Journal of Cell Science 108, 235-244 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995



# The role of protein phosphorylation in the assembly of a replication competent nucleus: investigations in *Xenopus* egg extracts using the cyanobacterial toxin microcystin-LR

**Jacqueline Murphy, Catherine M. Crompton, Sandra Hainey, Geoffrey A. Codd and Christopher J. Hutchison***

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, Scotland

*Author for correspondence

## SUMMARY

**Cell-free extracts of *Xenopus* eggs support nuclear assembly and DNA replication in vitro. Extracts supplemented with the protein phosphatase inhibitor microcystin-LR displayed various inhibitory effects at different concentrations of the toxin. In the presence of cycloheximide, additions of microcystin did not induce histone H1-kinase activity. Nevertheless, increasing concentrations of microcystin did sequentially prevent DNA replication, nuclear lamina assembly and nuclear envelope assembly. DNA replication was prevented when microcystin was added at 250 nM. Furthermore, this effect could be reversed after the addition of the catalytic sub-unit of protein phosphatase 2A to inhibited extracts. At a concentration of 250 nM microcystin, nuclear membrane assembly, nuclear lamina assembly and nuclear transport all occurred in egg extracts. In addition single-stranded M13 DNA replication was also permitted. However, it appeared that replicase assembly was not completed, since nuclei assembled in microcystin-treated extracts displayed an unusual distribution of proliferating cell nuclear antigen (PCNA). Although PCNA was located at sites that resembled pre-replication foci, this nuclear protein was readily solubilised when nuclei were isolated and extracted sequentially with Triton, nucleases and salts. Despite this, nuclei containing pre-assembled replication forks could synthesise DNA when transferred into microcystin-treated extracts.**

Key words: protein phosphorylation, DNA replication, microcystin, nuclear assembly

## INTRODUCTION

DNA replication in eukaryotes is a highly regulated event that is restricted to S-phase of the cell cycle. Entry into S-phase is controlled by cytoplasmic activities as demonstrated by fusion of a $G_1$ cell to an S phase cell (Rao and Johnson, 1970) or microinjection of DNA into amphibian eggs (Harland and Laskey, 1980). S-phase inducers accumulate through $G_1$ and, having reached a threshold level, act in two distinct ways: firstly, to co-ordinate the synthesis of gene products required for DNA synthesis (Dirick and Nasmyth, 1991; Lowndes et al., 1992); and secondly, to modify replication factors thus allowing the assembly of a replicase at a replication fork (Fotedar and Roberts, 1992; Dutta and Stillman, 1992).

While yeast cells have proved invaluable in elucidating mechanisms that control gene expression at the $G_1$/S-phase boundary (Nasmyth, 1993), modifications that control the assembly of a replicase have mostly been described in cell-free extracts that support DNA replication *in vitro*. Cell-free extracts of *Xenopus* eggs have proved to be useful experimental tools for studying the events associated with the initiation of DNA replication. In this system, specific DNA sequences are not required for the formation of replication origins, but instead the assembly of a nucleus around either chromosomal or plasmid DNA is an essential pre-requisite for the initiation of DNA replication (Blow and Laskey, 1986; Hutchison et al., 1987; Newport, 1987). Many of the steps in the assembly of a replication-competent nucleus have now been defined. Initially, the DNA template is modified in a process that is dependent upon nucleoplasmin (Newport, 1987; Philpott et al., 1991). A double-unit nuclear membrane containing nuclear pores then forms around the modified chromatin (Sheehan et al., 1988). The formation of the nuclear envelope then allows lamins to enter the nucleus, which leads to the assembly of a lamina (Newport et al., 1990; Meier et al., 1991). Soon after lamina assembly is completed, DNA replication is initiated (Hutchison et al., 1988). The cell cycle control of DNA replication and the co-ordination of initiation events both depend upon some aspect of nuclear envelope assembly. The best characterised of these events is the cell-cycle control of DNA replication in which initiation at each replication fork is limited to once every cell cycle. This is apparently achieved because an unidentified activity, termed licensing factor, binds to chromatin at mitosis and marks the sites of future replication forks. However, once the nuclear envelope has formed, additional licensing events cannot occur, since licensing factor

cannot cross the nuclear envelope. Furthermore, at initiation, the licensing factor that is already bound is destroyed so that it cannot be used again (Blow and Laskey, 1988; Leno et al., 1992; Coverly et al., 1993). In addition to the cell cycle control of DNA replication, nuclear envelope assembly appears to co-ordinate initiation events. Nuclei, once assembled, act as independent units of DNA replication (Blow and Watson, 1987). Within each nucleus initiation events apparently occur nearly synchronously from up to 50,000 pairs of replication forks, which are organised into 150-300 large replication centres (Mills et al., 1989; Cox and Laskey, 1991). How initiations are co-ordinated in this way is not fully understood, but there appears to be a requirement for both active nuclear transport (Cox, 1992) and the assembly of structural elements in the nucleus (Meier et al., 1991; Jenkins et al., 1993).

In egg extracts, entry into S-phase is probably controlled at several levels by protein phosphorylation. The activity of the cyclin-dependent kinase (cdk), cdk2 is required for the initiation of DNA replication (Blow and Nurse, 1990; Fang and Newport, 1991). In its nuclear form, the single-stranded DNA binding protein RP-A is phosphorylated by a cdk2-dependent mechanism. S-phase phosphorylation of RP-A appears to occur when it is bound to single-stranded DNA (Fang and Newport, 1993). RP-A associates with chromatin at sites that resemble replication foci prior to the assembly of a nuclear envelope (Adachi and Laemmli, 1992). Since it is required for the initiation of DNA replication (Fang and Newport, 1992), phosphorylation of RP-A at this stage may establish a pre-synthesis complex. In addition to cdk2, other protein kinase activities may be required for DNA replication. Treatment of egg extracts with the protein kinase inhibitor 6-DMAP prevents chromatin from being licensed at mitosis (Blow, 1993) but does not prevent nuclear assembly. However, the protein kinase activity that is inhibited by 6-DMAP and the protein target of this kinase are as yet undefined.

Unlike RPA, proliferating cell nuclear antigen (PCNA) is accumulated at replication centres only after nuclear envelope assembly and, in particular, lamina assembly are completed (Hutchison and Kill, 1989; Meier et al., 1991). Furthermore, there is a 15 minute delay between the time at which PCNA first appears at replication foci and the time at which DNA synthesis is first detected (Kill et al., 1991). By analogy with SV40 DNA replication, PCNA probably accumulates at replication forks after phosphorylation of RP-A (Dutta and Stillman, 1992). Thus, any delay between the time of appearance of PCNA at replication centres and the synthesis of DNA may represent a second rate-limiting step in the initiation of DNA synthesis. Furthermore, this second rate-limiting step may co-ordinate initiation events. We have tested this hypothesis using the protein serine/threonine phosphatase (PP) inhibitor microcystin-LR. Additions of microcystin-LR to *Xenopus* egg extracts, at concentrations that reduce PP1 and PP2A activity (see Materials and Methods) by 42%, prevent DNA synthesis. However, nuclei are still assembled in microcystin-treated extracts and PCNA is accumulated at sites that resemble pre-replication foci. Predictably, microcystin-LR does not prevent the synthesis of single-stranded M13 DNA in *Xenopus* egg extracts. Moreover, nuclei pre-assembled in extracts containing aphidicolin and then transferred to extracts containing microcystin-LR do replicate DNA. Since the capacity of microcystin-treated extracts to replicate DNA can be rescued by re-addition of the catalytic subunit of PP2A, we conclude that the phosphorylation status of one or more replication proteins or of one or more nuclear matrix proteins is important after the assembly of a pre-synthesis complex and a nuclear envelope. Moreover, this (these) phosphorylation event(s) may represent an additional level of control, which is required because of the complexity of co-ordinating initiation events within a nucleus and limiting these events within the context of the cell cycle.

## MATERIALS AND METHODS

### Preparation of egg extracts

Egg extracts were prepared according to the method described by Hutchison et al. (1988). Immediately after preparation, the extracts were mixed with glycerol (5% v/v), frozen and stored in liquid nitrogen. For assays, extracts were recovered from liquid nitrogen, thawed immediately and supplemented with an energy mix (150 μg/ml creatine phosphokinase, 60 mM creatine phosphate, 1 mM ATP), demembranated sperm heads and microcystin-LR. Microcystin was added at appropriate concentrations to 19.5 μl samples of extracts as a 0.5 μl volume. Alternatively, in control extracts an equal volume of buffer was added. Stock solutions of microcystin-LR were prepared in ethanol and stored at −20°C.

### DNA replication assays

DNA replication assays were carried out according to Hutchison et al. (1988). Volumes (20 μl) of extracts were prepared as described above. Each sample was supplemented with 1 μCi of [$^{32}$P]dCTP (3000 Ci/mmol) and incubated for varying time periods at 21°C. At the end of the incubation the reaction was terminated by adding stop solution and samples were processed for agarose gel electrophoresis by digestion with proteinase K. The samples were resolved on 0.9% agarose gels. After staining with ethidium bromide to detect molecular mass markers, the gels were dried under vacuum and exposed to X-ray film at −80°C.

Single-stranded M13 DNA replication assays were performed in either unfractionated egg extracts (LSS) or a high-speed supernatant of egg extracts (HSS: prepared by centrifugation of LSS at 200,000 ***g*** for 2 hours). Samples (90 μl) of either LSS or HSS were supplemented with 0.5 μg of M13 DNA, energy mix and 10 μCi of [$^{32}$P]dCTP, and made up to a final volume of 100 μl. The extract was divided into 19.5 μl samples and supplemented with microcystin or buffer to give a final volume of 20 μl. Incubations were for 90 minutes at 21°C. Samples were prepared for agarose gel electrophoresis as described above.

### Fluorescence microscopy

Nuclei were prepared for fluorescence microscopy by a modification of the method described by Hutchison et al. (1988). Extracts were prepared as described above and 20 μl volumes were supplemented with 4 μM biotin-11-dUTP. After incubation at 21°C for an appropriate period of time, the extracts were fixed by suspension in 400 μl of a solution containing 33 mM KCl, 7 mM HEPES (pH 7.5), 1.66 mM $MgCl_2$, 1 mM ethylene glycol-bis-(succinic acid *N*-hydroxysuccinimide ester) (EGS). Pronuclei were recovered from the suspension by centrifugation at 1,500 ***g*** through 25% sucrose and collected on glass coverslips. After air drying for 15 minutes the pronuclei were incubated in primary antibody solution (L6 5D5 to detect lamins, or a human autoantibody that detects PCNA) overnight at 4°C. After extensive washing, secondary antibody was applied (TRITC rabbit anti-mouse Ig or TRITC rabbit anti-human Ig) for 4 hours at 4°C. To detect biotin-11-dUTP incorporation, pronuclei were incubated with FITC-conjugated streptavidin for 4 hours at 4°C. To detect nuclear membranes, pronuclei were incubated with 1 μg/ml DHCC for 10 minutes at room

temperature. Slides were mounted in Mowiol containing 1 μg/ml DAPI and viewed with either a Zeiss Axioskop or a MRC 600 laser sharp confocal microscope. Image analysis was performed with COMOS software as described previously (Hutchison, 1994).

### Nuclear isolation and immunoblotting

Nuclei were isolated from egg extracts for immunoblotting according to the method described by Jenkins et al. (1993). Nuclei ($2\times10^5$) were assembled in 100 μl of extract as described above. The extract was then diluted in 2 ml of ice-cold nuclear isolation buffer (NIB: 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl (pH7.4), 1 mM 2-mercaptoethanol, 0.5 mM spermidine and 0.15 mM spermine). The diluted suspension was layered over a 1 ml 60% (v/v) Percoll cushion and separated by centrifugation at 3,000 ***g*** for 10 minutes at 4°C. The pronuclei were recovered at the interface and diluted in 1 ml of nuclear isolation buffer. The nuclear suspension was then layered over a 25% (w/v) sucrose cushion and recovered as a pellet after centrifugation at 4,000 ***g*** for 10 minutes. The nuclear pellet was either prepared directly for electrophoresis by heating to 95°C in SDS sample buffer or extracted to prepare nuclear matrix fractions. Nuclear matrix fractions were prepared according to a modification of the method of Nakayasu and Berezney (1989). This procedure is described in full by Jenkins et al. (1993). Samples prepared for immunoblotting were resolved on 12% SDS-PAGE. Proteins were then transferred to nitrocellulose paper and blotted with PC10 monoclonal anti-PCNA antibody supernatants (a kind gift from Prof. D. P. Lane). Detection was with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Jenkins et al., 1993).

### Nuclear transfer experiments

Nuclei were assembled in extracts treated with microcystin-LR or aphidicolin as described above. After 2 hours, the extracts were diluted by the addition of 1 ml of nuclear isolation buffer (NIB). Fresh extract was then added under the nuclear suspension. Nuclei were transferred to the fresh extract by centrifugation for 5 minutes at 600 ***g*** and the NIB was removed. The fresh extract was supplemented with [$^{32}$P]dCTP and the incubation continued for a further 3 hours at 21°C. Samples were processed as described above.

### Protein phosphatase assays

Protein serine/threonine phosphatase activity was determined by release of $^{32}PO_4^{2-}$ from phosphorylase *a* according to the method of Cohen (1989). To determine the ratio of PP1 to PP2A, assays were performed either in the presence of 2 nM okadaic acid or in the presence of 200 nM inhibitor-2 (okadaic acid and inhibitor-2 were kind gifts from Prof. Philip Cohen, MRC Protein Phosphorylation Unit, Dundee). Extracts at an initial protein concentration of 50 mg/ml were diluted 300-fold and incubated with 10 μM phosphorylase *a*. One milliunit of activity is defined as that amount that catalyses the dephosphorylation of 1 μmole of phosphorylase in one minute. PP1 was the phosphorylase phosphatase activity inhibited by inhibitor-2 or not inhibited by 2 nM okadaic acid. PP2A was the activity that was blocked by 2 nM okadaic acid but unaffected by 200 nM inhibitor-2. The relative contributions of PP1 and PP2A activity determined by both methods agreed to ±5%.

### H1-kinase assays

Histone H1-kinase assays were performed according to the method of Smythe and Newport (1990).

## RESULTS

### Microcystin-LR inhibits DNA replication in cell-free extracts of *Xenopus* eggs

*Xenopus* egg extracts, at an initial protein concentration of 50 mg/ml, contained a total phosphorylase phosphatase activity of 51 mU/ml, of which ~73% was PP2A and ~27% was PP1. When the cyclic heptapeptide microcystin-LR was added to egg extracts over a concentration range of 125 nM to 500 nM both phosphatases were inhibited equally. However, the degree of inhibition did vary from extract to extract. The effects of microcystin on a typical extract is illustrated in Fig. 1. Total phosphorylase phosphatase activity declined linearly over the concentration range to 12% of control levels at 500 nM microcystin. Since phosphatase inhibitors have been reported to induce cdc2/cyclin B activity in *Xenopus* egg extracts (Felix et al., 1990), we also measured histone H1-kinase activity in microcystin-treated extracts. Microcystin-LR was added to egg extracts at a final concentration of 250 nM in the presence or absence of cycloheximide (CHM). (Cycloheximide added at 10 μg/ml prevents the accumulation of cyclin B in these extract (Hutchison et al. (1989).) Samples were then collected at 20 minute intervals over a 2 hour period and H1-kinase activity was measured. In the absence of CHM, H1-kinase activity increased slowly over a 2 hour period (normally mitosis only occurs in these extracts after 3-4 hours). In contrast, in the presence of CHM, H1-kinase was not detectable above background levels. In the presence of microcystin-LR but in the absence of CHM, H1-kinase activity increased abruptly to 5× background levels and was maintained for 60 minutes. In contrast, in the presence of microcystin-LR and CHM, H1-kinase activity was not detectable over the 2 hour period (data not shown). These results are in broad agreement with previous studies, which suggest that protein phosphatase inhibitors such as okadaic acid stimulate cdc2/cyclin B activity. Since H1-kinase activity was not induced in the presence of CHM, the effects of microcystin on nuclear assembly and DNA replication were measured in the presence of CHM.

To investigate the effects of microcystin on DNA replication, egg extracts were supplemented with different concentrations of microcystin-LR before demembranated sperm heads were added. The amount of DNA replication in each extract was estimated by continuous labelling with [$^{32}$P]dCTP over a single 3-hour period. Samples were prepared for agarose gel

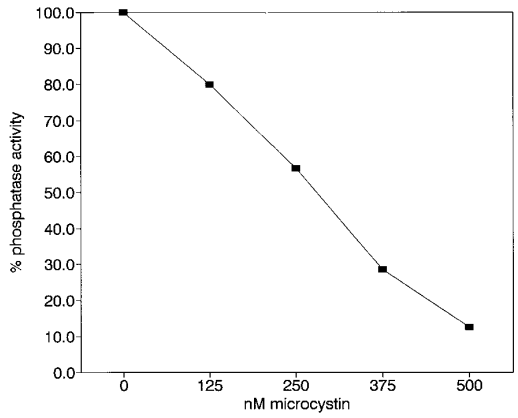


**Fig. 1.** Estimation of protein phosphatase activity in microcystin-LR-treated egg extracts. Total PP1/PP2A activity was estimated in egg extracts treated with increasing doses of microcystin-LR and expressed as a percentage of the activity in untreated extracts (0 nM: 51 mU/ml). Each point represents the mean of results obtained from three extracts.

electrophoresis and the relative amounts of newly synthesized DNA in each sample determined by autoradiography (Fig. 2). In control extracts (no microcystin added), there was extensive incorporation of radioactivity into bands that co-migrated with chromosomal DNA (lane 2). Incorporation of [$^{32}$P]dCTP in similar bands was also observed in extracts supplemented with 125 nM microcystin (lane 3). In contrast, in extracts containing 250 nM microcystin-LR (or greater, lanes 4-6) no incorporation of radioactivity into DNA was detected. As a control, parallel incubations were performed in which extracts were supplemented with aphidicolin (lane 1). Inhibition of DNA replication by microcystin at a concentration of 250 nM was as effective as aphidicolin at 25 μg/ml (Fig. 2, lanes 1 and 4).

Since additions of 250 nM microcystin to extracts inhibited DNA replication, we wished to confirm that this failure to replicate DNA resulted directly from a decline in phosphatase activity rather than through a non-specific action of the drug. To do this, we attempted to rescue DNA replicaton in microcystin-treated extracts by adding back catalytic subunits of either PP1 or PP2A. Egg extracts were supplemented with microcystin to a final concentration of 250 μM. After incubation for 20 minutes at 21°C (the time at which nuclear envelopes are first detected around decondensed sperm heads) the extracts were supplemented with catalytic subunits of PP1-α, PP1-γ or PP2A and [$^{32}$P]dCTP. After incubation for a further 180 minutes, samples were prepared for agarose gel electrophoresis and autoradiography as described above. As expected, in microcystin-treated extracts, no DNA replication was detected (Fig. 3, lane 4). In addition, convincing recovery of DNA replication was never obtained by additions of the catalytic subunits of PP-α and PP1-γ to microcystin-treated extracts (lanes 2 and 3). In contrast, when the catalytic subunit of PP2A was added to microcystin-treated extracts, their was a dramatic increase in DNA replication (Fig. 3 lane 1) to levels that were 54% of controls (Fig. 3, lane 5). Further additions of PP2A did not increase the level of DNA replication in microcystin-treated extracts. Because DNA replication was rescued by adding back PP2A activity to microcystin-treated extracts, we infer that the block in DNA replication resulted from a change in steady state level of phosphorylation of one or more nuclear proteins. Furthermore, inhibition of DNA replication was not obtained by additions of the PP1-specific inhibitor, inhibitor-2 (data not shown). Therefore, our results indicate that a PP2A-like activity is required for the assembly of an initiation-competent nucleus in *Xenopus* egg extracts.

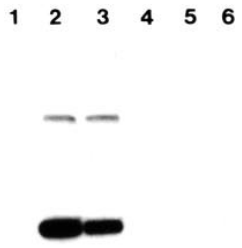


**Fig. 2.** Inhibition of DNA replication in microcystin-LR-treated *Xenopus* egg extracts. Extracts treated with increasing doses of microcystin were labelled with [$^{32}$P]dCTP. Samples were resolved on agarose gels. The gels were then dried under vacuum and exposed to autoradiograph plates overnight at –80°C. In each lane the slower-migrating band remained in the loading well, while the faster-migrating band had a molecular mass that was greater than the highest molecular mass marker (30 kDa; not shown). This is typical of the replication products of chromosomal DNA (Hutchison et al., 1987). Lane 1 contains labelled material recovered after 180 minutes from extracts treated with 25 μg/ml aphidicholin. Lane 2 contains labelled material recovered after 180 minutes from control extracts. Lanes 3, 4, 5 and 6 contain labelled material recovered after 180 minutes from extracts treated with 125 nM, 250 nM, 375 nM and 500 nM microcystin-LR, respectively.

## Different concentrations of microcystin have a range of effects on nuclear assembly

One explanation for the results described above is that by inhibiting PP1/PP2A activity in egg extracts, nuclear envelope assembly is prevented and as a consequence DNA replication does not occur. Indeed, the protein phosphatase inhibitor okadaic acid does prevent early events in nuclear membrane assembly in some assay systems (Pfaller et al., 1991). To eliminate this possibility, we used indirect immunofluorescence microscopy to investigate nuclear envelope assembly in microcystin-treated extracts. In these assays the presence of a nuclear membrane was confirmed both by phase-contrast microscopy (not shown) and by fluorescence microscopy using the lipophilic dye DHCC (Fig. 4B, E and H). The presence of a lamina was revealed by indirect immunofluorescence, using the monoclonal antibody L6 5D5 (Fig. 4C, F and I). The extent of nuclear envelope assembly in extracts varied with microcystin concentration. Pronuclei formed in the presence of 125-250 nM microcystin-LR (82-58% residual PP1/PP2A activity) were indistinguishable from control pronuclei: chromatin decondensation, nuclear membrane assembly and lamina assembly all appeared normal (Fig. 4A, B and C). However, at between 375 and 500 nM microcystin-LR (29-12% residual PP1/PP2A activity), while nuclear membrane assembly appeared normal, chromatin was not fully decondensed and the nuclei remained small. In addition, most nuclei failed to assemble a lamina (Fig. 4D, E and F). The most dramatic effects on nuclear envelope assembly were seen in extracts containing >500 nM microcystin (<12% residual PP1/PP2A activity). Chromatin decondensation was very limited and only some nuclei displayed DHCC fluorescence (Fig. 4G, H and I). These findings confirm and extend an earlier report that protein

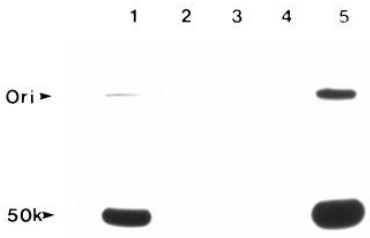


**Fig. 3.** Re-addition of protein phosphatase activity to microcystin-LR-treated *Xenopus* egg extracts. Extracts (20 μl) were supplemented with 250 nM microcystin and incubated for 20 minutes at 21°C. Each extract was then supplemented with 3 mU of PP2A (lane 1), 3 mU of PP1γ (lane 2), 3 mU of PP1α (lane 3), or an equal volume of buffer (lane 4). DNA replication was measured over 180 minutes by [$^{32}$P]dCTP incorporation and labelled samples were processed as described in Fig. 2. Lane 5 contains labelled material recovered from control (no microcystin added) extracts. k=kDa.

phosphatase inhibitors prevent nuclear envelope assembly in *Xenopus* egg extracts (Pfaller et al., 1991).

### DNA replication is inhibited in extracts that permit nuclear lamina assembly and nuclear transport

Since DNA replication was inhibited at microcystin concentrations that appeared to permit nuclear membrane assembly and lamina assembly (250 nM), we investigated lamina assembly more fully at this concentration. Pronuclei were assembled in extracts treated with 0, 250 or 500 nM microcystin and either prepared for indirect immunofluorescence (Fig. 5a-f) or isolated and extracted to prepare nuclear matrices. Nuclear matrix fractions were then resolved on 10% SDS-PAGE and either silver stained (Fig. 5g) or transferred to nitrocellulose and blotted with anti-lamin antibodies (Fig. 5h). In control extracts (Fig. 5a and d) and in extracts treated with 250 nM microcystin (Fig. 5d and e) nuclei contained uniformly decondensed DNA and were surrounded by a peripheral lamina. In contrast, at 500 nM microcystin, while DNA was deconsensed a lamina was mostly undetectable (Fig. 5c and f). These results were confirmed by the results of SDS-PAGE and immunoblotting. The protein profiles on 10% SDS-PAGE of nuclear matrix fractions prepared from pronuclei isolated from control or 250 nM microcystin-treated extracts appeared identical (Fig. 5g, lanes 1 and 2). Similarly, immunoblotting analyses indicated that the amounts of insoluble lamin in pronuclei assembled in control and 250 nM microcystin-treated extracts were also identical (Fig. 5h, lanes 1 and 2). In contrast, pronuclei isolated from 500 nM microcystin-treated extracts contained reduced levels of nuclear matrix proteins (Fig. 5g, lane 3) and undetectable levels of insoluble lamins (Fig. 5h, lane 3).

Since nuclear lamina assembly was completed in extracts containing 250 nM microcystin, we went on to investigate whether or not nuclei assembled in these exracts imported karyophilic proteins. We have previously used nuclear isolation and immunoblotting to demonstrate that PCNA is actively transported into in vitro assembled nuclei (Jenkins et al., 1993). Sperm pronuclei assembled in vitro were isolated from egg extracts by first collecting them on a 60% Percoll cushion and then by centrifugation through 30% sucrose. Karyophilic proteins were then resolved on 12% SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-PCNA antibodies. Comparable levels of PCNA were recovered from sperm pronuclei assembled in control extracts (Fig. 6g, lane 1) and in extracts containing 250 nM microcystin (Fig. 6g, lane 3). In contrast, little or no PCNA was recovered from sperm pronuclei assembled in extracts containing 2 mg/ml wheat germ agglutinin (WGA), which prevents nuclear transport (Fig. 6h, lane 2). Similar results were obtained by indirect immunofluorescence. Pronuclei assembled in control (Fig. 6a and b) or 250 nM microcystin-treated extracts (Fig. 6c and d) both displayed a granular distribution of anti-PCNA immunofluorescence. In contrast, pronuclei assembled in extracts containing 2 mg/ml WGA were not stained with anti-PCNA antibodies (Fig. 6e and f). Taken together, these results indicate that in the presence of 250 nM microcystin, egg extracts are capable of assembling nuclei that contain a complete nuclear lamina and which can transport karyophilic proteins. Nevertheless, DNA replication is inhibited. In view of this we standardised our assay conditions by supplementing extracts with 10 μg/ml CHM and 250 nM microcystin. These extracts are referred to as inhibitory extracts.

### Stable replication centres are not assembled in inhibitory extracts

We have previously reported a 15 minute delay between the appearance of PCNA at sites that resemble replication foci (pre-replication foci) and the time at which DNA synthesis is first detected in nuclei assembled in vitro (Kill et al., 1991). Since the assembly of pre-replication foci appeared to be a rate-limit-

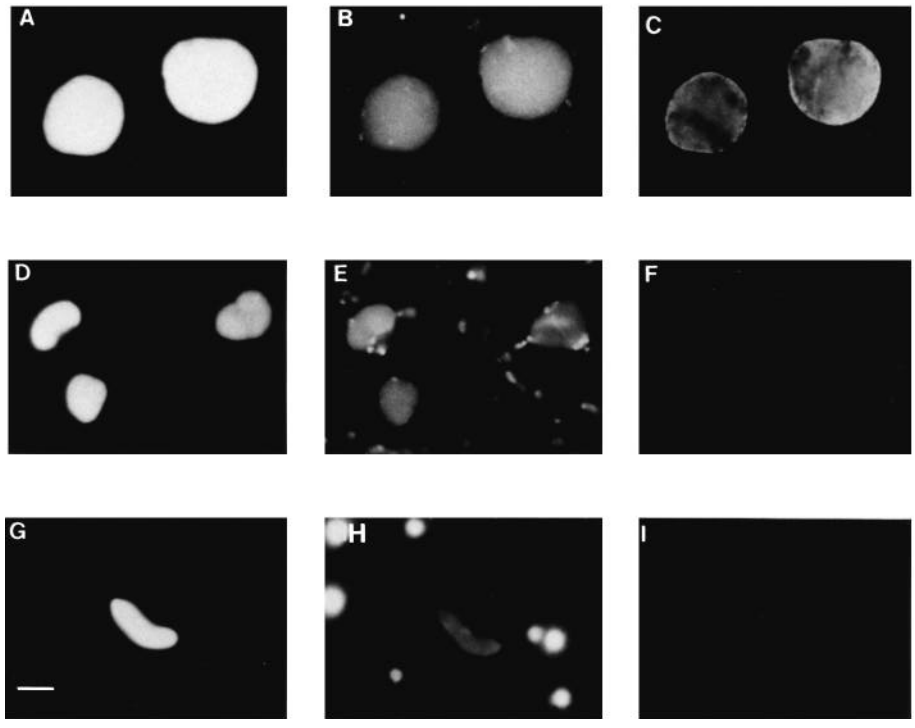


**Fig. 4.** Pronuclear assembly in microcystin-LR-treated *Xenopus* egg extracts. The morphology of sperm pronuclei assembled in microcystin-treated extracts was investigated by indirect immunofluorescence. After incubation in extracts containing 0 nM (A-C), 500 nM (D-F) or 1000 nM (G-I) microcystin for 180 minutes, pronuclei were fixed, recovered on glass coverslips and stained. Monoclonal anti-lamin antibodies (L6 5D5) and TRITC-conjugated rabbit anti-mouse Ig were used to detect lamina assembly (C, F and I), DHCC fluorescence was used to detect nuclear membrane assembly (B, E and H) and DAPI fluorescence was used to detect the distribution of DNA (A, D and G). Nuclei assembled in the presence of 125 nM and 250 nM microcystin were indistinguishable from controls (see Figs 5 and 6). Bar, 10 μm.

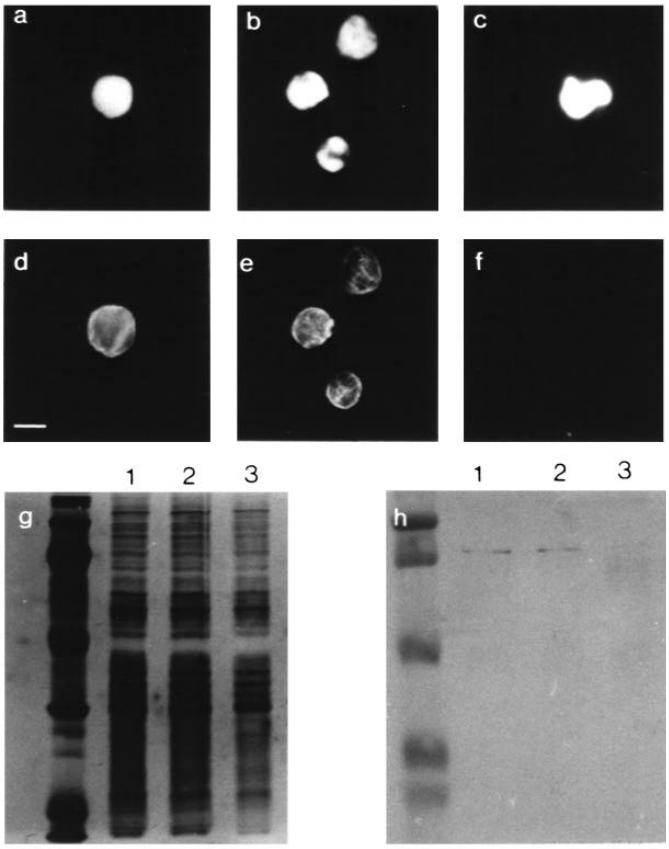


**Fig. 5.** Lamina assembly in microcystin-treated extracts. Egg extracts were supplemented with microcystin-LR to final concentrations of 0 nM (a, d, g: lane 1; and h: lane 1), 250 nM (b, e, g: lane 2; and h: lane 2) or 500 nM (c, f, g: lane 3; or h: lane 3). Sperm pronuclei were assembled in each extracts over a 3 hour period. Nuclei were then either fixed and prepared for indirect immunofluorescence microscopy (a-f) or isolated, extracted to prepare nuclear matrix fractions and analysed by SDS-PAGE and silver staining (g) or by immunoblotting (h). For immunofluorescence and immunoblotting analyses the monoclonal antibody L6 5D5 was used. (a-c) The distribution of DNA revealed by DAPI. (d-f) The distribution of anti-lamin immunofluorescence. Bars, 10 μm. Silver-stained molecular mass markers are 97.4 kDa, 66.4 kDa, 45 kDa, 31 kDa and 21.5 kDa. Pre-stained molecular markers are 106 kDa, 80 kDa, 49.5 kDa, 32.5 kDa and 27.5 kDa.

ing step in the pathway to DNA replication, we used indirect immunofluorescence and immunoblotting to investigate PCNA distribution in nuclei assembled in inhibitory extracts. Sperm heads were incubated for 3 hours in either untreated or inhibitory extracts. Nuclei formed in each extract were either fixed and re-isolated for indirect immunofluorescence, or collected for immunoblotting. Greater than 80% of pronuclei assembled in control extracts displayed a granular distribution of anti-PCNA immunofluorescence (Fig. 6a and b). An almost identical fraction of nuclei assembled in inhibitory extracts also displayed a granular distribution of PCNA (Fig. 6c and d). While we could detect no apparent differences between the nuclear distribution of PCNA in control and inhibitory extracts using indirect immunofluorescence, differences in the solubility of PCNA were detected by immunoblotting. Nuclei were isolated from both control and inhibitory extracts through Percoll and sucrose gradients. Isolated nuclei were then either prepared for SDS-PAGE immediately, or sequentially extracted with detergents, nucleases and $(NH_4)_2SO_4$ before being prepared for SDS-PAGE. Samples were resolved on 12% SDS-PAGE, transferred to nitrocellulose and probed with monoclonal anti-PCNA antibodies. The amount of PCNA detected was similar or identical in whole nuclei isolated from control and inhibitory extracts (Fig. 6g, lanes 1 and 3). However, while almost half of the nuclear PCNA was insoluble in control extracts (Fig. 6g, lane 2), little or no nuclear PCNA was insoluble in inhibitory extracts (Fig. 6g, lane 4). Since half of the nuclear PCNA was also insoluble in extracts treated with aphidicolin (data not shown), we infer that the changes in PCNA solubility observed in inhibitory extracts result from a failure to complete the assembly of replication centres.

To investigate further the distribution of PCNA foci in nuclei assembled in inhibitory extracts, we performed confocal microscopy on nuclei prepared for indirect immunofluorescence. We have recently reported that pre-initiation nuclei display widely spaced and discrete foci of anti-PCNA immunofluorescence, whereas in mid-S-phase nuclei PCNA foci fuse and become indistinguishable from neighbouring foci. Moreover, these changes can be detected with confocal microscopy (Hutchison, 1994). As expected, nuclei assembled in control extracts supplemented with biotin-11-dUTP had a uniform distribution of anti-lamin fluorescence at the nuclear periphery (Fig. 7A) and a distribution of PCNA that, while grainy, could not be resolved into discrete foci (Fig. 7C). In contrast, nuclei assembled in inhibitory extracts again had a uniform distribution of anti-lamin immunofluorescence at the nuclear periphery (Fig. 7E) but had a granular distribution of anti-PCNA immunofluorescence in which individual foci could easily be discerned (Fig. 7G). As expected, whereas nuclei isolated from control extracts displayed extensive biotin-11-dUTP incorporation (Fig. 7B and D), no biotin incorporation was detected in nuclei recovered from inhibitory extracts (Fig. 7F and H). These results in conjunction with the results of immunoblotting indicate that nuclei assembled in inhibitory extracts are arrested at a pre-initiation stage.

### Single-stranded DNA replication is not prevented in inhibitory extracts but complete DNA replication from pre-assembled replication forks is not achieved

Next we wished to eliminate the possibility that microcystin inhibits the elongation phase of DNA replication. Single-stranded DNA replication was tested in LSS and HSS over a range of microcystin concentrations. M13 DNA was incubated for 90 minutes in extracts containing [$^{32}$P]dCTP. The amount of DNA synthesis in each sample was estimated after agarose gel electrophoresis by autoradiography. Over the full range of microcystin concentrations the amount of radioactivity incorporated into form I and form II DNA, in HSS, was indistinguishable. In LSS there appeared to be a slight increase in the amount of form III DNA in the presence of microcystin. Finally, in both LSS and HSS a small amount of catenated DNA was detected in control but not in microcystin-treated extracts. No DNA synthesis was detected in extracts treated with 25 μg/ml aphidicolin (Fig. 8). These results indicate that microcystin does not inhibit processive DNA polymerases or supercoiling of double-stranded DNA.

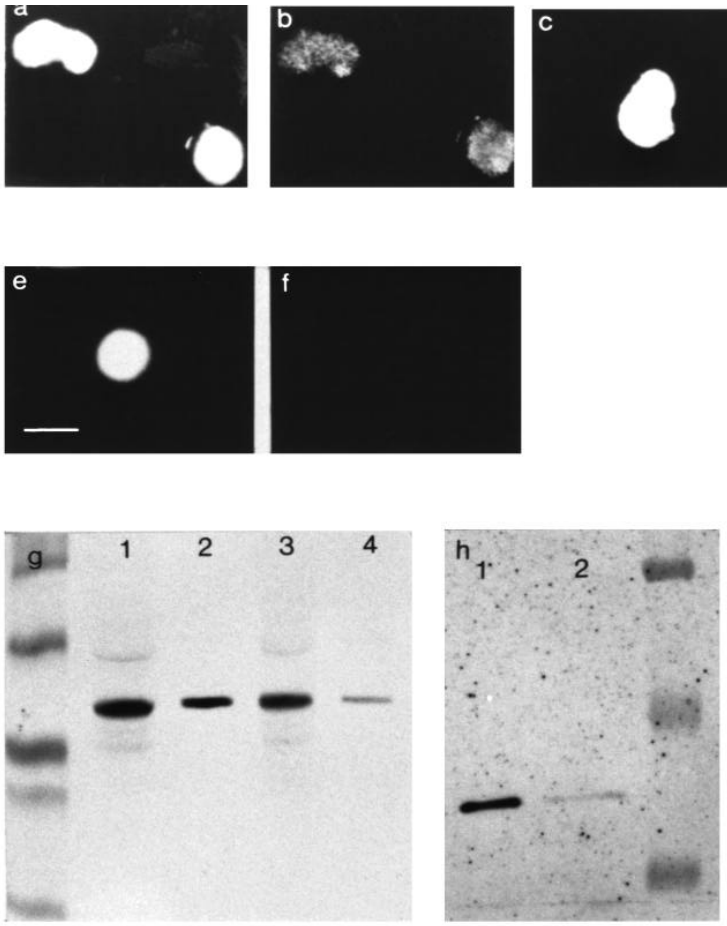


**Fig. 6.** Nuclear transport and PCNA distribution in microcystin-treated extracts. Extracts were supplemented with 0 nM (a, b, g: lanes 1 and 2; and h: lane 1) or 250 nM microcystin (c, d and g: lanes 3 and 4) or with 2 mg/ml wheat germ agglutanin (e, f and h: lane 2). Sperm pronuclei were assembled for 2 hours at 21°C and then prepared for indirect immunofluorescence (a-f) or isolated and prepared for immunoblotting (g, lanes 1 and 3; h), or isolated, extracted were used to prepare nuclear matrix fractions and then subjected to immunoblotting analysis (g, lanes 2 and 4). Immunofluorescence (b, d and f) was performed using human autoantibodies against PCNA (ImmunoConcepts). The distribution of DNA was revealed by DAPI fluorescence (a, c and e). Immunoblotting was performed with the monoclonal anti-PCNA antibody PC10. Bars, 10 μm. Pre-stained molecular mass standards are: (g) 80 kDa, 49.5 kDa, 32.5 kDa, 27.5 kDa and 18.5 kDa; (h) 80 kDa, 49.5 kDa and 32.5 kDa.

While the results described in Fig. 8 indicate that DNA synthesis is insensitive to microcystin, we wished to confirm this under conditions used to investigate chromosomal DNA replication. Nuclei containing pre-assembled replication forks were accumulated by incubating sperm heads for 2 hours in extracts supplemented with either 25 μg/ml or 5 μg/ml aphidicolin. These nuclei were then transferred to either control or inhibitory extracts by first diluting the aphidicolin-treated extracts, then underlaying the diluted extract with fresh extract and finally transferring the pre-assembled nuclei to the fresh extracts by low speed centrifugation. The fresh extracts were supplemented with [$^{32}$P]dCTP and incubated for a further 3 hours. As a positive control DNA replication was measured over a 5 hour period in untreated extacts (Fig. 9, lane 6). As a negative control, DNA replication was measured over a 5 hour period in extracts treated with aphidicolin (Fig. 9, lane 5). The amount of DNA synthesis in each sample was estimated by agarose gel electrophoresis and autoradiography. Quantification of DNA synthesis was achieved by counting the radioactivity emitted from each band. In our hands efficient rescue of pronuclei assembled in extracts treated with 25 μg/ml aphidicholin could not be achieved upon transfer to fresh untreated extracts. Since in the presence of 5 μg/ml aphidicholin DNA replication was not detectable in extracts after 5 hours (Fig. 9, lane 5), this concentration was used in most assays. The amount of radioactivity incorporated into DNA was 46% of control levels in nuclei transferred from aphidicolin-treated extracts to untreated extracts (compare Fig. 9, lanes 3 and 6). Similarly, the amount of radioactivity incorporated into DNA in nuclei transferred from inhibitory extracts to untreated extracts was 47% of control levels (compare Fig. 9, lanes 2 and 6). However, while extensive DNA replication was achieved when nuclei were transferred from aphidicholin-treated to inhibitory extracts, the level of incorporation was only 20% of controls. Since 75% of the nuclei transferred from extracts treated with 5 μg/ml aphidicholin to inhibitory extracts incorporated biotin-11-dUTP into multiple discrete foci (data not shown), we conclude that DNA synthesis can occur from pre-existing replication forks in microcystin-treated extracts, but additional inhibitory events prevent complete replication. Since the rescue from each inhibitory extract alone (microcystin or aphidicholin) is 50% of control (lanes 2 and 3) it seems likely that there is a background level of non-specific inhibition that cannot be reversed. Thus one explanation for the inability of microcystin-treated extracts to support complete replication from pre-existing forks is through the additive effects of non-specific inhibition upon transfer of nuclei from one inhibitory extract to another.

## DISCUSSION

### Microcystin-treated extracts reveal a novel regulatory step in the pathway to DNA replication

Recent reports from a number of laboratories indicate that protein phosphorylation controls the initiation of eukaryotic

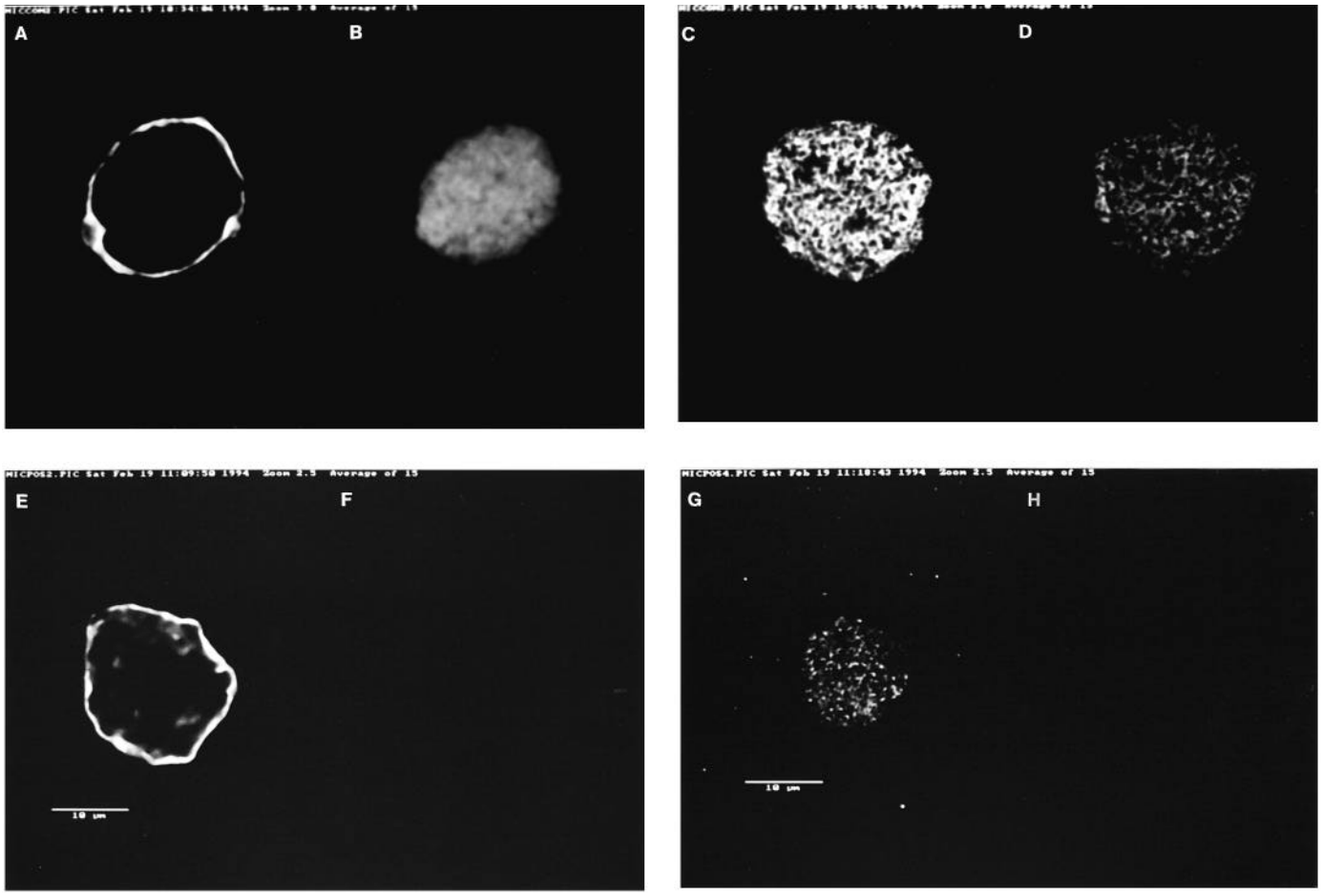


**Fig. 7.** Confocal microscope analysis of sperm pronuclei assembled in inhibitory extracts. Pronuclei assembled in control (A-D) or inhibitory (E-H) extracts were prepared for fluorescence microscopy as described above. The distribution of PCNA (C and G) and lamins (A and E), and the incorporation of biotin-11-dUTP (B, F, D and H), were detected as indicated for Figs 4 and 5. Representative nuclei were examined with a MRC600 confocal microscope. Z-series were collected, and the mid-section was projected and printed using a video printer. Normalisation was performed on each image but no other image enhancement function was used. Bar, 10 μm.

DNA replication. In SV40 DNA replication, phosphorylation of Tag and RPA, by one or more cdks is required for the assembly of a pre-synthesis complex and for progression from a pre-synthesis complex to an unwound complex (McVey et al., 1989; Fotedar and Roberts, 1992; Dutta and Stillman, 1992). In cell-free extracts of *Xenopus* eggs that replicate chromosomal DNA in vitro, phosphorylation of RPA-34 once the RPA complex is bound to DNA is also required for DNA replication (Fang and Newport, 1993). Moreover, extracts treated with the cdk inhibitor 6-DMAP are incapable of supporting DNA replication (Blow, 1993). The mechanism by which phosphorylation 'activates' RPA in egg extracts may be linked to the mechanism that prevents 'licencing of chromatin' during DNA replication in 6-DMAP-treated extracts. RPA binds to decondensing chromatin prior to nuclear envelope assembly at sites that resemble replication foci (Adachi and Laemmli, 1992). RPA acts as a substrate for interphase phosphorylation only when associated with DNA (Fang and Newport, 1993). Chromatin that is pre-incubated in untreated extracts will be assembled into replication-competent nuclei when transferred to 6-DMAP-treated egg extracts (Blow, 1993). Thus some phosphorylation events that activate DNA replication appear to occur prior to nuclear envelope assembly.

The data reported here demonstrate that by inhibiting protein phosphatase activity with microcystin-LR, DNA replication is prevented in *Xenopus* egg extracts. Inhibiting protein phosphatase activity is likely to increase the steady state phosphorylation levels of one or more nuclear proteins. Thus in contrast to previous reports, here an unknown protein kinase appears to be delaying DNA replication. Furthermore, nuclei assembled in microcystin-treated extracts and then transferred to untreated extracts will initiate DNA replication. This is in contrast to nuclei assembled in 6-DMAP-treated extracts, which will not initiate DNA replication when transferred to untreated extracts (Blow, 1993). Finally, while re-addition of the catalytic subunit of PP2A to microcystin-treated extracts 20 minutes into an incubation rescues DNA replication, 6-DMAP-treated extracts can only be rescued by addition of fresh extract before or at the same time as adding DNA (Blow, 1993). Thus it appears that the failure of nuclei to replicate DNA in microcystin-treated extracts indicates a novel regulatory step, which occurs after nuclear envelope assembly is

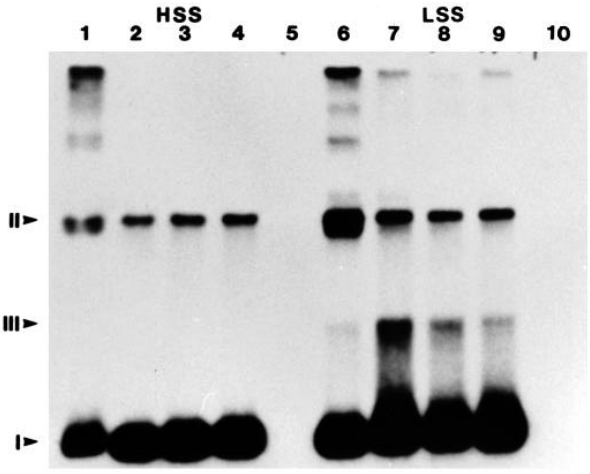


**Fig. 8.** M13 DNA replication in microcystin-LR-treated *Xenopus* egg extracts. A 100 ng sample of single-stranded M13 DNA was added to 20 μl of +CHM extracts in the presence of 0 nM (lanes 1 and 6), 250 nM (lanes 2 and 7), 500 nM (lanes 3 and 8) or 750 nM microcystin (lanes 4 and 9). As a negative control single-stranded M13 DNA was added to extracts treated with 25 μg/ml aphidicolin (lanes 5 and 10). +CHM extracts were prepared as low speed supernatants (LSS) or high speed supernatants (HSS). DNA replication was measured by [$^{32}$P]dCTP incorporation. Labelled material was detected after separation on agarose gels, by autoradiography as described in Fig. 2. I, form I, supercoiled DNA; II, form II, open circle DNA; and III, form III linear DNA.

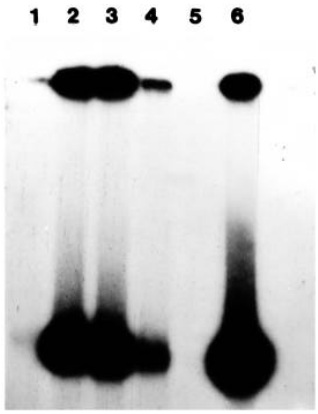


**Fig. 9.** Rescue of DNA replication in nuclei assembled in inhibitory extracts. Sperm pronuclei were assembled in inhibitory extracts or aphidicolin-treated extracts for 2 hours at 21°C. Preassembled nuclei were then transferred to untreated or microcystin-LR-treated extracts by centrifugation. Each extract was incubated for a further 3 hours at 21°C in the presence of [$^{32}$P]dCTP. DNA replication was analysed by agarose gel electrophoresis and autoradiography as described for Fig. 2. Lane 1, pronuclei incubated in inhibitory extracts for 5 hours at 21°C. Lane 2, pronuclei assembled in inhibitory extracts and transferred to untreated extracts. Lane 3, pronuclei assembled in aphidicolin-treated extracts and transferred to untreated extracts. Lane 4, pronuclei assembled in aphidicolin-treated extracts and transferred to inhibitory extracts. Lane 5, pronuclei incubated in aphidicolin-extracts for 5 hours at 21°C. Lane 6, pronuclei incubated in untreated extract for 2 hours at 21°C and then transferred to a fresh untreated extract and incubated for a further 3 hours at 21°C.

completed and which is unrelated to licensing of chromatin or modification of a pre-synthesis complex.

### In inhibitory extracts nuclear envelope assembly occurs but replicase assembly is prevented

Additions of okadaic acid prevent membrane vesicle binding to chromatin in fractions of *Xenopus* egg extracts (Pfaller et al., 1991). This result is achieved because PP2A antogonises a cdc2-activated protein kinase activity that disassembles nuclear membranes at mitosis. Thus in the absence of the phosphatase, interphase levels of the kinase are sufficient to prevent the binding of membrane vesicles to chromatin. In principle, the action of okadaic acid should be similar or identical to that of microcystin-LR (MacKintosh et al., 1989). Indeed, nuclear assembly is prevented in the presence of microcystin at concentrations >500 nM. Since, inhibition of PP2A by okadaic acid leads to the activation of cdk1/cyclin B (Felix et al., 1990), it is likely that lamina assembly and nuclear membrane assembly are prevented because of an increase in the activity of mitotic kinases and a decrease in the activity of antagonistic phosphatases. However, in the presence of CHM H1-kinase activity is undetectable in egg extracts even after the addition of 250 nM microcystin. In addition, lamina assembly is completed and PCNA is actively transported into sperm pronuclei and accumulated at multiple discrete foci. Since discrete PCNA foci are not accumulated in nuclei that lack a lamina (Meier et al., 1991; Jenkins et al., 1993), this confirms the view that nuclear envelope assembly and, more importantly, lamina assembly are completed in inhibitory extracts.

### Nuclei assembled in inhibitory extracts are arrested at a pre-initiation stage

In a recent study, we used laser scanning confocal microscopy to investigate changes in the distribution of PCNA through S-phase. We reported that PCNA was accumulated at sites that resembled replication centres before DNA synthesis was detected (Kill et al., 1991) but that these sites were distinct from active centres of DNA replication. Pre-replication centres were discrete and distributed ~1 μm apart. In contrast, active replication centres were closer together and as S-phase progressed appeared to fuse with neighbouring sites (Hutchison, 1994). In nuclei assembled in inhibitory extracts, PCNA is also distributed at widely spaced centres that do not fuse. Moreover, the majority of nuclear PCNA is soluble upon extraction of nuclei with Triton, DNase I and $(NH_4)_2SO_4$. This is in contrast to the behaviour of nuclear PCNA in replicating nuclei and in nuclei arrested with pre-formed replication forks. Thus we propose that, by inhibiting the activity of a PP2A-like phosphatase, the final step in the assembly of a functional replication centre is prevented. This conclusion is supported by experiments in which nuclei with pre-assembled replication forks are transferred to inhibitory extracts. While single-stranded DNA synthesis is permitted in inhibitory extracts, complete replication of these nuclei is not achieved. However, this result is easily explained. While replication centres are established at the beginning of S-phase (Mills et al., 1989), there appears to be continuous growth of each centre through S-phase leading

to the fusion of dicrete neighbouring centres into large rosette-shaped or string-like structures. These changes could occur if replication forks are continuously recruited into existing replication centres throughout S-phase (Hutchison, 1994). The result described above could then be explained if nuclei assembled in aphidicolin-treated extracts contain only a fraction of the assembled replication forks required to complete S-phase (in this instance 20% of the forks needed). When transferred to inhibitory extracts, DNA synthesis would occur from these forks but new forks would not be incorporated into existing centres. Thus DNA replication would not be completed. Therefore we propose that the activity of a PP2A-like phosphatase is required to recruit replication forks into replication centres and that this activity triggers initiation events in nuclei assembled in vitro.

The authors thank Prof. Philip Cohen for help and advice with protein phosphatase assays, Dr Patricia Cohen and Dr Bob Mackintosh for supplying us with purified catalytic subunits of protein phosphatase 1$\alpha$, 1$\gamma$ and 2A, Prof. David Lane for the supply of PC10 antibody supernatant, and Mr Kenneth Beattie for supplying purified microcystin-LR. We also thank Dr Carl Smythe and Dr William Whitfield for helpful discussions and for critical reading of the manuscript. This work was supported by the Cancer Research Campaign and the Medical Research Council.

## REFERENCES

**Adachi, Y. and Laemmli, U. K.** (1992). Identification of nuclear ore-replication centres poised for DNA synthesis in *Xenopus* egg extracts: Immunolocalization study of Replication protein A. *J. Cell Biol.* **119**, 1-15

**Blow, J. J.** (1993). Preventing re-replication of DNA in a single cell cycle: evidence for a Replication Licensing Factor. *J. Cell Biol.* **122**, 993-1002.

**Blow, J. J. and Laskey, R. A.** (1986). Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* **47**, 577-587.

**Blow, J. J. and Waston, J. V.** (1987). Nuclei act as independent and integrated units of replication within the cell cycle. *EMBO J.* **6**, 1997-2002.

**Blow, J. J. and Laskey, R. A.** (1988). A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* **332**, 546-548.

**Blow, J. J. and Nurse, P.** (1990). A cdc2-like protein is involved in the initiation of DNA replication in *Xenopus* egg extracts. *Cell* **62**, 855-862.

**Cohen, P.** (1989). The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453-508.

**Coverly, D., Downes, C. S., Romanowski, P. and Laskey, R. A.** (1993). Reversible effects of nuclear membrane permeabilization on DNA replication: Evidence for a positive licensing factor. *J. Cell Biol.* **122**, 985-992.

**Cox, L. S. and Laskey, R. A.** (1991). DNA replication occurs at discrete sites in psuedonuclei assembled from purified DNA *in vitro. Cell* **66**, 271-275.

**Cox, L. S.** (1992). DNA replication in cell-free extracts from *Xenopus* eggs is prevented by disrupting nuclear envelope function. *J. Cell Sci.* **101**, 43-53.

**Dirick, L. and Nasmyth, K.** (1991). Positive feedback in the activation of G1 cyclins in yeast. *Nature* **351**, 754-757.

**Dutta, A. and Stillman, B.** (1992). cdc2 family kinases phosphorylate a human cell DNA replication factor RPA and activate DNA replication. *EMBO J.* **11**, 2189-2199.

**Fang, F. and Newport, J. W.** (1991). Evidence that the $G_1$-S and $G_2$-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66**, 731-742.

**Fang, F. and Newport, J. W.** (1993). Distinct roles of cdk2 and cdc2 in RP-A phosphorylation during the cell cycle. *J. Cell Sci.* **106**, 983-994.

**Felix, M-A., Cohen, P. and Karsenti, E.** (1990). Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cycle: evidence from the effects of okadaic acid. *EMBO J.* **9**, 675-683.

**Fotedar, F. and Roberts, J. M.** (1992). Cell cycle regulated phosphorylation of RPA-32 occurs within the replication initiation complex. *EMBO J.* **11**, 2177-2187.

**Harland, R. M. and Laskey, R. A.** (1980). Regulated replication on DNA microinjected into eggs of *Xenopus laevis. Cell* **21**, 761-771.

**Hutchison, C. J., Cox, R., Drepaul, S., Gomperts, M. and Ford, C. C.** (1987). Periodic DNA synthesis in cell-free extracts of *Xenopus* eggs. *EMBO J.* **6**, 2003-2010.

**Hutchison, C. J., Cox, R. and Ford, C. C.** (1988). The control of DNA replication in a cell-free extract that recapitulates a basic cell cycle *in vitro*. *Development* **103**, 553-566.

**Hutchison, C. J. and Kill, I. R.** (1989). Changes in the nuclear distribution of PCNA/Cyclin and DNA polymerase alpha during the progress of the cell-cycle in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* **93**, 605-613.

**Hutchison, C. J., Brill, D., Cox, R., Gilbert, J., Kill, I. R. and Ford, C. C.** (1989). DNA replication and cell-cycle control in *Xenopus* egg extracts. *J. Cell Sci. Suppl. 12*, 197-212.

**Hutchison, C. J.** (1994). Local and global changes in the distribution of replication centres in rapidly expanding nuclei. *Chromosome Res.* (in press).

**Jenkins, H., Holleman, T., Lyon, C., Lane, E. B., Stick, T. and Hutchison, C. J.** (1993). Nuclei which lack a lamina accumulate karyophilic proteins and assemble an nuclear lamina. *J. Cell Sci.* **106**, 275-285.

**Kill, I. R., Bridger, J. M., Campbell, K. H. S., Moldonado-Codina, G. and Hutchison, C. J.** (1991). The timing of the formation and useage of replicases in nuclei of human dermal fibroblasts. *J. Cell Sci.* **100**, 869-876.

**Leno, G. H. and Laskey, R. A.** (1991). The nuclear membrane determines the timing of DNA replication in *Xenopus* egg extracts. *J. Cell Biol.* **112**, 557-566.

**Leno, G. H., Downes, C. S. and Laskey, R. A.** (1992). The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. *Cell* **69**, 151-158

**Lowndes, N. F., McInerny, C. J., Johnson, A. L., Fantes, P. A. and Johnston, L. H.** (1992). Control of DNA synthesis genes in fission yeast by the cell-cycle gene cdc10+. *Nature* **355**, 449-453

**MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A.** (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**, 187-192.

**McVey, D., Brizuela, L., Mohr, I., Marshak, D. R., Gluzman, Y. and Beach, D.** (1989). Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. *Nature* **341**, 503-507.

**Meier, J., Campbell, K. H. S., Ford, C. C., Stick. R. and Hutchison, C. J.** (1991). The role of lamin $L_{iii}$ in nuclear assembly and DNA replication in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* **98**, 271-278.

**Mills, A., Blow, White, J. G., Amos, W. B., Wilcock, D. and Laskey, R. A.** (1989). Replication occurs at discrete centres cpaced throughout nuclei replicating *in vitro. J. Cell Sci.* **94**, 471-477.

**Nakayasu, H. and Berezney, R.** (1989). Mapping replicational sites in the eukaryotic cell nucleus. *J. Cell Biol.* **108**, 1-11.

**Nasmyth, K.** (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* **5**, 166-179.

**Newport, J. W.** (1987). Nuclear reconstitution *in vitro*: stages of assembly around protein free DNA. *Cell* **48**, 205-217.

**Newport, J. W., Wilson, K. L. and Dunphy, W. G.** (1990). A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* **111**, 2247-2259.

**Pfaller, R., Smythe, C. and Newport, J. W.** (1991). Assembly/disassembly of the nuclear envelope membrane: cell cycle-dependent binding of nuclear membrane vesicles to chromatin *in vitro. Cell* **65**, 209-217.

**Philpott, A., Leno, G. H. and Laskey, R. A.** (1991). Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. *Cell* **65**, 569-578.

**Rao, P. N. and Johnson, R. T.** (1970). Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* **225**, 159-164.

**Sheehan, M. A., Mills, A. D., Sleeman, A. M., Laskey, R. A. and Blow, J. J.** (1988). Steps in the assembly of replication competent nuclei in a cell-free system from *Xenopus* eggs. *J. Cell Biol.* **106**, 1-12.

**Smythe, C. and Newport, J. W.** (1991). Systems for the study of nuclear assembly, DNA replication and nuclear breakdown in *Xenopus laevis* egg extracts. *Meth. Cell Biol.* **35**, 449-467.

(Received 27 July 1994 - Accepted 6 September 1994)