Determination of Initiation of DNA Replication before and after Nuclear Formation in *Xenopus* Egg Cell Free Extracts

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Abstract. *Xenopus* egg extracts prepared before and after egg activation retain M- and S-phase specific activity, respectively. Staurosporine, a potent inhibitor of protein kinase, converted M-phase extracts into interphase-like extracts that were capable of forming nuclei upon the addition of sperm DNA. The nuclei formed in the staurosporine treated M-phase extract were incapable of replicating DNA, and they were unable to initiate replication upon the addition of S-phase extracts. Furthermore, replication was inhibited when the staurosporine-treated M-phase extract was added in excess to the staurosporine-treated S-phase extract before the addition of DNA. The membrane-depleted S-phase extract supported neither nuclear formation nor replication; however, preincubation of sperm DNA with these extracts allowed them to form replication-competent nuclei upon the addition of excess staurosporine-treated M-phase extract. These results demonstrate that positive factors in the S-phase extracts determined the initiation of DNA replication before nuclear formation, although these factors were unable to initiate replication after nuclear formation.

THE regulation of DNA replication in eukaryotes, the initiation and termination of replication in particular, is a complex system. Both initiation and termination are controlled by the cell division cycle; reinitiation of replication is permitted only after mitosis, thus ensuring the exact duplication of genetic material during a single cell cycle. One problem that hinders the examination of molecular events regulating the replication of eukaryotic nuclear DNA is the apparent requirement of nuclear structures for replication (for review see Laskey et al., 1989). This situation makes it difficult to distinguish the components required for replication from those required for the formation of nuclear structures.

A further problem regarding the replication of eukaryotic nuclear DNA is that of the replication origin of the DNA. In the budding yeast, *S. cerevisiae*, DNA replication origins have been identified as short stretches of DNA (Huberman et al., 1987; Brewer and Fangman, 1987) and some origin binding proteins have been identified (Bell and Stillman, 1992; Diffley and Cocker, 1992). In mammalian cells (Vaughn et al., 1990), in *Drosophila* (Shinomiya and Ina, 1991), and in the fission yeast, *S. pombe* (Zhu et al., 1992), however, two-dimensional gel electrophoresis techniques suggest that replication origins contain fairly long stretches of DNA sequences and that the initiation of replication occurs in a broad zone of these DNA stretches. It is not known whether short stretches of specific DNA sequences function as the replication origin. These features make the study of

so-called origin-binding proteins difficult; these proteins are found universally in the replication of prokaryotic and viral DNA, and are possibly involved in the regulation of the initiation of DNA replication (Kornberg and Baker, 1992). The nuclear matrix also seems to play a fundamental role in regulating replication, but only few studies have shown the importance of the nuclear skeleton (Jackson and Cook, 1986; Newport et al., 1990; Meier et al., 1991). Lack of knowledge of such factors hinders the study of the regulation of DNA replication in eukaryotes.

Cell-free extracts of amphibian eggs provide a unique system for studying the regulation of the cell cycle. Egg extracts retain M- and S-phase specific activity, respectively, when they are prepared from unactivated and activated eggs (Lohka and Masui, 1983; Lohka and Maller, 1985). The essential role played by nuclear structures in the semiconservative replication of DNA has been demonstrated with S-phase extracts of *Xenopus* eggs (Blow and Laskey, 1986; Newport, 1987; Sheehan et al., 1988; Hutchison et al., 1988), and questions concerning the regulation of DNA replication have been addressed in studies of these extracts. Experimental results showing that the reinitiation of DNA replication requires the breakdown of the nuclear membrane led Blow and Laskey (1988) to propose the existence of positive factors regulating DNA replication. Such positive factor, termed the licensing factor, has been proposed to interact with chromatin only before nuclear formation, and thus it would contain no nuclear localization signals. Such a positive factor is inactivated at or during DNA replication. These characteristics fulfill the requirement for the precise regulation of the initiation of DNA replication; DNA replicates only once in a single cell cycle. Reinitiation requires the breakdown of the nuclear

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membrane, thus ensuring that DNA replication is reinitiated only after mitosis. The role of the nuclear membrane in preventing rereplication has been further confirmed in nuclei isolated from the G1 and G2 phases of human cells (Leno et al., 1992). Reinitiation of replication after permeabilization of the membrane in G2 nuclei suggests that the licensing factor may function in the normal cell cycle.

In this study, we found that the treatment of M-phase extracts with staurosporine led to a reversal of the cell cycle back to a G2-like phase; incubation of sperm DNA with the extracts led to the formation of nuclei that were incompetent in regard to DNA replication. In addition, we found that the M-phase extracts capable of forming nuclei inhibited the replication of DNA in S-phase extracts. Such extracts prompted us to study the possible determination of DNA replication before nuclear formation. Our results indicate (a) the presence of positive factors that interact with chromatin before nuclear formation, to allow the initiation of DNA replication and (b) the presence of negative factors, in the staurosporine-treated M-phase extracts that inactivated the positive factors in the S-phase extracts. The significance of these factors in regulating the cell cycle is discussed.

Materials and Methods

Preparation and Fractionation of Xenopus Egg Extracts

Mature *Xenopus laevis* females, primed by a single injection of pregnant mare gonadotropin (100 IU) 2–7 d before use, were ovulated by an injection of human chorionic gonadotropin (700 IU). Eggs were collected in 0.1 M NaCl; those that appeared degenerative were discarded. S-phase extracts were prepared by previously described methods (Newport, 1987), with some modification. Briefly, unfertilized eggs were dejellied in a solution consisting of 5 mM dithiothreitol, 110 mM NaCl, and 20 mM Tris-HCl at pH 8.5, washed in 1/5 MMR (MMR [0.1 M NaCl, 2 mM KCl, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 0.1 mM EDTA, and 25 mM Hepes-KOH at pH 7.5] diluted fivefold with 0.1 M NaCl), and activated with 0.5 μg/ml calcium ionophore A23187 in MMR. Activated eggs were washed with 1/5 MMR, followed by washes with ice-cold S buffer (0.25 M sucrose, 50 mM KCl, 2.5 mM MgCl₂, 2 mM β-mercaptoethanol, 15 μg/ml leupeptin, and 50 mM Hepes-KOH at pH 7.5) containing 5 mM EGTA. The washed eggs were packed into tubes by brief centrifugation for several seconds at 6,000 g. All excess buffer was removed and the eggs were crushed by centrifugation at 15,000 g for 10 min. The resulting supernatant between the lipid cap and pellet was collected and mixed with cytochalasin B (10 μg/ml final concentration), and recentrifugated at 50,000 g for 5 min to remove residual debris. M-phase extracts were prepared similarly, except that the calcium ionophore treatment of eggs was omitted. The egg extracts were supplemented with 60 mM creatine phosphate and 150 μg/ml creatine phosphokinase and used immediately.

The crude extracts were further fractionated by centrifugation at 100,000 g for 90 min to yield a pellet and supernatant (Sheehan et al., 1988). The supernatant was taken to be the membrane-depleted fraction. The pellets were resuspended in S buffer and collected by centrifugation at 15,000 g for 10 min. The resultant pellet was taken to be the membrane fraction.

Preparation of Sperm DNA

Sperm, prepared from mature *X. laevis* males by the method described by Gurdon (1976), were demembranated by treatment with 2 mg/ml lysophosphatidylcholine in SuNaSp buffer (250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, and 0.15 mM spermine). The permeabilization of the membrane was monitored by staining the sperm with 0.1% Trypan blue in PBS; permeabilization was stopped by adding ice-cold SuNaSp containing 3% BSA when most of the sperm were permeabilized. The demembranated sperm nuclei were washed with SuNaSp, suspended in SuNaSp containing 30% glycerol, frozen in liquid nitrogen, and stored at −80°C. These nuclei are referred to as sperm DNAs.

Measurement of Histone H1 Kinase Activity

Histone H1 kinase activity in the egg extracts was measured as described by Felix et al. (1989). Briefly, 2 μl of egg extracts was diluted by adding 6 μl of assay buffer (10 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 50 mM Tris-HCl at pH 7.4) containing 5 mM EGTA and 500 nM protein kinase inhibitor. Reactions were initiated by adding an equal volume of assay buffer containing [γ-³²P]ATP (10 kBq/nmol) and 4 mg/ml histone H1 in the presence of various concentrations of staurosporine. The mixtures were incubated for 10 min at 25°C and the reactions were stopped by pipetting the reaction mixtures onto P81 phosphocellulose papers (1 × 1 cm²). The papers were washed three times in 150 mM H₃PO₄ for 15 min each time, after which they were rinsed in absolute ethanol and dried. The radioactivity remaining on the papers was determined with a Beckman LS 1800 liquid scintillation counter.

Measurement of DNA Replication

DNA replication was measured as the incorporation of [α-³²P]dCTP (from 0.7 to 3.5 kBq/μl extract) into DNA. The formation of nuclei and the incorporation of dCTP were initiated by the addition of sperm DNAs at a concentration of about 2,000/μl of extract. Incubations were performed at 23°C. After an appropriate time, the samples (10 μl) were removed and added to 80 μl of buffer consisting of 0.5 mg/ml proteinase K, 0.5% SDS, 20 mM EDTA, and 20 mM Tris-HCl at pH 8. These mixtures were incubated for 30 min at 37°C; 100 μl of 10% TCA was then added. Acid-insoluble materials were collected by filtration through a Millipore filter (HAWP, 0.45-μm pore size) and the filters were washed twice with 10% TCA containing 30 mM NaPPi, followed by 10 mM HCl; the amount of [α-³²P]dCTP incorporated into the DNA was determined with the liquid scintillation counter.

Preparation of Samples for Microscopy

Samples of egg extracts containing about 2,000 sperm DNAs/μl extract were incubated for various times at 23°C. Reactions were stopped by pipetting 2-μl samples onto glass slides spotted with 3 μl of fixing solution (3% formaldehyde, 2 μg/ml HOECHST dye 33342, 80 mM KCl, 15 mM NaCl, 50% glycerol, and 15 mM Pipes at pH 7.2) containing 0.1 mg/ml 3,3'-diethylthiadicarbocyanine iodide. The fixed samples, covered with glass coverslips and sealed with nail varnish, were observed by phase contrast and fluorescence microscopy.

Immunoblotting

After SDS-PAGE, proteins were electrophoretically transferred from a polyacrylamide gel to nitrocellulose paper in a solution containing 125 mM Tris, 960 mM glycine, 0.05% SDS, and 20% methanol. The paper was washed for 10 min at room temperature with TTBS (0.9% NaCl, 0.1% Tween 20, and 100 mM Tris-HCl pH 7.5), and it was blocked with TTBS containing 5% skim milk for 1 h. Then, it was incubated with first antibodies at a dilution of 1:1,000 in TTBS containing 1% skim milk over night. After washing the paper with TTBS, it was incubated with the peroxidase-conjugated second antibody in TTBS containing 1% skim milk for 1–2 h. Immunoreactivity was detected with Konica Immunostain HRP.

Preparation of Samples for Indirect Immunofluorescence Microscopy

Sperm DNAs (2,000/μl extract) were incubated in 5 μl of egg extracts containing 2 μM biotin-11-dUTP at 23°C. After incubation, the samples were fixed by adding 50 μl of 3% formaldehyde in S buffer. After 15 min incubation at room temperature, the nuclei were recovered by centrifugation, at 1,200 g for 15 min, through S buffer containing 25% glycerol onto glass coverslips (Hutchison et al., 1988). The coverslips were washed with S buffer and then incubated overnight at 4°C with the first antibody (mouse anti-chick DNA polymerase α mAb, mouse anti-rabbit PCNA mAb, or rabbit anti-human lamin B antisera). The coverslips were then washed with S buffer and incubated further with a FITC-labeled second antibody (anti-rabbit or anti-mouse Ig) and /or Texas-red streptavidin. After the coverslips were washed in S buffer, they were mounted on glass slides in 5 μl of the fixing solution containing 0.5 M DTT.

Preparation of Fluorescent-labeled Proteins

Egg nucleoplasmin, prepared from heat-treated supernatant of egg extracts,

was purified by using protamine agarose. Briefly, egg extracts were heated in boiling water in a water bath for 10 min and denatured proteins were removed by centrifugation at 10,000 g for 15 min. The obtained supernatant was directly applied onto protamine agarose preequilibrated with S buffer containing 1 mM EGTA. After the column was thoroughly washed with the S buffer, nucleoplasmin was eluted by linear gradients of NaCl from 0 to 2.0 M. Nucleoplasmin was eluted at ∼1.2 M NaCl as a broad peak of more than 90% purity. Pooled fractions were further purified by passage through phenyl Sepharose (Dingwall et al., 1982). Proteins were labeled with TRITC (nucleoplasmin) or FITC (BSA and BSA conjugated with the nuclear localization signal of SV-40 large T-antigen), as described by Newmeyer et al. (1986), and free labels were removed with a centrifuge column (Penefsky, 1977).

Measurement of Nuclear Transport of Proteins

Samples of egg extracts containing 2,000 sperm DNAs/µl extract were incubated for 30 min at 23°C. Labeled proteins (final concentration, ∼0.1 mg/ml) were then added to the samples, and these were further incubated for 30 min (BSA) or 60 min (nucleoplasmin). After incubation, 2-µl samples were fixed with 3 µl of the fixing solution. The nuclear structures were identified by phase contrast microscopy. Those nuclei that had distinct accumulations of labeled proteins were counted as nuclear transport-positive.

Measurement of DNA Polymerase α Activity

DNA polymerase α activity in fractionated egg extracts was determined as described by Suzuki et al. (1989). Briefly, 5-µl samples diluted with S buffer were added to 25 µl of standard reaction mixture consisting of 3.3 mM 2-mercaptoethanol, 5 mM MgCl₂, 0.2 mg/ml BSA, 100 µM each of dATP, dCTP, and dGTP, 20 µM [³²P]dTTP (4.4 kBq/nmol), 0.5 mg/ml activated calf thymus DNA, and 20 mM Tris-HCl at pH 8.0. The radioactivity incorporated into acid-insoluble materials during 30-min incubation at 37°C was measured; one unit of activity was defined as the amount that catalyzed the incorporation of 1 nmol of deoxynucleoside monophosphate during 1-h incubation at 37°C.

Materials

BSA conjugated with the nuclear localization signal of SV-40 large T antigen and anti-human lamin B antisera were generous gifts from Dr. Y. Yoneda (School of Medicine, Osaka University, Osaka, Japan). Anti-chick polymerase α mAb (4-8 H) was a generous gift from Dr. A. Matsukage (Aichi Cancer Center Research Institute, Nagoya, Japan). Calf thymus activated DNA was a generous gift from Dr. F. Hanaoka (Riken Institute, Wako, Japan). FITC-labeled sheep anti-mouse IgG and donkey anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) were generous gifts from Dr. K. Kuroda in our department. Pregnant mare gonadotropin, human chorionic gonadotropin, A23187, phosphocreatine, creatine kinase, lysophosphatidylcholine, and FITC were obtained from Sigma Chem. Co. (St. Louis, MO); ³²P-labeled nucleotides and Texas-red streptavidin were from Amersham Japan (Tokyo, Japan); histone H1 and anti PCNA mAb were from Boehringer Mannheim Yamanouchi (Tokyo, Japan); deoxynucleotides were from Yamasa (Chiba, Japan). Staurosporine was obtained from Kyowa Medex Co., Ltd. (Tokyo, Japan); Konica Immunostain HRP was from Konica (Tokyo, Japan); TRITC was from Research Organics, Inc. (Cleveland, OH); biotin-11-dUTP was from ENZ Biochem. Inc. (New York, NY), and HOECHST dye 33342 was from Calbiochem Corp. (La Jolla, CA). All other chemicals were of reagent grade.

Results

Effects of Staurosporine on the Activity of S- and M-phase Extracts of Xenopus Eggs

We investigated possible involvement of protein kinases in retaining the activity of S- and M-phase egg extracts, using staurosporine as a kinase inhibitor. Fig. 1 A summarizes the results. S-phase extracts, prepared after egg activation, retained S-phase-specific activity. When demembranated sperm nuclei (sperm DNA) were added to the extracts, pseudonuclei were formed around the decondensed DNA, and

the incorporation of dCTP into DNA was initiated after nuclear formation (cf. Blow and Laskey, 1986). A micromolar concentration of staurosporine inhibited histone H1 kinase activity in the S-phase extracts by >60%, but the inhibition of replication was marginal. In the presence of 6 µM staurosporine, the time course of the incorporation of dCTP showed a delay at the onset (data not shown), while the final amount incorporated was as much as 70% of the control value obtained in the absence of staurosporine (Fig. 1, open square). In the presence of 10 µg/ml of aphidicolin, the amount of dCTP incorporated after 180-min incubation was <5% of the control value. Similar inhibition of dCTP incorporation was observed in the presence of 100 µM cytosine β-D-arabinofuranoside 5'-triphosphate (ara CTP), but not in the presence of 50 µM 3'-deoxythymidine 5'-triphosphate (data not shown).

Staurosporine markedly inhibited M-phase-specific activity. Both histone H1 kinase activity and condensation of the sperm DNA added to the extracts were dose dependently inhibited by this agent. In the absence of staurosporine, the histone H1 kinase activity in the extracts was more than 10 U/µl, a level similar to that reported in M-phase egg extracts (Felix et al., 1989). More than 80% of sperm DNA added to the extracts exhibited condensed chromatin with an apparent diameter of ∼1 µm, this being visualized by staining DNA with HOECHST (Fig. 1 B, a). Both activities were similarly decreased with increasing concentrations of added staurosporine. When the histone H1 kinase activity in the M-phase extracts dropped below that in S-phase extracts, almost all the sperm DNA in the extracts was decondensed (cf. Fig. 1 B, b). Additional increases in the concentration of staurosporine transformed the M-phase extracts into interphase-like extracts. In the presence of 5 µM staurosporine, >80% of the sperm DNAs added to the extracts were enclosed by nuclear membrane-like structures that were visualized under phase contrast microscopy (Fig. 1 B, c) and by staining the membrane with 3,3'-diethylthiadicarbocyanine iodide (Fig. 1 B, d). The concentration of staurosporine required for the formation of the nucleus-like structures varied in different preparations of the extracts, 2 µM staurosporine being sufficient for inducing nuclear formation in some cases. We observed no nuclear formation below 2 µM staurosporine.

Formation of Nuclei in M-phase Egg Extracts Treated with Staurosporine

Fig. 2 shows the morphology of nuclear lamin assembled around the decondensed DNA in staurosporine treated M-phase extracts compared with that in the S-phase extracts. In both samples, the decondensed DNAs were enclosed by distinct structures visualized under phase contrast microscopy (cf. Fig. 1 B). The assembly of lamin was investigated using anti-human lamin B antisera, which recognized a single polypeptide with an apparent molecular mass of 68-kD in both S- and M-phase egg extracts (Fig. 2 a). A similar molecular size has been reported for Xenopus egg lamin III, the only lamin found in the eggs in the early embryonic cell cycle (Krohne and Benavente, 1986). The antisera used in this study exclusively stained the nucleus-like structures formed in both extracts. In particular, cortical shell-like structures were found along the periphery of the decondensed DNA,
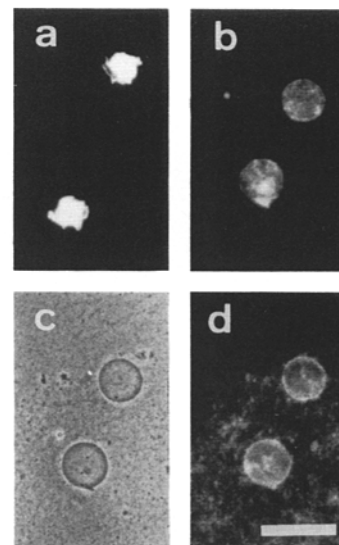
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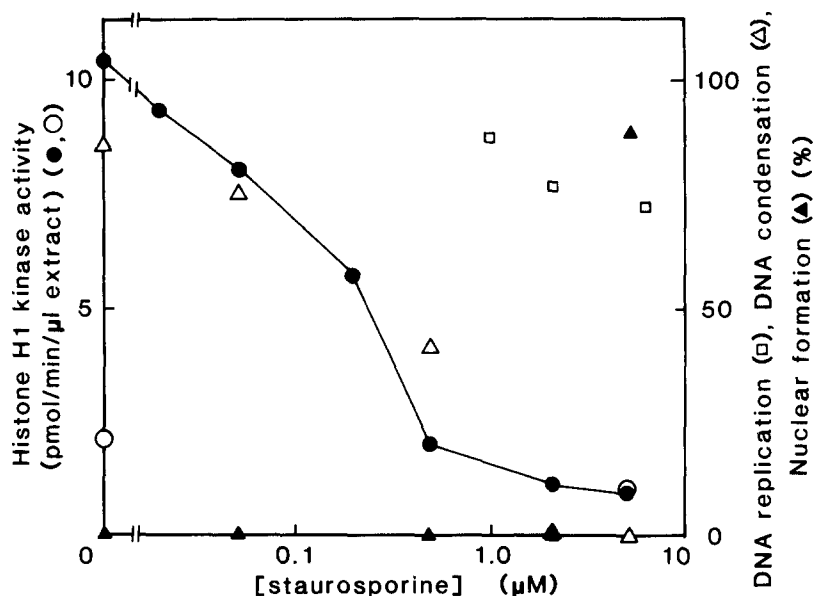
B

Figure 1. (*A*) Effects of staurosporine on S- and M-phase specific activity in egg extracts. H1 histone kinase activity in S-phase (○) and M-phase extracts (●) was measured as described in Materials and Methods. The DNA replicating activity of S-phase extracts (□) was measured as the incorporation of dCTP into sperm DNA. Means of the amount of dCTP incorporated after 120-, 150-, and 180-min incubation in the presence of 1, 2, and 6 μM staurosporine were normalized against that obtained in the absence of staurosporine, which was taken to be 100%. DNA condensation (△) and nuclear formation (▲) in M-phase extracts were measured by examining the morphology of sperm DNAs added to the extracts after 60-min incubation. More than 200 sperm DNAs were observed for determining the percentage of DNA with condensed chromatin and nuclear formation. (*B*) Micrographs of sperm DNAs added to M-phase extracts. Sperm DNAs were incubated for 30 min in M-phase extracts without (*a*) and with (*b, c,* and *d*) 5 μM staurosporine. Total DNAs (*a* and *b*) were visualized with HOECHST fluorescence. Phase contrast (*c*) and membrane staining with 3,3′-diethylthiadicarbocyanine iodide (*d*) are in the same field as *b*. Bar, 50 μm.
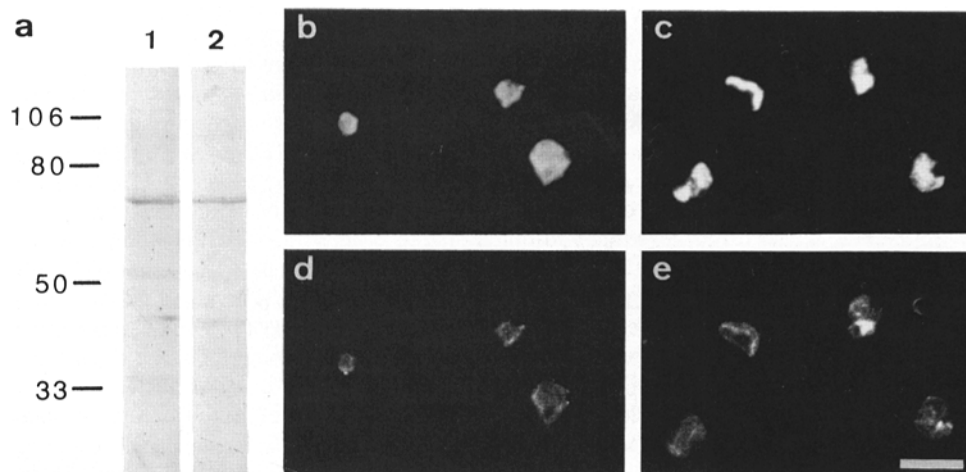

Figure 2. Fluorescent micrographs of nuclear structures formed in egg extracts. Sperm DNAs were incubated for 30 min in S-phase extracts (*b* and *d*) and in M-phase extracts (*c* and *e*), both of which contained 5 μM staurosporine. After incubation, the samples were fixed and processed as described in Materials and Methods. Total DNAs (*b* and *c*) were visualized with HOECHST fluorescence microscopy; the localization of lamin (*d* and *e*) was detected with an anti-human lamin B antisera followed by a FITC-labeled second antibody. Immunoblotting of S- (lane *1*) and M-phase egg extracts (lane *2*) by anti-human lamin B antisera was shown in *a*. Standard molecular masses were indicated as kD. Bar, 50 μm.

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while the condensed chromatins formed in the M-phase extracts were not stained by the antisera (data not shown).

The function of the nucleus-like structures formed in the egg extracts was examined by measuring the nuclear transport activity of several proteins. Fig. 3, *a* and *b* shows the specific accumulation of nucleoplasmin in the nucleus-like structures formed in M-phase extracts treated with staurosporine. In every sample of decondensed DNA surrounded by the nuclear membrane-like structures seen under phase contrast microscopy, fluorescent-labeled nucleoplasmin was accumulated during incubation. Fig. 3, *c* and *d* shows the transport of BSA conjugated with and without the nuclear localization sequence of SV-40 large T-antigen. Control BSA was not accumulated, rather being excluded from the nuclear structures formed in M-phase extracts treated with staurosporine, while BSA with the nuclear localization signal was accumulated in the nucleus-like structures. More than 80% of nuclei formed in the S- and staurosporine treated M-phase egg extracts had accumulated nucleoplasmin (42/51 and 64/70 nuclei formed in the S- and M-phase extracts, respectively) and BSA conjugated with the nuclear localization signal (57/65 and 75/80 nuclei formed in the S- and M-phase extracts, respectively). The nuclei formed in both extracts efficiently accumulated proteins containing nuclear localization signals.
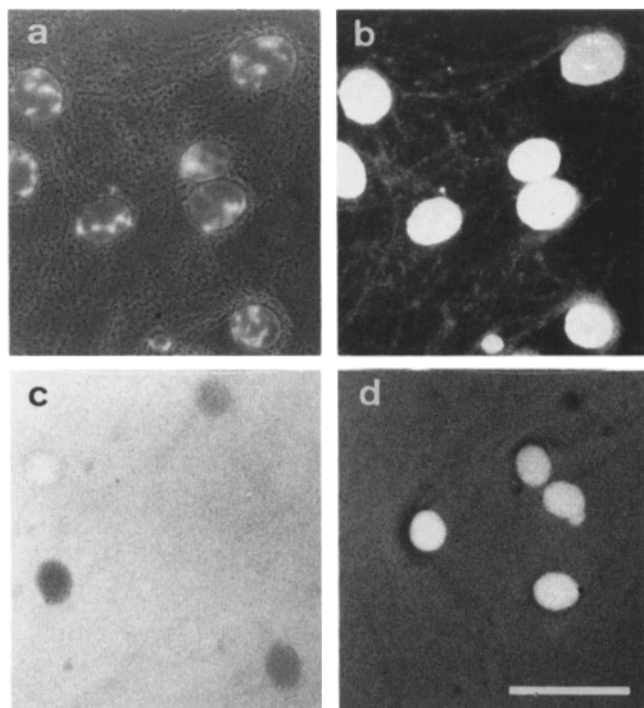

Figure 3. Transport of nuclear proteins into the nucleus-like structures formed in M-phase egg extracts. Sperm DNAs were incubated for 30 min in M-phase extracts treated with 5 μM staurosporine. Fluorescent-labeled protein was then added and the mixture was incubated for an additional 60 min to determine the transport of TRITC-labeled nucleoplasmin (*a* and *b*); the mixture was incubated for an additional 30 min to determine the transport of FITC-labeled BSA without (*c*) and with (*d*) the nuclear localization signal of SV-40 large T-antigen. The nuclei were fixed and viewed under a phase contrast microscopy with HOECHST staining (*a*), TRITC fluorescence (*b*), and FITC fluorescence (*c* and *d*). Bar, 100 μm.
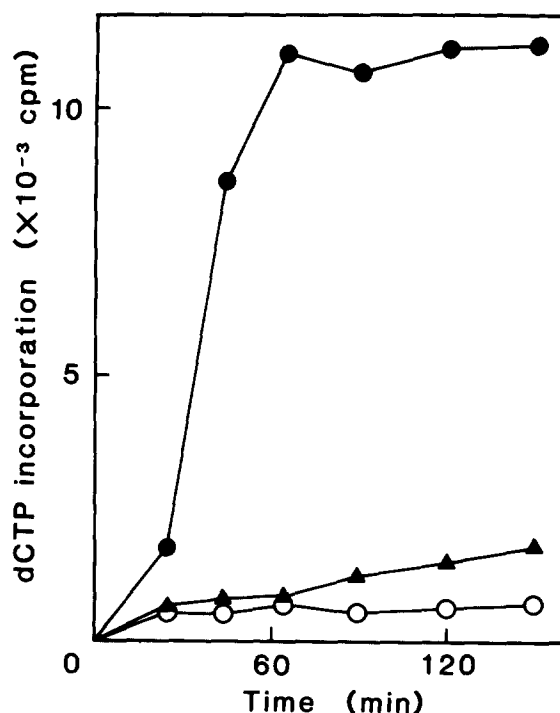

Figure 4. Time courses of incorporation of dCTP into sperm DNAs added to egg extracts. Sperm DNAs were incubated in S-phase (●) and M-phase extracts treated without (○) and with (▲) 5 μM staurosporine. After an appropriate time, the amounts of [α-^{32}P]dCTP incorporated into DNA were measured, as described in Materials and Methods.

Formation of Nuclei Unable to Replicate DNA in Staurosporine Treated M-phase Egg Extracts

The nuclei formed in M-phase extracts treated with staurosporine were incapable of replicating DNA. Fig. 4 shows the time courses of dCTP incorporation into the sperm DNA added to the egg extracts. When sperm DNAs were incubated in the S-phase extracts, the incorporation followed a typical time course, showing a lag period of ∼30 min, which corresponded to the time required for nuclear formation. However, with M-phase extracts, most sperm DNAs were condensed and no nuclear structures were formed around the DNA during incubation (cf. Fig. 1 *B*). With regard to DNA replication, the incorporation of dCTP was as low as that observed with the S-phase extracts including 10 μg/ml of aphidicolin (data not shown). When the M-phase extracts were treated with 5 μM staurosporine, nuclear structures were formed within 30 min after the addition of sperm DNA (cf. Fig. 1 *B*). However, dCTP incorporation occurred to the same extent as that observed upon the incubation of sperm DNA with M-phase extracts.

The low level of dCTP incorporation observed with the M-phase extract was not due to a deficiency in the activity of DNA polymerase α. When the polymerase activity was measured as the incorporation of dTTP into activated DNA that does not require initiation, the activity of the M-phase extract (20.0 ± 0.8 U/mg protein) was quite similar to that of the S-phase extract (19.3 ± 2.1 U/mg protein). In addition, staurosporine showed no appreciable effect on the polymerase activity. The polymerase activity of the M-phase ex-
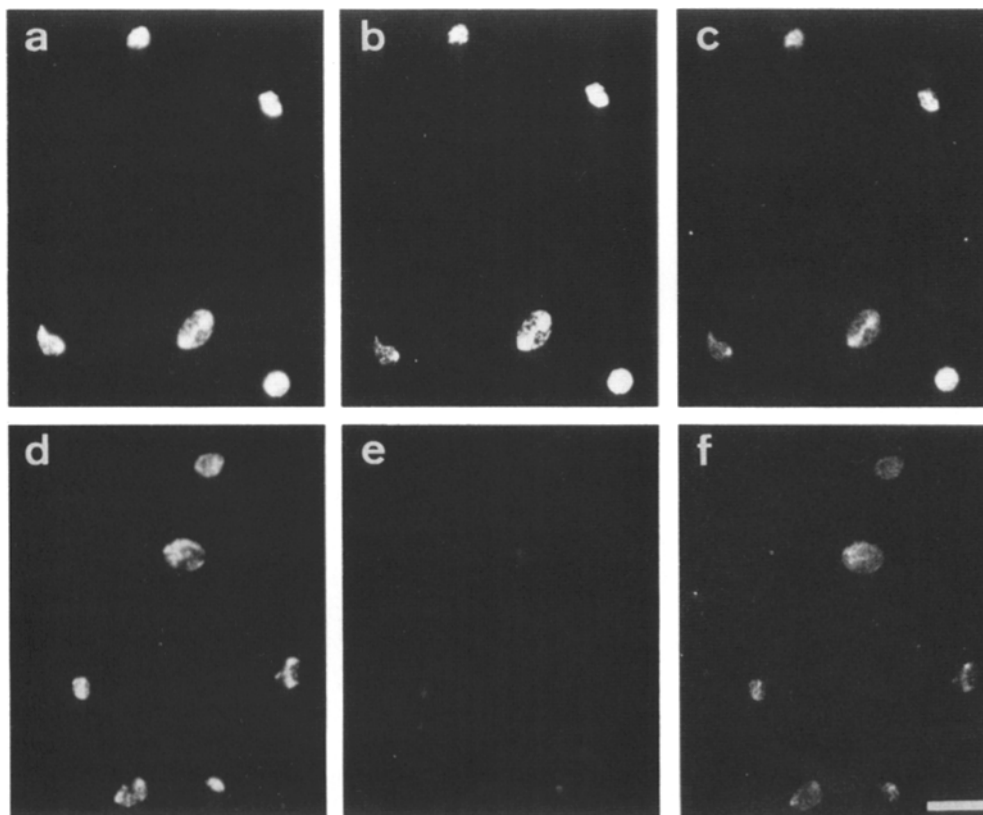
Figure 5. Fluorescent micrographs showing replicating activity of nuclei formed in egg extracts. Sperm DNAs were incubated in S-phase extracts (*a*, *b*, and *c*) and M-phase extracts (*d*, *e*, and *f*) both of which were treated with 5 μM staurosporine in the presence of 2 μM biotinylated dUTP. After 120-min incubation, samples were fixed and processed as described. Total DNAs (*a* and *d*) were stained with HOECHST. Incorporation of biotinylated dUTP (*b* and *e*) was detected with Texas-red–conjugated streptavidin. Localization of PCNA (*c* and *f*) was detected with anti-rabbit PCNA mAb followed by FITC-labeled second antibody. Bar, 50 μm.

tract observed in the presence of 5 μM staurosporine (21.0 ± 0.7 U/mg protein) was essentially the same as that in the absence of staurosporine. A similar result was obtained with the S-phase egg extract in the presence of 5 μM staurosporine (20.8 ± 2.2 U/mg protein), being consistent with the marginal effect of staurosporine on the activity of DNA replication in S-phase egg extract (Fig. 1 *A*).

We further examined the replicating activity of the nuclei formed in the egg extracts by visualizing the incorporation of biotinylated dUTP into the nuclei. In the following experi-
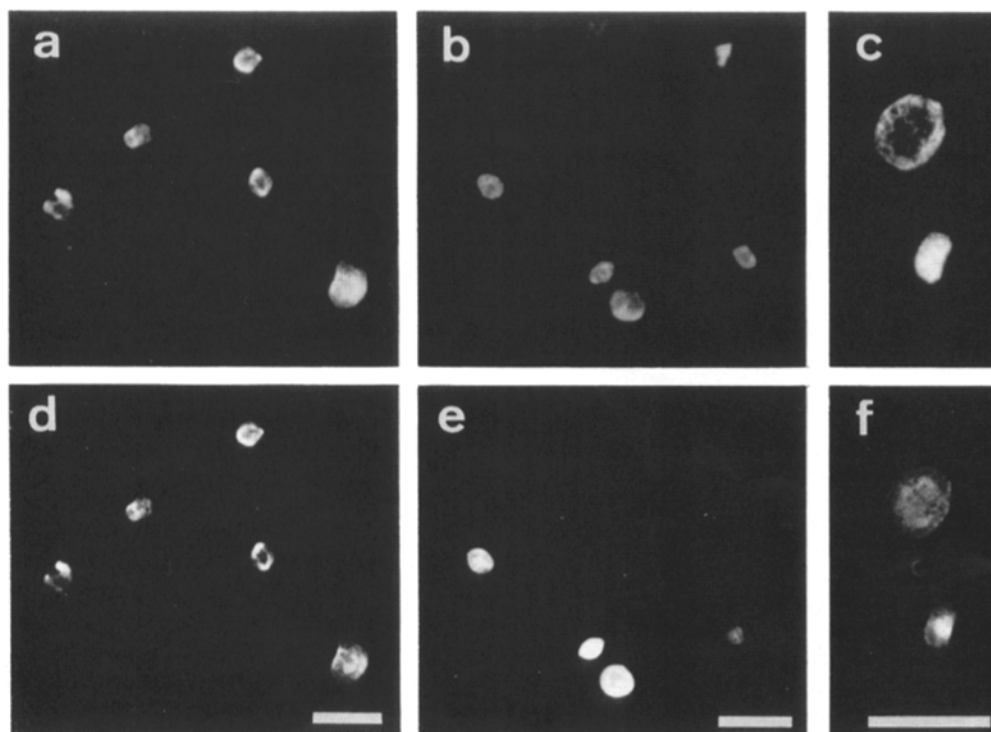

Figure 6. Accumulation of DNA polymerase α into nuclei formed in egg extracts. Sperm DNAs were incubated in S-phase extracts (*a* and *d*) and M-phase extracts (*b*, *c*, *e*, and *f*) both of which were treated with 5 μM staurosporine. After 120-min incubation, the samples were fixed and processed. The same fields were viewed by staining total DNA with HOECHST (*a*, *b*, and *c*) and DNA polymerase α with anti-chick DNA polymerase α mAb followed by FITC-labeled second antibody (*d*, *e*, and *f*). Bars, 40 μm.
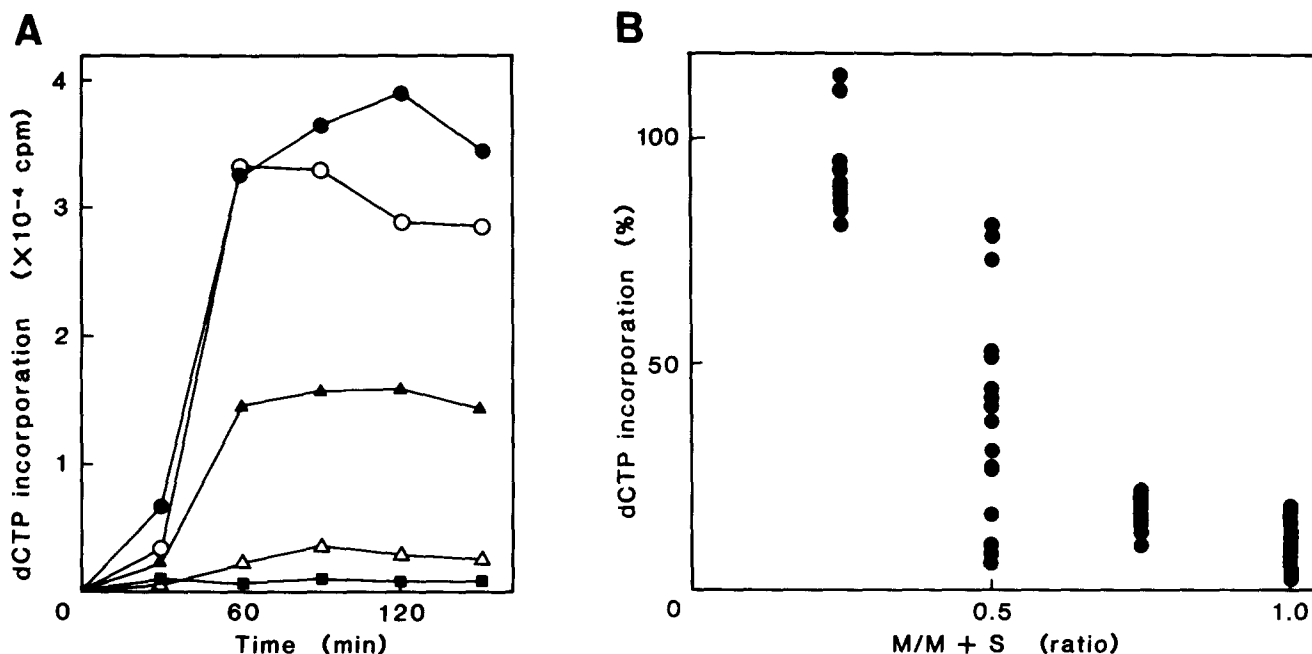
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Figure 7. Inhibition of DNA replication by M-phase extracts treated with staurosporine. (*A*) Time courses of dCTP incorporation into sperm DNA added to various mixtures of S- and M-phase extracts. Sperm DNAs were incubated in extracts that consisted of the following ratios of S- and M-phase extracts, both of which contained 5 μM staurosporine. ●, 1 : 0; ○, 3 : 1; ▲, 1 : 1; △, 1 : 3; ■, 0 : 1. After an appropriate time, the amounts of dCTP incorporated into DNA were measured. (*B*) Dose-dependent inhibition of replication in S-phase extracts by M-phase extracts. Sperm DNAs were incubated as in *A* and the maximal amounts of dCTP incorporated into the sperm DNAs were measured after 120-, 150-, and 180-min incubation. Each dot represents a mean value of the incorporation (SEM within 10% of mean value) and it is normalized against that obtained with S-phase extracts, which was taken to be 100%.

ments 5 μM staurosporine was added to both M- and S-phase egg extracts, to exclude any additional effect of staurosporine on the replication. Active incorporation of the label into DNA was detected in all the nuclei formed in S-phase extracts, but not in those formed in staurosporine treated M-phase extracts (Fig. 5). However, some factors essential to the replication of DNA were similarly accumulated in the nuclei formed in both extracts. One of the factors was PCNA (proliferating cell nuclear antigen), a subunit of DNA polymerase δ required for the processivity of the polymerase (Bravo et al., 1987; Prelich et al., 1987). PCNA was detected with an anti-rabbit PCNA mAb, which has been shown to recognize a *Drosophila* homologue in addition to mammals (Yamaguchi et al., 1991), and it recognized a single polypeptide of an apparent molecular mass of 36 kD in the egg extract (see Fig. 9). Accumulation of DNA polymerase α was also detected in the nuclei in both extracts (Fig. 6), using an anti-chick DNA polymerase α mAb (Hirose et al., 1988). This antibody (4-8H) could immunoprecipitate the activity of DNA polymerase α in the egg extract (data not shown), and it recognized a single polypeptide with an apparent molecular mass of 200 kD in the egg extract (see Fig. 9).

Close examination of the distribution of DNA polymerase α revealed some qualitative differences in the association of the polymerase with the chromatin. In the nuclei formed in the S-phase extracts, distribution of the polymerase coincided exactly with that of DNA. In the nuclei formed in staurosporine treated M-phase extracts, coinciding distribution of the polymerase and DNA was not reproducibly observed. Higher magnification view of the nuclei formed in staurosporine treated M-phase extracts shows inverse and partially coinciding distribution of the polymerase with DNA in some nuclei (Fig. 6, *c* and *f*).

Inhibitory Activity of Staurosporine Treated M-phase Extracts in the Replication of DNA in S-phase Extracts

M-phase extracts treated with staurosporine supported the formation of nuclei incapable of replicating DNA. Surprisingly, these extracts inhibited the replication of DNA in S-phase extracts. Fig. 7 *A* shows the time courses of the incorporation of dCTP after sperm DNA was added to egg extracts consisting of various proportions of S- and M-phase extracts. To avoid dilution of staurosporine, we added 5 μM staurosporine to both extracts throughout the experiments described below. At this concentration, staurosporine only marginally inhibited the replication of DNA in the S-phase extracts (see Fig. 1 *A*). In addition, we kept the concentration of sperm DNA in the extract mixture to be ~2,000 sperm nuclei per μl extract, since the replicating activity of the S-phase extract linearly increased with increasing concentration of sperm DNA up to about 6,000 sperm nuclei per μl extracts (data not shown). Under the experimental conditions described above, we found that the mixed extracts appeared to retain the capacity for DNA replication providing the content of the S-phase extracts exceeded that of the M-phase extracts. In contrast, when the M-phase extracts were in excess of the S-phase extracts the replication was inhibited. It should also be noted that the replication reached

the maximal level within 90 min after the addition of sperm DNA irrespective of the activity.

The dose-dependent inhibition of replication by staurosporine treated M-phase extracts is more clearly depicted in Fig. 7 B. In this figure, the replication activity of the extracts was measured as the average amount of dCTP incorporated into sperm DNA at 120, 150, and 180 min after starting the reaction. The replicating activity obtained in the extract containing S- and M-phases at various ratios was normalized against that of the S-phase extract alone. Most of the data obtained from up to 16 independent experiments per extract mixture tended to be the same as those shown in Fig. 7 A. The activity of the mixed extracts was dominated by the extract that was present in excess. At an equal proportion of the S- and M-phase extracts, the activity varied greatly depending on the preparation of the extracts.

Determination of Initiation of DNA Replication before and after Nuclear Formation

Mode of nuclear formation appeared to determine the replicating activity of the nucleus. Fig. 8 summarizes the results. Nuclei formed in staurosporine treated M-phase extracts failed to initiate DNA replication upon the addition of S-phase extracts that could overcome the inhibitory activity of the M-phase extracts. When threefold the amount of S-phase extract was added to the M-phase extract before sperm DNA was added, the replicating activity was similar to that observed with the S-phase extract alone. When threefold the amount of S-phase extract was added after nuclear formation, that is, 25 min after the addition of sperm DNA
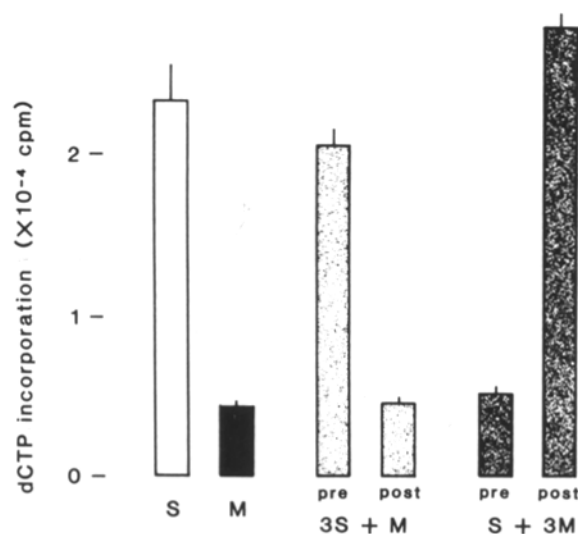

Figure 8. Determination of replicating activity after nuclear formation. Sperm DNAs (1,000/μl extract) were incubated in extracts mixed in the ratios indicated below the columns. In experiments indicated as post, sperm DNAs (4,000/μl) were preincubated with M- and S-phase extracts for 25 min, allowing the formation of nuclei; 3 vol of S- and M-phase extracts containing [^{32}P]dCTP were then added for initiating the incorporation of dCTP. In preexperiments, S- and M-phase extracts were mixed together, and sperm DNAs were added to initiate the incorporation of dCTP. Both extracts contained 5 μM staurosporine. The maximal amounts of dCTP incorporated into DNA were measured after 120-, and 150-, and 180-min incubation. Results are mean ± SEM.

to the M-phase extract, the incorporation of dCTP was suppressed to a level similar to that observed with the M-phase extracts alone.

In contrast, staurosporine treated M-phase extracts failed to inhibit replication once nuclei were formed in the S-phase extracts. The M-phase extracts inhibited replication when threefold amounts were added to the S-phase extracts before sperm DNA was added (see Fig. 7). The amount of dCTP incorporated into the DNA was essentially the same as that observed with staurosporine treated M-phase extracts alone. When the M-phase extracts were added after the nuclei had been formed in the S-phase extracts, that is, 25 min after sperm DNA had been added to the S-phase extracts, the incorporation of dCTP was as high as that observed with the S-phase extracts alone.

To explore the factors regulating the activity of DNA replication in the egg extracts, we separated the extracts into membranous and membrane-depleted fractions by centrifugation at 100,000 g for 90 min; reconstitution of the activity of nuclear formation requires both fractions (Lohka and Masui, 1984). Although the membrane-depleted fractions supported neither nuclear formation nor sperm DNA replication (cf. Newport, 1987), they contained the majority of DNA polymerase α. When the polymerase activity was measured with activated DNA as a substrate, the specific activity of S-phase membranous and membrane-depleted fractions was 0.2 and 13.3 ± 0.4 U/mg protein, respectively. Similar activity was found with staurosporine treated M-phase membranous and membrane-depleted fractions (0.3 and 12.6 ± 0.8 U/mg protein, respectively). Immunoblotting of these fractions with an anti-chick DNA polymerase α mAb showed that most of the 200-kD Xenopus homologue was found in the membrane-depleted fractions (Fig. 9). Fig. 9 also showed a similar enrichment of 36 kD Xenopus PCNA detected with anti-rabbit PCNA mAb, in the membrane-depleted fractions.

Preincubation of the sperm DNA with S-phase membrane-depleted fractions allowed the formation of nuclei competent for DNA replication upon addition of excess M-phase extracts treated with staurosporine. Fig. 10 A shows that the addition of the M-phase extracts to sperm DNA that had been preincubated with the S-phase membrane-depleted fractions for 10 min induced replication of DNA to the extent observed in the S-phase extracts alone. During preincubation, sperm DNA decondensed within a minute, but no nuclear structures were formed around the decondensed DNA in more than 60 min of preincubation. When staurosporine treated M-phase extracts were added to the S-phase membrane-depleted fractions just after the addition of sperm DNA, we observed only a low level of incorporation, similar to that seen with staurosporine treated M-phase extracts alone.

In contrast, preincubation of sperm DNA with membrane-depleted fractions of staurosporine treated M-phase extracts failed to inhibit replication upon the addition of excess S-phase extract. Fig. 10 B shows that the addition of excess S-phase extract induced replication when the sperm DNA had been preincubated with the M-phase membrane-depleted fractions for 30 min. Similar DNA replication activity was observed without preincubation. This activity was essentially the same as that observed with the S-phase extracts alone.
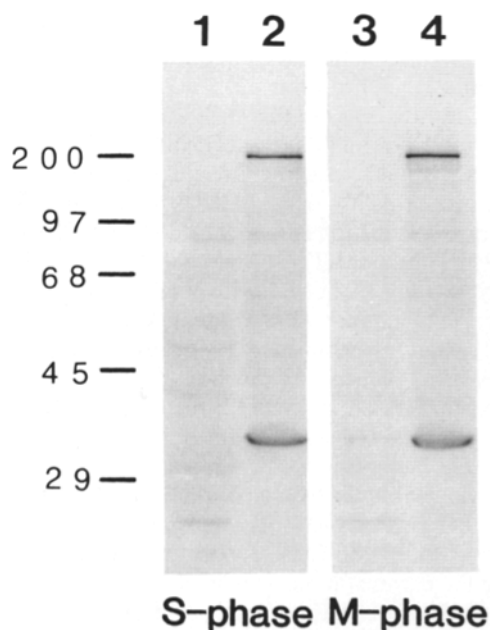
1 2 3 4

200 —

97 —

68 —

45 —

29 —

S-phase M-phase

Figure 9. Detection of DNA polymerase α and PCNA in membranous and membrane-depleted fraction of egg extracts. S- and M-phase membranous (lanes *1* and *3*) and membrane-depleted fractions (lanes *2* and *4*) were analyzed on SDS-PAGE and immunoblotting with anti-chick DNA polymerase α and anti-rabbit PCNA mAb were performed as described under Materials and Methods. Standard molecular masses were indicated as kD.
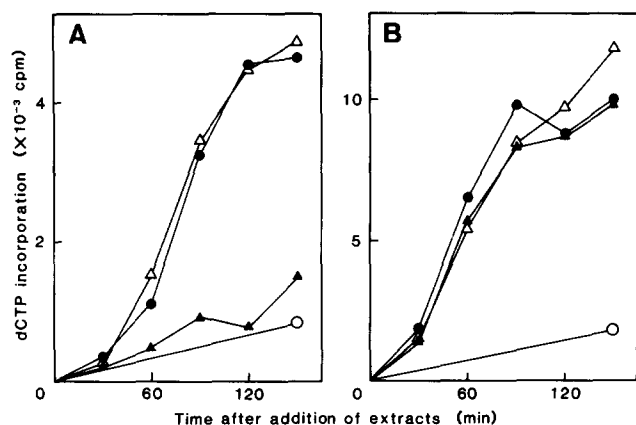

Figure 10. Determination of replicating activity before nuclear formation. (*A*) Sperm DNAs (4,000/μl) were preincubated with S-phase membrane-depleted fractions for 0 min (▲) and 10 min (△). After preincubation, 3 vol of M-phase extracts treated with 5 μM staurosporine were added to initiate nuclear formation. After an appropriate time, the amounts of dCTP incorporated into DNA were measured. (*B*) Sperm DNAs (4,000/μl) were preincubated with the membrane-depleted fractions of M-phase extracts treated with 5 μM staurosporine for 0 min (▲) and 30 min (△). After preincubation, 3 vol of S-phase extracts treated with 5 μM staurosporine were added to initiate nuclear formation. After an appropriate time, the amounts of dCTP incorporated were measured. Time courses of incorporation of dCTP in S-phase (●), and M-phase extracts (○), both of which were treated with 5 μM staurosporine, are shown as controls.

Discussion

Nuclear structure plays a central role in regulating the replication of eukaryotic genomes. Previous studies with a cell-free system of *Xenopus* eggs suggest that the nuclear structure plays a dual role: (1) initiating DNA replication (Blow and Laskey, 1986; Newport, 1987; Sheehan et al., 1988; Hutchison et al., 1988; Leno and Laskey, 1991) and (2) preventing reinitiation before mitosis (Blow and Laskey, 1988; Leno et al., 1992). The nature of the nuclear structure, however, makes it difficult to examine the molecular events that regulate the initiation of DNA replication. Our present findings that M-phase egg extracts treated with staurosporine formed nuclei that were incompetent with regard to replication, and that staurosporine treated M-phase extracts inhibited the replication of DNA in S-phase extracts, allowed us to distinguish the events required for the initiation of DNA replication into those occurring before and after nuclear formation.

The formation of a nucleus in staurosporine treated M-phase extracts was confirmed by examining the structure and the function of the nucleus-like structures formed upon the addition of sperm DNAs to the extracts. Comparing the structures formed in staurosporine treated M-phase with those formed in the S-phase extracts, we found that the structures enclosing the decondensed DNA were indistinguishable in respect to morphology, observed under phase contrast and fluorescence (for membrane staining) microscopy, and in respect to the assembly of nuclear lamin. Electron microscopic observations also revealed that double membrane structures typical of the nuclear membrane were formed around the decondensed DNA in both extracts (data not shown). In addition, the nuclear structures formed in staurosporine treated M-phase extracts retained a capacity to accumulate nuclear proteins similar to that observed in the nuclei formed in the S-phase extracts. These results indicate that the nucleus-like structures formed in staurosporine treated M-phase extracts were structurally and functionally intact nuclei.

The transformation of the M-phase extracts into interphase-like extracts by treatment with staurosporine suggests that protein kinase plays a central role in regulating M-phase-specific activity. Staurosporine inhibits a broad range of protein kinases (Ruegg and Burgess, 1989). Several reported Ki values (Tamaoki et al., 1986; Ruegg and Burgess, 1989) suggest that most kinases would be inactivated in the presence of micromolar concentrations of staurosporine. We found that an S-phase specific activity, i.e., the replication of DNA, was relatively insensitive to treatment with staurosporine, while M-phase specific activities were dose dependently inhibited by this agent. In particular, micromolar concentrations of staurosporine almost completely inhibited the activity of histone H1 kinase, previously attributed to an activated form of cdc2-cyclin complex (see reviews for Nurse, 1990; Lewin, 1990). The parallel inhibition of DNA condensation and histone H1 kinase activity suggests a close relationship between these two phenomena, while the apparent dissociation between H1 kinase activity and inhibition of nuclear formation supports a previous finding that nuclear formation is indirectly regulated by cdc2 kinase (Pfaller et al., 1991; Vigers and Lohka, 1992). A positive feedback system that amplifies the signal by protein kinase cascades may explain

the requirement of higher concentrations of staurosporine for the inhibition of M-phase-specific events.

The nuclei formed in staurosporine treated M-phase extracts were unable to replicate DNA. We found no difference in the activity of DNA polymerase α between S- and M-phase egg extracts. In addition, staurosporine had no appreciable effect on the polymerase activity, which was measured using activated DNA as a substrate. Since activated DNA requires no initiation of new replication forks for the replication reaction, the obtained results indicated that the nuclei formed in the staurosporine treated M-phase extract were unable to initiate DNA replication. This inability is possibly due to a deficiency of the proper association of DNA polymerase α with chromatin, but is not due to a deficiency in nuclear transport as well as in polymerase activity. In addition, an excess of S-phase extract, which overcame the inhibitory activity of staurosporine treated M-phase extracts, failed to initiate replication in the nuclei formed in staurosporine treated M-phase extracts. These results suggest that the nuclei formed in staurosporine treated M-phase extracts mimic the nuclei in the G2 phase (Leno et al., 1992). It is possible to speculate that the inhibition of M-phase-specific kinase activity by staurosporine reverses the cell cycle from M- to G2-phase.

Staurosporine treated M-phase extracts inhibited replication in S-phase extracts. The mode of inhibition indicates that a critical concentration of factor(s) regulates replication; either phase of an extract present in excess dominated activity in the mixed extracts. One of the possible explanations for the inhibition of the replication is the dilution of essential activity in the S-phase extract by addition of the staurosporine-treated M-phase extract. However, an excess of the M-phase extract did not inhibit the replication when added to sperm DNA preincubated with the S-phase to allow nuclear formation or with S-phase membrane-depleted fractions. These findings supported the view that the staurosporine-treated M-phase extract has inhibitory activity on the replication in the S-phase extract. We attempted to purify these factor(s) that inhibited replication. Preliminary experiments indicated that the soluble fraction of the M-phase extracts precipitated by ammonium sulfate contained the inhibitory activity. Addition of M-phase fractions, but not S-phase fractions or dialysis buffer, inhibited replication in the S-phase extracts (unpublished observation). This finding further indicates that the inhibition of DNA replication in the S-phase extract produced by the addition of the staurosporine-treated M-phase extract was not due to the dilution of factors required for replication in the S-phase extract.

Using M-phase extracts treated with staurosporine, we were able to distinguish the events regulating DNA replication into those that occurred before and after nuclear formation. We found that the formation of nucleus determined DNA replication activity. The addition of excess M- and S-phase extracts treated with staurosporine failed to alter the activity of the nuclei formed in the S- and M-phase extracts, respectively. More surprisingly, we found that the preincubation of DNA in the S-phase membrane-depleted fraction allowed the formation of nuclei that were capable of replication DNA upon the addition of excess M-phase extracts treated with staurosporine. The S-phase membrane-depleted fraction supported neither the formation of nuclei nor the replication of DNA, but it contained factors, such as DNA poly-

merase α, that are essential for replication. Our results indicate that the binding of some factors in the membrane-depleted fraction to DNA determined the state required for the initiation of DNA replication; this state was observed after a 10-min preincubation and was usually completed within 20 min of preincubation. Recently, Adachi and Laemmli (1992) reported the formation of replication protein A foci in sperm DNA upon incubation of the DNA with an S-phase membrane-depleted fraction. The formation of these foci, detected as early as within 30 min of the beginning of incubation, was implicated in the formation of a prereplication center. Our present findings show that the determination of replication appears to precede the formation of the so-called prereplication center. We are currently investigating the details regarding the determination of replication, and the identification of the factors involved in this process.

This present study has indicated that positive factors in the S-phase extracts determined the initiation of replication before the nucleus was formed. These positive factors did not appear to act on the chromatin once the nuclei were formed in staurosporine treated M-phase extracts, such characteristics resembling the behavior of the licensing factor proposed by Blow and Laskey (1988). Such a licensing factor was proposed as the reinitiation of DNA replication requires the breakdown of the nuclear membrane. Our present study has demonstrated that such factors licensing the initiation of DNA replication were present in the S-phase membrane-depleted fractions. The apparent absence of the activity in the M-phase extracts should aid in identifying the licensing factor.

The action of staurosporine treated M-phase extracts in inhibiting replication suggests that a negative factor acts either directly or indirectly on the positive factors to prevent their action. The following results support this idea: (a) The inability of staurosporine treated M-phase extracts to inhibit replication in S-phase membrane-depleted fractions after sperm DNA was preincubated with these fractions. (b) The failure of preincubation of sperm DNA with membrane-depleted fractions of staurosporine treated M-phase extracts to inhibit replication. The negative factor in staurosporine treated M-phase extracts may function to prevent the reinitiation of replication before the M-phase is completed. Further study both of the negative and the positive factors will enable us to answer several fundamental questions concerning the regulation of cell growth.

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