Journal of Cell Science 101, 43-53 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

DNA replication in cell-free extracts from *Xenopus* eggs is prevented by disrupting nuclear envelope function

LYNNE S. COX*

Cancer Research Campaign Molecular Embryology Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK

*Present address: CRC Cell Transformation Research Group, Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, UK

Summary

The lectin, wheat germ agglutinin (WGA), has previously been shown to prevent transport into the cell nucleus. This paper shows that WGA also inhibits nuclear DNA replication, under the same conditions that prevent transport. Although WGA eliminates sperm nuclear DNA replication in a cell-free extract of *Xenopus* eggs, DNA synthesis on a single-stranded template proceeds normally. Inhibition of nuclear DNA replication is partially reversed by addition of *N*-acetylglucosamine, and completely reversed by triacetylchitotriose. Sensitivity to inhibition by WGA is greatest during the nuclear assembly phase, and nuclear formation on sperm chromatin is blocked. DNA replication in preformed nuclear templates is also sensitive to WGA inhibition. I propose that WGA blocks DNA replication by preventing nuclear transport. The data presented here also indicate that, under certain circumstances, the elongation stage of DNA replication does not proceed in the absence of an intact nuclear envelope. The roles of the nuclear envelope and active nuclear transport in DNA replication are discussed.

Key words: DNA replication, nuclear transport, *Xenopus* egg extracts, lectins, WGA.

Introduction

Analysis of eukaryotic DNA replication using cell-free extracts of amphibian eggs (Lohka and Masui, 1983, 1984) has provided strong evidence that initiation of replication requires nuclear structures (Blow and Laskey, 1986; Hutchison et al., 1987, 1988; Sheehan et al., 1988). It was suggested by Blow and Watson (1987) that the nucleus is the fundamental unit of replication, on the basis of flow cytometry data showing synchronous initiation of replication within individual nuclei. Disruption of the normal processes of nuclear formation prevents initiation of replication (Sheehan et al., 1988; Newport et al., 1990; Meier et al., 1991). Purified DNA from various sources has been shown to be assembled into pseudonuclei in *Xenopus* egg extract (Blow and Laskey, 1986; Newport, 1987; Blow and Sleeman, 1990; Cox and Laskey, 1991) and, importantly, all replication of the added DNA occurs only within these nuclear structures (Blow and Sleeman, 1990).

What aspect of nuclear structure therefore determines the capacity to initiate DNA replication? The nuclear envelope may play an important part in controlling DNA replication, and a role for the nuclear envelope in excluding a cytoplasmic factor that can "license" replication when bound to DNA has been proposed (Blow and Laskey, 1988). Leno and Laskey (1991) have extended these observations, by showing that the nuclear envelope defines the unit of replication, and that chromatin from multiple nuclei replicates synchronously when surrounded by a common envelope, but asynchronously when compartmentalized by individual nuclear envelopes.

It is possible that the nuclear envelope controls replication by regulating those substances that enter the nucleus by active transport (Blow and Watson, 1987; Leno and Laskey, 1991). Nuclear transport occurs through nuclear pores (Feldherr et al., 1984; Dworetzky and Feldherr, 1988; Newmeyer and Forbes, 1988) and transport can be blocked using the lectin, wheat germ agglutinin (WGA) (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Newmeyer and Forbes, 1988; Wolff et al., 1988). WGA binds specifically to O-linked *N*-acetylglucosamine (O-GlcNAc), an abundant type of glycosylation on some nuclear pore proteins (reviewed by Gerace and Burke, 1988; Hart et al., 1989). Although binding of WGA to nuclear pore complexes prevents translocation of large proteins bearing nuclear localisation signals, it allows binding of such proteins to the nuclear periphery (Newmeyer and Forbes, 1988) and permits free diffusion of small

molecules through the pores (Finlay et al., 1987; Yoneda et al., 1987; Newmeyer and Forbes, 1988). The inhibition of transport by WGA is reversed by addition of competing sugars (Finlay et al., 1987; Yoneda et al., 1987).

If nuclear transport is necessary for DNA replication, then inhibiting transport using WGA would be expected to prevent replication. Here, I address the question of a requirement for nuclear transport in DNA replication. The data presented show that WGA blocks DNA replication of sperm chromatin and somatic cell nuclei, and that the inhibition is reversed by addition of the competing sugar, triacetylchitotriose (TAC). Since studies using inhibitors such as lectins are prone to artefacts, nuclear accumulation was also prevented by causing permanent disruption of the nuclear envelope. Again, DNA replication did not occur in the absence of a functionally transporting nuclear envelope.

These results indicate a requirement for accumulation of nuclear proteins across an intact nuclear envelope throughout S phase, and suggest that accumulation of such proteins may be necessary not only for the initiation of DNA replication, but also during the elongation phase.

Materials and methods

Xenopus *egg extracts*

Low-speed supernatant (LSS) was prepared as described by Blow and Laskey (1986) and high-speed supernatant (HSS) was made according to the method of Sheehan et al. (1988). LSS was made 5% with respect to glycerol, and HSS was made with 7% glycerol, then 15 μl samples were frozen and stored in liquid nitrogen.

Templates

Sperm nuclei were prepared according to the method of Gurdon (1976), and nuclei were isolated from exponential populations of HeLa cells, or G_1 populations of CHO cells, using low salt lysis buffer (Cox and Leno, 1990). Single-stranded phage M13 DNA was prepared from infected *Escherichia coli* cells as described by Maniatis et al. (1982). DNA was added to the extract to a final concentration of 2-5 ng/μl.

Replication reactions

Extract was thawed immediately prior to use, and supplemented with an energy-regenerating system (60 mM phosphocreatine, 150 μg/ml creatine phosphokinase), and 100 μg/ml cycloheximide. All incubations were carried out at 23°C. Microscopy reactions contained 20 μM biotin-16-dUTP (Boehringer-Mannheim). Alternatively, replication was analysed by incorporation of 0.1 μCi/μl [α-^{32}P]dATP or [^{3}H]dATP (Amersham) into material insoluble in 10% ice-cold trichloroacetic acid (TCA). For density substitution reactions, BrdUTP (Sigma) was added to a final concentration of 0.25 mM. DNA was purified and centrifuged to equilibrium on caesium chloride gradients (Blow and Laskey, 1986). Gradient fractions were collected and processed as described previously (Cox and Laskey, 1991). Wheat germ agglutinin (Sigma) was made up in distilled water, and stored at −20°C for up to one month. Samples for analysis by agarose gel electrophoresis were treated with 2.5 μg/ml proteinase K for 1 hour at 37°C, extracted with phenol-chloroform and precipitated with ethanol. A 1% agarose gel was run in 1×TBE buffer (Maniatis et al., 1982) at 35 mA for 14 h, then squashed and autoradiographed at −70°C for 1 day.

Nuclear transfer conditions (Fig. 5)

Samples shown in Fig. 5 (below) were prepared by incubating *Xenopus* sperm nuclei in egg LSS for 4 hours in the presence or absence of aphidicolin (Sigma) at 10 μg/ml in DMSO. Samples were diluted with 1 ml buffer A (Mills et al., 1989) and transferred into 20 μl of freshly thawed LSS, layered under the buffer, by centrifuging in a Beckman Centaur II rotor at 3500 rev./min for 4 min. The supernatant was discarded, and the extract was removed to clean tubes. WGA was added to 0.2 mg/ml in appropriate samples.

Nuclear transfer with permeabilisation (Figs 6, 7 and 8)

After incubation in egg LSS for the appropriate time, nuclei were diluted with 500 μl buffer A and pelleted by spinning at 3,000 rev./min for 3 min in a Beckman SW50 rotor. Supernatant was discarded, and 100 μl 500 μg/ml lysolecithin (Sigma) made up in buffer A, or 100 μl buffer A (unpermeabilised controls) was added to the tube. The nuclear pellet was resuspended by gentle mixing with a Pasteur pipette and incubated at 25°C for 10 minutes; 400 μl buffer A was added, fresh LSS was underlayered and samples centrifuged for 4 min at 3,500 rev./min. The supernatant was again discarded and samples placed in fresh tubes and incubated for 5 hours at 23°C.

Microscopy

Samples were fixed as described by Mills et al. (1989). Nuclei were stained with Hoechst 33258 (Eastman Kodak Co.) for total DNA. Samples for confocal microscopy were processed for immunofluorescence as described by Meier et al. (1991) and PCNA (proliferating cell nuclear antigen) was detected using anti-PCNA monoclonal antibodies (generous gift from Dr. Naushin Waseem) and sheep FITC-conjugated anti-mouse IgG polyclonal antibodies (Scottish Antibody Production Unit). Incorporated biotin-dUTP was stained with Texas red-streptavidin (Amersham). Confocal images were obtained using a BioRad-MRC 600 confocal scanning laser microscope and were then normalised. PCNA (green) and biotin-dUTP (red) signals were merged.

Results

WGA inhibits replication of sperm nuclei in vitro

A possible role for nuclear transport in DNA replication was examined by determining the effect of the transport inhibitor, wheat germ agglutinin (WGA) on replication of sperm nuclei incubated in *Xenopus* egg extract.

Demembranated sperm nuclei were incubated at 5 ng/μl in *Xenopus* egg low-speed supernatant (LSS) in the presence of [α-^{32}P]dATP. Various concentrations of WGA were added at the outset, and samples incubated for 3 hours. The extent of replication was assayed by incorporation of ^{32}P into acid-insoluble material, and the results are shown in Fig. 1A. WGA at concentrations greater than 0.02 mg/ml reduced sperm nuclear replication (filled symbols), and inhibition was complete at 0.1 mg/ml. WGA was used at 0.2 mg/ml in all

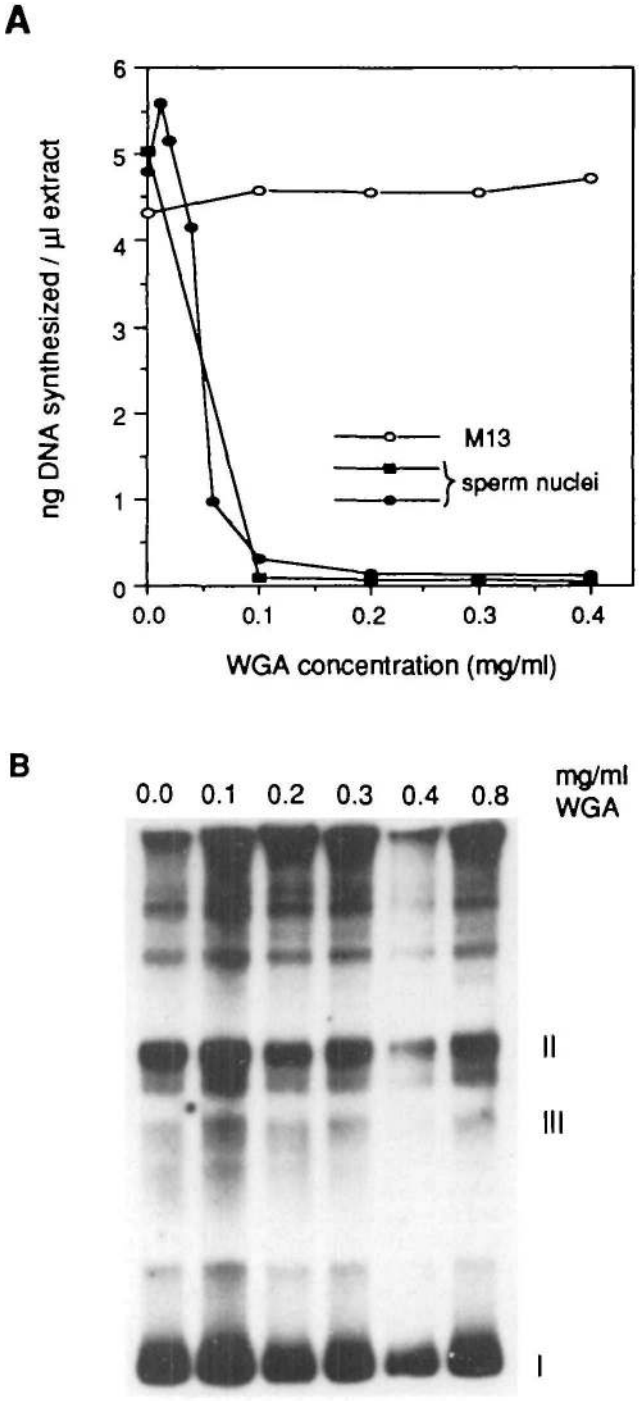

Fig. 1. Effect of WGA on replication of sperm nuclei and single-stranded M13 DNA. Wheat germ agglutinin (WGA) was added to egg LSS in the concentration range 0 to 0.4 mg/ml (A) or 0 to 0.8 mg/ml (B). Demembranated sperm nuclei (filled symbols) or ss M13 DNA (open symbols) were added to 5 ng/µl, and incubations carried out in the presence of [α-^{32}P]dATP for 90 min (M13) or 3 h (sperm) at 23°C. (A) The extent of replication was determined by incorporation of ^{32}P into acid-insoluble counts. Two different WGA concentration curves are shown for sperm chromatin. (B) DNA from the M13 reaction was purified, then analysed on a 1% agarose gel. Form I, double-stranded (ds) supercoiled plasmid; form II, ds circular relaxed DNA; and form III, ds linear M13 DNA.

subsequent experiments in this paper, to ensure complete inhibitory activity.

To determine whether this effect of WGA was due simply to interference with the DNA replication machinery, parallel incubations were set up containing single-stranded (ss) M13 DNA at 5 ng/µl, and incubated for 90 minutes at 23°C. Fig. 1A shows that WGA even at 0.4 mg/ml did not inhibit the second-strand synthesis reaction on ss M13 template (open symbols), which is thought to mimic lagging strand synthesis at a replication fork (Mechali and Harland, 1982). DNA was purified from the M13 incubations and analysed by agarose gel electrophoresis. The autoradiograph shown in Fig. 1B demonstrates that WGA had no detectable effect either on the extent of DNA replication or on chromatin assembly on this template. Forms I (double-stranded supercoiled), II (ds relaxed) and III (ds linear) were detected, in addition to some high molecular weight forms possibly representing concatemers. Therefore, WGA inhibited the replication of sperm nuclei without affecting the mechanism of DNA synthesis.

Inhibition of replication by WGA is reversible

WGA-induced inhibition of nuclear transport is reversed by treatment with 300-500 mM *N*-acetylglucosamine (GlcNAc) (Yoneda et al., 1987; Finlay et al., 1987) or 1 mM triacetylchitotriose (Finlay et al., 1987). To determine whether inhibition of nuclear DNA replication caused by WGA could be reversed under the same conditions that permitted reversal of transport inhibition, sperm nuclei at 5 ng/µl were incubated in egg LSS with [α-^{32}P]dATP, WGA was included to 0.2 mg/ml, and GlcNAc was added to various concentrations. The samples were incubated at 23°C for 5 h and replication was analysed by incorporation of ^{32}P into acid-insoluble material. The replication of sperm nuclei in the presence of GlcNAc but without WGA was also measured. The results are shown in Fig. 2A. As expected, no replication was detected in the presence of WGA without added sugar. GlcNAc concentrations up to 200 mM gradually increased the extent of sperm nuclear replication, but levels were at best 3-fold lower than the controls without WGA. At concentrations of GlcNAc higher than 200 mM, incorporation of ^{32}P was found to decrease, and at 300 and 500 mM, replication levels were extremely low. These results show that sperm nuclear replication was only partially rescued by GlcNAc concentrations previously used to relieve completely WGA-induced inhibition of nuclear transport. The levels of replication detected were too low for GlcNAc reversal of inhibition to be used reliably in further studies.

It was therefore decided to assay the effectiveness of the sugar triacetylchitotriose (TAC) on reversal of WGA-induced inhibition of DNA replication. This sugar contains multiple GlcNAc groups and has been shown to restore nuclear transport in WGA-blocked nuclei (Finlay et al., 1987). Sperm nuclei were again incubated in egg LSS with or without 0.2 mg/ml WGA, and replication was assayed by incorporation of [α-

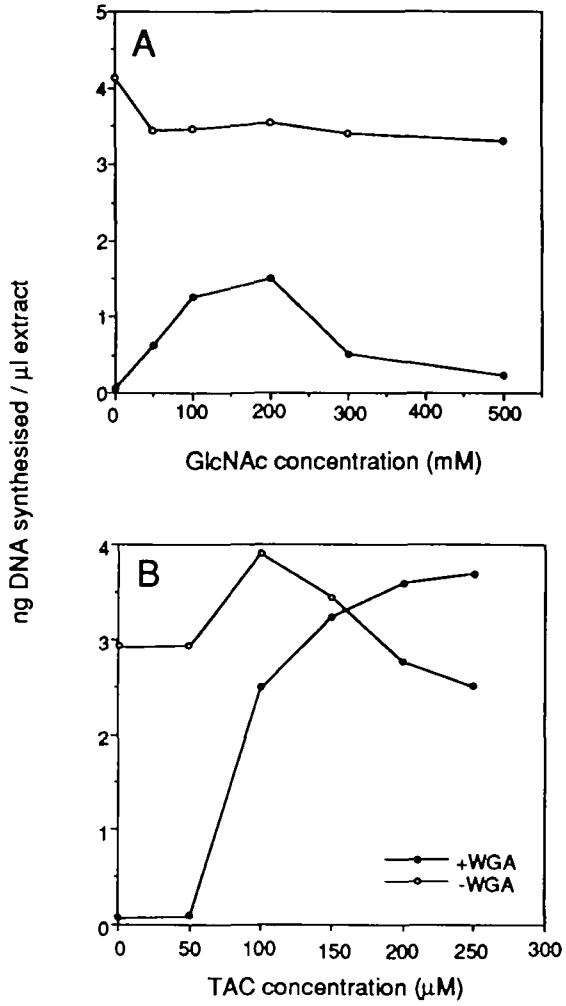

Fig. 2. Reversal of WGA inhibition of DNA replication by competing sugars. Sperm nuclei were incubated in egg LSS at 5 ng DNA/µl extract in the presence (filled circles) or absence (open circles) of 0.2 mg/ml WGA, with [α-^{32}P]dATP. Increasing concentrations of (A) *N*-acetylglucosamine (GlcNAc) and (B) triacetylchitotriose (TAC) were added. The extent of replication was assayed after 5 h by acid precipitation of insoluble counts. Note the 1000-fold difference in concentrations of the two sugars.

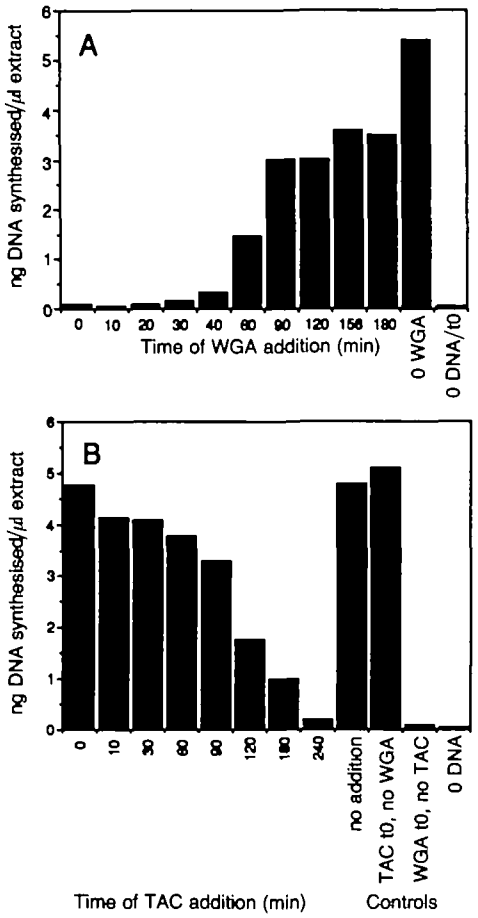

Fig. 3. Time of inhibition of replication by WGA. Sperm nuclei were incubated at 5 ng/µl at 23°C in egg LSS in the presence of [α-^{32}P]dATP. The extent of replication was determined by acid precipitation, and is expressed as ng DNA synthesized/µl extract. (A) WGA was added at various times to give a final concentration of 0.2 mg/ml. "0 WGA" control was incubated without WGA, and "0 DNA/t0" contained WGA added at the outset of the experiment but with no added DNA. (B) WGA at 0.2 mg/ml was added to all samples at the outset, then 250 µM TAC was added at various times. In the absence of added exogenous DNA ("0 DNA") no radiolabel incorporation was detected. WGA alone ("WGA t0, no TAC") inhibited replication completely, whilst triacetylchitotriose alone ("TAC t0, no WGA") had no significant effect on sperm nuclear replication compared with the "no addition" control.

^{32}P]dATP into acid-insoluble material. TAC was added to various concentrations. The results following a 5 hour incubation at 23°C are shown in Fig. 2B.

At 0-50 µM TAC, WGA inhibited replication of sperm nuclei. However, relief of inhibition was considerable at 100 µM TAC, and complete at 250 µM. In the absence of lectin, TAC had a variable effect on sperm nuclear replication. Although ^{32}P incorporation was slightly lower at higher TAC concentrations in this experiment, TAC was generally not found to affect sperm nuclear replication (see Fig. 3). Fig. 2B clearly demonstrates that TAC is effective in reversing WGA inhibition of replication at concentrations 1,000-fold lower than GlcNAc.

These results show that the WGA-induced inhibition of sperm nuclear replication can be reversed by treatment with low concentrations of a competing sugar, triacetylchitotriose, under conditions that have been reported to release the WGA transport block (Finlay et al., 1987).

Sensitivity of nuclear replication to WGA inhibition

Replication of sperm nuclei is prevented by WGA (Fig. 1A). In order to determine at what stage in the replication process this inhibition occurs, sperm nuclei were incubated in egg LSS with [α-^{32}P]dATP, and WGA was added at various times. Incorporation of radiolabel was assayed after a total incubation time of 5 hours at 23°C. The extent of replication, expressed as ng DNA synthesized per µl extract, is shown in Fig. 3A.

Addition of WGA before or at 40 minutes of incubation prevented replication of sperm nuclei. When WGA was added at 60 minutes, only 30% of the input template had replicated after 5 hours of incubation (1.47 ng/μl DNA synthesized from input 5 ng/μl). Later addition of WGA at, or after, 90 min of incubation of sperm nuclei in egg LSS permitted considerable DNA replication (61%), but WGA addition even after 3 hours decreased detectable replication to 65% of the positive control level. Fig. 3A therefore shows a major inhibition of sperm nuclear replication if WGA is added before or at 60 minutes of incubation.

Complementary to the time course of inhibition by WGA, the time at which TAC reversal of WGA inhibition occurred was also analysed. Fig. 3B shows the amount of DNA synthesized when sperm nuclei at 5 ng/μl and WGA at 0.2 mg/ml were incubated in egg LSS, with 250 μM TAC added at various times. The "WGA t0, no TAC" control shows that the concentration of WGA used here was totally inhibitory for replication of sperm nuclei. Addition of TAC at the outset of the incubation permitted almost the same extent of replication as was detected in the control sample without either WGA or sugar added ("no addition"), so TAC alone neither stimulated nor inhibited DNA replication. Although delayed sugar addition did not rescue replication completely, detectable DNA synthesis was considerable even when TAC was added as late as at 90 minutes. After this time, inhibition appeared to be less reversible, and by 4 hours of incubation of sperm nuclei with WGA, TAC addition did not lead to an increase in replication levels.

These data suggest that replication of sperm nuclei is very sensitive to WGA inhibition early in incubation, but that this type of inhibition is reversible. However, after long exposure to WGA, replication capacity is irreversibly lost.

WGA prevents nuclear formation

The most sensitive period for WGA inhibition of DNA replication (≤40 minutes, Fig. 3A) is also the time during which demembranated sperm nuclei usually decondense and acquire nuclear envelopes on incubation in egg extract. Since it was possible that WGA effectively depleted the egg extract of nuclear pore components (Finlay and Forbes, 1990), the morphology of sperm nuclei incubated in extracts with and without WGA was examined.

Fig. 4 shows typical nuclei observed on WGA addition at 0, 40 and 60 minutes, both at the time of addition (A) and after a total of 5 hours incubation at 23°C (B).

On addition of sperm nuclei to egg extract, rapid decondensation was observed, so that even "0" minute samples had decondensed partially. After 5 hours in egg LSS, nuclei incubated without WGA (0 WGA, Fig. 4B) had decondensed fully and become swollen, so that the DNA had become diffuse and dispersed, and had acquired a nuclear envelope, shown by the strong phase ring. By contrast, nuclei incubated with WGA added at zero time never progressed beyond the early stage of

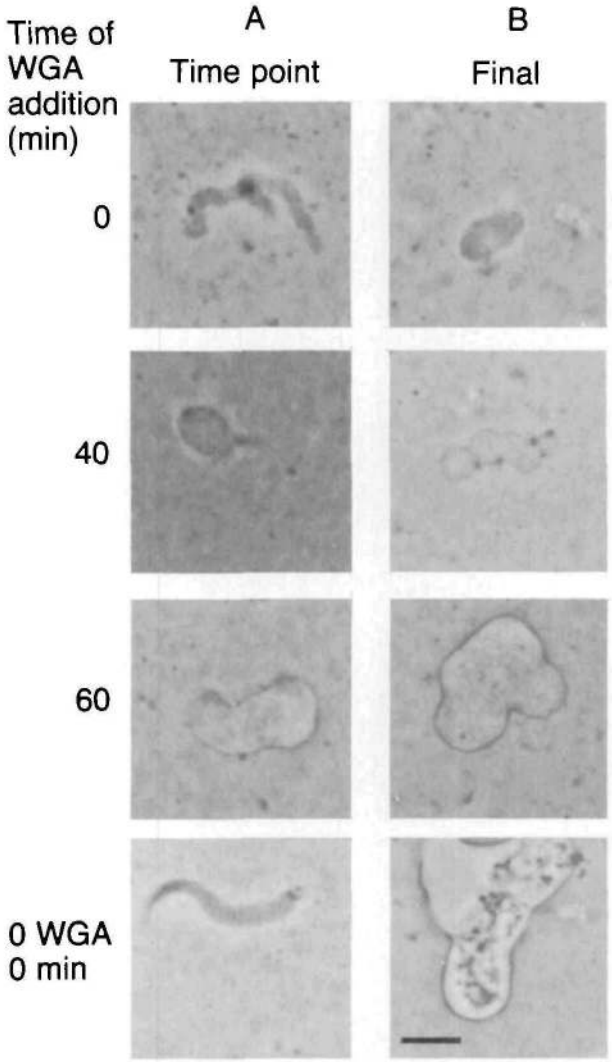

Fig. 4. WGA inhibits nuclear assembly. Sperm nuclei were incubated in egg LSS at 5 ng/μl. WGA was added to 0.2 mg/ml at 0, 40 or 60 min, and nuclear morphology was examined at the time of addition (A) or after 5 h total incubation time (B). A control sample without WGA (0 WGA, 0 min) was examined at 0 min and 5 h. Bar, 5 μm.

decondensation (0, Fig. 4B). Following 40 minutes of incubation in egg LSS, the sperm nuclei had started to round up, but although an outline could be discerned by phase-contrast microscopy, nuclear envelopes were not detected (40, Fig. 4A). After 5 hours of incubation, nuclei to which WGA was added at 40 minutes showed a nuclear outline that was weaker than that usually associated with an intact nuclear envelope (40, Fig. 4B). In addition, they did not show any appreciable increase in volume characteristic of normal nuclear swelling. After 60 minutes of incubation in untreated LSS, sperm nuclei had acquired nuclear envelopes (60, Fig. 4A), and the DNA had become diffuse (not shown). WGA added at 60 minutes permitted considerable subsequent changes in nuclear morphology (60, Fig. 4B). Although these nuclei showed distinct nuclear envelopes, the degree of swelling was less than that in 0 WGA

samples after 5 hours (0WGA, Fig. 4B). Addition of 250 μM TAC coincident with WGA at the start of the incubation, enabled normal nuclear assembly to proceed (data not shown).

These results show that WGA inhibits the formation of nuclear envelopes and subsequent swelling of sperm nuclei if it is added before or at 40 minutes of incubation. After that time, nuclear envelope formation appears insensitive to WGA, although nuclear swelling is impaired. On comparison with Fig. 3B, the period most sensitive to WGA inhibition of DNA replication is also the period in which nuclear formation is most affected.

WGA inhibits replication of preformed nuclei

The above data suggest that WGA inhibition of sperm nuclear replication could be accounted for entirely by prevention of nuclear formation. In order to determine if this is the case, two types of preformed nuclear templates were examined for replication in the presence of WGA.

Firstly, replication of nuclei prepared from exponentially growing HeLa cells was analysed. These nuclei were incubated in egg LSS at 2 ng/μl for a total of 4.5 hours at 23°C. WGA at 0.2 mg/ml was added at the start of the incubation, or after 35, 60 or 90 minutes. Replication was assayed by incorporation of [α-^{32}P]dATP into acid-insoluble counts, and the extent of replication is shown in Fig. 5A.

WGA added at the start of the incubation permitted a low level of nuclear replication, to 10% of that detected in the 90 minute sample. Late addition of WGA permitted further replication, and an inverse relationship was detected between time of WGA addition and extent of replication, but it should be emphasised that at all times analysed WGA inhibited replication to some extent.

The HeLa nuclei did not require assembly of complex structures *de novo*, and possessed nuclear pores, as determined by staining with fluorescein-conjugated WGA (data not shown). However, slight morphological changes such as gradual loss of heterochromatin and nucleolar staining were observed in the absence of WGA, but rarely in the presence of WGA (data not shown). It was therefore concluded that WGA inhibits somatic nuclear DNA replication, though the effects on replication and on nuclear morphology may not necessarily be separable.

Secondly, preformed sperm nuclei were used to determine if WGA inhibited the elongation stage of DNA synthesis. Sperm nuclei were assembled from demembranated sperm chromatin by incubation in egg LSS for 4 hours at 23°C, in the presence or absence of 10 μg/ml aphidicolin. Aphidicolin did not affect nuclear assembly (data not shown), and permitted establishment of stable initiated replication forks without elongation of DNA replication (Heintz and Stillman, 1988; Hutchison and Kill, 1989; Cox and Leno, 1990). Therefore, intact nuclei with functional pores could be assembled and initiated for replication, and arrested in that state. Nuclei so formed were then washed by

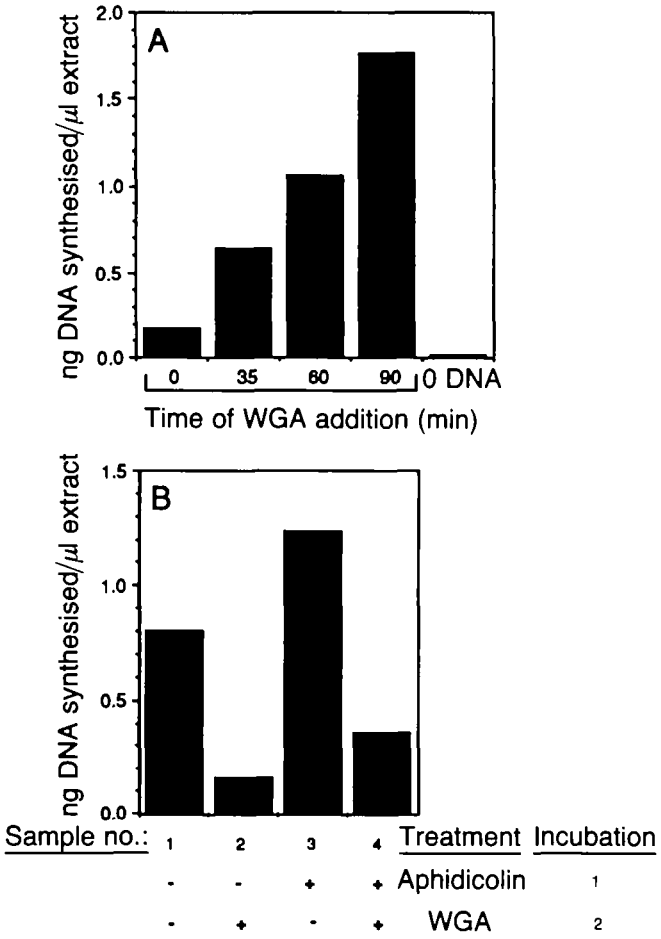

Fig. 5. WGA inhibits replication of pre-formed nuclei. (A) Nuclei prepared from exponentially growing HeLa cells were incubated in egg LSS at 2 ng/μl; 0.2 mg/ml WGA was added at various times, and the extent of replication assayed after a total incubation time of 5 h. In the absence of added template ("0 DNA"), no incorporation of label was detected. (B) Sperm nuclei were incubated in egg LSS with or without aphidicolin at 10 μg/ml. WGA was added to appropriate samples at 0.2 mg/ml. After 4 h incubation, nuclei were spun through buffer into fresh extract with [α-^{32}P]dATP that did or did not contain WGA at 0.2 mg/ml. Incubation in the second extract was allowed to proceed for 5 h at 23°C. Nuclei in mid S phase and nuclei that had only initiated were found to be sensitive to inhibition by WGA.

centrifugation through buffer and transferred to freshly thawed extract with or without WGA at 0.2 mg/ml. Replication of nuclei in the second incubation was detected by incorporation of [α-^{32}P]dATP into acid-insoluble material. Fig. 5B shows the results obtained.

Actively replicating nuclei showed a 6-fold inhibition of DNA synthesis when transferred to an extract containing WGA (sample 2), compared with similar nuclei transferred to an extract without WGA (sample 1). Likewise, nuclei initiated for replication, but stalled by aphidicolin treatment, were capable of only very limited DNA synthesis on transfer to a WGA-containing extract (sample 4), whereas similar nuclei replicated almost to completion in the absence of WGA (sample

3). The amount of synthesis observed in samples 1 and 3 probably represented complete single rounds of DNA replication. The detected level of replication is lower in sample 1 than sample 3, probably because some of the DNA in sample 1 had been replicated before transfer, whereas in sample 3, replication in the first incubation had been blocked by aphidicolin. Two lines of evidence suggest that the washing procedure used during nuclear transfer does not dislodge replication initiation complexes. Firstly, under very similar washing conditions, we have previously shown that an initiation-defective oocyte extract can elongate DNA synthesis from pre-initiated nuclei (Cox and Leno, 1990). Secondly, immunofluorescence microscopy has confirmed the presence of punctate replication complexes containing PCNA after identical washing procedures (data not shown).

These results show that WGA inhibits elongation of DNA replication from moving and stalled replication forks.

The elongation phase of nuclear DNA replication requires an intact nuclear envelope

WGA is known to block nuclear transport specifically (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Newmeyer and Forbes, 1988; Wolff et al., 1988). However, the capacity of WGA to bind many other intracellular components (reviewed by Hart et al., 1989) leaves the possibility that the observed inhibition of replication was not due solely to a transport block. Therefore, alternative methods were devised to block nuclear accumulation. Since an intact nuclear envelope is necessary for transport and accumulation (Zimmer et al., 1988), procedures were found that prevent resealing of a permeabilized nuclear envelope. Lysolecithin treatment of once-replicated nuclei permeabilises them (Blow and Laskey, 1988) and such nuclei remain permeable on incubation in a high-speed supernatant of frog eggs (HSS; Sheehan et al., 1988), which is devoid of membranes.

Sperm nuclei were incubated in egg LSS at 3 ng/μl for 5 hours in the presence of [α-^{32}P]dATP and 0.25 mM BrdUTP under conditions that permitted one full round of DNA replication. Nuclei were then pelleted and permeabilized with lysolecithin at 500 μg/ml, or mock treated with buffer. They were transferred by centrifugation to freshly thawed extract supplemented with [^{3}H]dATP and BrdUTP. On transfer to egg LSS, permeabilized nuclear envelopes became resealed, but permeabilized nuclei did not reseal when transferred to HSS. Mock-treated nuclei (not exposed to lysolecithin) stayed intact after transfer to either egg LSS or egg HSS. DNA was purified after the second incubation and centrifuged to equilibrium on caesium chloride (CsCl) gradients.

A typical result is shown in Fig. 6. Nuclei incorporated ^{32}P in the first incubation, giving a peak of incorporation at the density of heavy-light DNA (substituted at all T residues by BrdUTP on one strand). On transfer without permeabilisation to HSS (Fig. 6A) or LSS (data not shown), no incorporation of

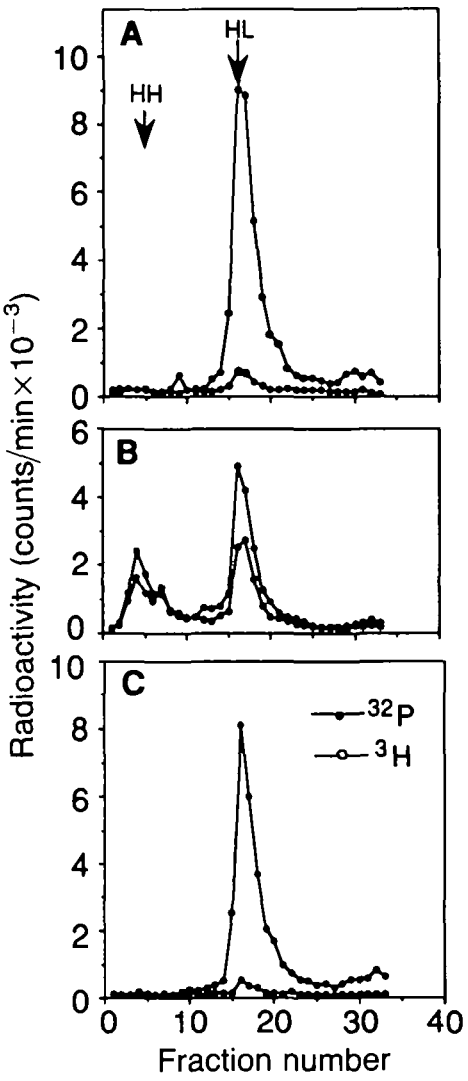

Fig. 6. Initiation of DNA replication is not detected in the absence of an intact nuclear envelope. Sperm nuclei were incubated at 5 ng/μl in egg LSS supplemented with [α-^{32}P]dATP and 0.25 mM BrdUTP for 5 h at 23°C. A small portion was taken from each sample to determine the extent of label incorporation at this time. Nuclei were transferred to fresh LSS (B) or HSS (A,C) labelled with [^{3}H]dATP and BrdUTP, with (B,C) or without (A) permeabilisation by lysolecithin treatment (see Materials and methods). They were incubated for a further 5 h at 23°C. DNA purified from each sample was centrifuged to equilibrium on a CsCl density gradient. Incorporation of ^{32}P (from the first incubation) and ^{3}H (second incubation) is shown. Heavy-heavy (HH; rereplicated) DNA banded at a caesium density of 1.802 g/cm^3 and heavy-light (HL; once replicated) DNA banded at 1.752 g/cm^3 on the caesium gradient.

^{3}H was detected, and there was no evidence of rereplication. In contrast, transfer of permeabilized nuclei to LSS (Fig. 6B) resulted in reinitiation of DNA replication, as shown by the heavy-heavy (HH) and heavy-light (HL) peaks. This result is consistent with earlier reports of reinitiation after nuclear envelope permeabilisation (Blow and Laskey, 1988). However,

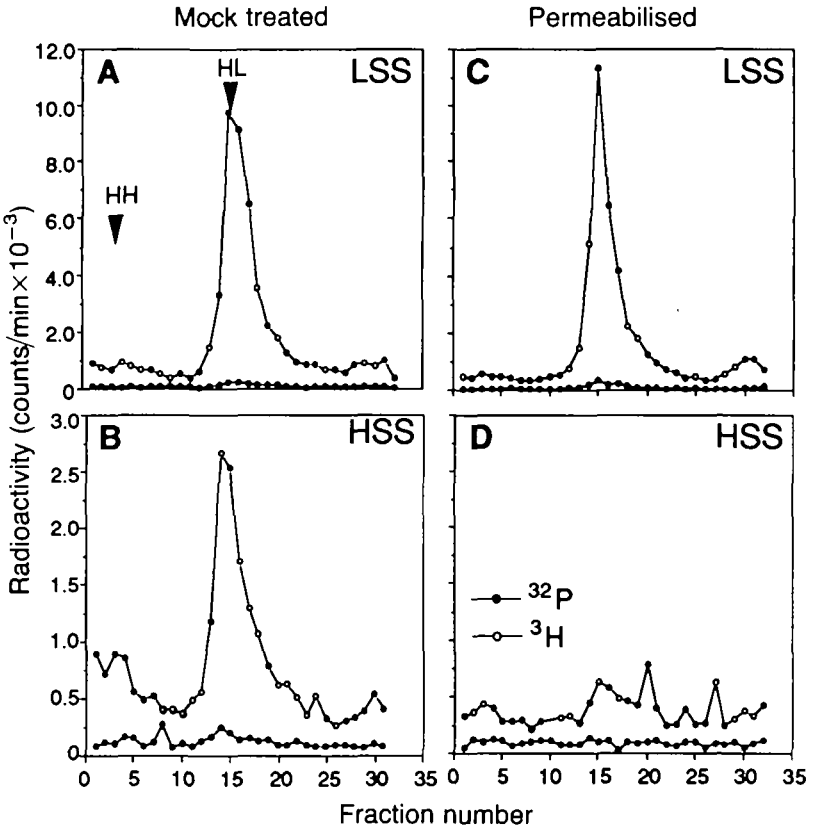

Fig. 7. The elongation stage of DNA synthesis requires an intact nuclear envelope. Sperm nuclei were incubated in egg LSS in the presence of [α-^{32}P]dATP, 0.25 mM BrdUTP and 10 μg/ml aphidicolin, for 1 h at 23°C. Nuclei were pelletted, resuspended in buffer (A,B) or lysolecithin (C,D), then transferred to LSS (A,C) or HSS (B,D) and incubated with [^{3}H]dATP and BrdUTP for a further 5 h at 23°C. Samples were then processed as described for Fig. 6. Heavy-light (HL) peaks represent a single semiconservative round of DNA replication; no appreciable rereplication (HH) was detected.

transfer of permeabilized nuclei to HSS (Fig. 6C) did not result in any further synthesis: no appreciable ^{3}H incorporation was detected and all ^{32}P-labelled DNA equilibrated at a CsCl density of 1.752 g/cm^3 (HL), indicating that only one complete round of DNA replication had occurred, in the first incubation.

These data show that reinitiation of DNA replication is detected only when the nuclear envelope of once-replicated nuclei is permeabilized and allowed to reseal. In the absence of an intact nuclear envelope, rereplication is not detected. However, this experiment cannot distinguish between an inability to reinitiate, and an inability to support elongation of reinitiated forks.

This question was addressed directly in Fig. 7. Sperm nuclei were incubated in egg LSS with [α-^{32}P]dATP, 0.25 mM BrdUTP and 10 μg/ml aphidicolin for 1 hour at 23°C. Nuclei were therefore formed and became initiated for replication, without further DNA synthesis (no ^{32}P incorporation was detected, although a low level of biotin-dUTP incorporation into punctate sites could be observed by fluorescence microscopy). Transfer of these intact nuclei directly to LSS (Fig. 7A) or HSS (Fig. 7B) enabled a single round of replication to proceed from the initiated forks, as detected by incorporation of [^{3}H]dATP after 5 hours of incubation into BrdUTP-labelled heavy-light peaks on CsCl gradients. Replication levels in HSS were consistently 3- to 4-fold lower than in LSS. Nuclei that were permeabilized with 500 μg/ml lysolecithin before transfer, then incubated in egg LSS (Fig. 7C), also underwent a single complete round of DNA replication. However, those nuclei that were permeabilized and transferred to HSS (so that they did not become resealed) did not support any significant replication (Fig. 7D).

It is possible that the lysolecithin permeabilisation step might have disrupted replication initiation complexes in the aphidicolin-treated nuclei. If complexes were reassembled in LSS but not HSS, then the results shown in Fig. 7 may be due to differential initiation rather than an effect of the nuclear envelope on elongation. In order to address this point, the presence of replication complexes, containing proliferating cell nuclear antigen (PCNA) at sites of biotin-dUTP incorporation, was examined using confocal scanning immunofluorescence microscopy. Sperm nuclei were incubated at 5 ng/μl in egg LSS supplemented with 20 μM biotin-dUTP and 10 μg/ml aphidicolin, for one hour at 23°C. Under such conditions, a low level of biotin-dUTP incorporation into nascent DNA can be detected (data not shown). Nuclei were then pelletted and permeabilised with 500 μg/ml lysolecithin or mock treated with buffer. After washing by centrifugation through buffer, the nuclei were fixed and processed for confocal microscopy. PCNA was detected with FITC-labelled antibodies (see Materials and methods) and incorporated biotin-dUTP was stained with Texas red-streptavidin. The two signals were merged to determine if PCNA remained at sites of DNA synthesis after permeabilisation. The results showed that permeabilisation did not dislodge the majority of the replication initiation complexes (data not shown).

The data presented above strongly suggest that an intact nuclear envelope is required for the elongation stage of DNA replication in sperm nuclei.

Discussion

This paper examines the requirement for an intact, functional nuclear envelope in DNA replication, using the lectin wheat germ agglutinin (WGA), or irreversible permeabilisation of the nuclear envelope, to prevent nuclear transport.

Replication of sperm nuclei is shown to be prevented completely by WGA concentrations as low as 0.1 mg/ml. Inhibition of replication is greatest if the lectin is added early in the incubation, at or before 40 minutes, and an effect of WGA on nuclear assembly on sperm chromatin is detected. However, non-nuclear DNA synthesis is not inhibited, since single-stranded M13 DNA acts as a template for second-strand synthesis in the presence of WGA.

Inhibition of replication can be reversed partially by *N*-acetylglucosamine (GlcNAc) and completely by triacetylchitotriose (TAC), both of which are bound by WGA. Reports of reversal of WGA-induced transport inhibition suggest that GlcNAc concentrations of 300-500 mM are sufficient (Yoneda et al., 1987; Finlay et al., 1987), but in the system described here, DNA replication requires higher GlcNAc concentrations to relieve WGA inhibition. It is possible that limited reversal of a transport block permits sufficient accumulation of nuclear proteins to give a strong fluorescent signal, without permitting accumulation of such proteins to the physiological level necessary for DNA replication.

However, triacetylchitotriose completely reverses the WGA-induced DNA replication block at concentrations as low as 250 μM. Reversal of WGA inhibition by TAC is most effective if the sugar is added coincidentally with WGA. Later sugar addition permits rescue of replication to lower levels until, after 4 hours, nuclei are irreversibly prevented from replicating. The WGA-sensitive period defined by these experiments is shown to occur during the early stages of nuclear assembly from demembranated chromatin. The presence of WGA during the time of early, critical events of decondensation and nuclear envelope formation prevents normal nuclear assembly.

It is conceivable that binding of WGA to nucleoporins (Gerace and Burke, 1988) in the egg extract prevents normal nuclear assembly. The observed absence of nuclear envelope structures (Fig. 4) may be accounted for by WGA binding to structural components of the formative nucleus, including the pore proteins. However, Finlay and Forbes (1990) have shown the formation of nuclei that appear morphologically normal, in egg extracts that have been depleted of pore components by batch adsorption with WGA-Sepharose. These authors suggest that nuclear pore complexes can form in the absence of GlcNAc-bearing pore proteins, but are non-functional for nuclear transport. The data presented in this paper do not contradict the findings of Finlay and Forbes (1990), since addition of WGA to extracts is very different from removal of WGA-binding proteins. Also, these authors depleted a soluble extract and added back untreated membranes that may have possessed sufficient nuclear envelope assembly capacity. In the LSS used in this paper, WGA may bind both to membranes and to soluble glycosylated proteins (K. Labib, personal communication). It is possible that, in the presence of WGA, nuclear pores form, with WGA bound to GlcNAc residues. Steric hindrance caused by bound lectin may then prevent the normal course of vesicle fusion in nuclear envelope formation, leading to the abortive nuclear assembly observed.

A subsequent report (Newport et al., 1990) has suggested that addition of WGA to egg extracts does not prevent nuclear assembly, a result that was not repeatable here. Several differences in technique may account for the apparent discrepancy. Firstly, Newport et al. (1990) showed only DNA and did not present data for the formation of intact nuclear envelopes. Secondly, they used a 3-fold lower nuclear concentration than is used here. If nuclear assembly is limited by the presence of WGA, a lower nuclear concentration may be more favourable for nuclear assembly.

Although evidence is presented here for WGA-induced inhibition of nuclear formation on sperm chromatin, the lectin is also found to inhibit the replication of fully formed nuclei with pores, prepared from cultured cells. Also, "pre-assembled" sperm nuclei that are initiated for DNA replication are prevented from synthesising DNA by WGA treatment. These observations suggest that the elongation phase of nuclear DNA replication is sensitive to inhibition of nuclear transport by WGA. It is significant that disruption of the nuclear envelope also prevents elongation synthesis and reinitiation of replication, as these data support the results obtained using WGA. Combination of the results from the two experimental approaches strongly suggests a requirement for functional integrity of the nuclear envelope in nuclear DNA replication in *Xenopus* egg extracts.

Current models of control of DNA replication focus on the importance of initiation (e.g. see Blow and Laskey, 1988) but the data presented here suggest that the elongation phase is also controlled. Since WGA does not block diffusion into the nucleus of small molecules such as nucleotides (Finlay et al., 1987; Yoneda et al., 1987; Newmeyer and Forbes, 1988), it appears that larger components, possibly proteins with nuclear localisation signals, are necessary for elongation. I suggest, therefore, that in addition to factors required for initiation of replication, proteins must be accumulated within the nucleus throughout S phase to support the elongation phase of DNA replication.

It is important to note here that, in some circumstances, the elongation phase of DNA replication can occur in the total absence of nuclear structures such as the nuclear envelope; for example, in the cell-free SV40 system (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985). This apparent contradiction may be resolved if one considers the crucial role of nuclear structure in replication within the *Xenopus* egg extract. In cases where replication proceeds within nuclei, treatments that interfere with nuclear structure,

such as WGA or lysolecithin, might be expected to affect adversely the efficiency of DNA replication. Conversely, such treatments would be expected to have no effect in soluble systems such as cell-free SV40 replication extracts or single-strand synthesis reactions in *Xenopus* egg extract, where replication occurs in the absence of nuclear structures. However, Newport and Kirschner (1984) and Hutchison and Kill (1989) have reported that considerable DNA synthesis can occur in meiotic metaphase-arrested eggs or in mitotic egg extracts (respectively), when nuclear envelopes are absent. The primary assay used by Hutchison and Kill (1989) was the incorporation of biotinylated-dUTP into nascent DNA. We have often observed such levels of incorporation under conditions where replication does not occur, e.g. in the presence of aphidicolin. Both groups also detected considerable incorporation of [α-^{32}P]dCTP in agarose gel assays, but did not distinguish between repair and replication. The slight degree of radiolabel incorporation shown in Fig. 7D is more indicative of repair-type DNA synthesis, and mitotic incorporation of label into plasmid DNA in egg extracts has already been demonstrated to represent repair synthesis (Blow and Sleeman, 1990).

An apparent contradiction still remains between the data presented here and other reports of replication elongation in permeabilized systems (e.g. see Jackson and Cook, 1986; Nakayasu and Berezney, 1989). At present, it is unclear how such differences may be resolved. However, it should be noted that electron microscopic analysis of nuclei encapsulated in agarose beads shows nuclear envelopes that appear to be intact (Jackson and Cook, 1985). Therefore the DNA synthesis reported by Jackson and Cook (1986) may have occurred within intact nuclei. It will be interesting to examine the relative contributions of repair and replication to the synthesis of DNA in such systems.

In conclusion, the data presented in this paper strongly suggest that nuclear DNA replication is dependent upon transport across an intact nuclear envelope.

I thank Julian Blow for his help and encouragement in initiating this project, Karim Labib for communication of unpublished results and Naushin Waseem for the gift of anti-PCNA monoclonal antibodies. I am grateful to Ron Laskey, Greg Leno and Julian Blow for critical reading of the manuscript, and to Chris Hutchison for subsequent discussion. This work was supported by the Cancer Research Campaign.

References

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 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 Sleeman, A. M. (1990). Replication of purified DNA in *Xenopus* egg extract is dependent on nuclear assembly. *J. Cell Sci.* **95**, 383-391.

Blow, J. J. and Watson, J. V. (1987). Nuclei act as independent and integrated units of replication in a *Xenopus* cell-free DNA replication system. *EMBO J.* **6**, 1997-2002.

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

Cox, L. S. and Leno, G. H. (1990). Extracts from eggs and oocytes of *Xenopus laevis* differ in their capacities for nuclear assembly and DNA replication. *J. Cell Sci.* **97**, 177-184

Dabauvalle, M.-C., Schultz, B., Scheer, U. and Peters, R. (1988). Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. *Exp. Cell Res.* **174**, 291-296.

Dworetzky, S. I. and Feldherr, C. M. (1988). Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* **106**, 575-584.

Feldherr, C. M., Kallenbach, E. and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* **99**, 2216-2222.

Finlay, D. R. and Forbes, D. J. (1990). Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. *Cell* **60**, 17-29.

Finlay, D. R., Newmeyer, D. D., Price, T. M. and Forbes, D. J. (1987). Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* **104**, 189-200.

Gerace, L. and Burke, B. (1988). Functional organisation of the nuclear envelope. *Annu. Rev. Cell Biol.* **4**, 335-374.

Gurdon, J. B. (1976). Injected nuclei in frog oocytes: fate, enlargement and chromatin dispersal. *J. Embryol. exp. Morph.* **36**, 523-540.

Hart, G. W., Haltiwanger, R. S., Holt, G. D. and Kelly, W. G. (1989). Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.* **58**, 841-874.

Heintz, N. H. and Stillman, B. W. (1988). Nuclear DNA synthesis *in vitro* is mediated via stable replication forks assembled in a temporally specific fashion *in vivo*. *Mol. Cell Biol.* **8**, 1923-1931.

Hutchison, C. J., Cox, R., Drepaul, R. 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. (1989). Changes in the nuclear distribution of DNA polymerase alpha and PCNA/cyclin during the progress of the cell cycle, in a cell-free extract of *Xenopus* eggs. *J. Cell Sci.* **93**, 605-613.

Jackson, D. A. and Cook, P. R. (1985). A general method for preparing chromatin containing intact DNA. *EMBO J.* **4**, 913-918.

Jackson, D. A. and Cook, P. R. (1986). Replication occurs at the nucleoskeleton. *EMBO J.* **5**, 1403-1410.

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.

Li, J. J. and Kelly, T. J. (1984). Simian virus replication *in vitro*. *Proc. Nat. Acad. Sci. USA* **81**, 6973-6977.

Lohka, M. J. and Masui, Y. (1983). Formation *in vitro* of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science* **220**, 719-721.

Lohka, M. L. and Masui, Y. (1984). Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations from amphibian eggs. *J. Cell Biol.* **98**, 1222-1230.

Maniatis, T., Fritsch, E. F. and Stambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, N.Y.

Mechali, M. and Harland, R. (1982). DNA synthesis in a cell-free system from *Xenopus* eggs: priming and elongation on single-stranded DNA *in vitro*. *Cell* **30**, 93-101.

Meier, J., Campbell, K. H. S., Ford, C. C., Stick, R. and Hutchison, C. J. (1991). The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* **98**, 271-279.

Mills, A. D., Blow, J. J., White, J. G., Amos, W. B., Wilcock, D. and Laskey, R. A. (1989). Replication occurs at discrete foci spaced 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.

Newmeyer, D. D. and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps *in vitro*: nuclear pore binding and translocation. *Cell* **52**, 641-653.

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

Newport, J. W. and Kirschner, M. W. (1984). Regulation of the cell cycle during early *Xenopus* development. *Cell* **37**, 731-742.

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.

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.

Stillman, B. W. and Gluzman, Y. (1985). Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Mol. Cell. Biol.* **5**, 2051-2060.

Wobbe, C. R., Dean, F., Weissbach, L. and Hurwitz, J. (1985). *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. *Proc. Nat. Acad. Sci. USA* **82**, 5710-5714.

Wolff, B., Willingham, M. C. and Hanover, J. A. (1988). Nuclear protein import: specificity for transport across the nuclear pore. *Exp. Cell Res.* **178**, 318-334.

Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M. and Uchida, T. (1987). Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* **173**, 586-595.

Zimmer, F. J., Dreyer, C. and Hansen, P. (1988). The function of the nuclear envelope in nuclear protein accumulation. *J. Cell Biol.* **106**, 1435-1444.

(Received 3 June 1991 – Accepted, in revised form, 3 October 1991)