Abstract. *Xenopus* egg extracts treated with the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) are unable to support the initiation of DNA replication. Nuclei assembled in 6-DMAP extracts behave as though they are in G2, and will not undergo another round of DNA replication until passage through mitosis. 6-DMAP extracts are functionally devoid of a replication factor that modifies chromatin in early G1 before nuclear envelope assembly, but which is itself incapable of crossing the nuclear envelope. This chromatin modification is capable of supporting only a single round of semiconservative replication. The behavior of this replication factor is sufficient to explain why eukaryotic DNA is replicated once and only once in each cell cycle, and conforms to the previous model of a Replication Licensing Factor. Cell cycle analysis shows that this putative Licensing Factor is inactive during mitophase, but becomes rapidly activated on exit from metaphase when it can modify chromatin before nuclear envelope assembly is complete.

The eukaryotic genome is replicated from $10^3$ to $10^4$ initiation events which are coordinated so that no section of the DNA is replicated more than once in each S phase. In order to ensure that re-replication cannot occur, replicated DNA must differ in some way from unreplicated DNA, so that re-initiation does not occur on replicated DNA. This can be observed directly in hybrids of G1 and G2 cells, where the G1 nucleus passes directly into S phase, while the G2 nucleus does not replicate until after the hybrid cell has passed through mitosis (Rao and Johnson, 1970).

Mechanisms which prevent DNA from replicating more than once in a single cell cycle have been investigated using cell-free extracts of *Xenopus* eggs (Blow and Laskey, 1988; Leno et al., 1992). These cell-free extracts support the efficient initiation and completion of DNA replication on added DNA templates (Blow and Laskey, 1986, 1987). However, no DNA replicates more than once in a single cell cycle in vitro (Blow and Laskey, 1986, 1988; Blow and Watson, 1987; Blow and Sleeman, 1990). DNA added to the cell-free extract is assembled into normal interphase nuclei (Lohka and Masui, 1983, 1984b; Newmeyer et al., 1986; Blow and Laskey, 1986; Newport, 1987), and the initiation of DNA replication occurs only on DNA that has been assembled into such nuclei (Newport, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990; Leno and Laskey, 1991; Cox, 1992).

When replicated (G2) nuclei are added to fresh G1 extract, they do not undergo a further round of DNA replication (Blow and Laskey, 1988). However, if the G2 nuclei are allowed to progress into mitosis in vitro, so that nuclear envelope breakdown and chromosome condensation occur, they can then undergo a further round of replication in fresh G1 extract. Therefore some metaphase function permits G2 nuclei to revert to the G1 state. G2 nuclei were subjected to various treatments to see whether they would permit the G2 nuclei to re-replicate in fresh extract (Blow and Laskey, 1988). Agents that caused nuclear envelope permeabilization, such as lysolecithin or phospholipase, left the G2 nuclei capable of re-replicating in fresh extract. This suggests that DNA replication is controlled by compartmentalization of replication factors between nucleus and cytoplasm. Similar results have been obtained in cell-free extracts of *Drosophila* eggs (Crevel and Cotterill, 1991) and on introduction of mammalian tissue culture nuclei into *Xenopus* eggs (De Roeper et al., 1977; Leno et al., 1992) or egg extracts (Leno et al., 1992). A simple model has been suggested (Blow and Laskey, 1988) whereby an essential replication factor, called Licensing Factor, can bind to DNA during mitosis. Licensing Factor cannot cross the nuclear envelope, so that once nuclear assembly is complete, Licensing Factor is present either free in the cytoplasm or bound to DNA in the nucleus. On entry into S phase, each molecule of Licensing Factor bound to DNA supports a single initiation event, after which it is inactivated or destroyed. Thus, in G2, no active Licensing Factor remains in the nucleus, and the nuclear envelope must be transiently permeabilized (as normally occurs during mitosis) to allow a further round of DNA replication to be Licensed.

If this model is correct in its general form, then Licensing Factor must display the following characteristics: (a) it must directly modify chromatin, either by binding to the chromatin or by causing an enzymatic modification such as phosphorylation; (b) this modification (the "Licence") must be required for the initiation of DNA replication; (c) Licensing Factor must be unable to cross the nuclear envelope, so it
is never found freely diffusible within the nucleus; (d) the Licence must be capable of supporting only a single initiation event, after which it is inactivated or destroyed.

In this paper we show that metaphase-arrested extracts treated with the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) become unable to support the initiation of DNA replication, although nuclear assembly and the elongation of previously initiated replication forks can still occur. 6-DMAP-treated extracts are functionally devoid of an activity that conforms to these four definitive features of Licensing Factor. The behavior of this factor is sufficient to explain why eukaryotic DNA is replicated once and only once in each cell cycle.

Materials and Methods

Preparation of Egg Extracts

Metaphase-arrested extracts were prepared by a modification of previous extraction procedures (Lohka and Masui, 1984a; Blow and Nurse, 1990). Eggs were collected in High Salt Barth solution (110 mM NaCl, 15 mM Tris HCl, pH 7.4, 2 mM KCl, 2 mM NaHCO3, 1 mM MgSO4, 0.5 mM Na2HPO4), and were washed in High Salt Barth containing 2 mM EGTA. Any degenerated or activated eggs were removed at this stage. Eggs were dejellied in 2% cysteine HCl, pH 7.8, 2 mM EGTA. Dejellied eggs were washed first in High Salt Barth containing 2 mM EGTA, and then in inactivating extraction buffer (UEB: 50 mM KCl, 50 mM HEPES-KOH, pH 7.6, 5 mM MgCl2, 5 mM EGTA, 2 mM β-mercaptoethanol) at 4°C. Eggs were packed by centrifugation in a rotor (HB-4; Sorvall Instruments, Newtron, CT) at 3,000 rpm for 1 min. All excess buffer was then removed along with any degenerated eggs, which float to the surface under these spin conditions. Eggs were spin crushed at 12,000 rpm for 10 min at 4°C in the HB-4 rotor. The entire cytoplasmic layer was taken with a pasteur pipette and was supplemented with cytochalasin B to a final concentration of 10 μg/ml and extraction dilution buffer (50 mM KCl, 50 mM HEPES-KOH, pH 7.5, 2 mM DTT, 0.4 mM MgCl2, 0.4 mM EGTA, 10 μg/ml each of leupeptin, pepstatin, and aprotonin) to 15% by volume. The extract was resuspended at 30,000 rpm in a rotor (SW50; Beckman Instruments, Palo Alto, CA) at 4°C for 15 min. This separates the cytoplasm into a golden fraction above a loose yellow vesicular fraction. The golden fraction was removed with a pasteur pipette, avoiding the yellow vesicular fraction which is inhibitory to extract function. Contaminating particles were removed by filtering the extract through a 25 μm Nybolt membrane (Plastok Ltd., Merseyside, UK) under gravity.

Interphase extracts were prepared by a modification of previous extraction procedures (Lohka and Masui, 1983; Blow and Sleeman, 1990). Eggs were collected and dejellied as for metaphase-arrested extracts. They were then washed in Barth solution (88 mM NaCl, 15 mM Tris HCl, pH 7.4, 2 mM KCl, 1 mM MgCl2, 0.5 mM CaCl2), activated for 5 min with 0.5 μM calcium ionophore at room temperature, and then washed with extraction buffer (UEB without EGTA) at 4°C for 15 min. All further steps were performed as for metaphase-arrested extracts except that the second spin was at 15,000 rpm.

Preparation of DNA Templates

Demembranated Xenopus sperm nuclei were prepared as described by Blow and Laskey (1986). Plasmid pXMA10A (Baggen et al., 1982) was a kind gift of R. Reed (Fred Hutchinson Cancer Research Centre, Seattle, WA). Aphiidicolin-blocked nuclei were prepared by incubating sperm nuclei for 2 h in interphase extract supplemented with 20 μg/ml aphidicolin (Blow and Nurse, 1990). Chromatin and nuclei were isolated from the extract by resuspending in 1 ml nuclear isolation buffer (NIBS: 50 mM KCl, 50 mM Hepes-KOH, pH 7.6, 5 mM MgCl2, 2 mM β-mercaptoethanol, 0.5 mM spermidine 3HCl, 0.15 mM spermine 4HCl, 1 μg/ml each of leupeptin, pepstatin, and aprotonin), after which they were underlayered with 100 μl 15% sucrose in NIBS and pelleted at 6,000 g for 5 min at 4°C in a swinging bucket rotor. All overlying buffer was removed, and nuclei were resuspended in 4 μl NIBS for transfer to fresh extract.

Isolated nuclei were permeabilized with 200 μg/ml lysolecithin as described by Blow and Laskey (1988).

Extract Use

After thawing, extracts were supplemented with 25 mM phosphocreatine, 5 μg/ml creatine phosphokinase, 250 μg/ml cycloheximide and 1 μCi ρ32P-labeled dATP, 1 μCi ρ3H-labeled dATP, 0.4 mM BrdUTP, or 50 μM biotin-11-UTP as appropriate. Demembranated sperm nuclei were added to give a final sperm DNA concentration of 3 ng DNA/μl extract (Blow and Laskey, 1986). Metaphase extracts were released into interphase by the addition of 0.3 mM CaCl2. Except where stated in the figure legend (see Fig. 7 and Table 3), cycloheximide was added to all extracts. Incubations were performed at 23°C.

TCA precipitation, CsCl density substitution, agarose gel electrophoresis, and autoradiography were performed as described by Blow and Laskey (1986). Analysis of biotin-11-UTP incorporation was as described by Blow and Watson (1987), except that the EGS fixation step was omitted. IgG exclusion was monitored as described (Blow and Sleeman, 1990).

Cyclin A and H1 Kinase

The vector containing X. laevis cyclin AΔ56 (Minshall et al., 1990) cloned in PET3 was a kind gift of Tim Hunt (ICRF Clare Hall Laboratories, UK). Cyclin AΔ56 kinase was expressed in E. coli and purified essentially as described for cdc25 (Strausfeld et al., 1991) and was a kind gift of Ulrich Strausfeld (ICRF Clare Hall Laboratories, UK).

H1 kinase levels were assayed by adding 1.5 μl extract to 25 μl kinase buffer containing 1 mg/ml histone H1, 200 μM ATP, 10 mM MgCl2, 20 mM Hepes, pH 7.5, 50 mM β-glycerophosphate, 5 mM EGTA, 100 μM Na Vanadate, 0.2 μCi ρ32P-labeled ATP and 50 μg/ml each leupeptin, pepstatin, and aprotonin. After incubation at 23°C for 5 min the reaction was stopped with 25 μl protein gel sample buffer. 15-μl aliquots were electrophoresed on 15% polyacrylamide minigels, which were then dried and autoradiographed. Bands corresponding to intact histone H1 were excised and counted by liquid scintillation.

Results

6-DMAP Treatment Blocks the Initiation of DNA Replication

Affinity- and immuno-depletion suggests that the initiation of DNA replication in Xenopus egg extracts is dependent on the function of cdc2-like protein kinases (Blow and Nurse, 1990; Fang and Newport, 1991). We assayed various inhibitors of cdc2-like kinases for their ability to block the initiation of replication in Xenopus egg extracts. In case there are activities that occur very early in the cell cycle, the inhibitors were added to metaphase-arrested extracts prepared from unactivated Xenopus eggs (Lohka and Masui, 1984a). These metaphase-arrested extracts were then released into interphase by the addition of CaCl2, after which DNA is normally assembled into nuclei and a single round of DNA replication occurs (Blow and Sleeman, 1990). 6-DMAP is a purine analogue that inhibits cdc2-like protein kinases such as the metaphase-inducing activity MPF (Maturation Promoting Factor) (Meijer and Pondaven, 1988; Neant and Guerrier, 1988). When 6-DMAP was added to metaphase-arrested extracts at concentrations higher than 2 mM, it caused an almost complete abolition of subsequent DNA replication (Fig. 1 A, filled squares). Inhibition of MPF activity was assessed by the spontaneous entry into interphase of metaphase-arrested extracts supplemented with 6-DMAP but not CaCl2 (Fig. 1 A, open circles). 6-DMAP at concentrations above 1 mM inhibited MPF kinase activity sufficiently to allow >95% of added sperm nuclei to be assembled into interphase nuclei as judged by acquisition of a 994
Figure 1. 6-DMAP inhibits DNA replication if added prior to exit from metaphase. (A) Metaphase-arrested Xenopus egg extracts were supplemented with sperm chromatin to a final DNA concentration of 3 ng/µl and various concentrations of 6-DMAP. (■—■) Extracts were released into interphase with CaCl₂, and the total DNA synthesis during a subsequent 3-h incubation was measured. (○—○) Nuclear assembly in the absence of added CaCl₂ was monitored under the light microscope. The percentage of nuclei surrounded by a phase-dense envelope is shown. (B) At various times after release into interphase with CaCl₂, metaphase-arrested extracts were supplemented with 6-DMAP to 3 mM. (■—■) Sperm chromatin was added to a final DNA concentration of 3 ng/µl at 10 min after CaCl₂ release. The dashed line shows the amount of DNA synthesized in a parallel incubation with no added 6-DMAP. (□—□) Sperm chromatin was added to a final DNA concentration of 3 ng/µl before CaCl₂ release; the extract was incubated for 15 min in metaphase, followed by addition of 6-DMAP and CaCl₂.

surrounding phase-dense envelope. Therefore concentrations of 6-DMAP required to inhibit DNA replication are slightly higher than those required to abolish MPF activity.

The ability of 6-DMAP to inhibit DNA replication is critically dependent on the time it is added relative to CaCl₂. Fig. 1 B (filled squares) shows that only when added to extract before CaCl₂ release does 6-DMAP inhibit subsequent DNA synthesis. Addition of 3 mM 6-DMAP 8 min after CaCl₂ release had virtually no effect on DNA replication. The ability of 6-DMAP to inhibit replication is dependent on the time it is added relative to CaCl₂ release, not relative to the time when template DNA is added. When DNA was pre-incubated in metaphase-arrested extract for 15 min, followed by 6-DMAP and CaCl₂ addition, DNA replication was still inhibited (Fig. 1 B, open square).

Similar results were also obtained using staurosporine, another inhibitor of cdc2-like protein kinases. When 30 µM staurosporine was added to metaphase-arrested extract, subsequent DNA synthesis was inhibited by 98% relative to the control; when the same concentration of staurosporine was added to extract 10 min after CaCl₂ release, inhibition was only 43%. Between 10–20 µM staurosporine was required to cause spontaneous exit from metaphase. In the experiments described in the following sections, “6-DMAP extract” refers to metaphase-arrested extract supplemented with 3 mM 6-DMAP before CaCl₂ release.

Fig. 2 shows that 6-DMAP does not inhibit the elongation stage of DNA replication, but appears to inhibit the initiation of new replication forks. Different DNA templates were incubated in untreated and 6-DMAP extracts, nascent DNA was labeled with ³²P-labeled dATP, and the products analyzed by agarose gel electrophoresis and autoradiography. Lanes 1 and 2 show that with sperm chromatin as template very little synthesis was seen in 6-DMAP extract. Similarly pXlr101A, a double-stranded plasmid DNA template whose replication also requires the initiation of new replication forks, did not replicate in 6-DMAP extract (lanes 3 and 4). However, DNA templates that do not require initiation were

Figure 2. 6-DMAP does not inhibit the elongation stage of DNA replication. DNA templates were incubated in metaphase-arrested extract supplemented with ³²P-labeled dATP plus (lanes 2, 4, 6, and 8) or minus (lanes 1, 3, 5, and 7) 3 mM 6-DMAP. 3 h after CaCl₂ release DNA was isolated, electrophoresed on a 0.6% agarose gel, and autoradiographed. DNA templates are: lanes 1 and 2, sperm nuclei at a final DNA concentration of 3 ng/µl; lanes 3 and 4, plasmid pXlr101A at a DNA concentration of 10 ng/µl; lanes 5 and 6, aphidicolin-blocked nuclei at a concentration of ~3 ng/µl; lanes 7 and 8, single-stranded M13 DNA at a concentration of 3 ng/µl. Lanes 3 and 4 have been exposed for approximately 10 times longer than the others due to the relative inefficiency of plasmid DNA replication in this system. Markers show migration of: pXlr101A form II (pXlr-II), high molecular weight linear DNA (hmwl), pXlr101A form I (pXlr-I), double-stranded M13 form II (M13-II), double-stranded M13 form I (M13-I), and the loading slot.
replicated efficiently in 6-DMAP extract. Sperm chromatin incubated in interphase extract supplemented with aphidicolin, a competitive inhibitor of replicative DNA polymerases, undergoes the initiation of replication, but the initiated forks cannot elongate. These "aphidicolin-blocked nuclei," containing stalled replication forks, were isolated and added to fresh extracts to assess their ability to support elongation of the preexisting replication forks (Blow and Nurse, 1990). Lanes 5 and 6 show that the replication forks present on aphidicolin-blocked nuclei elongated efficiently in 6-DMAP extract, demonstrating that 6-DMAP treatment does not significantly affect the extract's ability to support the elongation stage of DNA replication. Similarly 6-DMAP extract efficiently replicated single-stranded M13 DNA (lanes 7 and 8), a process that does not require the initiation of replication forks, but rather requires an active DNA primase and an active DNA polymerase. The generation of double-stranded form I (supercoiled) M13 DNA shows that the 6-DMAP extract, like the untreated extract, can synthesize a complete complementary DNA strand, remove the RNA primers, ligate nascent strands together, and assemble the replicated DNA into chromatin. These results show that 6-DMAP does not inhibit most of the important events occurring during the elongation stage of DNA replication, and so suggest that 6-DMAP extracts are defective in the initiation process.

Nuclear assembly is a prerequisite for the initiation of replication on double-stranded DNA in the Xenopus extract, so one possible way that 6-DMAP might inhibit initiation is by interfering with the process of nuclear assembly. To test this, nuclear assembly was compared in untreated (Fig. 3, A-C) and 6-DMAP (Fig. 3, D-F) extracts under the light microscope. After 60 min in either extract, the chromatin had decondensed (B and E) and had become surrounded by a phase-dense nuclear envelope (A and D). The nuclei assembled in either extract were capable of excluding fluorescent IgG (C and F), whilst remaining permeable to low molecular weight compounds such as Hoechst 33258 (B and E). This suggests that the nuclei assembled in 6-DMAP extract are essentially normal. One consistent difference, however, between nuclei assembled in the two extracts was that the chromatin in the 6-DMAP extract tended to be in a more reticular form, reminiscent of chromatin in the G2 phase of the in vitro cell cycle (Hutchison et al., 1988). When 6-DMAP nuclei underwent DNA replication after nuclear envelope permeabilization (see below), the chromatin became more typical of nuclei undergoing S phase in the Xenopus system (data not shown). It is not known whether this chromatin remodeling is a cause or a consequence of DNA replication.

**6-DMAP Treatment Abolishes Licensing Factor Activity**

G2 (replicated) nuclei cannot undergo further DNA replication in the Xenopus extract until their nuclear envelope has been transiently permeabilized (Blow and Laskey, 1988; Leno et al., 1992). Table I shows that nuclei assembled in 6-DMAP extracts behave in the same way as G2 nuclei. In Table I A, nuclei assembled in 6-DMAP extract were isolated and transferred either to metaphase-arrested extracts or to interphase extracts. On transfer to metaphase-arrested extract the 6-DMAP nuclei underwent nuclear envelope breakdown, after which CaCl2 was added to release the extract into interphase. The DNA was then reassembled into nuclei which replicated efficiently, as judged by incorporation of both 32P-labeled dATP and biotinylated dUTP. Similar results were obtained when 6-DMAP nuclei were permeabilized with lysolecithin treatment before transfer to interphase extract (data not shown). In contrast, intact 6-DMAP nuclei that were transferred to interphase extract and hence did not undergo a period of nuclear envelope breakdown, supported little DNA synthesis. The same conclusion can be drawn from Table I B, where replication in 6-DMAP extract was rescued by the addition of untreated extract. This rescue still occurred when the untreated extract was added after the 6-DMAP extract had been pre-incubated for 1 h, so long as the template DNA was added at the same time as the untreated extract. However, when the template DNA was added directly to the 6-DMAP extract, so that nuclear assembly was completed in unrescued 6-DMAP extract, very little DNA synthesis occurred when untreated extract was subsequently added. A time course of this effect showed that sperm chromatin incubated for 20 min or less in 6-DMAP extract could still replicate efficiently when untreated extract was subsequently added, but that DNA incubated for 30 min or more in 6-DMAP extract was replicated poorly (data not shown). Thus the time when DNA incubated in 6-DMAP ex-
Table 1. Nuclei Assembled in 6-DMAP Extract Cannot Replicate Even after Exposure to Untreated Extract

| Extract  | DNA synthesis none | weak | strong |
|----------|--------------------|------|--------|
| metaphase | 50.5               | 3    | 20     |
| interphase| 5.6                | 25   | 75     |

Nuclei assembled in 6-DMAP extract cannot replicate even after exposure to untreated extract. (A) Nuclei assembled in 6-DMAP extract for 60 min were isolated and equal aliquots (containing ~60 ng DNA) were transferred to either interphase or metaphase-arrested extract supplemented with either 32P-labeled dATP or biotin-11-dUTP. 10 min after receiving the nuclei the metaphase-arrested extract was released with CaCl2. Incubation was then performed for 3 h at 23°C. Nuclei were scored for biotin incorporation and classified into "none," "weak," or "strong." Most of the weakly incorporating nuclei in the interphase transfer resembled the very early-replicating nuclei described by Mills et al. (1989). (B) DNA synthesis was measured in mixes between 6-DMAP and untreated extracts. 6-DMAP extract was incubated for 0 or 60 min at 23°C with or without DNA before the addition of twice the volume of untreated extract. Each sample contained 80 ng template DNA.

If 6-DMAP extracts were lacking Licensing Factor, then chromatin that had been briefly exposed to untreated extract should become Licensed and hence able to replicate in 6-DMAP extract. Fig. 4 shows this to be the case. In the experiment shown in Fig. 4 A, sperm chromatin was pre-incubated in untreated interphase extract for different periods of time, and then re-isolated and transferred to 6-DMAP extract. On pre-incubation in untreated extract, DNA rapidly became competent to replicate in 6-DMAP extract, and after a 15-min pre-incubation was almost completely replication-competent. Fig. 4 B shows photomicrographs of DNA after different times in the untreated interphase extract. The first sections of nuclear envelope were first seen on DNA after about 20 min, and complete nuclear envelopes were seen after about 25-30 min. Therefore the ability of DNA pre-incubated for 15 min in control interphase extract to replicate in 6-DMAP extract (Fig. 4 A) is unlikely to be dependent on the integrity of the nuclear envelope at the time of re-isolation. This suggests that 6-DMAP extracts have lost the ability to make a chromatin modification that is required for the initiation of DNA replication.

To show that this modification is independent of nuclear envelope assembly, the experiments shown in Fig. 5 were performed. These compare the replication in 6-DMAP extract of chromatin treated in different ways. As before, sperm chromatin was replicated poorly in 6-DMAP extract ("sperm"), whereas sperm chromatin pre-incubated for 15 min in untreated extract ("preincubation") was replicated...
Nuclear envelope assembly is not required for preincubated chromatin to replicate in 6-DMAP extract. Sperm nuclei were subject to various treatments followed by incubation in 6-DMAP extract supplemented with 32P-labeled dATP. DNA synthesis was measured during a 3-h incubation and expressed as a percentage of the synthesis occurring in parallel using the same DNA templates in untreated extracts. (1, sperm) Untreated sperm nuclei. (2, preincubation) Sperm nuclei were incubated for 15 min in interphase extract. (3, lysolecithin) Sperm nuclei were incubated for 15 min in interphase extract, followed by isolation and treatment with lysolecithin. (4, metaphase) Sperm nuclei were incubated for 15 min in interphase extract, isolated, transferred to metaphase-arrested extract and incubated for 15 min before 6-DMAP and CaCl2 addition. (5, WGA) Sperm nuclei were incubated for 15 min in interphase extract supplemented with 250 μg/ml wheat germ agglutinin.

Figure 5. Nuclear envelope assembly is not required for preincubated chromatin to replicate in 6-DMAP extract. Sperm nuclei were subject to various treatments followed by incubation in 6-DMAP extract supplemented with 32P-labeled dATP. DNA synthesis was measured during a 3-h incubation and expressed as a percentage of the synthesis occurring in parallel using the same DNA templates in untreated extracts. (1, sperm) Untreated sperm nuclei. (2, preincubation) Sperm nuclei were incubated for 15 min in interphase extract. (3, lysolecithin) Sperm nuclei were incubated for 15 min in interphase extract, followed by isolation and treatment with lysolecithin. (4, metaphase) Sperm nuclei were incubated for 15 min in interphase extract, isolated, transferred to metaphase-arrested extract and incubated for 15 min before 6-DMAP and CaCl2 addition. (5, WGA) Sperm nuclei were incubated for 15 min in interphase extract supplemented with 250 μg/ml wheat germ agglutinin.

Figure 6. Permeabilized G2 nuclei are unable to re-replicate in 6-DMAP extract. Sperm nuclei were incubated in untreated extract supplemented with 32P-labeled dATP and BrdUTP for 3 h. Replicated nuclei were isolated, plus or minus lysolecithin treatment, and then transferred to fresh extract containing 3H-labeled dATP and BrdUTP. After a further 3-h incubation, DNA was isolated, and fractionated on CsCl equilibrium density gradients. The percentage of total 32P-labeled DNA in each fraction is shown. Arrows show the densities expected of light/light DNA (LL, 1.71 g/ml), heavy/light DNA (HL, 1.75 g/ml) and heavy/heavy DNA (HH, 1.79 g/ml). (A) DNA after the first incubation only. (B) Nuclei transferred directly to a second incubation in interphase extract. (C) Nuclei treated with lysolecithin after the first incubation and transferred to metaphase extract after release with CaCl2. (D) Nuclei treated with lysolecithin after the first incubation and transferred to metaphase extract that had been treated with 3 mM 6-DMAP before release with CaCl2.

Efficiently. Even when this pre-incubated chromatin was treated with lysolecithin to permeabilize any envelope that may have been assembled on the chromatin, it was still replicated efficiently in 6-DMAP extract ("lysolecithin"). More surprisingly, when pre-incubated chromatin was transferred to metaphase-arrested extract and isolated for 15 min before 6-DMAP and CaCl2 addition, so that any assembled nuclear envelope would be disassembled under the action of MPF, it was still replicated efficiently in 6-DMAP extract ("metaphase"). Another way of preventing complete nuclear envelope assembly is to supplement the pre-incubation extract with wheat germ agglutinin, a lectin that binds to nuclear pore components (Finlay et al., 1987; Finlay and Forbes, 1990) and prevents membrane vesicle fusion (Cox, 1992; and data not shown). Chromatin pre-incubated in extract supplemented with wheat germ agglutinin was still replicated efficiently in 6-DMAP extract ("WGA"). These results show that the ability of pre-incubated chromatin to replicate in 6-DMAP extract does not depend on the presence of an intact nuclear envelope during pre-incubation.

So far, these results have shown that 6-DMAP extracts behave as though they lack a replication factor that directly modifies chromatin, that is required for the initiation of DNA replication and that is unable to cross the nuclear envelope. This represents three out of the four features necessary and sufficient to define the missing activity as the Replication Licensing Factor. The remaining feature is that the activity can support only a single initiation event, so that chromatin must be exposed to it before each successive S phase can take place. This feature is demonstrated in Fig. 6. Nuclei were assembled and replicated in untreated extract supplemented with cycloheximide to block the extract in G2, as well as 32P-labeled dATP and BrdUTP to label nascent DNA. At this stage, all nascent DNA banded at the heavy/light position on a CsCl gradient, indicative of a single complete round of semi-conservative replication (Fig. 6 A; Blow and Laskey, 1986). When these nuclei were transferred to fresh G1 extract, no re-replication took place, as evidenced by the lack of labeled DNA at the heavy/heavy position (Fig. 6 B; Blow and Laskey, 1988). When the nuclear envelope was permeabilized with lysolecithin before transfer, significant re-replication occurred after transfer to fresh extract (Fig. 6 C; Blow and Laskey, 1988). When, however, the permeabilized nuclei were transferred to 6-DMAP extract, no re-replication occurred (Fig. 6 D). This means that the essential replication factor missing from 6-DMAP extract, which was present in the nuclei for the first round of replication and which binds to chromatin without the need for an intact envelope (Figs. 4 and 5), was not capable of supporting a second round of DNA replication in 6-DMAP extract. Instead, this essential replication factor must be reassembled into nuclei before each of successive rounds of replication. Taken together these results demonstrate that this essential replica-
Figure 7. Cell cycle variability of Licensing Factor activity. (A) Interphase extract was incubated for various times with no additions (O—O), cycloheximide (●—●—●), or with bacterially produced cyclin A protein (▲—▲—▲). M on the time axis refers to a metaphase-arrested extract plus minus cycloheximide. Sperm nuclei were then incubated in the extract for 15 min, and tested for its ability to replicate in 6-DMAP extract. DNA synthesis is expressed as a percentage of synthesis obtained with the same DNA template in untreated extract. (B) Time course of DNA synthesis (O—O) and histone H1 kinase activity (●—●) in the extract shown in A with no cycloheximide added. Nuclear envelope breakdown occurred at 100–120 min. (C) Interphase extract was incubated minus (O—O) or plus (●—●—●) cyclin A protein. At various times sperm nuclei were added, and the total amount of DNA synthesized in a further 3-h incubation was measured.

Cell Cycle Variation in Licensing Factor Activity

The pre-incubation technique described in Fig. 4 was used to make an approximate measurement of the amount of Licensing Factor activity present at different stages of the cell cycle in vitro (Fig. 7). Interphase extract can normally undergo a complete round of DNA replication without continuing protein synthesis, but requires synthesis of cyclin B protein to progress from G2 into M (Minshull et al., 1989; Murray and Kirschner, 1989). Extract without added DNA was incubated with or without cycloheximide (to block protein synthesis) or cyclin A protein (to force the extract into metaphase). At different times sperm chromatin was incubated in the extract for 15 min, after which the chromatin was isolated and tested for its ability to replicate in 6-DMAP extract (Fig. 7 A). This gives an indication of the extract's ability to Licence DNA at different cell cycle stages. The timing of some of the basic events of the in vitro cell cycle are shown in Fig. 7 B for comparison. In the normal cell cycle, with DNA added at the start of the incubation and with continuing protein synthesis, nuclear envelope assembly would be seen after 20–30 min (Fig. 4 B), DNA synthesis would start after 30–45 min (Fig. 7 B, open circles), histone H1 kinase levels would start to rise at about 90 min (Fig. 7 B, filled circles), and nuclear envelope breakdown would occur at about 100 min. In metaphase-arrested extracts, with high histone H1 kinase levels (Fig. 7 A and B, M on the time axis), measurable Licensing Factor activity was very low. On exit from metaphase into interphase, Licensing Factor levels rose rapidly, whether or not protein synthesis was inhibited with cycloheximide (Fig. 7 A, open and filled circles, and data not shown). In a normal cycle with continuing protein synthesis (Fig. 7 A, open circles), Licensing Factor activity then declined during S phase, so that by the time the extract was in G2, levels were as low as they were in metaphase. When protein synthesis was blocked with cycloheximide (Fig. 7 A, filled circles), Licensing Factor activity still declined during the cycle but at a slower, more monotonous rate than in the presence of protein synthesis. Addition of cyclin A protein at levels sufficient to cause nuclear envelope breakdown (Fig. 7 A, filled triangles) caused an immediate and profound inhibition of Licensing Factor activity, similar to the levels normally seen in metaphase.

The fall in Licensing Factor activity seen in Fig. 7 A is reflected by the ability of untreated extract to replicate DNA added to it at different stages of the in vitro cell cycle (Fig. 7 C). Extract was incubated for different times plus or minus cycloheximide, after which sperm chromatin was then added. The total amount of DNA synthesized during a further 3-h incubation was measured. The ability of extract to replicate newly added DNA fell with time, so that after a 60-min pre-incubation with continuing protein synthesis (open circles) it had fallen to virtually zero. In the presence of cycloheximide (filled circles) the ability of extract to replicate newly added DNA also fell with time, but at a slower rate.

| Preincubation          | DNA synthesis (ng/µl) | Percent synthesis (60 min/control) |
|------------------------|----------------------|-----------------------------------|
| Control extract        | 2.15                 | 0.06                              |
| 60-min extract         | 1.54                 | 0.51                              |
| 60 min/control         | 1.38                 | 0.06                              |

Licensing chromatin can replicate in 60-min extract. Sperm nuclei minus or plus a 15-min pre-incubation in untreated or 6-DMAP extract were isolated and transferred to either fresh control extract or to the extract used in Fig. 7 which had been pre-incubated for 60 min with continuing protein synthesis. Total DNA synthesis during a 3-h incubation is given, as well as the ratio of synthesis in control and 60-min extract. The final DNA concentration in the replication reactions was ~3 ng/µl, assuming 100% recovery from the pre-incubation.
These results are similar to the fall in Licensing Factor activity shown in Fig. 7 A. Similar results were obtained when two separate DNA additions were made, one aliquot at the start of the incubation and another aliquot after the different incubation times (data not shown). Further, Table II shows that extract pre-incubated for 60 min with continuing protein synthesis ("60-min extract") behaves like 6-DMAP extract in its response to pre-incubated chromatin. When sperm chromatin was pre-incubated in fresh untreated extract for 15 min and then transferred to 60-min extract it was partially replicated in the remaining part of interphase before the extract entered mitosis. In contrast, sperm chromatin that had not been pre-incubated, or sperm chromatin that had been pre-incubated in 6-DMAP extract, supported very little DNA synthesis in 60-min extract.

Discussion

This paper shows that when 3 mM 6-DMAP is added to metaphase-arrested Xenopus egg extracts, they become unable to initiate replication on added DNA templates. The 6-DMAP extracts behave as though they are functionally devoid of a factor whose activity is sufficient to explain why DNA is replicated only once in each cell cycle. This activity conforms to the four definitive features necessary and sufficient to define it as the Replication Licensing Factor of Blow and Laskey (1988): (a) The activity modifies DNA templates so that after a brief incubation in untreated extract they become competent to replicate in 6-DMAP extract (Fig. 4 A). This modification is made directly to the chromatin and does not require the integrity of the nuclear envelope (Figs. 4 B and 5). (b) This modification (the "Licence") is absolutely required for the initiation of DNA replication, both on sperm chromatin and on plasmid DNA. Very little DNA synthesis is seen in 6-DMAP extract (Figs. 1 and 2), and heavy/light DNA is undetectable after BrdUTP density substitution (Blow, J. J., and J. Chong, data not shown). The defect in 6-DMAP extract is unlikely to be at the elongation stage of replication, since a variety of templates can replicate fully in 6-DMAP extract, including aphidicolin-blocked nuclei (Fig. 2), single-stranded M13 DNA (Fig. 2) and DNA pre-incubated for 15 min in untreated extract (Figs. 4, 5, and 7). (c) The activity absent in 6-DMAP extract is unable to cross the nuclear envelope, since nuclei assembled in 6-DMAP extract are unable to replicate if subsequently exposed to untreated extract (Table I). This block to replication can be alleviated by transiently permeabilizing the nuclear envelope surrounding these nuclei (Table I A). (d) The chromatin modification absent in 6-DMAP extract is capable of supporting only a single initiation event, since nuclei replicated once in the Xenopus extract require a further exposure to the modifying activity before a second round of DNA replication can be initiated (Fig. 6). Results consistent with these are also obtained if Licensing Factor activity levels are allowed to fall spontaneously in the presence of continuing protein synthesis (Fig. 7 C and Table II).

The fulfillment of all four of these features suggests that 6-DMAP extracts are functionally devoid of Replication Licensing Factor, whose activity is sufficient to explain why DNA is replicated once and only once in each cell cycle. Fig. 8 summarizes the mode of action of Licensing Factor in a revised version of the original model of Blow and Laskey (1988). On exit from metaphase, inactive Licensing Factor is rapidly activated. Active Licensing Factor then licenses the decondensed DNA at future sites of initiation, either by binding it directly or by some enzymatic modification of the chromatin such as phosphorylation. Initiation cannot take place at this stage as the DNA is not assembled into an interphase nucleus. After nuclear assembly, a local signal is activated throughout the nucleus (Blow and Watson, 1987), causing initiation to take place at licensed sites. Each licensed site can support a single initiation event after which the Licence is inactivated or destroyed. Replicated DNA cannot re-replicate due to the exclusion of Licensing Factor from the DNA by the nuclear envelope, and because during the earlier parts of the cycle cytoplasmic Licensing Factor has been inactivated. It is only reactivated after exit from the next metaphase.

The model shown in Fig. 8 differs from the original model of Blow and Laskey (1988) in showing that the Licensing Factor activity undergoes marked variation with the cell cycle. No Licensing Factor activity can be detected in metaphase (Fig. 7), but it becomes activated very rapidly after exit into interphase (Figs. 1 and 7). This activation is not dependent on new protein synthesis, since the first round of DNA replication in the Xenopus eggs or egg extracts is not sensitive to protein synthesis inhibitors (Fig. 1; Harland and Laskey, 1980). Therefore, the activation of Licensing Factor on exit from metaphase must be due to posttranslational modification of preexisting proteins. 6-DMAP does not block the function of Licensing Factor once it has been activated, since when added more than 8 min after exit from metaphase, 6-DMAP has little effect on the replication of DNA added subsequently (Fig. 1). Instead, 6-DMAP must be added before release from metaphase to inhibit the appearance of Licensing Factor activity, suggesting that 6-DMAP interferes only with the activation process. Similar results were obtained with the protein kinase inhibitor staurosporine. Both inhibitors block the activation of Licensing Factor at concentrations slightly higher than is required to block MPF kinase activity and cause spontaneous exit from metaphase (Fig. 1 A). This correlation suggests that the target of these inhibitors might be a cdc2-like kinase. Since Licensing Factor is inactive in metaphase when MPF levels are high, it seems unlikely that MPF itself is responsible for activation of Licensing Factor. However, inhibition of MPF kinase by 6-DMAP prevents the degradation of cyclins that normally occurs on exit from metaphase (Felix et al., 1989; Luca and Ruderman, 1989), and it is possible that Licensing Factor activation is dependent on this proteolysis event.

Once activated in early G1, Licensing Factor activity appears to be unstable, and this instability is enhanced by ongoing protein synthesis. The decay of Licensing Factor activity is paralleled by the extract's inability to replicate newly added DNA at later stages of the in vitro cell cycle (Fig. 7 C and Table II). The absence of Licensing Factor activity during later stages of interphase provides a further mechanism preventing the re-replication of DNA in a single cell cycle. Even if the nuclear envelope were damaged after S phase had started, the absence of active Licensing Factor at these times means that no DNA could become licensed to re-replicate in the current cell cycle.

A number of other observations have been made concerning mechanisms that prevent re-replication of DNA in a sin-
Figure 8. Model showing mode of action and cell cycle variability of Licensing Factor. A schematic representation of the cell cycle events required for chromosome replication in the cell cycle; only a single chromosome is shown. (A) On exit from metaphase, inactive Licensing Factor (-) is rapidly activated (+) and the chromosomal DNA decondenses. (B) Decondensed DNA is Licensed at future sites of initiation. (C) DNA is assembled into a nucleus surrounded by a complete nuclear envelope. (D) A local signal is activated throughout the nucleus, causing initiation to take place at Licensed sites. Each Licensed site supports a single initiation event after which the Licence is inactivated. (E) The chromosome is replicated by forks initiated at Licensed sites. (F) Fully replicated G2 nuclei cannot re-replicate due to exclusion of Licensing Factor from the DNA by the nuclear envelope. (G) Entry into mitosis is accompanied by nuclear envelope breakdown and chromosome condensation. (H) Sister chromatids separate and are partitioned to the daughter cells. During the earlier parts of the cycle (A-E) cytoplasmic Licensing Factor activity has slowly decayed.

gle cell cycle (Roberts and Weintraub, 1986, 1988; Broek et al., 1991; Usui et al., 1991; Handeli and Weintraub, 1992). It is not clear at present how these other observations relate to the results presented here, since they come from other model systems. However, the behavior of Licensing Factor as presented here is strikingly similar to the behavior of a yeast cell cycle gene product, CDC46, which is required for a very early event in DNA replication and which has previously been suggested to represent a yeast Licensing Factor homologue (Hennessy et al., 1990). CDC46 protein is nuclear in G1, where it apparently associates with chromatin, but disappears from the nucleus at the start of S phase. It can then be seen in the cytoplasm throughout G2 and mitosis, but just at the end of mitosis it translocates back to the nucleus. CDC46 represents one of a large family of proteins present in a number of eukaryotic cells which share some as yet uncharacterized roles in DNA replication (Hennessy et al., 1990; Yan et al., 1991; Hennessy et al., 1991; Thömmes et al., 1992; Coxon et al., 1992).

This paper presents evidence that a Replication Licensing Factor is involved in the cell cycle control of DNA replication. Similar conclusions about the mode of action of Licensing Factor have also been reached by Coverley et al. (1993, in this issue) using a different approach involving the permeabilization and resealing of G2 nuclei. Previously, the most significant barrier to studying Licensing Factor had been the lack of a method for assembling nuclei without automatically Licensing the DNA. The ability of 6-DMAP to generate Licensing Factor-defective extracts provides an effective assay for Licensing Factor (Fig. 4), and we are currently using this assay to purify active Licensing Factor.

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