

# Reversible Effects of Nuclear Membrane Permeabilization on DNA Replication: Evidence for a Positive Licensing Factor

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**Abstract.** We have investigated the mechanism which prevents reinitiation of DNA replication within a single cell cycle by exploiting the observation that intact G2 HeLa nuclei do not replicate in *Xenopus* egg extract, unless their nuclear membranes are first permeabilized (Leno et al., 1992). We have asked if nuclear membrane permeabilization allows escape of a negative inhibitor from the replicated nucleus or entry of a positive activator as proposed in the licensing factor hypothesis of Blow and Laskey (1988). We have distinguished these possibilities by repairing permeabilized nuclear membranes after allowing soluble factors to escape. Membrane repair of G2 nuclei reverses the effects of permeabilization arguing that escape of diffusible inhibitors is not sufficient to allow replica-

tion, but that entry of diffusible activators is required. Membrane repair has no significant effect on G1 nuclei. Pre-incubation of permeable G2 nuclei in the soluble fraction of egg extract before membrane repair allows semiconservative DNA replication of these nuclei when incubated in complete extract. Addition of the same fraction after membrane repair has no effect. Our results provide direct evidence for a positively acting "licensing" activity which is excluded from the interphase nucleus by the nuclear membrane. Nuclear membrane permeabilization and repair can be used as an assay for licensing activity which could lead to its purification and subsequent analysis of its action within the nucleus.

**E**UKARYOTIC cells normally replicate their DNA only once between mitoses so reinitiation of replication in a single cell cycle must be blocked. In 1970 Rao and Johnson performed cell fusion experiments which demonstrated a fundamental difference in the ability of G1 and G2 phase cells to replicate their DNA (Rao and Johnson, 1970). They showed that G1 nuclei are induced to enter S phase prematurely when fused to S phase cells, but G2 nuclei are not. Similarly, in an exhaustive study of *Drosophila* embryos new replication bubbles were never observed on replicated DNA (Blumenthal et al., 1973). In this paper we investigate the mechanism which prevents reinitiation of replication within a single cell cycle.

The block to reinitiation of replication can be overcome by permeabilizing the nuclear membrane. *Xenopus* sperm nuclei undergo a single complete round of DNA replication without reinitiating when incubated in *Xenopus* egg extract (Blow and Laskey, 1986). However, when the nuclear membrane of a replicated sperm nucleus is made permeable by lysolecithin treatment, the replicated nucleus can initiate a new round of replication (Blow and Laskey, 1988). This observation demonstrated that an intact nuclear membrane is required to prevent reinitiation of replication and it led to the proposal of a "licensing factor" model (Blow and Laskey, 1988). The model speculates that a hypothetical factor which is essential for the initiation of DNA replication lacks a nuclear localization signal and therefore can only enter the nu-

cleus during mitosis when the nuclear membrane breaks down. The model assumes that active factor would be destroyed as a consequence of replication and that new factor would become available in the nucleus when the nuclear membrane breaks down again at the next mitosis. According to the model G1 nuclei would possess such a factor but G2 nuclei would not so DNA synthesis could occur only once between consecutive mitoses.

The model can explain the HeLa cell fusion experiments of Rao and Johnson (1970) and in addition it predicts that nuclear membrane permeabilization would allow a new round of replication in G2 nuclei. This possibility was tested by Leno et al. (1992) using nuclei isolated from synchronized G1 and G2 phase HeLa cells incubated in *Xenopus* egg extract. Permeabilization successfully induced G2 HeLa nuclei to replicate confirming the involvement of the nuclear membrane in the prevention of reinitiation.

These experiments establish clearly that treatments which permeabilize the nuclear membrane cause reinitiation of replication, and to that extent they support the licensing factor hypothesis. However, they do not distinguish between the two possibilities, that positive factor is allowed to enter by permeabilization or that a negative inhibitory factor is allowed to escape. In addition it remains possible that the permeabilization treatments also act on chromatin rather than just the membrane.

Here we investigate how an intact nuclear membrane pre-

vents reinitiation of replication and how permeabilization of the nuclear membrane causes over-replication. First, we show that permeabilized nuclear membranes can be repaired by membrane vesicles to restore selective protein import. Second, we show that membrane repair reverses the effects of permeabilization on DNA replication, confirming that permeabilization treatments exert their effects by acting on the nuclear membrane and not on the chromatin within. Third, and most importantly, we demonstrate that the nuclear membrane prevents reinitiation of replication by excluding an essential positive activator, rather than by retaining a negative inhibitor. Although Blow and Laskey (1988) had postulated that a positive factor might exist, it had not been demonstrated experimentally.

We argue that if replication in G2 nuclei is prevented by the existence of an inhibitory activity which can escape when the nuclear membrane is permeabilized, then repairing the membrane of permeabilized nuclei before addition to extract would not prevent replication because the inhibitor would still have had the opportunity to escape. However, if replication is the consequence of inward movement of a positive "licensing" activity, membrane repair before addition to extract would reimpose the block to replication present in intact nuclei. We present evidence that nuclear membrane repair reverses the effect of permeabilization unless the permeable nucleus is exposed to the soluble fraction of egg extracts before repair. Therefore we conclude that an essential replication activator is excluded by the nuclear membrane as proposed in the licensing factor hypothesis. We discuss how this might act directly or indirectly via other factors.

In addition, this work provides a functional assay for licensing activity as exposure of permeable G2 nuclei to protein fractions, before membrane repair and incubation in complete egg extract, will allow identification of essential activities which have only limited access to the nucleus *in vivo*.

## Materials and Methods

### Preparation and Fractionation of Extract

Low-speed *Xenopus* egg extracts (LSS)<sup>1</sup> were prepared essentially according to the procedure described by Blow and Laskey (1986). Extraction buffer (50 mM Hepes-KOH, pH 7.4, 50 mM KCl, 5 mM MgCl<sub>2</sub>) was thawed and supplemented with 1 mM DTT, 1 μg/ml leupeptin, pepstatin A, chymostatin, aprotinin, and 10 μg/ml cytochalasin B (Sigma Immunochemicals, St. Louis, MO) immediately before use. Extracts were supplemented with 2% glycerol and snap-frozen as 10–20-μl beads in liquid nitrogen or subjected to further fractionation.

High speed supernatant (HSS) and membrane fractions were prepared from low-speed egg extract as described (Sheehan et al., 1988). Membranous material, isolated by centrifugation of 1–2 ml of low-speed extract, was washed at least two times by dilution in 5 ml extraction buffer. Diluted membranes were centrifuged for 10 min at 10 krpm in an SW50 rotor (SW50; Beckman Instruments, Inc., Palo Alto, CA) to yield vesicle fraction 1. The supernatant was then centrifuged for a further 30 min at 30 krpm to yield vesicle fraction 2. Washed membranes were supplemented with 5% glycerol and snap-frozen in 5-μl beads in liquid nitrogen. Vesicle fractions 1 and 2 were mixed in equal proportions before use in nuclear membrane repair reactions.

1. *Abbreviations used in this paper:* BrdUTP, bromodeoxyuridine; HSS, high speed supernatant; LSS, low speed supernatant; SLO, streptolysin O.

### Preparation of Synchronous Populations of Nuclei

Cell culture and synchronization were carried out as previously described (Leno et al., 1992). Nuclei were prepared as described except that all incubations were carried out in HE buffer (50 mM Hepes-KOH, pH 7.4, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 1 μg/ml aprotinin, pepstatin, leupeptin, chymostatin).

### Nuclear Membrane Permeabilization

SLO-prepared nuclei (Leno et al., 1992) were incubated with 20 μg/ml lysolecithin (Sigma Immunochemicals) and 10 μg/ml cytochalasin B in HE at a concentration of  $\sim 1.5 \times 10^4$  nuclei/ml for 10 min at 23°C with occasional gentle mixing. Reactions were stopped by the addition of 1% nuclease free BSA (Sigma Immunochemicals). Nuclei were gently pelleted by centrifugation in a RC5B rotor (Sorvall Instruments, Newton, CT) at 500 rpm for 5 min and then washed three times by dilution in 1 ml HE. Pelleted nuclei were recovered in a small volume of buffer and resuspended to  $\sim 1 \times 10^4$  nuclei/μl.

### Nuclear Membrane Repair

Lysolecithin-permeabilized nuclei were repaired by incubation with membrane components prepared from *Xenopus* egg extracts. Nuclei at a concentration of  $\sim 5,000/\mu\text{l}$  were mixed with an equal volume of pooled vesicular fractions 1 and 2 and supplemented with 1 mM GTP and ATP. 10–20-μl reactions were incubated at 23°C for up to 90 min with occasional gentle mixing. Aliquots were taken at intervals and assayed for nuclear permeability.

### Permeability Assays

**Exclusion of Fluorescently Labeled Molecules.** Permeability of the plasma membrane after SLO treatment and of the nuclear membrane after lysolecithin treatment were both assessed by monitoring the extent of inclusion of tetramethyl rhodamine linked 40 kD neutral dextran molecules (Sigma Immunochemicals). Nuclei in HE were incubated with dextran at 200 μg/ml and visualized by fluorescent confocal microscopy. Determination of the extent of membrane permeability was made after 2-min incubations with dye. For determination of the extent of membrane repair, incubations were for 10–20 min. This longer incubation allows visualization of any nuclei which remain slightly permeable after incubation in membrane repair reactions.

**Protein Import into the Nucleus.** Nuclear protein import in repaired nuclei was assessed by incubation in *Xenopus* egg LSS supplemented with T7 polymerase with or without a functional nuclear targeting signal (Dunn et al., 1988) (kindly provided by Colin Dingwall, University of Cambridge at Wellcome/CRC Institute). After 30 min at 23°C nuclei were fixed with 4% paraformaldehyde, and spun through a 30% sucrose cushion onto polylysine-coated coverslips (Mills et al., 1989). Coverslips were incubated with anti-T7 polyclonal antiserum (a kind gift of Paul Fisher, State University of New York, Stony Brook, NY), and then fluorescein-conjugated anti-rabbit antibody (Vector Laboratories, Burlingame, CA).

### Replication Reactions

Typical replication reactions contained  $\sim 500$  HeLa nuclei per μl of *Xenopus* egg LSS in a final volume of 15–30 μl. Reactions were supplemented with a 150 μg/ml creatine phosphokinase and a 10× reaction mix containing the following (concentration in reaction): 50 μM dATP, dCTP, dGTP, and TTP, 60 mM phosphocreatine, 100 μg/ml cycloheximide, 1 mM ATP, and 20 mM Hepes-KOH, pH 7.8. Biotin-dUTP (Sigma Immunochemicals) at 20 μM or [ $\alpha^{32}\text{P}$ ]-labeled dATP at 2 μCi/10 μl of extract (3,000 Ci/mM; New England Nuclear, Boston, MA) were included to allow quantitation of DNA replication. Reactions were allowed to proceed for 4–5 h except for the experiment shown in Fig. 5 which was the result of an overnight incubation. Biotin-labeled reactions were stopped, fixed, stained, and visualized as described (Mills et al., 1989). Where results are expressed as the percent of nuclei which stained positively for biotin, between one and two hundred nuclei were scored. DNA replication reactions which contained [ $\alpha^{32}\text{P}$ ]-labeled dATP were stopped by the addition of 80 μl of 0.5% SDS, 20 mM Hepes-KOH, pH 7.4, 20 mM EDTA. After incubation for 1 h at 37°C with 1 mg/ml Proteinase K (Sigma Immunochemicals) reactions were extracted two times with phenol/chloroform, ethanol precipitated, and resuspended in water. Nuclear DNA was separated from RNA and any remaining unincorporated label by electrophoresis through a 0.5% agarose gel in TBE in the presence of 0.5 μg/ml ethidium bromide. DNA synthesis was

visualized by autoradiography and quantified by liquid scintillation counting of bands cut from dried agarose gels. Results were normalized for DNA content by densitometry of photographs of stained gels and sometimes expressed as the percent of input DNA which was replicated. For the experiment shown in Fig. 5, 250  $\mu$ M bromodeoxyuridine triphosphate was included in the replication reaction (Sigma Immunochemicals). Substituted DNA was separated from unsubstituted DNA by centrifugation through a CsCl gradient as described by Blow and Laskey (1986).

## Results

### *An Intact Nuclear Membrane Is Required to Prevent Reinitiation of Replication in G2 Nuclei*

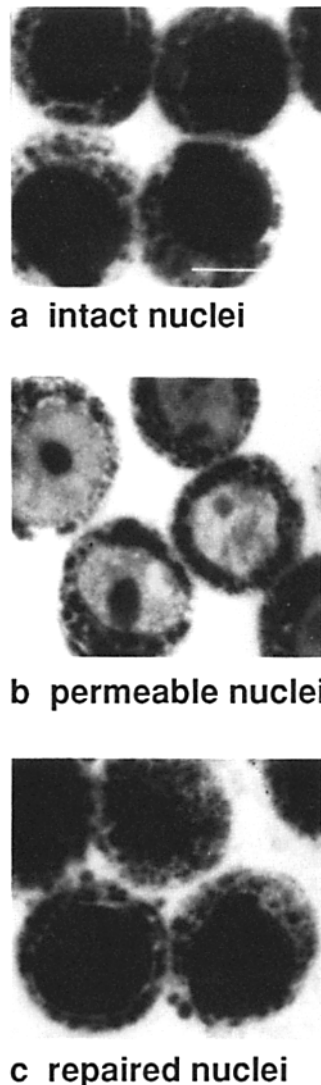
To investigate the role of the nuclear membrane in controlling DNA replication, nuclei were prepared from a synchronized G2 population of HeLa cells using the bacterial exotoxin streptolysin O (SLO) (Alouf, 1980; Duncan and Schlegel, 1975; Prigent and Alouf, 1976). Under the conditions described by Leno et al. (1992) SLO generates a cell population with uniform plasma membrane permeability while >95% of nuclear membranes remain intact. This high specificity for the plasma membrane allows direct investigation of the role of nuclear membrane integrity in the control of DNA replication.

G2 nuclei which possess an intact nuclear membrane are unable to replicate their DNA when incubated in *Xenopus* egg extract, however when the nuclear membrane is permeabilized by treatment with NP-40 they become competent for replication (Leno et al., 1992). We have confirmed these results by permeabilizing with low concentrations of the phospholipid lysolecithin (data shown below) and extended them to distinguish positive models of replication control from negative ones by reversing lysolecithin treatment by specific membrane repair.

### *A Vesicle Fraction Prepared from Xenopus Egg Extracts Repairs the Membrane of Lysolecithin-treated HeLa Nuclei*

Nuclei prepared by the SLO method were permeabilized using low concentrations of lysolecithin (Blow and Laskey, 1988; Gurdon, 1976) and then repaired by incubation with membrane components isolated from *Xenopus* egg extract. Fractionation of *Xenopus* egg low-speed extract by centrifugation yields a heterogeneous vesicle population (Sheehan et al., 1988) which was collected and extensively washed (see Materials and Methods) to yield a vesicle fraction capable of repairing nuclear membranes permeabilized by treatment with lysolecithin. The plasma membrane of SLO-prepared nuclei was never detectably affected by this vesicle fraction. Nuclei treated with low lysolecithin concentrations (up to  $\sim$ 20  $\mu$ g/ml) were fully repaired by incubation with the vesicle fraction, but nuclei treated with higher concentrations were repaired comparatively poorly. Permeabilization with lysolecithin at 20  $\mu$ g/ml allows reinitiation of replication in G2 nuclei, but at lower levels than those achieved by higher concentrations. As efficient repair is essential for the experiments described here all permeable populations of nuclei were generated by treatment with lysolecithin at 20  $\mu$ g/ml.

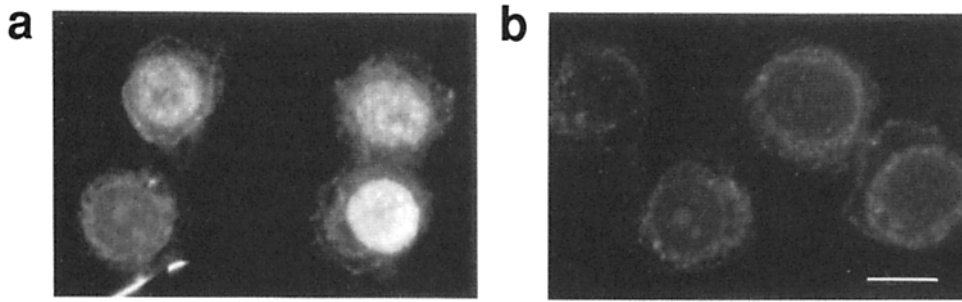
Intact, permeable and repaired populations of nuclei were tested for nuclear permeability before (Fig. 1) and immediately after (not shown) addition to egg extract. Nuclei were incubated with fluorescently labeled 40-kD dextran mole-



**Figure 1.** Repaired nuclear membranes exclude fluorescently labeled dextran molecules. G2 HeLa nuclei were prepared with (a) intact nuclear membranes, (b) lysolecithin-permeabilized membranes, and (c) nuclear membranes which had been permeabilized and then repaired. The three populations were incubated with 200  $\mu$ g/ml rhodamine-conjugated 40-kD dextran molecules and diffusion into the nucleus was assessed by fluorescent confocal microscopy. All populations of nuclei used for subsequent experiments were screened in this way. Bar, 10  $\mu$ m.

cules then viewed using a confocal microscope. Routinely, >90% of all nuclei prepared with SLO showed nuclear integrity by excluding fluorescent dextran from the nucleus (Fig. 1 a). After permeabilization with 20  $\mu$ g/ml lysolecithin almost 90% of nuclei allowed entry of fluorescent dextran molecules (Fig. 1 b), although the degree of permeability varied between nuclei. After nuclear membrane repair by treatment with membrane vesicles in the presence of ATP and GTP (see Materials and Methods) fluorescent dextran is excluded again (Fig. 1 c). Repair was routinely assessed on the basis of dextran exclusion and only those populations in which essentially all nuclei excluded dextran were used for further study. In addition, dextran exclusion provided a measure of the degree of membrane repair as the intensity of nuclear fluorescence decreased with increasing incubation time in repair reactions.

Assessment of the state of the nuclear membrane by a means independent from its effect on DNA replication is essential in order to draw conclusions about the role of nuclear membrane integrity in replication control. Therefore all populations used for the replication studies described here were first subjected to microscopic analysis with fluorescent probes.



T7 polyclonal antiserum followed by fluorescein-conjugated second antibody. T7 polymerase with a functional nuclear localization signal accumulated in >80% of nuclei. In the control reaction (T7 polymerase lacking an NLS) 9% of nuclei showed some fluorescence though this was significantly paler than in the test sample. Bar, 10  $\mu$ m.

### Repaired Nuclear Membranes Allow Selective Nuclear Entry

Although repaired nuclei exclude dextran molecules to the same extent as intact (untreated) nuclei (Fig. 1, *a* and *c*) it is important to show that they are still able to import nuclear proteins selectively, and that repair has not sealed the nucleus within an impermeable barrier.

Repaired nuclei were tested for their ability to accumulate nuclear proteins by active transport through nuclear pores. Nuclei were incubated in *Xenopus* egg extract (Newmeyer et al., 1986) in the presence of T7 polymerase to which an SV-40 T-antigen nuclear localization signal had been added (Dunn et al., 1988). Accumulation of T7 polymerase was detected using anti-T7 polyclonal antisera followed by a fluorescently labeled second antibody (Fig. 2 *a*). In control incubations, T7 polymerase without a nuclear localization signal was excluded from most (91%) of the repaired nuclei (Fig. 2 *b*). These results demonstrate that vesicle-mediated repair restores selective permeability to the nuclear membrane.

### Repairing the Nuclear Membrane of G2 Nuclei Reimposes the Block to Replication

Permeabilizing the nuclear membrane might cause reinitiation of replication in G2 nuclei by allowing a positive licensing factor to enter as hypothesized by Blow and Laskey (1988) or by allowing a negative inhibitory factor to escape. Alternatively, it could be acting via secondary damage to chromatin. These possibilities can be distinguished by repairing permeable nuclei before incubation in egg extract. Fig. 3 *a* shows the results of incubating intact, permeable, and repaired G2 nuclei in *Xenopus* egg extract in the presence of biotin-dUTP. Incorporation into nuclear DNA was visualized using fluorescently labeled streptavidin which allowed the number of nuclei replicating under each condition to be assessed. In this experiment 37% of intact G2 nuclei showed some degree of replication (Fig. 3 *a*, *I*). This is consistent with previous observations which showed that late S phase contaminants within an identically prepared G2 population represent ~30% of nuclei and that these account for the biotin-labeled ones (Leno et al., 1992). In the population used here, 26% of all nuclei were shown directly by bromodeoxyuridine pre-labeling to be late S phase contaminants rather than true G2 nuclei. This accounts for most of the biotin labeled nuclei in Fig. 3 *a* (*I*) and presumably in Fig. 3 *a* (*R*). When a sample of this G2 population was

permeabilized by treatment with lyssolecithin then incubated in extract, the number of nuclei incorporating detectable amounts of biotin-dUMP increased to 71% as a result of initiation in true G2 nuclei (Fig. 3 *a*, *P*). In other experiments where a higher concentration of lyssolecithin was used, the number of nuclei incorporating biotin-dUMP increased to ~90%. Replication of all permeabilized nuclei was never

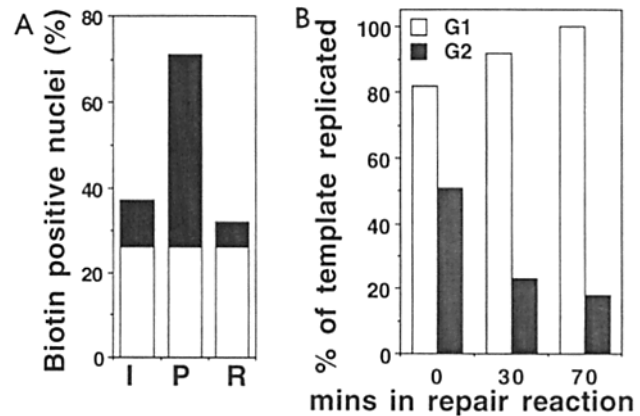
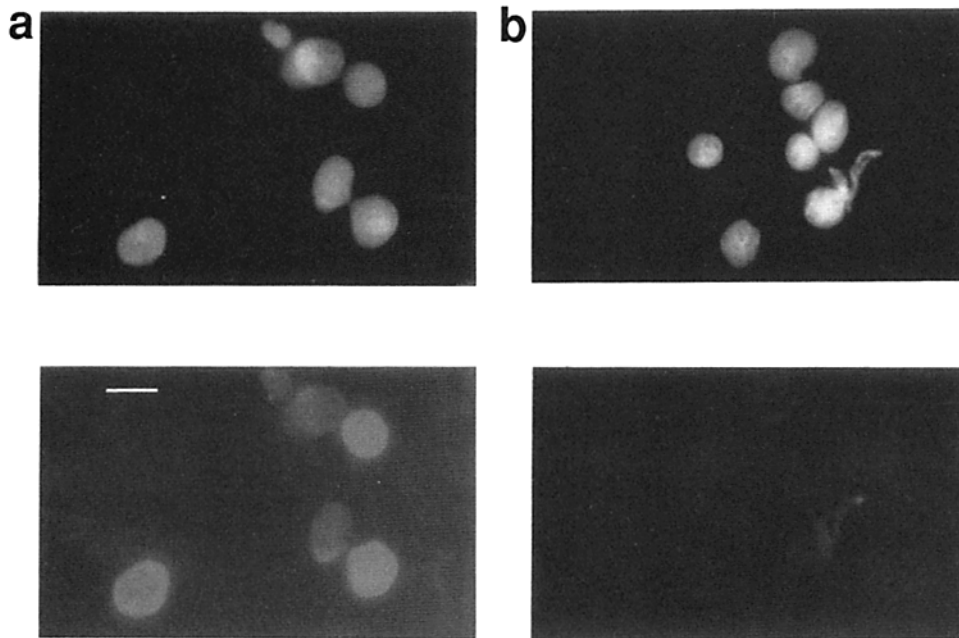


Figure 3. Repair of G2 nuclei reimposes the block to replication present in intact G2 nuclei. (A) The replication capacity of SLO-prepared G2 nuclei was tested by incubation in *Xenopus* egg extract in the presence of biotin-dUTP. Nuclei were tested while intact (*I*), after permeabilization with lyssolecithin (*P*), and after membrane repair by incubation with a vesicle fraction prepared from *Xenopus* egg extracts (*R*). DNA synthesis was monitored using fluorescein-conjugated streptavidin to allow the number of nuclei incorporating biotin-dUTP to be scored (>100 nuclei were scored for each sample). Results are expressed as percent of nuclei which were biotin positive in each sample. 26% of the biotin positive nuclei in the intact population are accounted for by the presence of late S phase contaminants within the G2 population (see text). These are represented by the lower (unshaded) portion of each column. (B) Timecourse for the effect of membrane repair on the replication capacity of permeabilized G1 and G2 nuclei. Lyssolecithin-permeabilized nuclei were incubated with vesicle fraction in parallel incubations (~3,700 nuclei/ $\mu$ l). After 0, 30, and 70 min, samples (containing 3,700 G2 nuclei or 7,400 G1 nuclei) were transferred to egg extract supplemented with [ $\alpha$ - $^{32}$ P]-labeled dATP. The extent of DNA synthesis in each sample was assessed by liquid scintillation counting of gel purified DNA. Results were normalized for the amount of DNA in each reaction by densitometry of photographs of ethidium bromide stained gels. Results are plotted as the percentage of template replicated.



**Figure 4.** Preincubation of permeable nuclei with *Xenopus* egg HSS licenses G2 nuclei for replication. Permeable G2 nuclei ( $5,000/\mu\text{l}$ ) were preincubated in the presence (a) or absence (b) of  $3 \mu\text{l}$  of HSS in  $6\text{-}\mu\text{l}$  reactions. After 20 min an equal volume of vesicle fraction was added. Repaired nuclei (judged by dextran exclusion) were transferred to complete *Xenopus* egg extract supplemented with biotin-dUTP. Total nuclei were stained with Hoechst 33258 (top) and those incorporating biotin were visualized using fluorescein conjugated streptavidin (bottom). 87% of nuclei that were preincubated with HSS incorporated biotin brightly compared to pale fluorescence in only 26% of the control sample. Bar,  $10 \mu\text{m}$ .

observed perhaps because some nuclei are damaged by the presence of unrepairable SLO-induced holes in the nuclear membrane. Repair of the nuclear membrane with isolated membrane vesicles before incubation in extract successfully reimposed the block to replication which was present in the intact population and reduced the number of nuclei incorporating biotin into their DNA to 32% (Fig. 3 a, R). The number of nuclei incorporating biotin after membrane repair is very similar to that observed with the intact population and to the known number of S phase contaminants (26%). This is consistent with replication forks which are active in intact S phase nuclei remaining active after permeabilization and repair.

The observation that the effects of lysolecithin treatment on DNA replication in G2 nuclei are fully reversible by repairing nuclei with membrane vesicles demonstrates that this agent does indeed act on the nuclear membrane rather than by a secondary effect on chromatin.

To show that repair of G2 nuclei does not prevent replication by a nonspecific mechanism, membrane repair reactions were also carried out on G1 nuclei (Fig. 3 b). In this experiment replication was monitored by measuring the incorporation of radiolabeled nucleotides into nuclear DNA after incubation for various times with vesicle fraction. Nuclei prepared from synchronized populations of G1 and G2 HeLa cells were permeabilized with lysolecithin then incubated with vesicle fraction in parallel reactions. Samples were removed after 0, 30, and 70 min, and then transferred to *Xenopus* egg extract to assess their replication capacity. After 70 min both populations of nuclei were fully repaired as judged by dextran exclusion (not shown). DNA synthesis in G1 nuclei did not decrease as a consequence of membrane repair whereas synthesis in G2 nuclei dropped to less than half the unrepaired level (Fig. 3 b). It can be concluded that the reduction in synthesis in G2 nuclei cannot be a nonspecific consequence of incubation with the vesicle fraction, but that

membrane repair reimposes the specific differences between G1 and G2 nuclei. G1 nuclei retained their competence for replication after permeabilization and repair so "licensing" factor or the consequences of its action must remain stable in the nucleus throughout the treatment.

Replication of the G1 template increased from 80% to almost 100% with increasing time in the vesicle repair reaction. The first step in *Xenopus* egg extract-mediated replication is the formation of a complete and intact nuclear envelope. This takes in the order of 30 min and results in a lag before DNA synthesis is detected (Sheehan et al., 1988). We interpret the small increase in DNA synthesis in G1 nuclei to be the consequence of removing this time lag by performing an intact nuclear membrane during the vesicle repair reaction.

These results argue strongly for the existence of at least one positive factor which can only enter the nucleus when the nuclear membrane is in a permeable state.

#### ***The Soluble Fraction of Xenopus Egg Extract Will License DNA Replication in Permeable G2 Nuclei***

The experiments described so far cannot be explained by a negative inhibitory activity which escapes from the nucleus upon permeabilization, because repairing the membrane reimposes the block to initiation, even though an inhibitor has had the opportunity to escape. However, these data remain fully consistent with the possibility that a positive factor must enter while the nuclear membrane is permeable.

Therefore, we have asked if it is possible to license permeable G2 nuclei by incubating them in active protein fractions before repairing the membrane and assaying in complete extract.

Lysolecithin-permeable G2 nuclei were incubated with *Xenopus* egg HSS (Sheehan et al., 1988), repaired, and then assayed for replication competence in complete extract by

monitoring the incorporation of biotin-dUMP and scoring the number of positive nuclei after staining with fluorescein-conjugated streptavidin (Fig. 4). 87% of G2 nuclei that were pre-exposed to HSS before being repaired incorporated biotin-dUMP into their DNA when added to complete extract (Fig. 4 a). If incorporation of biotin was monitored in nuclei that were not transferred to complete extract, only trace levels of DNA synthesis were observed, in a similar number of nuclei. This is in line with previous observations on the dependence of DNA synthesis on extract concentration. In the control incubation, repaired G2 nuclei that were not pre-exposed to HSS, but instead supplemented with HSS after vesicle-mediated repair, only 26% incorporated biotin-dUMP (Fig. 4 b). Therefore pre-incubation of permeable nuclei in HSS, before membrane repair, stimulated the initiation of DNA synthesis.

This conclusion was confirmed in two ways and extended to show that the DNA synthesis induced by pre-exposure to HSS is true semi-conservative DNA replication. First, the effect of preincubation in increasing amounts of egg HSS before membrane repair was monitored by measuring incorporation of radiolabeled nucleotides into DNA. Increasing volumes of HSS resulted in increased replication (data not shown). Second, repaired nuclei with and without pre-exposure to HSS were replicated in the presence of the dense nucleotide analogue bromodeoxyuridine triphosphate (BrdUTP) as well as radiolabeled dATP. The extent of incorporation of BrdUMP was assessed by centrifugation of replicated DNA through a CsCl density gradient (Fig. 5). Almost all radiolabel incorporated into both DNA samples equilibrated in the position expected of heavy/light DNA (at a CsCl density of 1.75 g/ml) indicating the occurrence of semi-conservative DNA synthesis. Very little incomplete strand synthesis or rereplication was observed in either sample. The extent of DNA synthesis in the nuclei that were prelicensed by exposure to HSS before nuclear membrane repair is approximately tenfold greater than that in the nuclei that received HSS only after nuclear membrane repair.

These data provide the first direct evidence for a factor(s) in the egg HSS which is required for the initiation of DNA replication, but which is unable to enter the interphase nucleus. Further fractionation of *Xenopus* egg HSS should allow identification of this activity.

## Discussion

Previous experiments have shown that permeabilizing the nuclear membrane of a replicated nucleus allows a second complete round of replication (Blow and Laskey, 1988; Leno et al., 1992). To explain this Blow and Laskey (1988) suggested that a hypothetical positively acting "licensing factor" was necessary for DNA synthesis to begin, but that this factor was unable to enter the nucleus when the nuclear membrane was intact. A number of other explanations remained possible such as escape of a negative inhibitor of replication or secondary effects on chromatin caused by the permeabilization procedure, either of which might be involved in allowing reinitiation of replication.

By reversibly manipulating the nuclear membrane of G2 HeLa cells we have resolved these possibilities. First, we have shown that the effects of nuclear membrane permeabilization on DNA replication are reversed by membrane repair

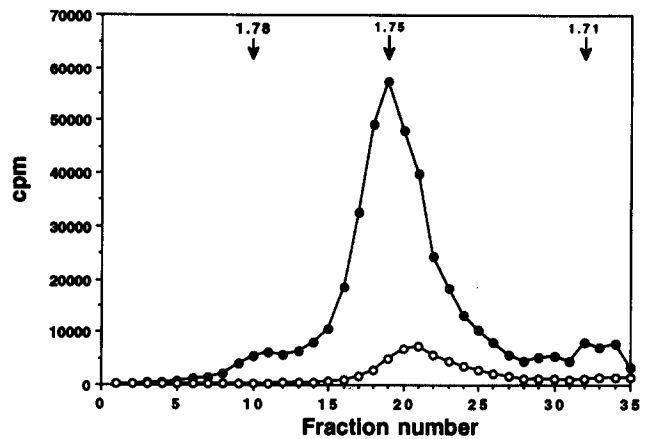
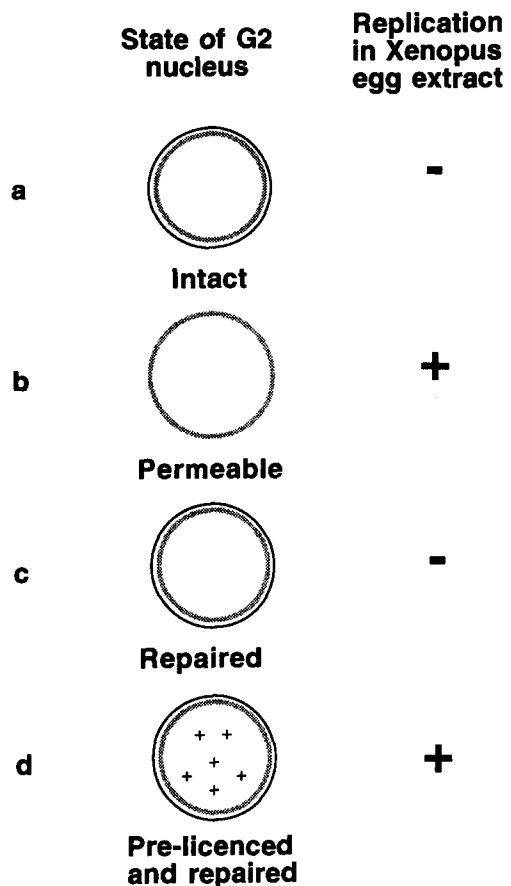


Figure 5. Pre-licensed repaired G2 nuclei carry out true semi-conservative DNA synthesis. Permeable G2 nuclei were repaired after 30-min preincubation with (●) and without (○) an equal volume of *Xenopus* egg HSS and then replicated in complete egg extract in the presence of [ $\alpha$ - $^{32}$ P]-labeled dATP and BrdUTP. Replicated DNA was isolated and centrifuged to equilibrium through a CsCl density gradient. Fractions were collected and acid insoluble counts were determined by liquid scintillation counting.

with isolated membrane vesicles, arguing against the possibility of secondary effects on chromatin. Second, we have shown that nuclear membrane permeabilization acts by allowing entry into the nucleus of a positive activator of DNA replication, present in the soluble fraction of *Xenopus* egg extract (Fig. 5), rather than allowing escape of a negative inhibitor.

The experiments described here confirm the licensing factor model by providing direct evidence for a positive regulatory activity. The nature of this activity is not known, nor is its mode of action, but two main possibilities exist. Licensing factor could bind directly to the chromosome and persist there until it is used at the start of S phase as originally proposed by Blow and Laskey (1988). In this case it could be a component of replication complexes or of replication-competent chromatin. The factor would be necessary but not sufficient for initiation which would be triggered by another event at the start of S phase, resulting in the destruction of licensing activity and the prevention of reinitiation.

An alternative interpretation is the existence of a positive catalytic activity capable of modifying structural components of chromatin, or proteins within the replication complex. Reversible modification occurring at, or as a consequence of mitosis, would provide a tight control on reinitiation. In this case the factor need not persist throughout G1, instead it could have a "hit and run" effect where only the products of its action would persist. A catalytic factor of this type could either activate replication proteins which would then be inactivated again by DNA synthesis (see below), or alternatively it could cancel a bound inhibitor of reinitiation, generated as a consequence of DNA synthesis. This last possibility could explain how the licensing effect can persist through a long G<sub>0</sub> phase in quiescent cells. This question will be addressed experimentally elsewhere (Cox, L., M. Madine, and R. A. Laskey, manuscript in preparation). All three types of positive model are completely compatible with our data and with earlier studies (Blow and Laskey, 1988; Leno et al., 1992).



**Figure 6.** Schematic summary of the results. (a) G2 nuclei with an intact nuclear membrane cannot replicate their DNA when incubated in *Xenopus* egg extract. (b) However, if their nuclear membranes are first permeabilized they become competent for replication in extract (Leno et al., 1992), suggesting that the nuclear membrane acts as a barrier to diffusion of a factor involved in the control of replication. (c) Repair of permeabilized nuclei before incubation in egg extract reimposes the block to replication that was originally present in intact G2 nuclei. This suggests that the nuclear membrane acts to exclude a positively acting "licensing" activity from G2 nuclei rather than to prevent loss of a negative inhibitor of replication which could have escaped while the membrane was permeable. (d) This conclusion is confirmed directly by exposing permeable nuclei to *Xenopus* egg HSS before repair and incubation in complete extract. They then become competent to replicate semi-conservatively. In contrast, HSS has no effect if added after membrane repair. Therefore a positive component required for DNA replication is able to act only when the nuclear membrane is permeable.

A catalytic activity capable of modifying essential replication proteins is an attractive possibility in view of the mechanisms which control the onset of mitosis. The activity of the viral initiator protein, T antigen, is regulated by a cycle of phosphorylation and dephosphorylation (McVey et al., 1989) raising the possibility that the cellular initiator protein itself might be regulated by modification. Cyclical phosphorylation and dephosphorylation has also been described for RPA (Din et al., 1990; Dutta et al., 1991; Fotedar and Roberts, 1991; Fotedar and Roberts, 1992) and a requirement for a *cdc2*-like activity for the efficient initiation of DNA replication has been reported in a number of studies (Blow and Nurse, 1990; D'Urso et al., 1990; Fang and New-

port, 1991). It remains to be seen whether the licensing activity we report here exerts its control over initiation as part of a pathway of protein phosphorylation and dephosphorylation.

A catalytic licensing factor model can also be reconciled with negative models such as that proposed by Roberts and Weintraub (1986). By studying hybrid replication origins of bovine papilloma virus and SV-40, they argued that reinitiation of replication is prevented by the presence of a *cis*-acting negative inhibitor bound to replicated chromatin. A catalytic licensing factor could act by cancelling a chromatin bound inhibitor, but a negative inhibitor on its own is not an adequate explanation of our results. To explain them, it is still necessary to invoke a positive factor which is excluded by the nuclear membrane. Conversion of inhibitory structural components of G2 chromatin into a conformation suitable for a new round of DNA synthesis could provide efficient dual control if the passage of a replication fork could reverse the effect of the positive factor, thereby blocking new initiations.

Blow and Laskey (1988) proposed that an essential activity could enter the nucleus only when the nuclear membrane breaks down during mitosis. However, the nuclear membrane does not break down in yeast and other lower eukaryotes, but they are still able to limit replication to only one round per cell cycle. Despite the closed mitosis, one member of a family of yeast DNA replication proteins, the CDC46 gene product of *Saccharomyces cerevisiae* (Hennessy et al., 1990), shows a cyclical pattern of nuclear entry entirely consistent with that predicted for licensing factor. It accumulates in the nucleus as mitosis is completed and rapidly disappears again at the G1/S boundary. In this case accumulation must occur as a consequence of regulated nuclear transport, raising the possibility that this is the normal route of entry into the nucleus for licensing factor in organisms other than yeast. Experimentally induced nuclear membrane permeabilization may bypass this process rather than mimic the effects of nuclear envelope breakdown at mitosis.

Members of the CDC46 gene family have been identified in *S. cerevisiae* (Gibson et al., 1990; Hennessy et al., 1990, 1991; Yan et al., 1991), *Schizosaccharomyces pombe* (Coxon et al., 1992), *Xenopus* (C.-Y. Khoo, personal communication), human, and mouse cells (Thommes et al., 1992). Two other members of this family, MCM2 and MCM3, have also been implicated in initiation in *S. cerevisiae* (Gibson et al., 1990; Yan et al., 1991). The conserved mammalian P1 protein shows strong sequence homology to MCM3 (Thommes et al., 1992) and it is associated with complex forms of DNA polymerase  $\alpha$ , further implicating this protein family in DNA replication. Their implication in DNA replication and the similarity between the nuclear entry cycles observed for CDC46 and predicted for licensing factor make this protein family good candidates for further study.

Regulated entry of a licensing activity in yeast can also be used to reconcile the data of Broek et al. (1991) with our observations. They report the isolation of *ts p34<sup>cdc2</sup>* mutants which can be induced to undergo two rounds of DNA synthesis without an intervening mitosis. Both of these mutants specifically effect the G2 function of *cdc2* resulting in a lack of induction of mitosis. It would be particularly interesting to know the nuclear accumulation pattern of *cdc21* and other homologues of CDC46 in the *ts p34<sup>cdc2</sup>* mutants.

We cannot distinguish yet between stable binding or "hit and run" models for the mode of action of licensing factor. However, the experiments described here demonstrate that a positively acting licensing factor exists. This conclusion is supported by the results of recent experiments which use specific protein kinase inhibitors (Blow, 1993). In addition the experiments described here provide an assay for identification of the licensing activity.

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### References

- Alouf, J. E. 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther.* 11:661-717.
- 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.
- Blumenthal, A. B., H. J. Kriegstein, and D. S. Hogness. 1973. The units of DNA replication in *Drosophila melanogaster* chromosomes. *CSH Symp. Quant. Biol.* 38:205-223.
- Broek, D., R. Bartlett, K. Crawford, and P. Nurse. 1991. Involvement of p34<sup>cdc2</sup> in establishing the dependency of S-phase on mitosis. *Nature (Lond.)* 349:388-393.
- Coxon, A., K. Maundrell, and S. E. Kearsey. 1992. Fission yeast cdc21 + belongs to a family of proteins involved in an early step of chromosome replication. *Nucleic Acids Research.* 20:5571-5577.
- 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.
- Duncan, J. L., and R. Schlegel. 1975. Effect of streptolysin O on erythrocyte membranes, liposomes and lipid dispersions. A protein cholesterol interaction. *J. Cell Biol.* 67:160-173.
- Dunn, J. J., B. Krippel, K. E. Bernstein, H. Westphal, and F. W. Studier. 1988. Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. *Gene.* 68:259-266.
- D'Urso, G., R. L. Marraccino, D. R. Marshak, and J. M. Roberts. 1990. Cell cycle control of DNA replication by a homologue from human cells of the p34 cdc2 protein kinase. *Science (Wash. DC)* 250:786-791.
- Dutta, A., S. Din, S. J. Brill, and B. Stillman. 1991. Phosphorylation of replication protein A: A role for cdc2 kinase in G1/S regulation. *CSH Symp. Quant. Biol. LVI*:315-324.
- Fang, F., and J. W. Newport. 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell.* 66:731-742.
- Fotedar, R., and J. M. Roberts. 1991. Association of p34cdc2 with replicating DNA. *CSH Symp. Quant. Biol. LVI*:325-333.
- Fotedar, R., and J. M. Roberts. 1992. Cell cycle regulated phosphorylation of RPA-32 occurs within the replication initiation complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2177-2187.
- Gibson, S. I., R. T. Surosky, and B. K. Tye. 1990. The phenotype of the mini-chromosome maintenance mutant mcm3 is characteristic of mutants defective in DNA replication. *Mol. Cell Biol.* 10:5707-5720.
- Gurdon, J. B. 1976. Injected nuclei in frog oocytes: fate, enlargement, and chromatin dispersal. *J. Embryol. Exp. Morphol.* 36:523-540.
- Hennessy, K. M., C. D. Clark, and D. Botstein. 1990. Subcellular localization of yeast CDC46 varies with the cell cycle. *Genes & Dev.* 4:2252-2263.
- Hennessy, K. M., A. Lee, E. Chen, and D. Botstein. 1991. A group of interacting yeast DNA replication genes. *Genes & Dev.* 5:958-969.
- 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.
- McVey, D., L. Brizuela, I. Mohr, D. R. Marshak, Y. Gluzman, and D. Beach. 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. *Nature (Lond.)* 341:503-507.
- 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.
- Newmeyer, D. D., D. R. Finlay, and D. J. Forbes. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* 103:2091-2102.
- Prigent, D., and J. F. Alouf. 1976. Interaction of streptolysin O with sterols. *Biochim. Biophys. Acta.* 443:288-300.
- Rao, P. N., and R. T. Johnson. 1970. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature (Lond.)* 225:159-164.
- Roberts, J. M., and H. Weintraub. 1986. Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. *Cell.* 46:741-752.
- Sheehan, M. A., A. D. Mills, A. M. Sleeman, R. A. Laskey, and J. J. Blow. 1988. Steps in the assembly of replication competent nuclei in a cell-free system from *Xenopus* eggs. *J. Cell Biol.* 106:1-12.
- Thommes, P., R. Fett, B. Schray, R. Burkhardt, M. Barnes, C. Kennedy, N. C. Brown, and R. Knippers. 1992. Properties of the nuclear P1 protein, a mammalian homologue of the yeast Mcm3 replication protein. *Nucleic Acids Res.* 20:1069-1074.
- Yan, H., S. Gibson, and B. K. Tye. 1991. Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. *Genes & Dev.* 5:944-957.