Replication of purified DNA in *Xenopus* egg extract is dependent on nuclear assembly

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Summary

Purified DNA undergoes a single round of semiconservative replication when incubated in extracts of *Xenopus* eggs. These extracts also assemble purified DNA into pseudo-nuclei, structures closely resembling normal interphase nuclei. In this paper we show that although <60% of purified DNA is assembled into pseudo-nuclei, DNA replication takes place only within these pseudo-nuclei. Further, when nuclear assembly is prevented, the initiation of replication on purified DNA molecules does not occur. In contrast to previous reports, we show that the initiation of DNA replication occurs only during interphase and not during mitosis, even when very high concentrations of purified DNA are used. These experiments show that nuclear formation is a general requirement for the initiation of DNA replication in this system.

Key words: DNA replication, nuclear assembly, *Xenopus* eggs, cell cycle.

Introduction

Extracts of gently homogenised frog eggs can support a range of cell cycle events that normally occur during the first mitotic cell divisions of the fertilised egg (Lohka and Masui, 1983). Cell-free extracts support the assembly of demembranated sperm nuclei into interphase nuclei surrounded by a double unit envelope studded with nuclear pores and capable of accumulating soluble nuclear proteins (Lohka and Masui, 1983, 1984; Blow and Laskey, 1986; Newmeyer et al. 1986; Hutchison et al. 1987; Sheehan et al. 1988). These extracts also support the efficient semiconservative replication of demembranated sperm nuclei (Blow and Laskey, 1986; Blow and Watson, 1987; Hutchison et al. 1987). Replication starts after a lag corresponding to the time taken for nuclei to acquire complete surrounding envelopes (Blow and Laskey, 1986). If vesicular material is removed from the extract by centrifugation, it can no longer assemble or replicate sperm nuclei (Lohka and Masui, 1984; Sheehan et al. 1988). Reconstitution of soluble and vesicular fractions restores both these activities.

Flow cytometry using the thymidine analogue biotin-dUTP suggests that nuclear assembly may be involved in the coordination of the thousands of initiation events required to replicate each sperm nucleus fully (Blow and Laskey, 1987). Different nuclei in the same extract start to replicate at different times. However, as each nucleus starts to replicate it undergoes a near-synchronous burst of initiations to reach its maximal rate of replication rapidly. Thus when an individual nucleus starts to replicate, some sort of signal must act locally throughout it in order to coordinate many thousands of initiation events (Blow and Watson, 1987; Blow, 1988).

It is possible that the replication of sperm nuclei may involve a series of events not required in subsequent cell cycles (Hutchison et al. 1988). Sperm nuclei are maintained in a highly organised and condensed state. It is possible that this nuclear organisation makes the sperm nucleus dependent on nuclear assembly before it can replicate. For example, replication origins might be masked by sperm proteins, that cannot be removed until the DNA is assembled into an interphase nucleus. To examine whether nuclear assembly is a general requirement for the initiation of DNA replication in this system, or whether it is specifically required for the replication of sperm nuclei, the behaviour of purified DNA in frog egg cytoplasm can be studied.

When purified DNA is microinjected into eggs of the frog *Xenopus laevis* it is replicated semiconservatively in step with the cell cycle of the early embryo (Harland and Laskey, 1980). No special DNA sequences seem to be required to support this controlled replication, since all DNA molecules so far tested behave in this manner (Harland and Laskey, 1980; Mechali and Kearsey, 1984). However, replication is usually inefficient, comprising...
<20% of injected molecules. Further, purified DNA microinjected into *Xenopus* eggs is assembled into structures resembling interphase nuclei (Forbes et al., 1983). These 'pseudo-nuclei' consist of many thousands of DNA molecules surrounded by a double unit envelope studded with nuclear pores.

These events can also be recreated in cell-free extracts. Purified DNA can be assembled into pseudo-nuclei lined with lamins (Newport, 1987) and capable of accumulating soluble nuclear proteins in *vitro* (Newmeyer et al., 1986a). Cell-free extracts also support the semiconservative replication of purified DNA at efficiencies comparable to those achieved in *vitro* (Blow and Laskey, 1986; Blow et al., 1987, 1988; Newport, 1987). However, it is not known what proportion of purified DNA molecules is assembled into pseudo-nuclei, either in *vitro* or in *vitro*, or whether DNA replication occurs only within pseudo-nuclei.

In this paper we show that although less than 60% of purified DNA molecules are assembled into pseudo-nuclei, DNA replication takes place only in pseudo-nuclei. When nuclear assembly is prevented from taking place, either by removing vesicular components from the extract or by blocking it in mitosis, semiconservative replication of plasmid DNA does not take place. This suggests that whether DNA is added as sperm nuclei or as purified DNA, nuclear assembly is a general requirement for the initiation of DNA replication in this system.

**Materials and methods**

**Preparation of extracts**

Activated egg extracts were prepared by a modification of previously published methods (Blow and Laskey, 1986, 1988). Unfertilized *Xenopus* eggs were collected in high salt Barth solution (110 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 0.5 mM Na₂HPO₄, 2 mM NaHCO₃, 15 mM Tris-HCl, pH 7.4), and any showing degenerative changes were discarded. The remaining eggs were dejellied in 2% cysteine-HCl, pH 7.8, and washed in Barth solution (88 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 15 mM Tris-HCl, pH 7.4), and activated for 5 min with 0.5 µg/ml calcium ionophore A23187 at room temperature. Activated eggs were washed again in Barth solution, and then in Extraction Buffer (50 mM KCl, 50 mM Hepes-KOH, pH 7.4, 5 mM MgCl₂, 2 mM β-mercaptoethanol) at 4°C. Washed eggs were transferred to Beckman SW50 centrifuge tubes and prespun at 15000 revs min⁻¹, 2 min, 4°C. All excess buffer was removed, and eggs were spun crushed at 10000 revs min⁻¹, 10 min, 4°C. The cleared supernatant was taken, made 2% with respect to glycerol, and frozen by dropping 15-µl samples directly into liquid nitrogen.

Extracts were fractionated into soluble and vesicular components as described (Sheehan et al., 1988). Unactivated egg extracts were prepared by the method of Lohka and Masui (1985). They were made 10% with respect to glycerol and frozen by dropping 15-µl samples directly into liquid nitrogen.

After thawing, extracts were supplemented with 50 mM creatine phosphate and 15 μg ml⁻¹ creatine phosphokinase. Unactivated extracts were activated by the addition of CaCl₂ to a final concentration of 3 mM. Low-speed supernatants were supplemented with cycloheximide to a final concentration of 250 μg ml⁻¹. Samples (30 µl) were supplemented with 10 µCi of [α³²P]dATP (Amersham International), 1.5 µl biotin-19-dUTP (Calbiochem), 0.5 mM BrdUUTP and 3 ng ml⁻¹ DNA as appropriate, and incubated at 23°C.

**Preparation of DNA templates**

Plasmid DNA was prepared by an alkali lysis protocol (Maniatis et al., 1982), and form I DNA was further purified on sucrose gradients (Blow and Laskey, 1986).

pUCm54 is a 12 kb (kb=kilobase-pairs) pUC plasmid containing part of the *Xenopus α-cardiac* actin gene, and was a gift from Dr T. Mohun.

RK2 is a naturally occurring 60 kb broad host-range plasmid (Thomas, 1981), and was a gift from Dr F. H. C. Franklin.

**Microscopy**

Samples for electron microscopy were stopped by overlaying with 100 µl of cold fixative (0.25% tannic acid, 1% glutaraldehyde, 2% paraformaldehyde, 2% dimethylsulphoxide, 0.1 M sodium cacodylate buffer, pH 7.2) and kept on ice for 1 h. A further 500 µl of cold fixative was then added and the samples were kept at 4°C overnight. The samples were spun at 600 g for 5 min and the pellets washed three times with 0.1 M sodium cacodylate buffer, pH 7.2. Pellets were then washed three times in this buffer, followed by three washes in water before block staining overnight in 2% uranyl acetate. The samples were dehydrated through an ethanol series and embedded using acrylic resin (LR White; London Resin Co., London, England). Blocks were sectioned to give silver to pale gold sections that were taken onto colloidion- and carbon-coated 300-mesh grids before double staining with uranyl acetate and Reynolds' lead citrate (10 min each). The sections were viewed using a Phillips EM300 at 60 kV.

Fluorescence microscopy was performed on unfixed pseudo-nuclei in situ in the cell-free extract. Samples (3 µl) of extract were placed onto a microscope slide and gently mixed with 1 µl fluorescein-tagged streptavidin (Amersham), 0.5 µl 50 µg ml⁻¹ Hoechst 33258, and 0.5 µl rhodamine-tagged rabbit IgG (Sigma). A coverslip was dropped onto the sample, and it was viewed wet under fluorescence and phase-contrast optics. Unfixed nuclei retained a normal morphological appearance for only 10–15 min on the microscope stage under these conditions.

**Sucrose and CsCl gradient centrifugation**

Samples for sucrose gradient centrifugation were resuspended in 10 volumes of Buffer A (60 mM KCl, 15 mM Tris-HCl, pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermine-3HCl, 0.15 mM spermine-4HCl) at 4°C, and were layered on top of a 10% to 30% sucrose gradient in Buffer A. Some gradients had a 70% sucrose cushion at the bottom. Gradients were spun in a Beckman SW50.1 rotor at either 2000 revs min⁻¹ (375 g average), or 6000 revs min⁻¹ (3360 g average) for 10 min at 4°C. After gradient fractionation, the bottom of the tube was washed out with StopB (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.5% NP-40) to release pelleted DNA. Fractions were supplemented with 0.5 µg ml⁻¹ proteinase K and 0.1 volume of 10XStopB, incubated at 37°C for 30 min, and extracted with phenol. Incubations containing [α³²P]dATP were spotted onto GF/C glass fibre filters, precipitated with trichloroacetic acid and scintillation-counted. For incubations containing [α³²P]dATP parallel gradients were run with and without added plasmid DNA; the minus DNA gradient gave a background ³²P profile, which was subtracted from the plus DNA profile. Incubations to be slot-blotted were further extracted
with phenol/chloroform, chloroform and ether before being denatured in 0.5 M NaOH, blotted onto Hybond N (Amer sham) and fixed with ultraviolet light. Profiles for RK2 chromatin were obtained by incubating RK2 DNA in the soluble component of the *Xenopus* egg extract for 1.5 h before resuspension in Buffer A and sucrose gradient centrifugation; profiles for RK2 DNA were obtained using RK2 DNA in Buffer A. Blots were probed with RK2 labelled with \( ^{32}P \) dATP by random oligonucleotide priming. After autoradiography of the blots, individual bands were cut out and quantified by scintillation counting.

CsCl gradient centrifugation for BrdUTP density substitution was performed as described (Blow and Laskey, 1986).

**Results**

Purified DNA is assembled into pseudo-nuclei in vitro

Electron microscopy shows that on incubation in the cell-free extract purified DNA templates are assembled into pseudo-nuclei, clearly surrounded by double unit envelopes (Fig. 1); A, B and C show structures assembled from RK2 DNA, a 60 kb naturally occurring broad host-range plasmid (Thomas, 1981), whilst D, E and F show structures assembled from pUCm54, a 12 kb pUC plasmid containing part of the *Xenopus* \( \alpha \)-cardiac actin gene.

The number of nuclear pores seen in each section varies from zero (Fig. 1A) to five or more (Fig. 1F), considerably fewer than are assembled onto sperm chromatin (Blow et al. 1987). Pseudo-nuclei are assembled within 1 h (data not shown), but at early times tend to be small (approx. 2 \( \mu m \) in diameter) with few pores (Fig. 1A,D). At later times, larger pseudo-nuclei with more abundant pores are observed (Fig. 1C,F).

At early time points partially assembled pseudo-nuclei are also observed (Fig. 2). Large aggregates unassociated with vesicular material are frequently observed (Fig. 2A), which are similar to structures previously observed by light microscopy (Newport, 1987). Some pseudo-nuclei with incomplete sections of surrounding envelope can also be seen (Fig. 2B). Larger nuclei can be seen that have infoldings of the inner nuclear envelope (Fig. 1C), closely resembling the ‘tunnels’ seen during

![](image)

**Fig. 1.** Electron micrographs of pseudo-nuclei assembled from plasmid DNA. Plasmids RK2 (A,B,C) or pUCm54 (D,E,F) were incubated in egg extract at 3 ng/\( \mu l \) for 3 h (A,B,D) or 5 h (C,E,F), and then prepared for electron microscopy. Small arrowheads show nuclear pores. Large arrowhead (C) shows an annulate lamellus.
Fig. 2. Electron micrographs of partially assembled pseudo-nuclei. pUCm54 was incubated in egg extract at 3 ng ml⁻¹ for 1 h and then prepared for electron microscopy. A. A large aggregate of chromatin unassociated with vesicular material. B. Pseudo-nucleus with incomplete surrounding envelope. Arrowhead shows nuclear pore.

the assembly of nuclei from sperm chromatin (Sheehan et al. 1988). However, no 'pre-pores' have been observed on partially assembled pseudo-nuclei. Annulate lamellae are sometimes seen in close proximity to the outer envelope of the pseudo-nuclei, possibly transferring pore material to the pseudo-nuclei (Fig. 1C). Large structures surrounded by only a single envelope are occasionally observed, but are also observed in extracts to which no DNA had been added, and so are unlikely to represent intermediates in nuclear assembly (data not shown).

Replication takes place within pseudo-nuclei

Like other DNA templates (Blow and Laskey, 1986; Blow et al. 1987) RK2 undergoes a single round of semiconservative replication in the egg extract (Fig. 3). In this experiment 29% of the DNA molecules were fully replicated, comparable to the efficiency in vivo (Harland and Laskey, 1980), but clearly less than the 70–100% achieved with sperm nuclei (Blow and Laskey, 1986).

Since RK2 is a naturally occurring prokaryotic plasmid, this shows that the egg extract does not need eukaryotic DNA sequences either to initiate DNA replication or to prevent re-replication in a single cell cycle, consistent with results in vivo (Harland and Laskey, 1980; Mechali and Kearsey, 1984).

The light microscopic appearance of pseudo-nuclei assembled from RK2 is shown in Fig. 4. The DNA fluorescence signal suggests that they each contain several thousand copies of the RK2 plasmid. The size of the pseudo-nuclei is variable, though most are between 2 and 8 μm in diameter, consistent with the observations by electron microscopy (Fig. 1). Under phase-contrast optics they are clearly seen to be surrounded by an envelope (Fig. 4D,E,F). Use of the thymidine analogue biotin-dUTP (Blow and Watson, 1987) shows that pseudo-nuclei are sites of DNA replication (Fig. 4J,K). Not all pseudo-nuclei in an incubation undergo a significant amount of replication (Fig. 4L). However, structures not surrounded by a phase-dense envelope never incorporate biotin-dUTP. The envelope surrounding the pseudo-nuclei has the properties of an active nuclear envelope in being permeable to streptavidin (60K, K=10⁷ M⁻¹; Fig. 4J,K), whilst larger nuclei (>4 μm in diameter) can be seen to exclude fluorescent IgG (150K; Fig. 4G,H,I). Smaller pseudo-nuclei displace too small a volume to determine unequivocally whether they also exclude IgG.

Replication does not take place outside pseudo-nuclei

 Incorporation of biotin-dUTP shows that DNA replication takes place in pseudo-nuclei, and not in other large structures. However, it is possible that DNA can replicate both inside pseudo-nuclei and also free in solution in the cytoplasm. To examine this, pseudo-nuclei were separated from naked DNA and chromatin by sucrose gradient centrifugation. The sedimentation velocity of RK2, both as naked DNA and after assembly into chromatin, was measured. Centrifugation conditions were then devised either to separate pseudo-nuclei of different sizes (Fig. 5) or to separate naked DNA and chromatin from pseudo-nuclei (Fig. 6).

Fig. 5 shows the heterogeneous sedimentation of structures containing RK2 DNA replicated in egg extract. RK2 DNA was replicated in extract containing [α³²P]dATP, and then the extract was diluted in buffer
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and centrifuged gently (375 g, 10 min) on a sucrose gradient. Gradient fractions were precipitated with trichloroacetic acid to measure the quantity of replicated RK2 present. Under these conditions naked RK2 DNA or RK2 assembled into chromatin would not be expected to migrate out of the loading volume (arrow L). However, more than 95% of the replicated DNA migrates into the sucrose gradient, and 8% pellets at the bottom of the tube. This shows that all replicated DNA is in large structures of a heterogeneous size. This is consistent with the observed size of pseudo-nuclei (Figs 1 and 4).

In order to resolve RK2 DNA and RK2 chromatin, the gradients were spun harder (3360 g, 10 min; Fig. 6). More than 70% of replicated DNA pellets into a cushion at the bottom of the tube under these conditions, whilst a further 20% migrates significantly ahead of the position of RK2 chromatin (Fig. 6A). The amount of replicated DNA migrating with chromatin or naked DNA is very close to background and could be accounted for by a small amount of breakage of the nuclei.

A very similar profile is obtained when RK2 is pulse-labelled with [α-32P]dATP for 30 min prior to sucrose gradient centrifugation (Fig. 6C). This shows that all ongoing replication takes place in rapidly sedimenting material.

Fig. 6B shows the migration of total RK2 DNA in

Fig. 4. Light microscopic appearance of pseudo-nuclei assembled from RK2 DNA. RK2 DNA was incubated in egg extract at 3 ng μl⁻¹ for 1.5 h with biotin-19-dUTP. Samples were supplemented with Hoechst 33258 to show DNA (A,B,C), fluorescein-tagged streptavidin to show biotin (J,K,L), or rhodamine-tagged IgG to demonstrate nuclear exclusion (G,H,I), and were then viewed unfixed under fluorescence (A,B,C,G,H,I,J,K,L) or phase-contrast optics (D,E,F). Bar, 5 μm.
Low-speed sucrose gradient fractionation of RK2 DNA replicated in the cell-free system. RK2 DNA was incubated in egg extract at 3 ng/ml with [α-32P]dATP for 3 h, resuspended in buffer and centrifuged on a 10% to 30% sucrose gradient at 375 g for 10 min. Fractions were precipitated with trichloroacetic acid, and the proportion of all replicated DNA present in each fraction was calculated. The arrow shows the vertical extent of the loading volume; under these centrifugation conditions RK2 DNA and RK2 chromatin are not expected to migrate significantly out of this volume. Fraction P, replicated DNA pelleted to the bottom of the tube.

These gradients, as determined by dot-blotting. About 60% of total RK2 DNA migrates faster than expected of either naked RK2 DNA or RK2 assembled into chromatin. However, at the time of centrifugation only 8% of the RK2 DNA had replicated. At later times, up to 29% of the template goes on to replicate (Fig. 3). Therefore, a large proportion of RK2 DNA is found in a rapidly sedimentable form without having been replicated. This may be accounted for by DNA in partially assembled pseudo-nuclei (Fig. 2) and by DNA in pseudo-nuclei that have not yet started to replicate (Fig. 4L).

Replication does not occur without nuclear assembly
The above experiments demonstrate that when purified DNA is incubated in egg extract, DNA replication occurs only within pseudo-nuclei. Does this reflect a causal dependence of DNA replication on nuclear assembly, or is it a chance correlation? This question was addressed by specifically inhibiting nuclear formation in the extract.

Extracts can be separated by high-speed centrifugation (>100,000 g) into soluble and vesicular fractions. Assembly of nuclei or pseudo-nuclei from sperm chromatin or purified DNA requires both soluble and vesicular components (Lohka and Masui, 1984; Newport, 1987; Sheehan et al. 1988). Fig. 7 (filled symbols) shows that soluble fraction alone is incapable of replicating RK2 DNA. However, the soluble fraction contains active DNA polymerase and primase activities and can fully replicate single-stranded DNA molecules (data not shown). When the soluble and vesicular fractions are reconstituted, the extract regains the ability to support a single round of semi-conservative replication of RK2 DNA (Fig. 7, open symbols).

Initiation of replication only occurs in interphase
During mitosis nuclear assembly does not occur. Neither does the initiation of DNA replication occur at this stage in vivo. Fig. 8 shows that this cell cycle dependency is maintained in the cell-free system. Extracts prepared from eggs blocked in mitosis do not assemble nuclei (Lohka and Masui, 1985) and do not replicate purified DNA.
Fig. 7. Density substitution of RK2 DNA incubated in soluble and reconstituted fractions of egg extract. RK2 was incubated at 3 ng/μl with [α-32P]dATP and BrdUTP for 3 h in soluble fraction alone (••) or soluble plus vesicular fractions (□□). DNA was isolated and fractionated on CsCl equilibrium density gradients. Vertical arrows show the expected densities of heavy/light (1.75) and light/light DNA (1.71).

Fig. 8. Replication of pUCm54 in mitotic and interphase extracts. pUCm54 was incubated at 3 ng/μl with [α-32P]dATP and BrdUTP in extract prepared from eggs blocked in mitosis. DNA was isolated and fractionated on CsCl equilibrium density gradients. (••) Extract blocked in mitosis; <1% of the plasmid DNA was replicated. (□□) Extract released into interphase by the addition of CaCl2; approximately 29% of the plasmid DNA was replicated. Vertical arrows show the expected densities of heavy/light (1.75) and light/light DNA (1.71).

DNA added to them (Fig. 8, filled symbols). When these extracts are allowed to progress into interphase by the addition of Ca2+, they become able to assemble nuclei (Lohka and Masui, 1985) and to replicate purified DNA efficiently (Fig. 8, open symbols).

However, Newport and Kirschner (1984) have reported that when very large quantities of plasmid DNA (>0.5 μg/egg) are microinjected into Xenopus eggs blocked in mitosis, incorporation of [α-32P]dATP in DNA is observed. They interpreted this incorporation as replication of the plasmid DNA, and suggested that the large quantity of DNA had titrated out an inhibitor of replication that normally prevents the initiation of DNA replication during mitosis. This interpretation would suggest that the initiation of DNA replication can occur in Xenopus eggs without the assembly of the template DNA into pseudo-nuclei.

To test this point, pUCm54 was incubated at high concentration in extracts blocked in mitosis. Consistent with the report of Newport and Kirschner (1984), incorporation of [α-32P]dATP into DNA is observed. However, when this incorporation is examined by BrdUTP density substitution, labelled DNA is found mainly as light/light (unsubstituted) DNA, with a smear running down towards the heavy/light position (Fig. 9A). This is the profile expected of repair-type incorporation of label, rather than of semi-conservative replication. The possibility that DNA molecules of intermediate density might represent replicative intermediates is ruled out by agarose gel electrophoresis (Fig. 9B). Labelled pUCm54 runs either as form II plasmid (nicked or relaxed circular) or as high molecular weight concatemers (lane 1). If the plasmid is linearised before electrophoresis, it runs as monomer plasmid, and not as a smear as expected of replicative intermediates (lane 2). Therefore, significant semi-conservative replication does not take place under these conditions. Fluorescence microscopy shows plasmid DNA in condensed networks and not in interphase nuclei (data not shown). Thus, in mitosis, a physiological situation where nuclear assembly does not occur, the initiation of DNA replication on double-stranded DNA cannot occur.

Discussion
We have used two plasmids, RK2 (60 kb) and pUCm54 DNA replication dependent on nuclear assembly 389
to investigate the possible requirement for nuclear assembly in the semi-conservative replication of DNA in *Xenopus* egg extract. These purified plasmid DNAs are rapidly assembled by interphase extracts into pseudo-nuclei, surrounded by a double envelope studded with nuclear pores. The size of the pseudo-nuclei is variable: most are 2–8 \( \mu \)m in diameter, but some are much larger. The abundance of nuclear pores in the envelope is lower than that seen when sperm chromatin is incubated in identical extracts (Blow et al. 1987). It is not possible to determine exactly the proportion of plasmid DNA that is assembled into functional pseudo-nuclei, though about 60% of the plasmid DNA becomes assembled into structures larger than monomer chromatin. However, a significant proportion of DNA is seen in large unenveloped chromatin aggregates (Fig. 2A; Newport, 1987). Therefore, the proportion of DNA in functional pseudo-nuclei is likely to be closer to 30%, which is approximately the proportion of DNA molecules replicated by the extract.

Light microscopy reveals pseudo-nuclei as structures surrounded by a phase-dense envelope. These envelopes are permeable to streptavidin (60K) but exclude IgG (150K). These are the properties of functional nuclei, which exclude proteins larger than 60K unless the proteins possess a nuclear localisation signal (Dingwall and Laskey, 1986). Pseudo-nuclei assembled in similar extracts have been shown to be lined with lamins (Newport, 1987), and to accumulate nucleoplasmin, a 165K protein possessing a nuclear localisation signal (Dingwall et al. 1982; Newmeyer et al. 1986a).

Plasmid DNA undergoes semiconservative replication in these *Xenopus* egg extracts (Blow and Laskey, 1986; Blow et al. 1987). Incorporation of biotin-dUTP into nascent DNA (Blow and Watson, 1987) shows that pseudo-nuclei are sites of DNA replication. No biotin incorporation is seen into structures that are not surrounded by a phase-dense envelope. Sucrose gradient centrifugation shows that >90% of replicated DNA labelled with \[^{32}P\]dATP pellets significantly faster than monomer chromatin, with a broad distribution as expected of pseudo-nuclei. Therefore, within the reasonable limits of accuracy in these experiments, all the replication of plasmid DNA in this system takes place within pseudo-nuclei.

Under conditions where nuclear assembly does not take place, the initiation of DNA replication on double-stranded DNA does not take place either. This can be demonstrated by clearing the extract of vesicular material, so that nuclear formation cannot occur (Lohka and Masui, 1984). Such cleared extracts do not initiate DNA replication on double-stranded DNA (Mechali and Harland, 1982; Blow and Laskey, 1986; Newport, 1987; Sheehan et al. 1988). Newport (1987) has shown that when vesicular material is added back to cleared extracts they regain the ability to both assemble pseudo-nuclei and incorporate \[^{32}P\]dATP into purified DNA. However, such incorporation data do not unequivocally demonstrate semiconservative replication, as they are also consistent with repair processes (e.g. see Fig. 9). In this paper we show that only after reconstitution with vesicular material can the soluble fraction support a single round of semiconservative replication.

The cell-free system also maintains the same dependency of DNA replication on the cell cycle stage as is seen *in vivo*. Semiconservative replication of purified DNA occurs only in interphase extracts, and not in extracts unable to assemble pseudo-nuclei because they are blocked in mitosis. In apparent contrast to this, Newport and Kirschner (1984) reported that when very large amounts of purified DNA (>0.5 \( \mu \)g/egg) are micro-injected into *Xenopus* embryos blocked in mitosis, \[^{32}P\]dATP is incorporated into DNA. They interpreted this incorporation as semiconservative replication. However, we show in this paper that it represents repair-type nucleotide turnover instead. The quantity of DNA repair observed is much higher than is observed with lower concentrations of plasmid DNA, possibly because DNA at these concentrations is not assembled into chromatin, and so may be more exposed to nucleases.

**The role of nuclear formation**

Why is DNA replication dependent on nuclear assembly in this system? High-speed supernatants of *Xenopus* eggs (extracts lacking vesicular components) can fully replicate single-stranded and alkali-denatured plasmid DNA, and can elongate simian virus 40 (SV40) replicative intermediates (Richter et al. 1981; Mechali and Harland, 1982; Blow and Laskey, 1986). It therefore seems likely that the requirement for nuclear assembly is for the initiation of replication forks on native double-stranded DNA.

Sperm chromatin that has been fully replicated in the *Xenopus* egg extract is incapable of further rounds of replication when added back to fresh extract (Blow and Laskey, 1988). However, once replicated chromatin has passed into a mitosis-like state, it becomes capable of efficient re-replication in fresh extract. The effect of passage into mitosis can be mimicked by treatments with agents, such as lyssolecithin and phospholipase, that permeabilise the nuclear envelope. This suggests that DNA replication is controlled in this system by the compartmentalisation of initiation factors between the nucleus and the cytoplasm. The initiation of DNA replication in this system may therefore require the creation of a special intranuclear microenvironment by the selective accumulation of nuclear proteins.

The requirement for nuclear assembly may also reflect a requirement for the assembly of replication forks into higher-order structures. Sites of DNA synthesis in cultured mammalian cells have a distinctive punctate organisation, showing that replication forks are organised into clusters scattered throughout the interior of the nucleus (Nakamura et al. 1986; Nakayasu and Berezney, 1989). Similar patterns are seen in nuclei replicated in the *Xenopus* cell-free system (Mills et al. 1989; Hutchison and Kill, 1989). Nuclear run-off experiments with immobilised HeLa cell nuclei also suggest that active replication forks are associated with very large intranuclear structures (Jackson and Cook, 1986a,b,c). The assembly of replication forks into higher-order structures may
represent a further requirement before complete semi-conservative replication can occur.

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