Initiation of DNA Replication in Nuclei from Quiescent Cells Requires Permeabilization of the Nuclear Membrane

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Abstract. We have investigated the replication capacity of intact nuclei from quiescent cells using *Xenopus* egg extract. Nuclei, with intact nuclear membranes, were isolated from both exponentially growing and contact-inhibited BALB/c 3T3 fibroblasts by treatment of the cells with streptolysin-O. Flow cytometry showed that >90% of all contact-inhibited cells and ∿50% of the exponential cells were in G0/G1-phase at the time of nuclear isolation. Intact nuclei were assayed for replication in the extract by incorporation of [α-^{32}P]dATP or biotin-dUTP into nascent DNA. Most nuclei from exponential cells replicated in the egg extract, consistent with previous results showing that intact G1 nuclei from HeLa cells replicate in this system. In contrast, few nuclei from quiescent cells replicated in parallel incubations. However, when the nuclear membranes of these intact quiescent nuclei were permeabilized with lysophosphatidylcholine prior to addition to the extract, nearly all the nuclei replicated under complete cell cycle control in a subsequent incubation. The ability of LPC-treated quiescent nuclei to undergo DNA replication was reversed by resealing permeable nuclear membranes with *Xenopus* egg membranes prior to extract incubation demonstrating that the effect of LPC treatment is at the level of the nuclear membrane. These results indicate that nuclei from G1-phase cells lose their capacity to initiate DNA replication following density-dependent growth arrest and suggest that changes in nuclear membrane permeability may be required for the initiation of replication upon re-entry of the quiescent cell into the cell cycle.

THE regulation of eukaryotic DNA replication is a complex process. Analysis of the regulatory mechanisms governing the replication of cellular DNA has been greatly facilitated by the development of a cell-free system from amphibian eggs (Lohka and Masui, 1983, 1984). In *Xenopus* egg extracts, initiation of DNA replication is dependent upon nuclear structure (Blow and Laskey, 1986; Newport, 1987; Hutchison et al., 1987; Blow and Watson, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990). One particular feature of nuclear structure, namely the nuclear envelope, is directly involved in regulating DNA replication in at least three ways (for review see Leno, 1992). First, the initiation of replication is dependent upon the assembly of an intact, functional nuclear envelope (Newport et al., 1990; Meier et al., 1991; Cox, 1992). Second, the nuclear membrane determines the timing of initiation of replication (Leno and Laskey, 1991), and, third, the nuclear membrane prevents re-replication of DNA within a single cell cycle (Blow and Laskey, 1988; Leno et al., 1992).

The cell fusion studies of Rao and Johnson (1970) clearly demonstrated that mammalian G1 nuclei differ from G2 nuclei regarding their capacities for DNA replication. Recently, Leno et al. (1992) showed that the nuclear membrane defines the different replication capacities of these pre-S-phase (G1) and post-S-phase (G2) nuclei in *Xenopus* egg extract suggesting that this structure may limit DNA replication to a single round per cell cycle in mammalian cells. The mechanism by which the nuclear membrane prevents re-replication within a single cell cycle has not been completely defined. According to one model (Blow and Laskey, 1988), an essential replication factor binds to DNA only at mitosis when the nuclear membrane is broken down and is destroyed after replication initiates. In this way, the factor would "license" the DNA to replicate once and only once following nuclear re-assembly, but further replication could not occur until the nuclear membrane again breaks down in the next mitosis.

Recently, the existence of a positive "licensing" activity in egg extract has been confirmed (Coverley et al., 1993; Blow, 1993; Kubota and Takisawa, 1993), although its mechanism of action remains unclear. There are at least two possible ways in which a positive activity could license the DNA for replication: first, by direct binding of the factor to the DNA, as was originally proposed (Blow and Laskey, 1988), or second, by the catalytic conversion of inactive replication components into active forms (Coverley et al., 1993; Blow, 1993). In either case, the net result is removal of the block

to re-replication on G2 nuclei, thereby rendering G1 nuclei competent for DNA replication in the ensuing cell cycle.

In the adult vertebrate organism, the majority of cells exist in a quiescent state often referred to as "G0" (Pardee, 1989). Most of these G0 cells contain unduplicated DNA, demonstrating that quiescence occurs prior to entry into S-phase. Untransformed 3T3 fibroblasts can be induced to exit the cell cycle early in G1-phase (Zetterberg and Larsson, 1991) and enter a reversible growth-arrested state either by contact inhibition (Holley and Kienan, 1968) or serum deprivation (Larsson et al., 1985). Presumably, this quiescent state is similar to the in vivo G0 state. Quiescent fibroblasts differ from cycling G1 fibroblasts in several ways, including cell size, metabolic activity, and ribosome structure (Pardee, 1989). Along with these differences is the specific expression of several growth-arrest–specific (gas) genes in quiescent cells which demonstrates that the "out of cycle" quiescent state is biologically distinct from the "in cycle" G1-phase (Schneider et al., 1991). When contact-inhibited 3T3 cells are re-plated at a lower cell density, or when serum-starved cells are re-fed serum or specific growth factors, the quiescent state is reversed, and these cells re-enter the cell cycle in G1. Thus, like cycling G1 cells, these previously arrested G1 cells eventually undergo DNA replication.

We were interested in determining whether intact nuclei from contact-inhibited cells, which exit the cell cycle at G1, retain the capacity for initiating DNA replication or whether G1 nuclei lose this capacity following growth arrest. Here we show that most intact quiescent nuclei do not initiate DNA replication in the *Xenopus* egg extract, even after short periods of growth arrest. However, if their nuclear membranes are permeabilized with lysophosphatidylcholine (LPC)[1] before addition to the extract, virtually all nuclei replicate under complete cell cycle control in a subsequent incubation. The ability of LPC-treated quiescent nuclei to undergo DNA replication can be reversed by resealing permeable nuclear membranes with *Xenopus* egg membranes prior to extract incubation demonstrating that the effect of LPC treatment is at the level of the nuclear membrane. These data indicate that nuclei from G1 cells lose their capacity to replicate following growth arrest and suggest that changes in nuclear membrane permeability may be required for the initiation of replication upon re-entry of the quiescent cell into the cell cycle.

Materials and Methods

Preparation of Egg Extracts

Xenopus egg extracts were prepared according to the procedure used by Leno and Laskey (1991) with the modifications described by Leno et al. (1992). Specifically, female frogs were injected with 50 IU pregnant mare's serum gonadotropin (Sigma Chemical Co., St. Louis, MO) 6–7 d before injection with 400–500 IU human chorionic gonadotropin (Sigma Chemical Co.). Eggs were collected from high-salt Barth's (Blow and Laskey, 1986) and de-jellied with 2% cysteine hydrochloride, pH 8.0. De-jellied eggs were rinsed several times in Barth's (Blow and Laskey, 1986) and activated in Barth's containing 0.5 μg/ml calcium ionophore A23187 (Sigma Chemical Co.). Activated eggs were rinsed several times in Barth's without calcium

and transferred to ice-cold extraction buffer (Blow and Laskey, 1986). The protease inhibitors aprotinin, leupeptin, and pepstatin (Sigma Chemical Co.), all at 1 μg/ml, were included in the final extraction buffer rinse. Eggs were packed by centrifugation at 1,500 rpm for 1 min in a SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA), and all excess buffer and discolored eggs were removed. Packed eggs were crushed by centrifugation at 9,000 rpm for 10 min in a SW 50.1 rotor. The cytoplasmic layer was removed, supplemented with cytochalasin B (Sigma Chemical Co.) to a final concentration of 10 μg/ml, and centrifuged at 15,000 rpm for 10 min in a SW 50.1 rotor. The low-speed supernatant was supplemented with glycerol to a final concentration of 2% and frozen as beads in liquid nitrogen.

Preparation and Fractionation of Mitotic Vesicles

Mitotic extracts from unactivated *Xenopus* eggs were prepared as described by Newport and Spann (1987). Mitotic vesicles were isolated and fractionated according to the procedures described by Wilson and Newport (1988). The "light" vesicles collected from the 1.1 M/0.9 M and the 0.9/0.7 M interfaces of the discontinuous sucrose gradient were combined and incubated with *Xenopus* sperm chromatin in a membrane-depleted "high-speed" supernatant derived from activated eggs (Sheehan et al., 1988). Nuclear envelope formation around sperm chromatin was confirmed by phase contrast microscopy and staining with the lipophilic dye Nile red (Leno and Laskey, 1991). The protein concentration of the combined "light" mitotic vesicle fraction was determined using the DC protein assay kit (Bio-Rad Laboratories, Cambridge, MA).

Cell Culture

BALB/c 3T3 cells (No. CCL 163; American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% calf serum (GIBCO BRL), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (GIBCO BRL). HeLa cells were grown and synchronized in G1-phase as previously described (Leno et al., 1992).

Quiescent cells were obtained as follows. Each of six 75 cm^2 flasks were seeded with an equal number of exponentially growing 3T3 cells, and all cultures were fed fresh medium daily. Cells in all cultures reached confluence ($\sim$6 $\times$ 10^4 cells/cm^2) on the same day (confluent day 0; C0). Nuclei, with intact nuclear membranes, were isolated from the C0 culture and from the remaining cultures every 2 d for up to 10 d (C2–C10) (see below). Intact nuclei were also isolated from exponentially growing 3T3 cells ($\sim$6 $\times$ 10^3 cells/cm^2) as described below. To label nuclei from S-phase cells in both the confluent and exponentially growing cell cultures, each culture was pulse-labeled for 1 h with 100 μM bromodeoxyuridine (BrdU) before nuclear isolation.

Preparation of Nuclei

Nuclei, with intact nuclear membranes, were isolated from exponentially (EXP) growing and confluent, contact-inhibited 3T3 cultures (C0–C10) and from G1-phase HeLa cultures using the bacterial exotoxin streptolysin-O (SLO; Murex Diagnostics Inc., Norcross, GA) (Leno et al., 1992). Specifically, BrdU-labeled cells were rinsed with Dulbecco's phosphate-buffered saline without calcium chloride or magnesium chloride (D-PBS; GIBCO BRL) and detached from the flask by incubation with nonenzymatic cell dissociation solution (Sigma Chemical Co.) or with trypsin-EDTA (GIBCO BRL). The detached cells were pelleted by centrifugation for 10 min at 190 g. The cell pellet was resuspended in ice-cold Pipes buffer (50 mM K-Pipes, pH 7.0, 50 mM KCl, 5 mM MgCl$_2$, 2 mM EGTA) containing 1 μg/ml each aprotinin, leupeptin, and pepstatin and 1 mM DTT (Sigma Chemical Co.). Cell numbers were determined using a hemacytometer, and Pipes buffer was added to yield $\sim$5 $\times$ 10^5 cells/ml. An equal volume of ice-cold Pipes buffer containing SLO at a concentration of 1.5 IU/ml was added to give a final concentration of SLO equal to 0.75 IU/2.5 $\times$ 10^5 cells/ml. Cells were held on ice for 30 min and the tubes were inverted every 10 min. SLO-treated cells were pelleted by centrifugation at 0°C as described above. The supernatant, containing unbound SLO monomer, was removed and the cell pellet resuspended in Pipes buffer. Cells were pelleted and rinsed twice in Pipes buffer. The final cell pellet was resuspended in 1 ml Pipes buffer and incubated 5 min at 37°C. The permeability of both plasma and nuclear membranes was determined by incubating an aliquot of SLO-treated cells with affinity purified TRITC-IgG (Sigma Chemical Co.) for 5 min. Samples were viewed with a laser-scanning confocal microscope (Noran) with an excitation wavelength of 529 nm through a 20$\times$ objective lens.

The nuclear membranes of intact exponential (EXP) and intact contact-inhibited (C0-C10) 3T3 nuclei and G1-phase HeLa nuclei were permeabilized by treatment with lysophosphatidylcholine (LPC; Sigma Chemical Co.). Specifically, an aliquot from each intact nuclear population was incubated on ice for 15 min with 200 μg LPC/1 × 10^5 nuclei/ml Pipes buffer. Membrane permeabilization was stopped by the addition of BSA (Sigma Chemical Co.) to a final concentration of 1%. Samples were centrifuged at 47 g for 10 min at 0°C. The nuclear pellets were rinsed three times by re-suspension in Pipes buffer followed by centrifugation. An aliquot of LPC-treated nuclei from each sample was incubated with TRITC-IgG and viewed as described above.

The concentration of DNA in each 3T3 sample was estimated by counting nuclei with a hemacytometer and assuming a DNA mass of 10 pg per hypotetraploid nucleus. This value is based upon a DNA mass of 5.1 pg per diploid fibroblast nucleus (Patel and Rickwood, 1992). Our assumption of 10 pg DNA per 3T3 nucleus would result in an underestimate of the actual concentration of DNA in the exponential population, relative to the quiescent samples, due to the number of cells in S- and G2/M-phases in the former population (see Fig. 1 C). However, these small differences in the concentration of DNA between exponential and quiescent samples should have had little, if any, effect on our results. Both intact and permeabilized nuclei that were not used immediately in replication assays were frozen using cryo 1°C freezing containers (Nalgene Co., Rochester, NY) following addition of dimethylsulfoxide to a final concentration of 5%.

The concentration of DNA in each HeLa sample was estimated by counting nuclei and assuming a DNA mass of 14.4 pg per G1-phase nucleus (Fasman, 1976).

Flow Cytometry

Approximately 1 × 10^5 intact nuclei from each cell population were fixed in −80°C methanol for 10 min, pelleted, and resuspended in 0.5 ml D-PBS in preparation for analysis by flow cytometry. The DNA was stained by incubating each nuclear sample with 0.25 ml of a 0.2% propidium iodide solution in PBS for 30 min at room temperature. Samples were analyzed at ∼20 nuclei/s on a Becton-Dickinson FACScan using CellFIT software for doublet discrimination and the RFIT (rectangular fit) model for cell cycle staging. LYSYS II was used for graphic representation of the composite histograms. Each histogram represents an analysis of ∼15,000 nuclei.

Resealing of Permeable Nuclear Membranes

Approximately 15,000 LPC-permeabilized quiescent (C2) 3T3 nuclei or G1-phase HeLa nuclei were incubated with "light" mitotic membranes (0.1 μg protein) in a total volume of 20 μl Pipes buffer supplemented with ATP and GTP to a final concentration of 1 mM. Samples were incubated for 1 h at 23°C and the nuclei were pelleted by centrifugation at 47 g for 10 min at 4°C. The nuclei were resuspended in Pipes buffer and counted. The percentage of nuclei excluding TRITC-IgG was determined.

In Vitro Replication

Egg extract was thawed and supplemented with an energy-regenerating system (Blow and Laskey, 1986) and cycloheximide as previously described (Leno and Laskey, 1991). Previously unfrozen or freshly thawed intact, LPC-permeabilized or -resealed nuclei were added at ∼1 ng DNA/μl extract and labeled with either 100 μCi/ml [α-^{32}P]dATP (800 Ci/mmol; New England Nuclear, Boston, MA) or 20 μM 5-biotin-16-deoxyuridine triphosphate (Boehringer Mannheim GmbH, Mannheim, Germany). Samples were incubated for 6 h at 23°C. Determination of the extent of [α-^{32}P]dATP incorporation was as previously described (Leno and Laskey, 1991). Biotin-labeled nuclei were spun onto polylysine-coated coverslips, rinsed with D-PBS and fixed in ice-cold methanol for 15 min.

To detect both incorporated biotinylated dUTP and BrdU within the same nuclei, coverslips of fixed nuclei were rinsed with Tween-Tris-buffered saline (TTBS; 25 mM Tris base, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20; Sigma Chemical Co.) containing 1% BSA and incubated with anti-BrdU monoclonal antibody containing nuclease (Amersham Corp., Arlington Heights, IL) for 1 h at 23°C. Excess antibody was removed by rinsing with TTBS, and the coverslips were then incubated with sheep anti-mouse Texas Red-conjugated immunoglobulin (Amersham Corp.), diluted 1:25 in TTBS, for 2 h. After rinsing with buffer A (Blow and Watson, 1987), each coverslip was incubated in 0.5 ml buffer A containing 5 μl fluorescein streptavidin (Amersham Corp.) and 1 μl Hoechst 33258 (Sigma Chemical Co.) at 100 μg/ml for 10 min. Coverslips were rinsed in deionized water and mounted in phenylenediamine (Sigma Chemical Co.)

mountant. For detection of biotinylated dUTP alone, coverslips of fixed nuclei were rinsed in buffer A, and each was incubated for 15 min in 0.5 ml buffer A supplemented with 5 μl fluorescein streptavidin, 0.5 μl propidium iodide at 1 mg/ml and 0.5 μl RNase A at 10 mg/ml. Coverslips were rinsed in buffer A and mounted as described above.

Density substitution experiments were conducted as previously described (Leno et al., 1992) with the following modifications. 3T3 nuclei were incubated at 1 ng DNA/μl extract supplemented with 0.25 mM BrdUTP (Sigma Chemical Co.) and 100 μCi/ml [α-^{32}P]dATP for 6 h at 23°C. Substituted DNA was separated from unsubstituted DNA by centrifugation to equilibrium in a cesium chloride gradient using a 70.1 Ti rotor (Beckman Instruments Inc., Palo Alto, CA) run at 35,000 rpm for 48 h at 20°C. The refractive index of every fifth gradient fraction was determined and each fraction was counted by liquid scintillation.

Results

Isolation of Intact Nuclei from Exponentially Growing and Contact-inhibited Mouse 3T3 Cells

Mammalian G1-phase nuclei, with intact nuclear membranes, initiate DNA replication when incubated in *Xenopus* egg extract (Leno et al., 1992). We were interested in determining if intact nuclei from quiescent cells, which exit the cell cycle at G1, retain the ability to initiate DNA synthesis in extract even following extended periods of growth arrest. To address this question, mouse 3T3 cells were grown to confluence (i.e., saturation density), and nuclei, with intact nuclear membranes, were isolated from both confluent cultures and exponentially growing cultures by treatment of the cells with the bacterial exotoxin, SLO (Leno et al., 1992). Using a procedure similar to that described by Hugo et al. (1986) and Ahnert-Hilger et al. (1989), SLO selectively permeabilizes the plasma membrane, leaving the underlying nuclear membrane intact (Leno et al., 1992). Intact nuclei were isolated from confluent cells on the day that the cells reached confluence (C0) and every second day thereafter for up to 10 d (C2–C10).

Nuclear membrane integrity was determined by incubating aliquots of SLO-treated cells with TRITC-IgG (Sigma Chemical Co.). TRITC-IgG diffuses across an SLO-permeabilized plasma membrane but not across an intact, selectively permeable nuclear membrane. Thus, exclusion of TRITC-IgG from within the nucleus indicates nuclear membrane integrity (Leno et al., 1992). Fig. 1 A is a composite of three representative fields of SLO-treated, quiescent 3T3 cells, from the 2-d confluent culture (C2), incubated with TRITC-IgG, and viewed by confocal microscopy. The majority of cells showed bright cytoplasmic fluorescence but little or no nuclear fluorescence indicating diffusion of TRITC-IgG across the plasma membrane but not across the intact nuclear membrane. The uniform fluorescence observed within some nuclei (double arrowheads) demonstrates the loss of nuclear membrane integrity presumably during nuclear isolation.

Over 100 nuclei from each cell population were examined by confocal microscopy and the percentage excluding TRITC-IgG was determined (Fig. 1 B). 92% of the nuclei isolated from the exponentially growing cell culture excluded the labeled IgG. The percentage of confluent cell nuclei that excluded label ranged from a high of 87% in the 2-d culture (C2) to a low of 74% in the 6-d culture (C6). We were consistently able to isolate a greater percentage of intact nuclei from exponentially growing cells than from confluent cells. This may indicate that the nuclear membranes from
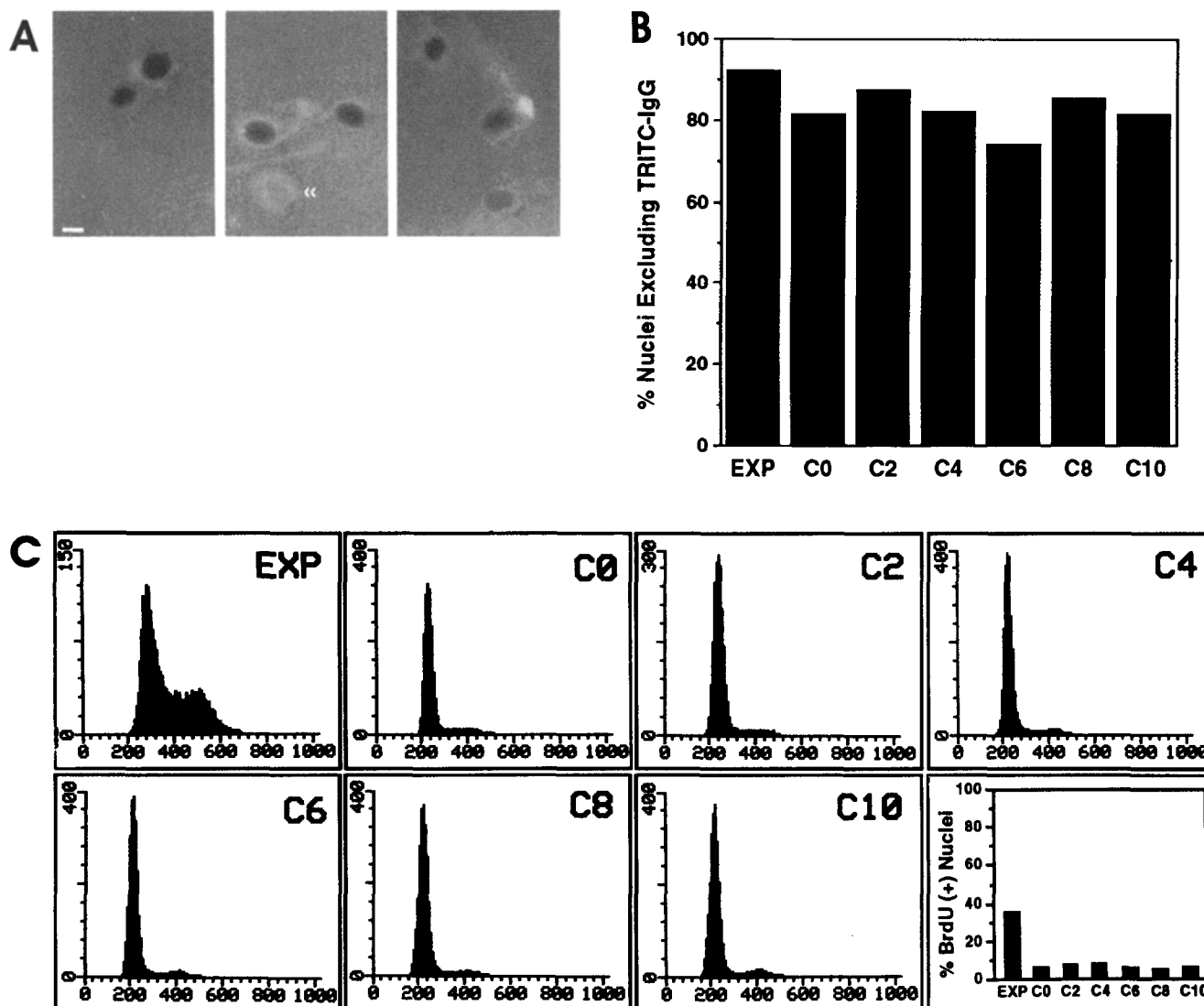
Figure 1. Isolation of intact nuclei from BALB/c 3T3 fibroblasts. Cultures of exponentially-growing and confluent 3T3 cells (*C0-C10*; indicating the days post-confluence) were treated with the bacterial exotoxin SLO as described in Materials and Methods. Aliquots of SLO-treated cells were incubated with TRITC-IgG and viewed, without fixation, by confocal microscopy. (*A*) Three representative fields of SLO-treated cells from the 2-d (*C2*) culture are shown. Most cells showed a bright cytoplasmic fluorescence but little or no nuclear fluorescence demonstrating nuclear membrane integrity, i.e., intact nuclei. However, some cells did show both cytoplasmic and nuclear fluorescence (*double arrowheads*) demonstrating the loss of nuclear membrane integrity presumably during nuclear isolation. (*B*) Over 100 randomly selected cells from each culture were examined for exclusion of TRITC-IgG from the nucleus. The percentage of cells showing nuclear exclusion from each population is shown. (*C*) Intact nuclei, isolated from exponentially growing and confluent cell cultures, were also stained with propidium iodide and analyzed by flow cytometry. Histograms show relative DNA content (x-axis) and nuclear number (y-axis). Approximately 90% of the nuclei from the confluent cell cultures were arrested with a G0/G1 DNA content (*C0-C10*). Nearly 50% of the nuclei from the exponential culture were derived from cells in G1 (*EXP*). All cell cultures were pulse-labeled with BrdU prior to nuclear isolation to identify S-phase cells. In separate experiments, nuclei were probed with an anti-BrdU monoclonal antibody and a fluorescein-conjugated anti-mouse secondary antibody. 100 nuclei from each culture were counted and the percentage of BrdU positive (+) (S-phase) cells was determined (*lower right*). Less than 10% of the nuclei from each confluent culture were BrdU positive. Bar (in *A*), 10 μm.

confluent cells may be more fragile than the nuclear membranes from dividing cells.

To confirm that the 3T3 cells in each confluent population were arrested in G0/G1-phase, we analyzed the DNA content of the isolated nuclei by flow cytometry (Fig. 1 *C*). The results show that ~90% of the nuclei from each confluent cell culture, i.e., from day 0 (C0) through day 10 (C10), were arrested with a diploid DNA content. No discernible increase in the number of S-phase nor G2/M-phase nuclei was

observed throughout this 10-d period suggesting that most confluent cells were indeed quiescent. Flow cytometric analysis of nuclei derived from exponentially growing 3T3 cells (EXP) showed that ~50% of these cells were in G1 at the time of nuclear isolation.

All 3T3 cultures were pulse-labeled with BrdU for 1 h before nuclear isolation. To specifically determine the number of S-phase cells in each culture, an aliquot of each nuclear sample was fixed in methanol and probed with an anti-BrdU

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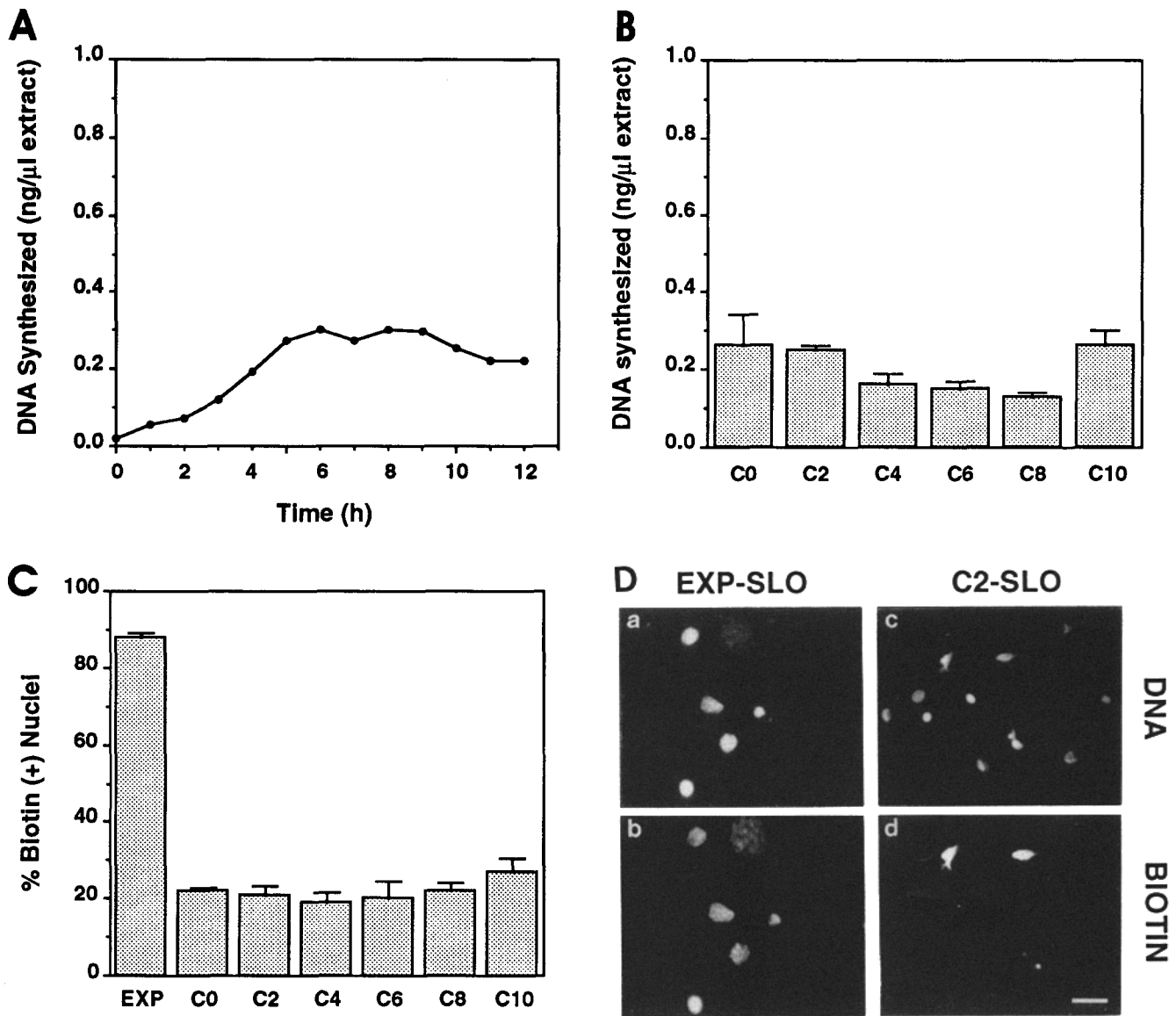
Figure 2. Replication of intact exponential and quiescent cell nuclei in *Xenopus* egg extract. (*A*) Intact quiescent nuclei isolated from the C0 3T3 culture were incubated at 1 ng DNA/μl extract supplemented with 100 μCi [α-^{32}P]dATP for up to 12 h. At hourly intervals, the samples were processed as described in Materials and Methods. The mass of DNA synthesized at each time point is expressed as nanograms per microliter of extract. (*B*) Intact quiescent nuclei isolated from each culture were incubated at 1 ng DNA/μl extract supplemented with 100 μCi [α-^{32}P]dATP. After 6 h, the samples were processed as described in Materials and Methods. The value shown for each culture is the mean of three separate experiments and the bar represents the standard error of each mean. (*C*) Intact exponential and quiescent cell nuclei were incubated for 6 h at 1 ng DNA/μl extract supplemented with 20 μM biotinylated-dUTP. Nuclei were spun onto coverslips, fixed with methanol, stained with propidium iodide to show total DNA, and then stained with fluorescein streptavidin to detect biotin-dUTP incorporated into nascent DNA. More than 100 nuclei from each sample were examined for fluorescein fluorescence by confocal microscopy. The mean percentage of fluorescein-labeled, i.e., biotin-positive (+) nuclei from three separate experiments are shown for each culture. The standard error of each mean is also shown. (*D*) A representative field of intact exponential nuclei (*EXP-SLO*; *a* and *b*) and of intact quiescent nuclei (*C2-SLO*; *c* and *d*) are shown following incubation in egg extract. Nuclei were stained with propidium iodide (*DNA*; *a* and *c*) and probed with fluorescein streptavidin to detect incorporated biotin-dUTP (*Biotin*; *b* and *d*). Bar, 35 μm.

primary antibody followed by a Texas Red–conjugated secondary antibody. 100 nuclei from each sample were analyzed by fluorescence microscopy and scored as BrdU positive or negative. The percentage of BrdU-positive (+) nuclei from each culture is shown in the lower right-hand panel of Fig. 1 *C*. 36% of the exponential nuclei (EXP) were BrdU positive while <10% of the nuclei from each of the confluent cell cultures had incorporated label. These data confirm that

few confluent cells were in S-phase at the time of nuclear isolation.

Most Intact Quiescent Nuclei Do Not Initiate DNA Replication in Xenopus Egg Extract

To determine the extent to which intact quiescent nuclei replicate in the egg extract we conducted time course experi-

ments using nuclei isolated from the C0 culture. Intact nuclei were incubated for up to 12 h at ~1 ng DNA/μl of extract supplemented with [α-^{32}P]dATP. At hourly intervals, the reactions were stopped and the DNA was purified and precipitated with trichloroacetic acid. The results of a typical experiment are shown in Fig. 2 A where the extent of replication is expressed as nanograms of DNA synthesized per microliter of extract. DNA synthesis increased during the first 6 h of incubation and then reached a plateau after which replication decreased slightly. In this case, only 0.3 ng of DNA was synthesized representing replication of 30% of the input DNA. While the extent of replication of intact C0 nuclei varied slightly among experiments (see Fig. 2 B), in nearly all cases, the amount of DNA synthesized did not increase significantly after 6 h of incubation. Therefore, we used a single 6 h time point in all subsequent experiments.

To determine if the extent of replication of intact nuclei was correlated with the duration of growth arrest, we incubated nuclei from each cell population for 6 h in egg extract supplemented with [α-^{32}P]dATP. The concentration of DNA added was ~1 ng/μl of extract. Fig. 2 B shows the mean values from three separate experiments with each bar indicating the standard error of the mean. In this set of experiments, the average mass of DNA synthesized ranged from a low of 0.13 ng/μl (C8) to a high of 0.26 ng/μl (C0 and C10) representing 13 and 26% of the total template replicated, respectively. These data illustrate two important points. First, unlike intact G1 HeLa nuclei which replicate extensively in the egg extract (Leno et al., 1992; Coverley et al., 1993), intact quiescent nuclei undergo limited replication in this system. Second, the replication capacity of these intact quiescent nuclei does not appear to be correlated with the duration of growth arrest (Fig. 2 B; compare mean values for C0 and C10 samples).

The small amount of DNA synthesis observed with intact quiescent nuclei could be due to either complete replication of a small subset of nuclei or to limited replication within most nuclei. To distinguish between these two possibilities, we used an approach that allows for the identification of individual nuclei undergoing DNA replication. Specifically, we incubated intact nuclei from exponential (EXP) and quiescent (C0-C10) 3T3 cells in egg extract supplemented with 20 μM biotinylated-dUTP. We chose to use exponential cells as a source for our G1 "control" nuclei in these experiments primarily to avoid inducing a temporary G0 arrest in our G1 cells as a result of synchronization (Zickert et al., 1993). Following a 6-h incubation in extract, samples were spun onto coverslips, fixed in methanol, and stained with both propidium iodide to label total DNA and fluorescein streptavidin to detect the biotinylated dUTP incorporated into nascent DNA. The percentage of biotin-positive (+) nuclei in each sample was determined. Fig. 2 C shows the mean values from three separate experiments along with the standard error of each mean. In these experiments, an average of 88% of the intact exponential nuclei (EXP) incorporated biotin as judged by fluorescein fluorescence. A representative field of exponential nuclei from one experiment is illustrated in Fig. 2 D (EXP-SLO; a and b). This observation, coupled with the fact that ~50% of these nuclei were derived from G1 cells (Fig. 1 C), suggests that most intact G1 nuclei replicated during extract incubation. This idea is consistent with previous results showing that intact G1 nuclei from

HeLa cells initiate replication in this system (Leno et al., 1992).

In contrast to the results obtained with exponential nuclei, few intact quiescent nuclei incorporated biotin in the extract. The mean percentage of biotin positive quiescent nuclei ranged from only 19% (C4) up to 27% (C10) (Fig. 2 C). A representative field of quiescent nuclei from one experiment is illustrated in Fig. 2 D (C2-SLO; c and d). To determine how many of the intact quiescent nuclei that replicated in egg extract were actually S-phase contaminants, we incubated nuclei from each cell population in extract supplemented with biotin-dUTP and then probed the fixed nuclei with Texas Red–streptavidin, to detect incorporated biotin, and with anti-BrdU monoclonal antibody and fluorescein-conjugated secondary antibody to detect incorporated BrdU. We found that an average of 8% of all intact quiescent nuclei that incorporated biotin in extract had previously incorporated BrdU in vivo (data not shown) suggesting that these S-phase nuclei were undergoing residual DNA synthesis in the extract. Taken together, these data demonstrate that most intact nuclei from quiescent cells do not initiate DNA replication in egg extract, irrespective of the duration of growth arrest.

Nuclear Membrane Permeabilization Allows Replication of Quiescent Cell Nuclei in Egg Extract

Mammalian G2-phase nuclei, with intact nuclear membranes, are unable to initiate DNA replication in the egg extract. However, if the nuclear membranes of these G2 nuclei are permeabilized with detergent prior to addition to the extract, virtually all of these nuclei initiate replication during a subsequent extract incubation (Leno et al., 1992). Thus, we were interested in determining if nuclear membrane integrity was also preventing replication of quiescent cell nuclei in this system.

To address this question, we first treated intact nuclei from exponential and quiescent cell populations with lysophosphatidylcholine (LPC). Aliquots of LPC-treated nuclei were then incubated with TRITC-IgG to determine nuclear membrane integrity. Greater than 99% of all nuclei from each population included TRITC-IgG within the nucleus (see Fig. 4 A, b) demonstrating that the nuclear membranes of virtually all nuclei were permeabilized by this treatment. Permeable nuclei from each quiescent cell population were then incubated in extract supplemented with [α-^{32}P]dATP for 6 h. In contrast to the low levels of replication observed with intact quiescent nuclei (Fig. 2, A and B), permeable quiescent nuclei replicated extensively (Fig. 3 A). In this set of experiments, the average mass of DNA synthesized ranged from 0.79 ng/μl (C4 and C6) to 0.93 ng/μl (C8) representing 79 and 93% of the total template replicated, respectively. Consistent with these results was the observation that nearly all permeable quiescent nuclei incorporated biotinylated dUTP into nascent DNA during a similar 6-h incubation in the extract (Fig. 3 B). The mean number of biotin positive nuclei from three separate experiments ranged from 84 (C2) to 95% (C6 and C10). An average of 96% of the permeable exponential nuclei replicated after 6 h in the extract (Fig. 3 B). Fig. 3 C shows a representative field of permeable exponential nuclei (EXP-LPC; a and b) and of permeable quiescent nuclei (C2-LPC; c and d) following incubation in extract.
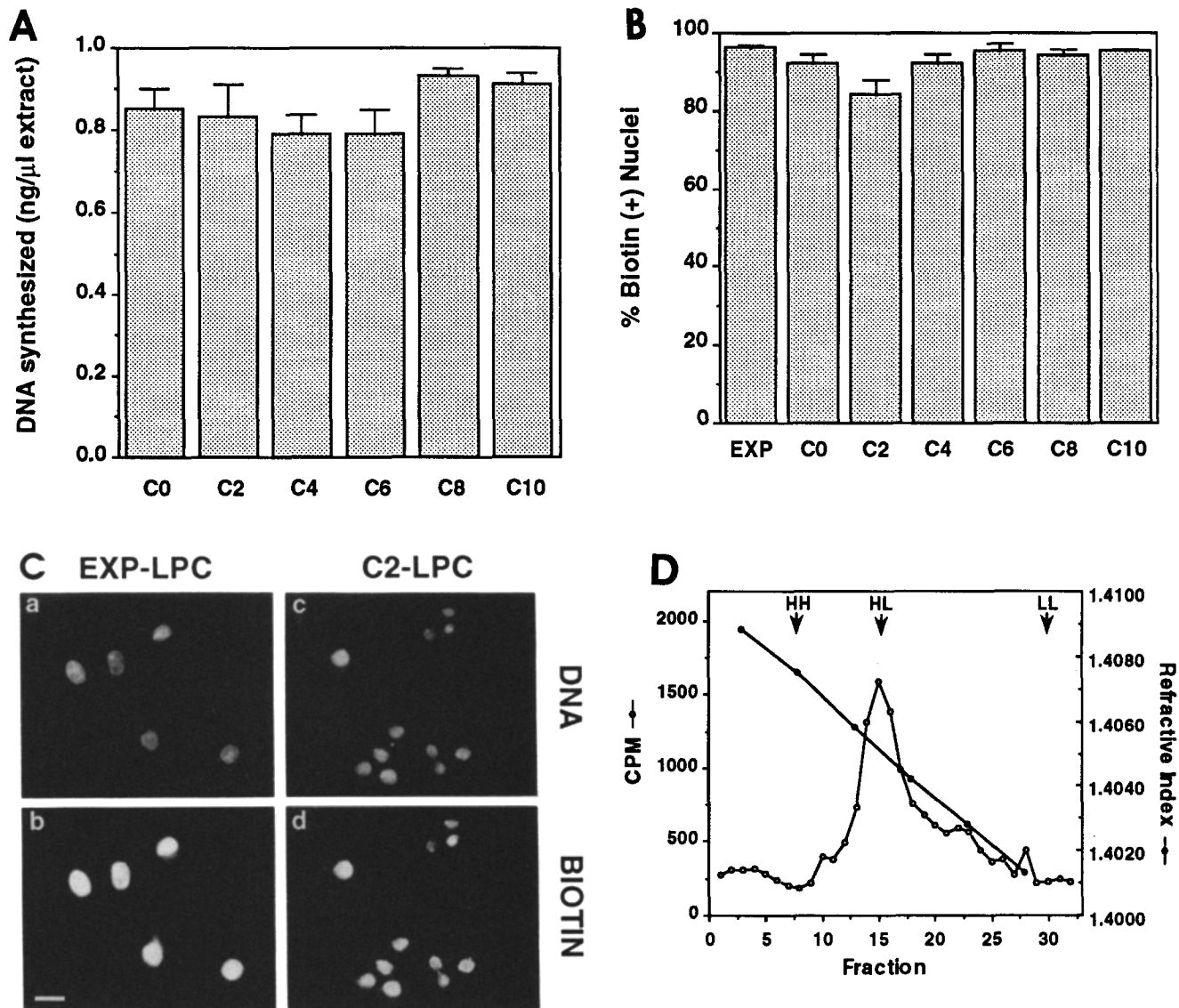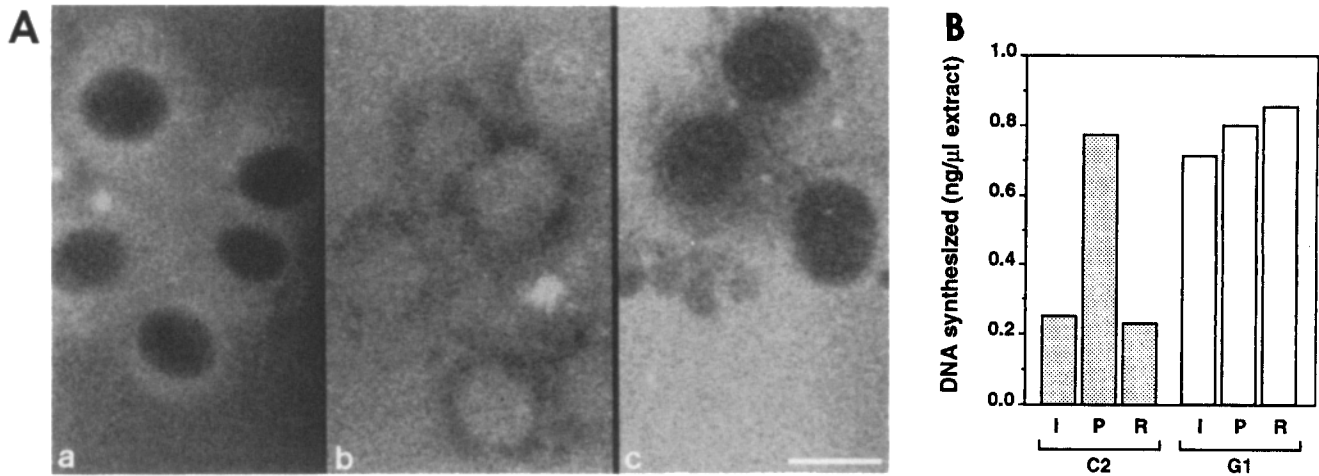
Figure 3. Permeabilizing the nuclear membranes of intact quiescent nuclei allows replication in egg extract. Intact exponential and quiescent cell nuclei were permeabilized with LPC (for example see Fig. 4 *A*, *b*) and incubated at 1 ng DNA/μl extract supplemented with 100 μCi [α-^{32}P]dATP (*A*) or 20 μM biotin-dUTP (*B*) for 6 h at 23°C. Incubated nuclei were processed as described in the legend for Fig. 2, *B* and *C* and in Materials and Methods. (*A*) The mass of DNA synthesized is expressed as nanograms per microliter of extract. The value shown for each culture is the mean of three separate experiments and the bar represents the standard error of each mean. (*B*) Over 100 nuclei from each sample were examined for fluorescein fluorescence by confocal microscopy. The mean percentage of fluorescein-labeled, i.e., biotin-positive (+), nuclei from three separate experiments are shown for each culture. The standard error of each mean is also shown. (*C*) A representative field of permeable exponential nuclei (*EXP-LPC*; *a* and *b*) and of permeable quiescent nuclei (*C2-LPC*; *c* and *d*) are shown following incubation in egg extract. Nuclei were stained with propidium iodide (*DNA*; *a* and *c*) and probed with fluorescein streptavidin to detect incorporated biotin-dUTP (*Biotin*; *b* and *d*). (*D*) Permeable quiescent nuclei (C2) were incubated at 1 ng DNA/μl extract supplemented with 0.25 mM BrdUTP (Sigma Chemical Co.) and 100 μCi/μl [α-^{32}P]dATP for 6 h at 23°C. Substituted DNA was separated by centrifugation to equilibrium in a cesium chloride gradient. Gradient fractions were collected and the refractive index of every fifth fraction was determined (●). The counts per minute (*CPM*) in each fraction were determined by liquid scintillation (○). The peak of incorporation was observed at a density of ∼1.76 g/cm^3 indicating heavy/light (*HL*) DNA. The expected densities of heavy/heavy (*HH*) and unsubstituted light/light (*LL*) DNA are ∼1.79 g/cm^3 and ∼1.71 g/cm^3, respectively. Bar (in *C*), 25 μm.

These results demonstrate that permeabilizing the nuclear membranes of intact quiescent cell nuclei prior to incubation in the extract allows nearly complete replication of all of these nuclei during a subsequent extract incubation. Furthermore, density substitution experiments confirmed that 3T3 nuclear DNA was replicated semi-conservatively and limited to one round in the extract. In this experiment, permeable quiescent 3T3 nuclei from the 2-d confluent culture (C2) were incubated in extract supplemented with 0.25 mM BrdUTP and 100 μCi/ml [α-^{32}P]dATP. The DNA was purified and separated by centrifugation to equilibrium in a cesium chloride gradient. A single prominent peak of ^{32}P

Figure 4. Resealing the nuclear membranes of permeable quiescent nuclei prior to extract incubation prevents DNA replication in a subsequent incubation. Intact quiescent (C2) 3T3 nuclei or G1-phase HeLa nuclei were permeabilized with LPC and subsequently "resealed" following incubation for 1 h with membranes isolated from *Xenopus* eggs. (A) Aliquots of intact, permeable and resealed C2 3T3 or G1 HeLa (not shown) nuclei were combined with TRITC-IgG and examined by confocal microscopy. TRITC-IgG was excluded from ~80% of the nuclei in this intact C2 population (a). LPC permeabilization resulted in a uniformly bright nuclear fluorescence in >99% of these nuclei (b). Incubation of these permeable nuclei with *Xenopus* egg membranes resulted in exclusion of TRITC-IgG from 80 to 85% (c) indicating resealing (i.e., repair) of their nuclear membranes. (B) Intact, permeable and resealed C2 nuclei (C2, shaded) or G1-phase HeLa nuclei (G1, unshaded) were incubated at 1 ng DNA/μl extract supplemented with 100 μCi [α-^{32}P]dATP for 6 h at 23°C. The samples were processed as described in Materials and Methods. The mass of DNA synthesized is expressed as nanograms per microliter of extract. The value shown for each sample is the mean of two separate experiments. *I*, intact; *P*, permeable; and *R*, resealed. Bar (a–c) 13 μm.

incorporation was observed at a density of ~1.76 g/cm³ (Fig. 3 D; heavy/light DNA; HL) demonstrating a single round of semi-conservative DNA replication in the egg extract.

The Effect of Lysophosphatidylcholine Treatment on DNA Replication in Quiescent Cell Nuclei Is Reversible

To determine if the effect of LPC treatment on DNA replication in quiescent 3T3 nuclei is directly related to nuclear membrane permeability rather than the alteration of some other aspect of nuclear structure, we conducted nuclear membrane resealing (i.e., repair) experiments similar to those described by Coverley et al. (1993). Initially, intact quiescent (C2) 3T3 or G1-phase HeLa nuclei were permeabilized with LPC and subsequently incubated with a membrane fraction from *Xenopus* eggs for 1 h. Aliquots of intact, permeable, and resealed quiescent 3T3 nuclei (Fig. 4 A) or G1 HeLa nuclei (not shown) were then incubated with TRITC-IgG to determine nuclear membrane integrity. In the intact quiescent 3T3 sample, ~80% of the nuclei excluded TRITC-IgG (Fig. 4 A, a). After permeabilization with LPC, >99% of these nuclei included the labeled IgG demonstrating permeabilization of their nuclear membranes (Fig. 4 A, b). After incubation of these permeable nuclei with egg membranes, ~80% again excluded TRITC-IgG (Fig. 4 A, c) demonstrating that their nuclear membranes had been resealed. Approximately 86% of intact G1 HeLa nuclei excluded TRITC-IgG; however, >99% of these nuclei included the label following LPC treatment. Over 80% of these permeable nuclei again excluded labeled IgG following incubation with egg membranes demonstrating nuclear membrane repair.

Intact, permeable or resealed C2 or G1 nuclei were added

to egg extract at 1 ng DNA/μl and the extract was supplemented with 100 μCi/ml [α-^{32}P]dATP. Samples were then incubated for 6 h at 23°C. The mass of DNA synthesized in each sample was determined as described in Materials and Methods. The results shown in Fig. 4 B are mean values from two independent experiments in which permeable nuclei were resealed with a similar efficiency, i.e., ~80%. Consistent with previous results, intact quiescent nuclei (Fig. 4 B, C2, I) underwent limited replication in the extract while permeable quiescent nuclei (C2, P) replicated extensively in this system (compare Fig. 4 B with Figs. 2 B and 3 A). However, when permeable nuclei were resealed before the addition to the extract, replication in a subsequent incubation was reduced approximately fourfold relative to the permeable sample (Fig. 4 B, C2, R) but it was similar to the extent of replication observed with intact nuclei. These results are in striking contrast to the results observed with resealed G1 nuclei. In this latter case, resealed G1 nuclei (Fig. 4 B, G1, R) replicated as extensively as did permeable nuclei (Fig. 4 B, G1, P) consistent with the results of Coverley et al. (1993). Taken together, these results demonstrate two important points. First, the effect of LPC on DNA replication in quiescent cell nuclei is at the level of the nuclear membrane. Second, the inability of resealed quiescent nuclei to replicate is not due to a nonspecific effect of egg membranes on permeable nuclei, as evidenced by the replication of resealed G1 nuclei, but rather it appears to be related to some specific difference in replication capacity between the nuclei of quiescent and cycling G1 cells.

Discussion

We have investigated the replication capacity of intact nuclei from quiescent cells using *Xenopus* egg extract. Surpris-

ingly, we find that most intact quiescent nuclei, unlike nuclei from G1-phase cells, do not initiate DNA replication in the extract (Fig. 2). However, when the nuclear membranes of these intact nuclei are permeabilized with LPC before addition to the extract, nearly all nuclei undergo a single round of replication during the subsequent extract incubation (Fig. 3). Furthermore, resealing the nuclear membranes of these permeable quiescent nuclei prior to addition to the extract resulted in the loss of replication capacity during the subsequent extract incubation, demonstrating that the effect of LPC treatment on DNA replication is indeed at the level of the nuclear membrane (Fig. 4). Taken together, these data indicate that G1 nuclei lose the capacity to initiate replication following density-dependent growth arrest and that changes in nuclear membrane permeability may be required for the initiation of replication upon re-entry of the quiescent cell into the cell cycle.

There are at least three general ways in which intact quiescent nuclei could be prevented from initiating DNA replication in the extract. First, replication of quiescent nuclei could be prevented by a growth-arrest–specific inhibitor of initiation which remains within the nucleus following nuclear isolation. This inhibitor could be acting directly at the level of initiation by binding to the DNA or alternatively, it could act by binding and inactivating an essential replication protein such as a "licensing" factor (Blow and Laskey, 1988). A growth-arrest–specific inhibitor could also indirectly prevent initiation of replication through a variety of mechanisms. Second, quiescent nuclei may not be "licensed" for replication and if this replication license is a DNA-bound initiation factor (Blow and Laskey, 1988), loss of initiation capacity following growth arrest could result from inactivation or degradation of this factor. Thus, an intact nuclear membrane could be preventing initiation of replication in quiescent cell nuclei either by preventing the loss of a diffusible inhibitor or by excluding fresh "licensing" factor from the nucleus. The demonstration that resealed quiescent nuclei do not replicate in the extract (Fig. 4) suggests that the inability of intact nuclei to replicate is not due to a diffusible inhibitor of initiation. Rather our results suggest that exposure of permeable quiescent nuclei to the egg extract may be required for initiation. However, it is important to point out that these data do not exclude the possibility that a nondiffusible, chromatin-associated inhibitor is present within the nuclei of quiescent cells and that extract exposure is required for inactivation of this inhibitor.

Third, quiescent cell nuclei could be prevented from replicating in egg extract by a variety of specific growth arrest–related changes in nuclear structure and/or function. One intriguing possibility involves the reduced capacity for signal-mediated nuclear transport in growth-arrested cells (Feldherr and Akin, 1990, 1991, 1993). It has been suggested that changes in cell shape may alter the nucleocytoplasmic transport properties of the nuclear envelope and, in turn, the capacity of the nucleus for DNA replication (Ingber and Folkman, 1989; Ingber, 1990). This view is supported by the observations that flattened 3T3 cells have a greater functional pore size than rounded cells and that more flattened cells synthesize DNA than rounded cells (Feldherr and Akin, 1993). Thus, the changes in cell shape that accompany density-dependent growth arrest could alter nuclear pore structure which, in turn, could lead to a reduction in enve-

lope permeability and the inability of intact quiescent nuclei to initiate DNA replication. However, Feldherr and Akin (1991) found that the functional nuclear pore diameter in confluent cells appears to be sufficiently large to allow the import of most nuclear proteins suggesting to us that the inability of quiescent cell nuclei to initiate replication in extract may not be due to a reduction in their capacity for import of nuclear proteins. Yet, it is possible that replication is dependent upon the import of a large protein complex and that this complex is unable to cross the nuclear membrane of a quiescent nucleus.

Whichever of these or other possibilities are correct, the observation that nuclear membrane permeabilization is necessary for replication of quiescent nuclei in egg extract suggests that changes in membrane permeability may be required for entry of quiescent cells into S-phase following release from growth arrest. Such permeability changes could be due to alterations in either signal-mediated nuclear transport or in the passive diffusion of molecules through the nuclear pores (Jiang and Schindler, 1988).

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