mu$ l of fluorescein-streptavidin (Amersham Corp. Arlington Heights, IL), 1  $\mu$ g/ml HOECHST 33258, and 15  $\mu$ g/ml RNase. After incubation for 30 min at room temperature, the samples were washed four times with PBS buffer.

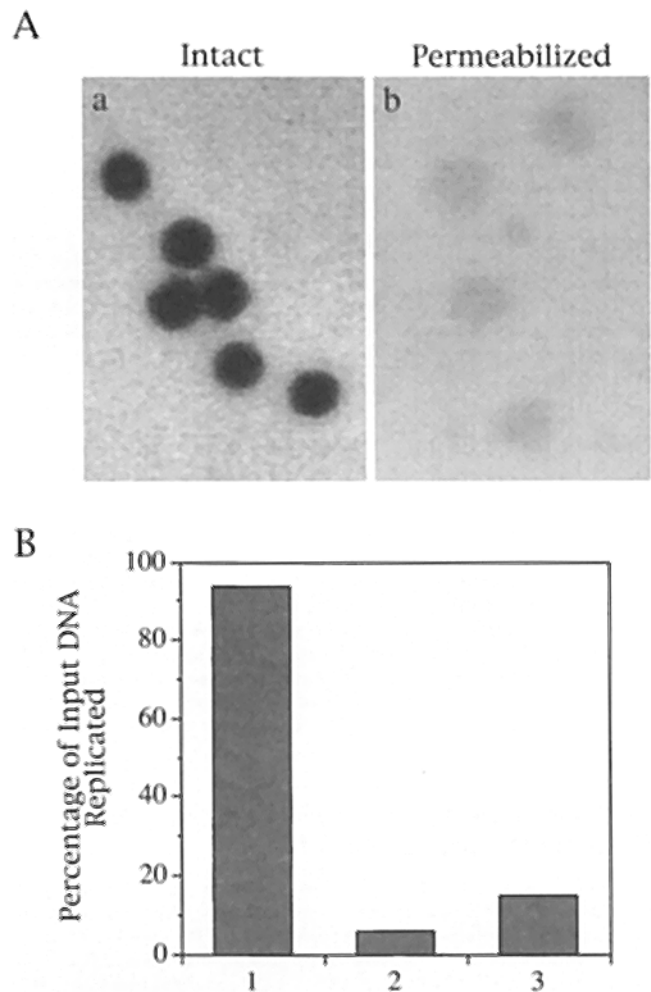
The coverslips prepared above were loaded onto glass slides with loading buffer (95% glycerol, 2.5% diazabicyclo [2.2.2] octane in PBS), and examined under either an Olympus BHS microscope or an Odyssey Real Time Laser Scanning confocal system.

## Results

### Nuclei from Quiescent *Xenopus* Cells Did Not Replicate in *Xenopus* Egg Extracts

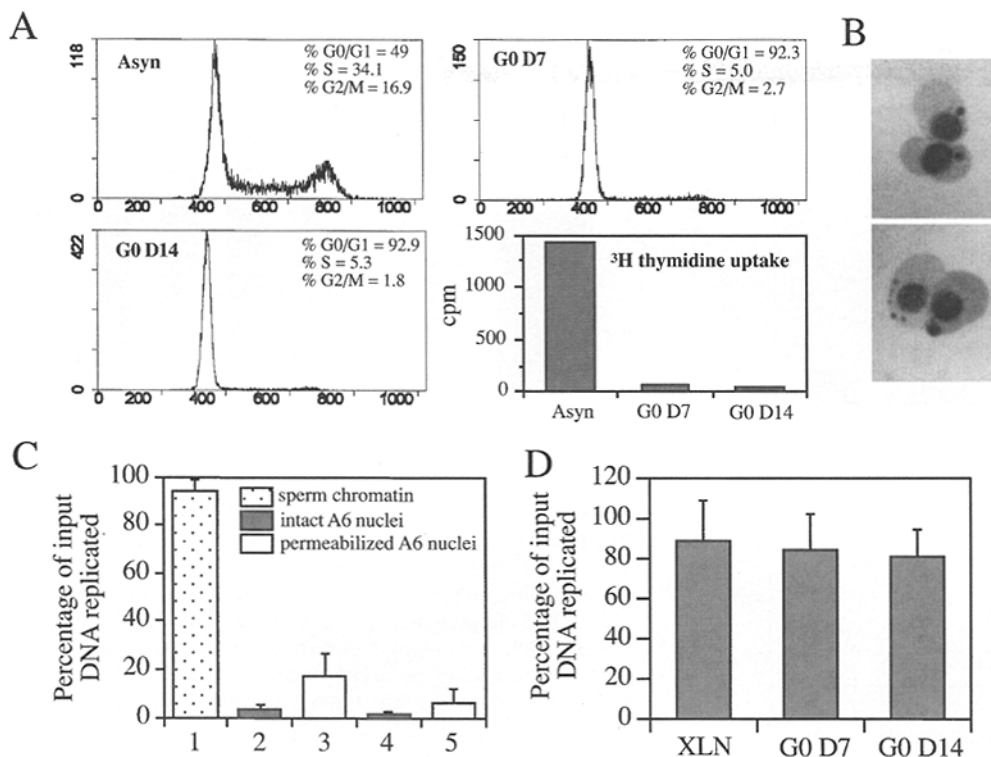
When *Xenopus* sperm chromatin is added to *Xenopus* egg extracts, DNA replication ensues rapidly and efficiently after formation of nuclear envelopes around decondensed sperm chromatin (Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison et al., 1987; Newport, 1987). Intact G1-phase nuclei of HeLa cells are also capable of replicating in egg extracts (Leno et al., 1992). However, intact G2-phase nuclei of HeLa cells (Leno et al., 1992) and intact G0-phase (quiescent) nuclei of mouse BALB/c 3T3 cells (Leno and Munshi, 1994) have been reported not to undergo DNA replication unless the nuclei are permeabilized. It should be noted that this system is heterologous, involving mammalian cell nuclei in *Xenopus* extracts.

To study G0 phase arrest and G0 to G1 phase transition, we first investigated the potential of quiescent *Xenopus* liver nuclei to replicate in *Xenopus* egg extracts. The integ-



**Figure 1.** Intact or permeabilized *Xenopus* liver nuclei fail to replicate in *Xenopus* egg extracts. (A) Preparation of intact and permeabilized nuclei from *Xenopus* liver cells. To make permeabilized nuclei, isolated liver nuclei were treated with an equal volume of lysolecithin (10 mg/ml) for 5 min. Aliquots of intact and permeabilized nuclei were combined with FITC-dextran and examined by fluorescence microscopy. FITC-dextran was excluded from >80% of the untreated liver nuclei (a), whereas >99% of lysolecithin-treated nuclei were stained by FITC-dextran (b). (B) 0.15  $\mu$ g *Xenopus* sperm chromatin (1), intact liver nuclei (2), or permeabilized liver nuclei (3) were incubated in 30  $\mu$ l egg extract with 2  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP for 4 h. After incubation, the samples were treated with proteinase K, electrophoresed, and autoradiographed. DNA synthesis was monitored by the incorporation of [ $\alpha$ -<sup>32</sup>P]dAMP. Percentage of input DNA replicated in egg extracts was calculated as described in Materials and Methods.

riety of each preparation of isolated liver nuclei was examined by exclusion of FITC-dextran, and only those >80% intact were used (Fig. 1 A). As shown in Fig. 1 B (compare column 1, control *Xenopus* sperm chromatin, vs 2, an equivalent amount of intact *Xenopus* liver nuclei), only ~6% of DNA in intact liver nuclei was replicated in egg extracts compared with ~100% replication of sperm nuclei. We also examined replication of liver nuclei that was permeabilized by treatment with lysolecithin (Fig. 1 A). The efficiency of replication in permeabilized liver nuclei was marginally increased, and ~15% of the DNA ultimately



**Figure 2.** Intact or permeabilized quiescent *Xenopus* A6 cells fail to replicate in *Xenopus* egg extracts. (A) Asynchronous (Asyn) and quiescent *Xenopus* A6 cells (G0 D7 and G0 D14) were fixed, stained with propidium iodide, and analyzed by flow cytometry. Quiescent cells were examined after culturing in L-15 medium with 0.5% FBS for 7 d (G0 D7) and 14 d (G0 D14). The percentage of cells in each phase of the cell cycle are shown beside each histogram. DNA synthesis in the asynchronous and quiescent cells was monitored by [<sup>3</sup>H]thymidine uptake (lower left side of A) as described in Materials and Methods. (B) Two representative fields of SLO-treated quiescent *Xenopus* A6 cells after being incubated with FITC-dextran. Bright cytoplasmic fluorescence and dark nuclei indicate that nuclei

were intact while plasma membrane was permeabilized. (C) DNA replication in nuclei of quiescent A6 cells was not observed in egg extracts. Sperm chromatin (1), day 7- (2 and 3) or day 14- (4 and 5) quiescent A6 nuclei were incubated in egg extracts for 6 h. [<sup>32</sup>P]dATP was added to monitor replication. After incubation, the samples were treated with proteinase K, electrophoresed, and autoradiographed. Percentage replication of input DNA was quantitated as described in Materials and Methods. (D) Trypsinization restores the capacity of quiescent nuclei to replicate in egg extracts. *Xenopus* liver nuclei (1 and 2), day 7-quiescent nuclei (G0 D7) and day 14-quiescent A6 nuclei (G0 D14) were treated with trypsin (10  $\mu$ g/ml) for 5 min, and incubated in egg extracts for 6 h. [<sup>32</sup>P]dATP was added to monitor replication.

was replicated (Fig. 1 B, column 3). Therefore, neither intact nor permeabilized quiescent *Xenopus* liver nuclei were replicated efficiently in *Xenopus* egg extracts.

To investigate whether nuclei of cultured *Xenopus* cells in quiescence can be induced to replicate, quiescent *Xenopus* A6 cells were generated after confluence by incubation in media containing 0.5% FBS. Analysis by flow cytometry established that 92% of the cell population had entered G0 phase by day 7 of serum starvation (Fig. 2 A). [<sup>3</sup>H]Thymidine uptake confirmed that the quiescent A6 cells were not synthesizing DNA (Fig. 2 A). To prepare intact nuclei, quiescent A6 cells were harvested at day 7 or day 14 of serum starvation, and the plasma membranes of the cells were permeabilized by the bacterial exotoxin, SLO (Leno et al., 1992). The integrity of the nuclear membrane was verified by FITC-dextran exclusion: as shown in Fig. 2 B, SLO-treated cells exhibited bright cytoplasmic staining and little or no nuclear fluorescence. Only 4% of intact quiescent A6 nuclei were replicated in egg extracts (Fig. 2 C). In quiescent A6 nuclei permeabilized by lysolecithin, only 10–20% of nuclei were replicated in egg extracts (Fig. 2 C).

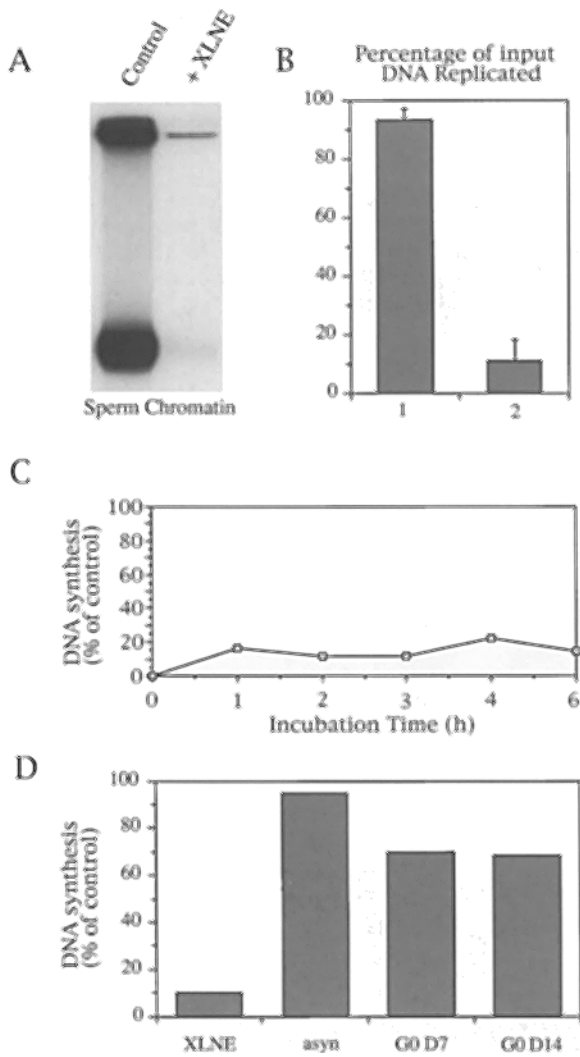
Since *Xenopus* egg extracts contain everything necessary for naked DNA to form a nucleus competent for DNA replication, and since permeabilized G0 phase nuclei from mouse BALB/c 3T3 cells are replicated in these extracts, it seemed likely that species-specific proteins in the quies-

cent *Xenopus* nuclei are responsible for the inability of nuclei from quiescent *Xenopus* cells to replicate. To examine this possibility, permeabilized nuclei from quiescent *Xenopus* cells were treated with trypsin (Coppock et al., 1989), and incubated in egg extracts. After trypsin treatment, ~80% of both liver nuclei and quiescent A6 nuclei regained the ability to replicate in egg extracts (Fig. 2 D).

#### **Replication of *Xenopus* Sperm Chromatin in Egg Extracts Was Inhibited by Extracts of Adult *Xenopus* Liver Nuclei**

If inhibition of replication of intact or permeabilized nuclei from quiescent *Xenopus* cells was due to inhibitors specific to quiescent cells, it is likely that the inhibitors might suppress positive modulators of the replication machinery in *Xenopus* egg extracts. To investigate this possibility, extracts of quiescent *Xenopus* nuclei were prepared. As shown in Fig. 3, A and B, replication of *Xenopus* sperm chromatin was strikingly inhibited when extracts of nuclei from quiescent liver cells were added to the egg extracts.

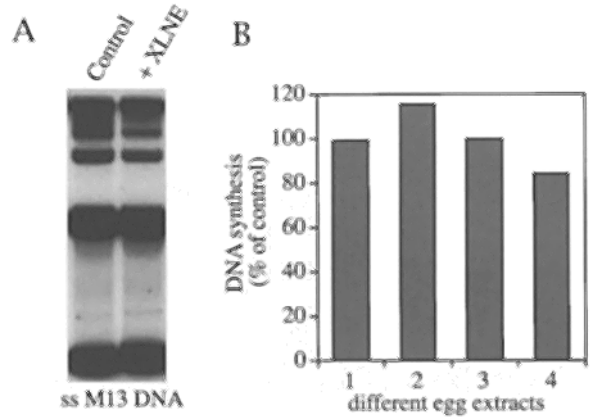
Since nuclei from G0 cells require longer to enter S phase than G1 nuclei (Baserga, 1985; Rossini et al., 1988; Owen et al., 1990), it is possible that the inhibition of DNA replication may be due to a delay in the onset of replication rather than *bona fide* inhibition. To investigate this possibility, extracts of liver nuclei and sperm chromatin were in-



**Figure 3.** Inhibition of DNA replication of *Xenopus* sperm chromatin in egg extracts by *Xenopus* liver nuclear extracts. (A) Sperm chromatin was incubated in egg extracts in the presence of extracts of *Xenopus* liver nuclei (+ XLNE) or NIBB buffer (Control) for 3.5 h. After treatment with proteinase K, DNA was electrophoresed on a 0.8% agarose gel and autoradiographed. DNA synthesis was quantitated by incorporation of [ $\alpha$ - $^{32}$ P]dAMP. (B) Incorporation of [ $\alpha$ - $^{32}$ P]dAMP shown in panel A was quantitated as described in Materials and Methods. (C) Time course of DNA synthesis in egg extracts. Sperm chromatin was incubated in egg extracts with *Xenopus* liver nuclear extracts or NIBB buffer for the indicated time. (D) Effect of extracts of asynchronously growing or quiescent *Xenopus* A6 nuclei on DNA replication in egg extracts. *Xenopus* sperm chromatin was incubated in egg extracts for 3.5 h in the presence of nuclear extracts prepared from *Xenopus* liver (XLNE), or from asynchronous A6 nuclei (asyn), or from day 7 quiescent A6 nuclei (G0 D7) or from day 14-quiescent A6 nuclei (G0 D14). Calculation of percentage DNA synthesized was described in Materials and Methods. Standard errors of the mean are indicated by bars.

cubated in egg extracts for up to 6 h. Inhibition of DNA replication by extracts of *Xenopus* liver nuclei persisted at about the same level throughout the 6-h incubation (Fig. 3 C).

If quiescent cell nuclei contain endogenous inhibitors, the inhibitory activity may be absent from proliferating



**Figure 4.** Effect of extracts of *Xenopus* liver nuclei on complementary-strand synthesis in egg extracts. (A) ss M13 DNA was incubated in egg extracts with [ $\alpha$ - $^{32}$ P]dATP in the presence of NIBB buffer (Control) or liver nuclear extracts (+ XLNE) for 3 h. The samples were treated with proteinase K, electrophoresed, and autoradiographed as described in Materials and Methods. (B) Effect of the same liver nuclear extract upon DNA synthesis on ss M13 DNA templates in different egg extracts. ss M13 DNA was incubated in four different batches of egg extracts (1, 2, 3, and 4) in the presence of the same liver nuclear extract or NIBB buffer for 3 h.

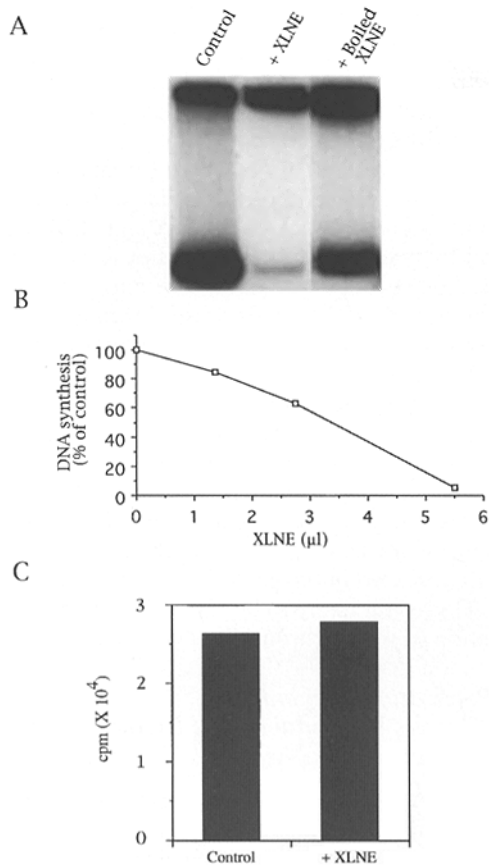
cells. In support of this possibility, nuclear extracts from exponentially growing *Xenopus* A6 cells were shown not to inhibit replication of *Xenopus* sperm chromatin in egg extracts (Fig. 3 D). In contrast, nuclear extracts of quiescent A6 cells showed inhibition of replication, although the level was less than that observed with extracts of *Xenopus* liver nuclei (Fig. 3 D). Because inhibition of replication by extracts of *Xenopus* liver nuclei was more dramatic, and because it is far easier and cheaper to obtain large quantities of liver cells, we focused on the endogenous inhibitor(s) in *Xenopus* liver nuclei.

#### Complementary-strand Synthesis on Single-stranded DNA Templates in Egg Extracts Was Not Inhibited by Extracts of *Xenopus* Liver Nuclei

Two steps in the replication of *Xenopus* sperm chromatin in egg extracts have been identified: initiation on duplex DNA and subsequent chain elongation (Blow and Laskey, 1986; Hutchison et al., 1988; Benbow et al., 1992). When single-stranded (ss) M13 DNA is added to egg extracts, efficient priming and elongation of nascent DNA chains occurs without any requirement for initiation as defined above (Mechali and Harland, 1982; Zhao and Benbow, 1994). To investigate whether the endogenous inhibitors in *Xenopus* liver nuclei inhibited chain elongation, their effect on DNA synthesis on ss M13 DNA templates was examined. Complementary-strand synthesis was not inhibited by addition of extracts of *Xenopus* liver nuclei (Fig. 4). This result also minimizes the possibility that the observed inhibition is caused by degradation of DNA templates in extracts of *Xenopus* liver nuclei.

#### Properties of the Inhibitors from *Xenopus* Liver Nuclei

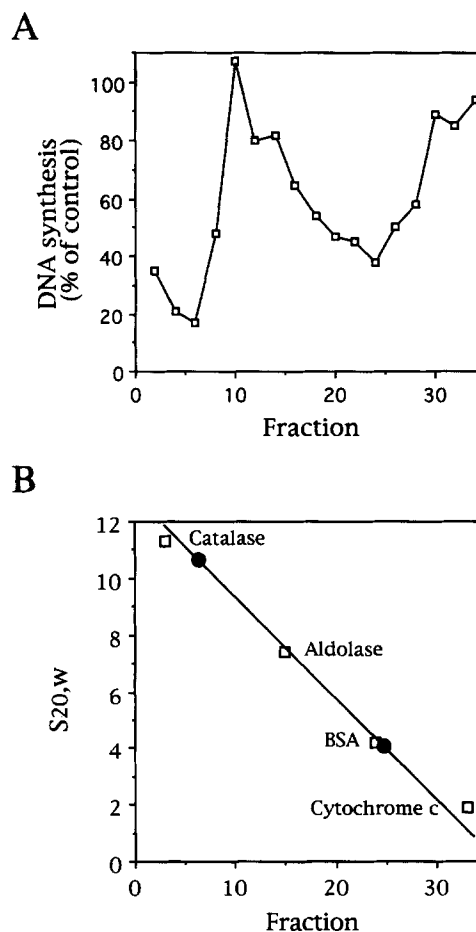
The sensitivity of inhibition of DNA replication to trypsin (Fig. 2 D, above) raised the possibility that the inhibitors



**Figure 5.** Biochemical characteristics of the inhibitors in *Xenopus* liver nuclear extracts. (A) Effect of heated extracts of liver nuclei upon DNA replication. Liver nuclear extract was boiled for 5 min, and precipitated proteins were removed by centrifugation. *Xenopus* sperm chromatin was incubated in egg extracts with [ $\alpha$ - $^{32}$ P]dATP in presence of NIBB buffer (Control), liver nuclear extracts (+ XLNE), or boiled liver nuclear extracts (+ Boiled XLNE) for 3 h. The samples were then treated with proteinase K, run in 0.8% agarose gel, and autoradiographed as above. (B) Dose dependence of inhibition of DNA replication by liver nuclear extracts. Sperm chromatin were incubated in egg extracts for 3 h in the presence of different amounts of liver nuclear extracts as indicated. (C) Template digestion assay for liver nuclear extracts.  $^3$ H-labeled  $\lambda$  phage DNA was incubated in egg extracts in the presence of NIBB buffer (Control) or liver nuclear extracts (+ XLNE) for 2 h. Undergraded  $^3$ H-labeled DNA was quantitated by trichloroacetic acid precipitation described under Materials and Methods.

might be proteins. Moreover, extracts of *Xenopus* liver nuclei prepared in the absence of protease inhibitors show much less inhibition (data not shown). Inhibition was also diminished if the nuclear extracts were subject to frequent freeze-thaw cycles. The inhibitory effect of extracts of *Xenopus* liver nuclei on DNA replication was lost after heating (Fig. 5 A), and inhibition of DNA replication was concentration-dependent (Fig. 5 B).

Although the efficient DNA synthesis on ss M13 DNA templates in the presence of the nuclear extracts makes it unlikely that the DNA templates are degraded (which would cause apparent inhibition), to directly examine this possibility we incubated  $^3$ H-labeled bacteriophage  $\lambda$  DNA with egg extracts to which extracts of liver nuclei had been



**Figure 6.** Glycerol gradient sedimentation. (A) A profile of a fractionated glycerol gradient. *Xenopus* liver nuclear extracts were subjected to glycerol gradient centrifugation and fractions examined for inhibition of DNA replication in *Xenopus* egg extracts as described in Materials and Methods. (B) Sedimentation coefficient determination of inhibitors in *Xenopus* liver nuclear extracts. Standard proteins were indicated by open squares, the inhibitory fractions were shown by closed circles.

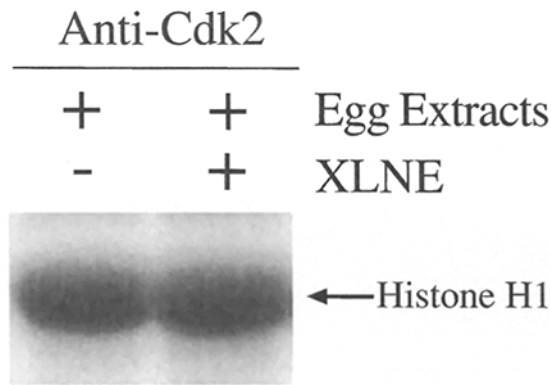
added. The input  $^3$ H-labeled  $\lambda$  DNA was not detectably degraded in 2 h at room temperature (Fig. 5 C).

The size of the inhibitor(s) in extracts of *Xenopus* liver nuclei was examined by glycerol gradient sedimentation to ask whether one or more endogenous quiescent cell-specific molecule(s) was involved in the inhibition we observed. Two sizes of macromolecules, with sedimentation coefficients of  $\sim 10.7$  S and 4.1 S, respectively (Fig. 6), were found to inhibit replication of sperm chromatin in egg extracts, and in addition, both resulted in similar abnormal nuclear morphology (as shown in Fig. 8 below).

#### ***Inhibition of cdk2 Activity Was Not Observed in Egg Extracts Mixed with Extracts of Xenopus Liver Nuclei***

Cyclin-dependent kinases (cdks) play a key role for cells at every checkpoint of the cell cycle (for review see Morgan, 1995). Among these, cdk2 is critical during G1-S phase transition and S phase in mammalian cells (Dulic et al., 1992; Pagano et al., 1992). In *Xenopus* egg extracts, depletion of cdk2 prevents *Xenopus* sperm chromatin from being replicated (Fang and Newport, 1991). Recently, several





**Figure 7.** Effect of liver nuclear extracts upon cdk2 kinase activity in egg extracts. Mixtures of egg extracts and liver nuclear extracts were incubated at room temperature for 30 min. After incubation, cdk2 kinase was immunoprecipitated by anti-cdk2 antibody and agarose-protein A. Histone H1 kinase assays were performed on the agarose beads at 30°C for 30 min as described in Materials and Methods. The proteins were separated by 12% SDS-PAGE gel, which were dried and autoradiographed.

small proteins have been found to be negative regulators of cdk kinases (for review see Morgan, 1995). One of these, p21, has been shown to inhibit DNA replication by inhibiting both cdk2 kinase and PCNA-dependent DNA polymerase  $\delta$  (Adachi and Laemmli, 1994; Waga et al., 1994; Yan and Newport, 1995a).

To investigate the possibility that the inhibition we observed may be due to cdk2 inhibitors like p21, a mixture of interphase egg extracts and extracts of liver nuclei was incubated for 30 min at room temperature. After incubation, cdk2 kinase was immunoprecipitated from the mixture, and its activity was measured by its ability to phosphorylate histone H1. As shown in Fig. 7, inhibition of cdk2 kinase activity was not detected.

#### **Extracts of *Xenopus* Liver Nuclei Caused Morphological Changes in Nuclei Formed in Interphase Egg Extracts**

When *Xenopus* sperm chromatin is added to interphase egg extracts, decondensation is observed within minutes. Nuclear envelopes are formed around the decondensed chromatin by about 30 min (Newport, 1987; Blow, 1993), and an intact nuclear envelope is essential for DNA replication in egg extracts (Cox, 1992). Conversely, aberrant DNA replication is often accompanied by abnormal nuclear morphology (Blow, 1993; Kornbluth et al., 1994; Zhao and Benbow, 1994).

We investigated the effect of extracts of *Xenopus* liver nuclei on the morphology of nuclei formed in egg extracts: extracts of liver nuclei did not affect formation of nuclear envelope around sperm chromatin (Fig. 8 A). However, the subsequent growth of nuclei in egg extracts was greatly diminished in comparison with control nuclei (Fig. 8 A). After 60 min incubation in egg extracts, chromatin inside the nuclei were shown to be more condensed in presence of extracts of liver nuclei than that in the control egg extracts (Fig. 8 A). Heated extracts of liver nuclei (Fig. 5 A) did not have any effect on the morphology of sperm nuclei in egg extracts.

#### **Inhibitors in *Xenopus* Liver Nuclei Do Not Block DNA Decondensation in Cytosol of Egg Extracts**

Two stages of chromatin decondensation are observed after *Xenopus* sperm chromatin is added to cytosol of interphase egg extracts from which nuclear membrane vesicles have been depleted (Kornbluth et al., 1994). Initial decondensation occurs within 5 min and is mainly due to removal of protamines and assembly of histones H2A and H2B on sperm chromatin by nucleoplasmin (Philpott et al., 1991). The second stage of decondensation is slow and persists for 30 min (Kornbluth et al., 1994). Because chromatin decondensation inside nuclei in egg extracts might play a role in growth of nuclei formed in egg extracts, and that process seems to be especially related to the second stage of decondensation (Kornbluth et al., 1994), chromatin decondensation in cytosol of egg extracts was examined. As shown in Fig. 8 B, extracts of liver nuclei did not interfere with either the rate or extent of sperm chromatin decondensation in the cytosol of egg extracts, suggesting that the early block in nuclear growth observed in the presence of liver nuclear extracts (Fig. 8 A) was not likely to be the result of abnormal decondensation of sperm chromatin.

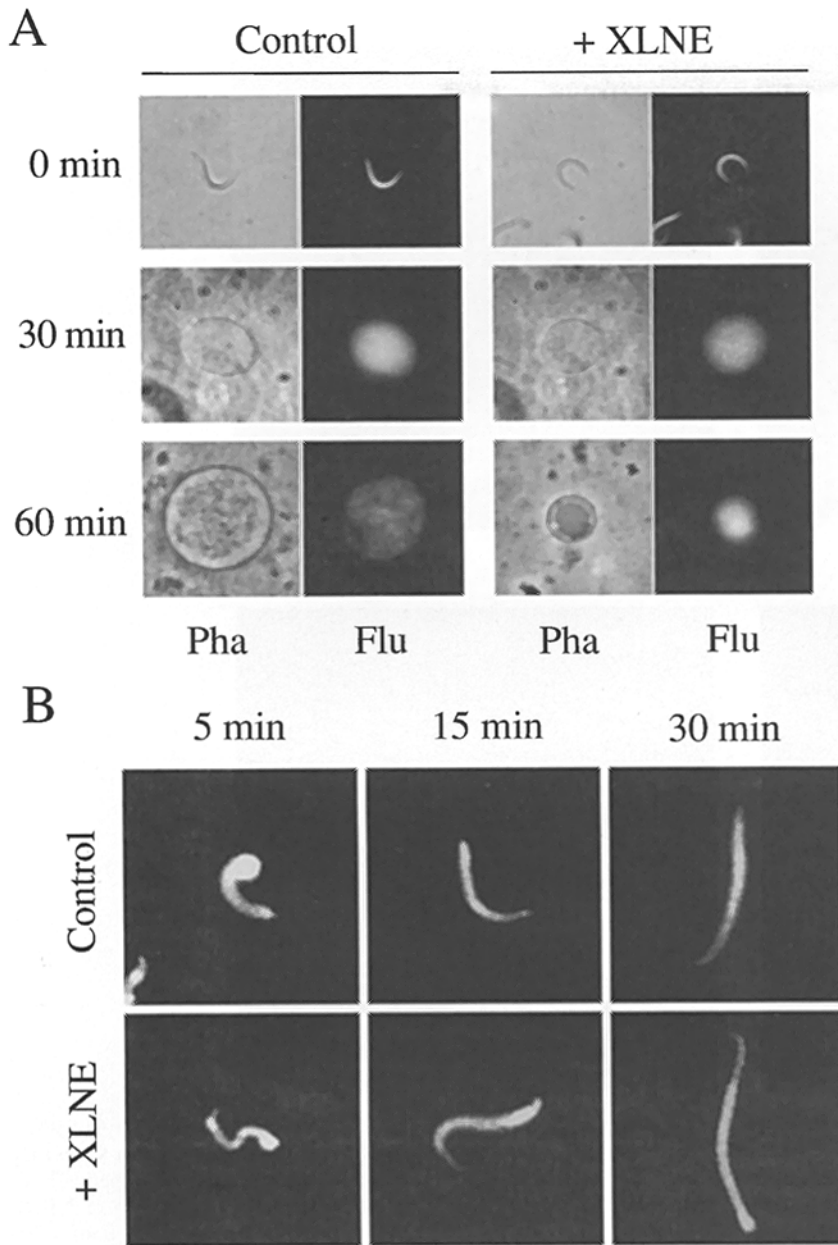
#### **Formation of Replication Foci but Not Prereplication Centers Was Inhibited by Extracts of *Xenopus* Liver Nuclei**

Replication protein A (RP-A), which was originally identified in a mammalian cell-SV40 DNA replication system, has been shown to be an essential factor for DNA replication (Fairman and Stillman, 1988; Wold and Kelly, 1988). Using anti-RP-A antibody, discrete prereplication centers (RP-A foci) have been identified either in newly formed sperm nuclei in egg extracts or in decondensed sperm chromatin in cytosol of egg extracts (Adachi and Laemmli, 1992, 1994; Yan and Newport, 1995a, b). It has been suggested that these prereplication centers (RP-A foci) are the precursors of replication centers which are identified by fluorescent labeling of dUMP incorporation into nascent DNA (Adachi and Laemmli, 1994; Yan and Newport, 1995a).

Since formation of prereplication centers and replication foci are both required for initiation of DNA replication, either step (or both) could be blocked by the inhibitors in extracts of *Xenopus* liver nuclei. To examine the first possibility, sperm chromatin was added to cytosol of interphase egg extracts in the presence of extracts of liver nuclei and incubated for 60 min before being fixed and stained with anti-RP-A antibody. As seen in Fig. 9 A, formation of prereplication (RP-A foci) centers was not inhibited by extracts of *Xenopus* liver nuclei. Inhibition of DNA replication by the extracts was, therefore, not caused by interference with the formation of prereplication foci.

To examine the effect of the putative inhibitors on the formation of active DNA replication centers, sperm chromatin was incubated in egg extracts with extracts of liver nuclei for 45 min and pulse-labeled with biotin-dUTP before being fixed and stained with fluorescein-streptavidin. Fig. 9 B shows that incorporation of dUMPs at replication foci in sperm nuclei was blocked by the extracts. Therefore, the inhibitors appear to function, at least in part, by inhibiting formation of functional replication centers.





**Figure 8.** (A) Changes in nuclear morphology induced by extracts of liver nuclei. (A) Egg extracts were incubated at room temperature with *Xenopus* sperm chromatin in the presence of liver nuclear extracts (+ XLNE) or NIBB buffer (Control). Samples were stained with HOECHST 33258 at 0, 30, and 60 min of incubation for phase-contrast (Pha) and fluorescence (Flu) microscopy. (B) Decondensation of sperm chromatin in the presence of extracts of liver nuclei. A cytosol of interphase egg extracts was prepared as described in Materials and Methods. Sperm chromatin was incubated in the cytosol with extracts of *Xenopus* liver nuclei (+ XLNE) or NIBB buffer (Control) at room temperature for 5, 15, and 30 min. Samples were stained with HOECHST 33258 for phase-contrast (Pha) and fluorescence (Flu) microscopy.

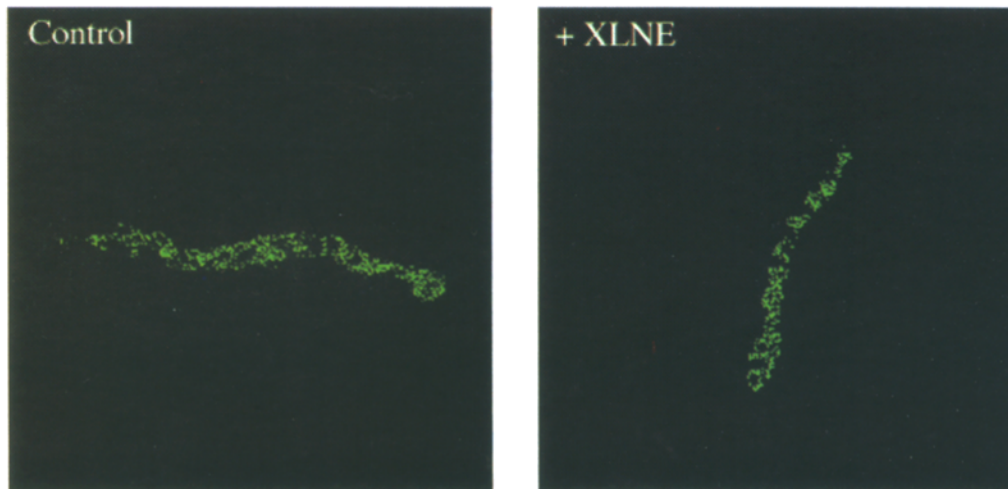
#### ***Inhibition of DNA Replication by Inhibitors in *Xenopus* Liver Nuclei Was Alleviated during the Transition of Egg Extracts from Metaphase to Interphase***

If quiescence-specific inhibitors are present in liver cells (which are not terminally differentiated), these inhibitors may be inactivated before S phase once the cells are stimulated to reenter the cell cycle. To examine this possibility, we prepared metaphase egg extracts from unactivated eggs in the presence of EGTA (Lohka and Masui, 1984). Metaphase egg extracts contain high levels of cytostatic factor (CSF) and maturation promoting factor (MPF) (for review see Lewin, 1990). Addition of  $Ca^{++}$  ions to metaphase egg extracts triggers the inactivation of CSF and MPF, releasing the metaphase egg extracts to interphase, thus making them competent for DNA replication (Lohka and Masui, 1984; Lohka and Maller, 1985; Blow and Nurse, 1990).

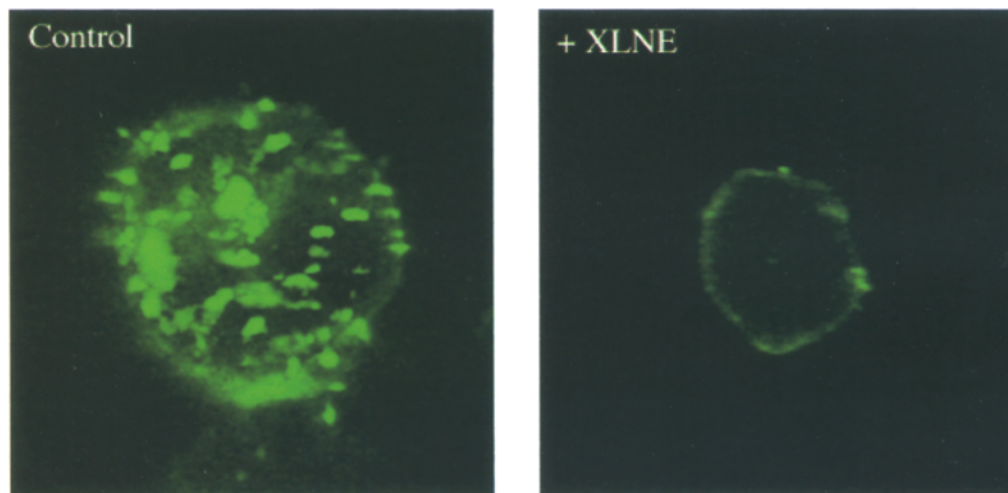
Extracts of *Xenopus* liver nuclei were added to egg extracts either before or after transition from metaphase to interphase, and replication of sperm chromatin was examined. As shown in Fig. 10, A and B, inhibition by extracts of *Xenopus* liver nuclei was relieved if they were added to metaphase egg extracts either 5 min before or 5 min after addition of  $Ca^{++}$  ions. However, nuclear extracts inhibited DNA replication when they were added 40 min after addition of  $Ca^{++}$  ions (that is, when metaphase egg extracts had been completely released to interphase). Thus, the inhibitors in *Xenopus* liver nuclei are negatively regulated during transition of egg extracts from metaphase to interphase.

If the inhibitors are cell cycle-regulated and suppressed during metaphase to interphase transition of egg extracts, quiescent *Xenopus* nuclei should be able to regain replication competence after addition of  $Ca^{++}$  ions. To examine this possibility, permeabilized quiescent nuclei were incu-

A



B



**Figure 9.** Effect of extracts of *Xenopus* liver nuclei on formation of prereplication centers (RP-A foci) and replication foci. (A) Sperm chromatin was added to cytosol of egg extracts in the presence of extracts of *Xenopus* liver nuclei (+ *XLNE*) or NIBB buffer (*Control*) and incubated for 1 h. Decondensed sperm nuclei were fixed and stained for RP-A by indirect immunofluorescence as described in Materials and Methods. (B) Sperm chromatin was incubated in egg extracts with extracts of *Xenopus* liver nuclei (+ *XLNE*) or NIBB (*Control*) for 45 min. Nuclei were pulse-labeled with biotin-dUTP for 3 min immediately before fixation. DNA synthesis is visualized by staining of incorporated biotin-dUTP with fluorescein-streptavidin.

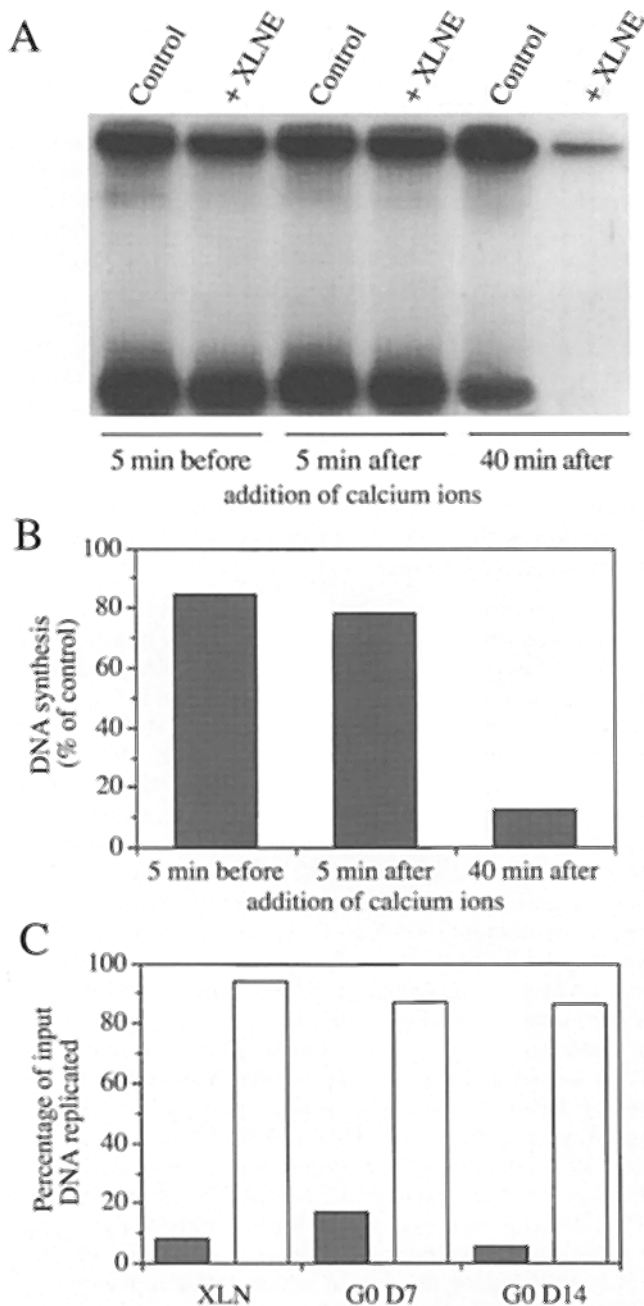
bated in either interphase or metaphase egg extracts, and  $\text{Ca}^{++}$  ions were then added to metaphase egg extracts. As shown in Fig. 10 C, both *in vivo* *Xenopus* liver nuclei and cultured quiescent *Xenopus* A6 nuclei became replication competent after metaphase to interphase transition in egg extracts whereas replication of quiescent nuclei in interphase egg extracts remained blocked.

## Discussion

### *Do Nuclei of Quiescent Xenopus Cells Replicate Efficiently in Xenopus Egg Extracts?*

Using cell-free extracts of *Xenopus laevis* eggs, we have investigated the ability of intact nuclei isolated from quiescent *Xenopus* liver cells to replicate in egg extracts, and found that they do not. In addition, permeabilization of nuclei

from quiescent BALB/c 3T3 cells resulted in efficient replication (Leno and Munshi, 1994), permeabilized *Xenopus* liver nuclei failed to replicate efficiently in egg extracts during 6 h of incubation. It should be noted that efficient replication of nuclei isolated from livers of young (~6 mo) *Xenopus* in egg extracts and extracts of other proliferating cells has been reported previously (Benbow and Ford, 1975; Jazwinski et al., 1976; Floros et al., 1978). In the Leno and Munshi (1994) study, only ~13–26% of intact quiescent 3T3 nuclei replicated in *Xenopus* egg extracts during 6 h incubation. Once the nuclear membrane was permeabilized, nearly 100% of quiescent 3T3 nuclei were replicated in egg extracts. One explanation they proposed for these results is the lack of “replication licensing factor” in quiescent nuclei. Resealing of permeabilized quiescent 3T3 nuclei prevented the nuclei from being replicated in egg extracts (Leno and Munshi, 1994).



**Figure 10.** Inhibitors in quiescent *Xenopus* nuclei were inactivated during metaphase to interphase transition of egg extracts. Transition from metaphase to interphase was induced by adding CaCl<sub>2</sub> to a final concentration of 0.4 mM to metaphase egg extracts. (A) Extracts of *Xenopus* liver nuclei (+ XLNE) or NIBB buffer (Control) were added with sperm chromatin to metaphase egg extracts either 5 min before or 5 min and 40 min after addition of CaCl<sub>2</sub>. The reactions were continued at room temperature for 3.5 h. The samples were treated by protease K, electrophoresed, and autoradiographed. DNA synthesis was monitored and quantitated by incorporation of [ $\alpha$ -<sup>32</sup>P]dAMP. (B) The [ $\alpha$ -<sup>32</sup>P]dAMP radioactivity incorporated shown in A was quantitated as described in Materials and Methods. (C) Permeabilized *Xenopus* liver nuclei (XLN), or day 7-quiescent (G0 D7) or day 14-quiescent (G0 D14) nuclei of *Xenopus* A6 cells were added to either interphase egg extracts (filled bars) or metaphase egg extracts (unfilled bars), respectively. The reactions were continued for 6 h after addition of CaCl<sub>2</sub>. Replication of input DNA was quantitated as described in Materials and Methods.

To understand better the difference between quiescent *Xenopus* nuclei and quiescent mouse 3T3 nuclei in their ability to replicate in egg extracts, we examined quiescent nuclei isolated from cultured *Xenopus* A6 cells at day 7 or day 14 of quiescence, and found that both intact and permeabilized quiescent A6 nuclei did not replicate in *Xenopus* egg extracts. In contrast, control permeabilized nuclei from mammalian (rat) quiescent cells did replicate in the same egg extracts (data not shown), confirming the results of Leno and Munshi (1994) using permeabilized nuclei from quiescent (mouse 3T3) cells. It is reasonable to conclude that species-specific quiescence inhibitors may be responsible for at least part of the inhibition observed in *Xenopus* quiescent cell nuclei in our study. However, since extracts of rat liver nuclei induced slight to moderate inhibition of replication of sperm chromatin in egg extracts, it is also possible that there are nonspecific inhibitors as well. In the case of quiescent 3T3 cells, lack of essential replication factors may account for the failure of intact quiescent nuclei to replicate in egg extracts. After permeabilization of the nuclear membrane, mouse quiescence inhibitors might be unable to efficiently block the positive *Xenopus* replication factors in egg extracts which initiate DNA replication in 3T3 nuclei. Similar results have been obtained previously using quiescent *Xenopus* erythrocytes (Coppock et al., 1989): permeabilized *Xenopus* erythrocyte nuclei did not replicate in egg extracts unless they had been treated with trypsin. However, permeabilized quiescent erythrocyte nuclei isolated from chicken replicated efficiently in *Xenopus* egg extracts (Leno and Laskey, 1991). This further confirms some species-specificity in the regulation of quiescence in different organisms. Our results also show that ~80% of trypsin-treated nuclei of *Xenopus* quiescent cells subsequently replicated in egg extracts, which is consistent with the existence of inhibitors within quiescent *Xenopus* nuclei.

#### ***Do Nuclei of Quiescent Xenopus Cells Contain Negative Regulatory Molecules?***

Since nuclei in G<sub>0</sub>, G<sub>1</sub>, and G<sub>2</sub> phases of cell cycle, including those isolated from different organisms, replicate efficiently in *Xenopus* egg extracts, either before or after permeabilization depending on the phase of the cell cycle, one possible explanation for the failure of both in vivo and cultured quiescent *Xenopus* nuclei to replicate in *Xenopus* egg extracts is that they may be specifically blocked from replicating their DNA by endogenous negative modulators that accumulate during prolonged quiescence. To explore this possibility, we tested whether replication of *Xenopus* sperm chromatin in egg extracts was inhibited by extracts of quiescent *Xenopus* nuclei. Efficient inhibition of DNA replication in egg extracts was observed with addition of extracts of *Xenopus* liver nuclei which persisted throughout 6 h incubation. Similar extracts prepared from quiescent *Xenopus* A6 nuclei showed moderate inhibition of replication, but nuclear extracts from asynchronously growing *Xenopus* A6 cells did not show inhibition. This suggests that the inhibitors found in quiescent *Xenopus* nuclei may be not expressed, or may be modified or inactivated in proliferating cells. The different degree of inhibition of DNA replication by extracts of *Xenopus* liver nuclei and

quiescent A6 nuclei may be explained by various stages of quiescence. When WI-38 cells are quiescent for more than three months, they enter a "deep" G0 state characterized by a prolonged prereplicative phase and specific biochemical changes compared with "early" quiescence (Rossini et al., 1988; Owen et al., 1990). The nuclei of quiescent cultured A6 cells were probably in early quiescence (only up to 14 d contact inhibition and serum deprivation), and there may be qualitative or quantitative differences in the factors that block DNA replication in quiescent A6 cells vs the ordinarily permanent block in adult *Xenopus* liver cells.

Complementary-strand synthesis on single-stranded DNA in egg extracts is a measure of chain elongation, priming and synthesis by DNA polymerase  $\alpha$  primase (Mechali and Harland, 1982), probably augmented by synthesis catalyzed by DNA polymerases  $\delta$  and  $\epsilon$  on the primed templates. In our study, complementary-strand synthesis on single-stranded DNA was blocked by aphidicolin (data not shown) but not affected by extracts of *Xenopus* liver nuclei (Fig. 4). This is consistent with cell fusion studies that show fusion of a quiescent cell with an S phase cell does not affect DNA synthesis by the latter (Stein and Yanishevsky, 1981), and, therefore, that the contents of a quiescent cell do not inhibit ongoing chain elongation. In contrast, fusion of a quiescent cell with a proliferating cell, several hours before S phase, usually results in the failure of the latter to enter S phase (Rabinovitch and Norwood, 1980; Stein and Yanishevsky, 1981). It may be interpreted that this quiescent cell is blocked at some step in initiation of DNA replication. In *Xenopus* egg extracts, replication of sperm chromatin or double-stranded DNA templates is a measure of initiation. The results in Figs. 1, 2, and 3 are thus consistent with the cell fusion studies, and imply that initiation of DNA replication in egg extracts is perturbed by inhibitors in *Xenopus* nuclei isolated from quiescent cells, but chain elongation is not (Fig. 4).

### ***Effect of the Inhibitors on Nuclear Morphology***

Initiation of DNA replication is a complicated process involving coordination of many events and factors (for reviews see Newport, 1987; Benbow et al., 1992; Coverley and Laskey, 1994). The morphology of sperm nuclei formed in egg extracts is one factor that appears to be very important for efficient initiation of DNA replication. Using *Xenopus* egg extracts, it has been postulated that assembly of nuclei may involve several steps (Newport and Dunphy, 1992). According to this model, membrane vesicles first bind to chromatin-associated proteins, and fusion of adjacent vesicles results in formation of a complete nuclear envelope around condensed chromatin. Further nuclear growth may be accomplished through fusion of vesicles directly to the outer membrane of the nuclear envelope, decondensation of chromatin inside the nuclei, and continuous buildup of an internal nuclear structure which is competent for DNA replication. In our study the inhibitors from *Xenopus* liver nuclei did not affect the formation of nuclear envelope around sperm nuclei as indicated both by light microscopy (Fig. 8 A) and FITC-dextran exclusion (data not shown). Therefore, binding of the vesicles to chromatin and subsequent fusion of adjacent vesicles may not be blocked by the inhibitors. Failure of nuclear growth in the presence of

inhibitors suggests a later step may be affected. However, our data show that chromatin decondensation is also normal in presence of the inhibitors (Fig. 8 B). Therefore, it is possible that the inhibitors may block the subsequent buildup of an internal nuclear structure which is necessary for efficient DNA replication. Similar changes in nuclear morphology in egg extracts have also been observed in previous studies of factors that inhibit DNA replication. When either Ran/TC4 mutant protein (Kornbluth et al., 1994), *Xenopus* oocyte extracts (Zhao and Benbow, 1994), or p245 (Zhao, J., and R.M. Benbow, manuscript submitted for publication) were added to egg extracts, both the growth of newly formed nuclei and DNA replication were concomitantly inhibited. It seems likely that the two processes are tightly coupled and may not be separable.

### ***Do the Inhibitors in Xenopus Liver Nuclei Affect Formation of Prereplication Centers (RP-A Foci) and Active Replication Foci?***

Replication protein A (RP-A) was first identified as a single-stranded DNA-binding protein which is essential for DNA synthesis in a mammalian cell-SV40 DNA replication system (Fairman and Stillman, 1988; Wold and Kelly, 1988). The function of RP-A has been extensively studied using *Xenopus* egg extracts (Adachi and Laemmli, 1992; Fang and Newport, 1993; Adachi and Laemmli, 1994; Yan and Newport, 1995a, b). RP-A was shown to be essential for initiation of replication in egg extracts (Fang and Newport, 1993; Adachi and Laemmli, 1994). RP-A was shown by immunofluorescence to be located transiently in discrete prereplication centers (RP-A foci) before inception of DNA synthesis, and disassociated from chromatin after replication (Adachi and Laemmli, 1994; Yan and Newport, 1995a). Incubation of sperm chromatin with cytosol of interphase egg extracts also results in efficient assembly of RP-A foci (Adachi and Laemmli, 1992; Yan and Newport, 1995b). RP-A has been shown to be phosphorylated during G1-S phase transition (Din et al., 1990; Fang and Newport, 1993). Recently, a *Xenopus* foci-forming activity 1 (FFA-1) has been identified (Yan and Newport, 1995b). This 170-kD protein is essential for assembly of RP-A foci in cytosol of egg extracts and was suggested to be an integral component of the foci at which DNA replication is initiated (Yan and Newport, 1995b). Given the above, any factor in liver nuclear extracts which interferes with the functions of either FFA-1 or RP-A would result in blockage of RP-A assembly to form prereplication centers and therefore block DNA replication. Nevertheless, our results indicate that formation of RP-A foci in decondensed sperm chromatin in cytosol of egg extracts is not affected during 60 min incubation with extracts of liver nuclei. This suggests that the inhibitors function downstream of RP-A foci assembly. One such downstream step is the assembly of active replication foci. Fluorescence assays of pulse-labeled dUMP incorporation into nascent DNA has established that DNA synthesis starts at numerous discrete replication foci both in cultured mammalian nuclei and the nuclei formed in *Xenopus* egg extracts (Nakamura et al., 1986; Hutchison et al., 1988; Mills et al., 1989; Hassan and Cook, 1993; Yan and Newport, 1995a). The replication foci are maintained throughout S phase and become at-

tached to the nucleoskeleton (Hassan and Cook, 1993; Hozak et al., 1993). Colocalization of RP-A foci with replication foci has suggested that RP-A foci may be precursors to replication foci (Adachi and Laemmli, 1994; Yan and Newport, 1995a). In our experiments, pulse-labeling with biotin-dUTP showed that the functioning of replication foci in egg extracts was severely impaired by inhibitors isolated from liver nuclei. This might be the result of abnormal assembly or altered components of the replication foci. Thus, formation of functional replication foci but not prereplication centers (RP-A foci) was altered by inhibitors in *Xenopus* liver nuclei.

### ***Is Inhibition Due to Inhibitors of cdk2?***

One of the cyclin-dependent kinases, cdk2, has proven to be essential for DNA replication in mammalian cells and *Xenopus* egg extracts (for review see Morgan, 1995). Immunodepletion of cdk2 from *Xenopus* egg extracts blocks DNA replication (Fang and Newport, 1991). One of cdk2 inhibitors, p21, inhibits cdk2 kinase activity by forming quaternary complexes with cdk2, cyclin E, or cyclin A and proliferating cell nuclear antigen (PCNA) (for review see Morgan, 1995). Using a mammalian cell-SV40 DNA replication system, p21 was found to directly inhibit DNA replication by blocking the ability of PCNA to activate DNA polymerase  $\delta$ , and inhibition by p21 was shown to be heat-resistant (Waga et al., 1994). Inhibition of DNA replication by p21 in *Xenopus* egg extracts has also been confirmed (Adachi and Laemmli, 1994; Chen et al., 1995; Yan and Newport, 1995a). It has also been reported that p21 inhibits DNA replication primarily through its inhibition of cdk2 in *Xenopus* egg extracts (Chen et al., 1995). To investigate the possible involvement of cdk2 inhibitors in the inhibition observed in our study, cdk2-related histone H1 kinase activity was examined in a mixture of interphase egg extracts and liver nuclear extracts. No evidence of inhibition of cdk2 kinase activity was detected (Fig. 7). In addition, unlike inhibition by p21, the inhibitors we observed were shown to be heat-sensitive. Therefore, on the basis of heat sensitivity (and also size; see below), the quiescent cell inhibitors in our study can not be p21 or other cdk2 inhibitors.

### ***Is the Inhibitor Cell Cycle Dependent?***

Although liver is composed of a population of quiescent cells which usually remain in G0 phase, in response to mass organ loss created by partial hepatectomy, liver cells begin to proliferate and restore the liver to its original size within 1–2 wk (Baserga, 1985). Therefore, it seems reasonable that the postulated inhibitors in *Xenopus* liver nuclei could be regulated during progression through the cell cycle. To investigate this possibility we used transition of egg extracts from metaphase to interphase after addition of  $\text{Ca}^{++}$  ions (Lohka and Masui, 1984). Inhibition by *Xenopus* liver nuclear extracts was suppressed when they were added to egg extracts before or at early stage of metaphase to interphase transition. However, liver nuclear extracts inhibit DNA replication if they are added 40-min after  $\text{Ca}^{++}$  release. The 40-min incubation of metaphase extracts with  $\text{Ca}^{++}$  ions ensures the metaphase egg extract has entered into interphase completely before the addition

of the inhibitors (Blow and Nurse, 1990; Murray, 1991). Based on the fact that both *Xenopus* liver cells and cultured A6 cells exit from or reenter into the cell cycle at G1 phase, it is unlikely that the inhibitors from quiescent *Xenopus* nuclei are inactivated by metaphase egg extracts. Rather, our results indicate that the inhibitors may be negatively regulated during metaphase to interphase transition in egg extracts, implying the likelihood of cell cycle regulation. For quiescent cells in vitro changes in culture medium to a high concentration of serum and/or culturing cells at lower density reactivates quiescent cells to reenter the cell cycle and begin DNA synthesis. Therefore, it is likely that the inhibitors which abrogate DNA replication in *Xenopus* A6 quiescent cells may also be inactivated before the onset of DNA synthesis. As shown in Fig. 10 C, ~90% of both quiescent *Xenopus* A6 nuclei as well as liver nuclei were replicated in egg extracts after the egg extract has undergone metaphase to interphase transition. This confirms that inhibitors in quiescent *Xenopus* nuclei may be cell cycle regulated.

### ***Comparison of the Inhibitor(s) in Quiescent Xenopus Nuclei with Other Negative Regulatory Molecules***

In addition to the quiescence-specific and negative regulatory proteins described in the Introduction, several additional proteins have been identified as negative regulators of eukaryotic DNA replication. Mutations in the *Drosophila* maternal genes *giant nuclei*, (*gnu*), *pan gu* (*png*), and *plutonium* (*plu*) uncouple DNA replication from mitosis, resulting in inappropriate initiation and giant polyploid nuclei (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). *plutonium* (*plu*) encodes an ankyrin repeat protein of ~20 kD which negatively regulates S phase during early embryogenesis (Axton et al., 1994). Mutation of *plu* leads to multiple rounds of DNA replication without intervening mitoses in early embryo. *plu* transcripts were found to be expressed only in ovary and during first third of embryogenesis (Axton et al., 1994). In mammalian cells, the 50-kD tumor suppressor/developmental regulator protein WT1 was found to be an inhibitor of TAG- and SV40 origin-dependent replication (Anant et al., 1994). It has been suggested that this zinc-finger protein might inhibit SV40 replication by binding to the GC box promoter motifs of the SV40 21-bp repeat replication auxiliary sequence (Anant et al., 1994). The *Xenopus* quiescent cell inhibitors do not share most properties of these negative regulatory molecules.

Oocytes of *Xenopus laevis* are arrested at the G2/prophase of first meiosis. It has been reported that *Xenopus* oocyte extracts inhibited DNA replication of *Xenopus* sperm chromatin in egg extracts (Zhao and Benbow, 1994) and a 245-kD (p245) metaphase-related inhibitor was isolated from *Xenopus* oocyte extracts which inhibits replication of sperm chromatin but not complementary-strand synthesis (Zhao, J., and R.M. Benbow, manuscript submitted for publication). The activity of p245 declined gradually during oocyte maturation and was inactivated during M to S phase transition in egg extracts. In our study the glycerol gradient sedimentation experiments (Fig. 6) suggest that at least two sizes of macromolecules can lead to inhibition. One has sedimentation coefficient of around 10.7S, which



is close to beef liver catalase (11.3S, 240 kD) and very close to that of metaphase related inhibitor (245 kD) from *Xenopus* oocytes. However, we cannot easily reconcile the inhibitor from *Xenopus* oocytes with the soluble quiescent cell inhibitors in our study. The oocyte protein is likely to be a nuclear matrix protein that binds tenaciously to DNA and is nearly insoluble under the conditions employed in our study. The second inhibitory species had a sedimentation coefficient of  $\sim 4.1S$ , similar to that of bovine serum albumin (4.22S, 68 kD). In our study the larger species could be a tetramer of the 68-kD protein, but there is no direct evidence for this; no low molecular weight inhibitor was seen in the oocyte extracts (Zhao, J., and R.M. Benbow, manuscript submitted for publication). Further purification of the quiescent cell inhibitors is needed to establish whether the inhibition we observe is caused by one or several proteins.

### How Do the Quiescent Cell Inhibitors Function?

At least two possibilities might account for the blockage of DNA replication in quiescent cells. One that has been proposed is the absence of "licensing factor" (or any other essential positive replication factors) in quiescent cells (Leno and Munshi, 1994). Licensing factor was postulated to be required for initiation of DNA replication (Blow and Laskey, 1988; Blow, 1993; Coverley et al., 1993). Replication licensing factor M (RLF M) in *Xenopus* eggs apparently consists of three at least proteins of about 92, 106, and 115 kD, related to the yeast MCM proteins (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). More specifically, p106 (p100 of Kubota and colleagues) is the *Xenopus* homologue (XMCM3) of yeast MCM 3 protein (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). It was reported that the level of XMCM3 is low in quiescent cells (Kubota et al., 1995). Blow and colleagues (Chong et al., 1995) have suggested that a second component of licensing factor is a DNA-binding protein, termed replication licensing factor B (RLF B), which is postulated to load RLF M onto chromatin to form the active complex (Chong et al., 1995; Madine et al., 1995). However, since Laskey has recently suggested that the term licensing factor is no longer useful (Huberman, 1995), it seems no longer germane to discuss its possible direct interaction with the quiescent cell inhibitor(s).

Another possibility for blockage of DNA replication is the existence of specific inhibitors in nuclei of quiescent cells that are able to annul the activity of the certain positive replication factors, thereby abrogating the initiation of chromosomal replication in quiescence. In this view, expression of positive replication factors is suppressed during long-term quiescence. The quiescence-specific inhibitors abrogate DNA replication in quiescence by preventing positive replication factors from executing their normal functions. In order for DNA replication to commence, the level of the positive replication factors must exceed the level of the inhibitors so that cells have sufficient excess of the positive replication factors to initiate DNA replication. Thus, the inhibitors may act as a threshold to prevent cells from starting DNA synthesis in either in vivo or in cultured cell quiescence. Reentry into the cell cycle of quiescent cells is presumably accompanied by inactivation of the

quiescence-specific inhibitors and expression of positive replication factors, which eventually leads to breakdown of the inhibitor-threshold and inception of DNA replication. The data in our study are most easily explained by this hypothesis; the key difference between our hypothesis and others is that we propose that the negative regulatory molecules rather than positive regulatory molecules are dominant in *trans*.

It is not unreasonable that replication of *Xenopus* nuclei in somatic or cultured cells and *Xenopus* sperm nuclei in egg extracts may be carried out by the same mechanism using similar replication machinery. Since there are quiescence-specific inhibitors in quiescent *Xenopus* nuclei that actively block DNA replication in *Xenopus* egg extracts, it is not unreasonable that they also play a crucial role to abrogate DNA replication in vivo and in cultured *Xenopus* cells in quiescence.

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### References

- Adachi, Y., and U.K. Laemmli. 1992. Identification of nuclear pre-replication centers poised for DNA synthesis in *Xenopus* egg extracts: 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 centers in *Xenopus* egg extracts. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4153-4164.
- Anant, S., S.A. Axenovich, S.L. Madden, F.J. Rauscher III, and K.N. Subramanian. 1994. Novel replication inhibitory function of the developmental regulator/transcription repressor protein WT1 encoded by the Wilms' tumor gene. *Oncogene.* 9:3113-3126.
- Axton, J.M., F.L. Shamanski, L.M. Young, D.S. Henderson, J.B. Boyd, and T.L. Orr-Weaver. 1994. The inhibitor of DNA replication encoded by the *Drosophila* gene *plutonium* is a small, ankyrin repeat protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:462-470.
- Barone, M.V., A. Crozat, A. Tabae, L. Philipson, and D. Ron. 1994. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. *Genes & Dev.* 8:453-464.
- Baserga, R. 1985. *The Biology of Cell Reproduction*. Harvard University Press, Cambridge, MA. 245 pp.
- Bedard, P.A., S.D. Balk, H.S. Gunther, A. Morisi, and R.L. Erikson. 1987. Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by Rous Sarcoma virus. *Mol. Cell. Biol.* 7:1450-1458.
- Benbow, R.M., and C.C. Ford. 1975. Cytoplasmic control of nuclear DNA synthesis during early development of *Xenopus laevis*: a cell-free assay. *Proc. Natl. Acad. Sci. USA.* 72:2437-2441.
- Benbow, R.M., J. Zhao, and D.D. Larson. 1992. On the nature of origins of DNA replication in eukaryotes. *BioEssays.* 14:661-670.
- Blobel, G., and V.R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science (Wash. DC).* 154:1662-1665.
- Blow, J.J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. *J. Cell Biol.* 122:993-1002.
- 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.
- Blow, J.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.
- 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.
- Chong, J.P.J., H.M. Mahubani, C.Y. Khoo, and J.J. Blow. 1995. Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature (Lond.)* 375:418-421.
- Coppeck, D.L., R.A. Lue, and L.J. Wangh. 1989. Replication of *Xenopus* erythrocyte nuclei in a homologous egg extract requires prior proteolytic treatment. *Dev. Biol.* 131:102-110.
- Coverley, D., and R.A. Laskey. 1994. Regulation of eukaryotic DNA replication. *Annu. Rev. Biochem.* 63:745-776.
- Coverley, D., C.S. Downes, P. Romanowski, and R.A. Laskey. 1993. Reversible effects of nuclear membrane permeabilization on DNA replication: evidence for a positive licensing factor. *J. Cell Biol.* 122:985-992.
- Cox, L.S. 1992. DNA replication in cell-free extracts from *Xenopus* eggs is prevented by disrupting nuclear envelope function. *J. Cell Sci.* 101:43-53.
- Del Sal, G., M.E. Ruaro, L. Philipson, and C. Schneider. 1992. The growth arrest-specific gene, *gas 1*, is involved in growth suppression. *Cell* 70:595-607.
- Din, S., S.J. Brill, M.P. Fairman, and B. Stillman. 1990. Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. *Genes & Dev.* 4:968-977.
- Dulic, V., E. Lees, and S.I. Reed. 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science (Wash. DC)* 257:1958.
- Fairman, M.P., and B. Stillman. 1988. Cellular factors required for multiple stages of SV40 replication *in vitro*. *EMBO (Eur. Mol. Biol. Organ.) J.* 7: 1211-1218.
- Fang, F., and J.W. Newport. 1991. Evidence that 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.
- Floros, J., H. Chang, and R. Baserga. 1978. Stimulated DNA synthesis in frog nuclei by cytoplasmic extracts of temperature-sensitive mammalian cells. *Science (Wash. DC)* 201:651-652.
- Freeman, M., and D.M. Glover. 1987. The *gnu* mutation of *Drosophila* causes inappropriate DNA synthesis in unfertilized eggs. *Genes & Dev.* 1:924-930.
- Gorski, D.H., D.F. LePage, C.V. Patel, N.G. Copeland, N.A. Jenkins, and K. Walsh. 1993. Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G0/G1 transition in vascular muscle cells. *Mol. Cell Biol.* 13:3722-3733.
- Gorski, K., M. Carneiro, and U. Schibler. 1986. Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* 47:767-776.
- Hassan, A.B., and P.R. Cook. 1993. Visualization of replication sites in unfixed human cells. *J. Cell Sci.* 105:541-550.
- Hozak, P., A.B. Hassan, D.A. Jackson, and P.R. Cook. 1993. Visualization of replication factories attached to a nucleoskeleton. *Cell* 73:361-373.
- Huberman, J.A. 1995. Prokaryotic and eukaryotic replicons. *Cell* 82:535-542.
- 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 (Eur. Mol. Biol. Organ.) J.* 6:2003-2010.
- Hutchison, C.J., R. Cox, and C.C. Ford. 1988. The control of DNA replication in a cell-free extract that recapitulates a basic cell cycle *in vitro*. *Development* 103:553-566.
- Jazwinski, S.M., J.L. Wang, and G.M. Edelman. 1976. Initiation of replication in chromosomal DNA induced by extracts from proliferative cells. *Proc. Natl. Acad. Sci. USA* 73:2231-2235.
- Kornbluth, S., M. Dasso, and J. Newport. 1994. Evidence for a dual role for TC4 protein in regulating nuclear structure and cell cycle progression. *J. Cell Biol.* 125:705-719.
- Kubota, Y., S. Mimura, S. Nishimoto, H. Takisawa, and H. Nojima. 1995. Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. *Cell* 81:601-609.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Leno, G.H., and R.A. Laskey. 1991. The nuclear membrane determines the timing of DNA replication in *Xenopus* extracts. *J. Cell Biol.* 112:557-566.
- Leno, G.H., and R. Munshi. 1994. Initiation of DNA replication in nuclei from quiescent cells requires permeabilization of the nuclear membrane. *J. Cell Biol.* 127:5-14.
- Leno, G.H., C.S. Downes, and R.A. Laskey. 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. *Cell* 69:151-158.
- Lewin, B. 1990. Driving the cell cycle: M phase kinase, its partner, and substrates. *Cell* 61:743-752.
- Lohka, M.J., and Y. Masui. 1983. Formation *in vitro* of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science (Wash. DC)* 220:719-721.
- Lohka, M.J., and Y. Masui. 1984. Effects of Ca<sup>2+</sup> ions on the formation of metaphase chromosomes and sperm pronuclei in cell-free preparations from unactivated *Rana pipiens* eggs. *Dev. Biol.* 103:434-442.
- 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.
- Madine, M.A., C.Y. Khoo, A.D. Mills, and R.A. Laskey. 1995. MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. *Nature (Lond.)* 375:421-424.
- Mechali, M., and R.M. Harland. 1982. DNA synthesis in a cell-free system from *Xenopus* eggs: priming and elongation of single-stranded DNA *in vitro*. *Cell* 30:93-101.
- Mills, A.D., J.J. Blow, J.G. White, W.B. Amos, D. Wilcock, and R.A. Laskey. 1989. Replication occurs at discrete foci spaced throughout nuclei replicating *in vitro*. *J. Cell Sci.* 94:471-477.
- Morgan, D.O. 1995. Principles of CDK regulation. *Nature (Lond.)* 374:131-134.
- Murray, A.W. 1991. Cell cycle extracts. In *Methods in Cell Biology*. B. Kay and H.B. Peng, editors. Academic Press, Inc., San Diego, CA. 581-603.
- Nakamura, H., T. Morita, and C. Sato. 1986. Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* 165:291-297.
- Newmeyer, D.D., and K.L. Wilson. 1991. Egg extracts for nuclear import and nuclear assembly reactions. In *Methods in Cell Biology*. B. Kay and H.B. Peng, editors. Academic Press, Inc., San Diego, CA. 607-634.
- Newport, J. 1987. Nuclear reconstitution *in vitro*: stages of assembly around protein-free DNA. *Cell* 48:205-217.
- Newport, J., and W. Dunphy. 1992. Characterization of the membrane and fusion events during nuclear envelope assembly using purified components. *J. Cell Biol.* 116:295-306.
- Nuell, M., D.A. Stewart, L. Walker, V. Friedman, C.M. Wood, G.A. Owens, J.R. Smith, E.L. Schneider, R.D. Orco, C.K. Lumpkin, et al. 1991. Prohibitin, an evolutionary conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. *Mol. Cell Biol.* 11:1372-1381.
- Owen, T.A., C. Ruth, M.M. Whitman, D.R. Soprano, and K.J. Soprano. 1990. Evidence that density-dependent growth arrest is a two-stage process in WI-38 cells. *J. Cell Physiol.* 142:137-148.
- Padmanabhan, R., T.H. Howard, and B.H. Howard. 1987. Specific growth inhibitory sequences in genomic DNA from quiescent human embryo fibroblasts. *Mol. Cell Biol.* 7:1894-1899.
- 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 (Eur. Mol. Biol. Organ.) J.* 11:961-971.
- Pardee, A.B. 1989. G1 events and regulation of cell proliferation. *Science (Wash. DC)* 246:603-608.
- Philpott, A., G.H. Leno, and R.A. Laskey. 1991. Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. *Cell* 65:565-578.
- Rabinovitch, P.S., and T.H. Norwood. 1980. Comparative heterokaryon study of cellular senescence and the serum-deprived state. *Exp. Cell Res.* 130:101-109.
- Ron, D., and J.F. Habener. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcriptional factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes & Dev.* 6:439-453.
- Rossini, M., J. Lin, and R. Baserga. 1988. Effects of prolonged quiescence on nuclei and chromatin of WI-38 fibroblasts. *J. Cell Physiol.* 88:1-12.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 2.60-2.80.
- Schneider, C., R.M. King, and L. Philipson. 1988. Genes specifically expressed at growth arrest of mammalian cells. *Cell* 54:787-793.
- Shamanski, F.L., and T.L. Orr-Weaver. 1991. The *Drosophila plutonium* and *pan gu* genes regulate entry into S phase at fertilization. *Cell* 66:1289-1300.
- Smith, J.C., and J.R. Tata. 1991. *Xenopus* cell lines. In *Methods in Cell Biology*. B. Kay and H.B. Peng, editors. Academic Press, Inc., San Diego, CA. 635-654.
- Stein, G.H., and R.M. Yanishevsky. 1981. Quiescent human diploid cells can inhibit entry into S phase in replicative nuclei in heterokaryons. *Proc. Natl. Acad. Sci. USA* 78:3025-3029.
- Stein, G.H., and L. Atkins. 1986. Membrane-associated inhibitor of DNA synthesis in senescent human diploid fibroblasts: Characterization and comparison to quiescent cell inhibitor. *Proc. Natl. Acad. Sci. USA* 83:9030-9034.
- Waga, S., G.J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature (Lond.)* 369:574-578.
- Wold, M.S., and T.J. Kelly. 1988. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA* 85:2523-2527.
- Yan, H., and J. Newport. 1995a. An analysis of the regulation of DNA synthesis by *cdk2*, Cip1, and licensing factor. *J. Cell Biol.* 129:1-15.
- Yan, H., and J. Newport. 1995b. FFA-1, a protein that promotes the formation of replication centers within nuclei. *Science (Wash. DC)* 269:1883-1885.
- Zhao, J., and R.M. Benbow. 1994. Inhibition of DNA replication in cell-free extracts of *Xenopus laevis* eggs by extracts of *Xenopus laevis* oocytes. *Dev. Biol.* 164:52-62.