The control of DNA replication in a cell-free extract that recapitulates a basic cell cycle in vitro

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Summary

Cell-free extracts prepared from Xenopus eggs support chromosome decondensation and pronuclear formation on demembranated sperm heads. \(^{32}\)P-dCTP pulse-labelling studies demonstrate that DNA synthesis occurs in multiple bursts of 30–40 min in extracts containing pronuclei, each burst being followed by a period of 20–50 min during which no synthesis occurs. Density substitution with bromodeoxyuridine indicates that the synthesis in each burst is semiconservative and results from new initiations, and that, following multiple bursts of synthesis, reinitiation events can occur. Changes in nuclear morphology have been characterized in the extract by phase-contrast microscopy and by fluorescence microscopy following pulse labelling with biotin-11-dUTP and staining with anti-lamin antibodies. Lamin accumulation occurs as DNA decondenses and parallels the acquisition of membrane structures. Biotin-11-dUTP incorporation is first observed in small nuclei having decondensed DNA and an extensive lamina. While DNA synthesis is occurring nuclei remain relatively small, but rapid swelling accompanied by chromosome condensation occurs when biotin incorporation ceases. Nuclear swelling and chromatin condensation is followed by nuclear membrane breakdown, lamin dispersal and chromosome formation. Mitosis lasts for approximately 20 min. Nuclear reassembly is recognized by the appearance of membrane vesicles around small pieces of decondensed DNA, which parallels the appearance of lamin islands within a chromatin mass. These 'islands' incorporate biotin, indicating that DNA synthesis is occurring, and apparently fuse as larger S-phase nuclei are formed. Extensive protein synthesis occurs for at least 4 h in most extracts. This synthesis is required for the initiation of mitotic events and the reinitiation of DNA synthesis.

Key words: Xenopus egg extracts, DNA replication, mitosis, nuclear assembly, cell cycle, decondensation.

Introduction

The study of many of the events of the cell cycle have been facilitated in recent years by the development of cell-free systems which induce nuclear assembly in vitro. These extracts, mostly prepared from amphibian eggs, are characterized by their ability to decondense chromatin and assemble a bilayered nuclear envelope on demembranated sperm heads (Lohka & Masui, 1983, 1984; Lohka & Mailer, 1985; Miake-Lye & Kirschner, 1985). Such extracts have the capacity to assemble plasmid DNA into chromatin and subsequently into nuclear structures with nuclear lamina and bipartite membrane structures (Blow & Laskey, 1986; Newport, 1987). Nuclei formed in vitro appear to have pore complexes and accumulate nuclear proteins by an ATP-dependent mechanism (Lohka & Masui, 1984; Newmeyer et al. 1986). Such nuclei initiate and complete efficient DNA synthesis (Blow & Laskey, 1986; Blow & Watson, 1987; Newport, 1987) and respond to the addition of mitotic-inducing factors such as maturation-promoting factor (MPF) by undergoing nuclear envelope breakdown, lamin disassembly and chromosome formation (Lohka & Masui, 1984; Lohka & Maller, 1985; Miake-Lye & Kirschner, 1985; Newport & Spann, 1987).

These extracts therefore provide a means to study the enzymology associated with the events of DNA synthesis and mitosis. However, because mitotic factors are known to induce the nuclear events of mitosis precociously in S-phase nuclei (Johnson et al. 1970) the use of these types of cell-free extracts may...
not be ideal for a study of the relationship between DNA synthesis and mitosis and specifically exclude studies on the role of protein synthesis in the control of cell cycles in early embryos. In an attempt to overcome this problem, we have described an extract prepared from Xenopus eggs that supported periodic DNA replication in sperm pronuclei (Hutchison et al. 1987). The periodic properties of this extract appeared to be dependent on protein synthesis, and multiple bursts of replication led to reinitiation events occurring on some of the DNA. However, we were unable to detect any mitotic events occurring between the bursts of DNA synthesis (Hutchison et al. 1987). We have now modified our procedure and report here the properties of an extract that recapitulates the nuclear events of the early embryonic cell cycles of Xenopus laevis. In these extracts, bursts of DNA synthesis are separated by mitoses in which the nuclear lamina breaks down, chromatin condensation occurs and chromosomes form. The time course of these periodic events can be altered by partial inhibition of protein synthesis form.

Materials and methods

Preparation of extracts

Extracts were prepared from X. laevis eggs using a modification of the method described by Lohka & Maller (1985). Small mature female frogs were used at three month intervals and stimulated to lay eggs by injecting 500—600 i.u. of human chorionic gonadotrophin (Chorulon, Intervet Laboratories) into their dorsal lymph sacs, 15 h before the removal of the jelly coats, the eggs were rinsed twice in saline tap water and then examined in the same medium. At this stage the eggs were generally very stable with few or no necrotic ones. Batches of eggs that were not like this were discarded. The eggs were next rinsed twice in distilled water and leupeptin (6 jig ml⁻¹). The final extract was stored on ice, but used within 45 min of preparation.

Preparation of sperm

Demembranation of sperm was performed as previously described (Hutchison et al. 1987).

In vitro assays

Additions of sperm, nucleotides and amino acids to the extracts were in SuNaSp (0-25 m-sucrose, 75 mm-NaCl, 0-5 mm-spermidine, 0-15 mm-spermine, Gurdon, 1976), and were at concentrations such that the total volume of the additions did not exceed 20 % of the volume of the extract.

Pulse-labelling studies with ³²P-dCTP

Pulse-labelling studies using ³²P-dCTP (3000 Ci mmol⁻¹ DuPont/NE) were carried out as previously described (Hutchison et al. 1987). Samples labelled with ³²P-dCTP were prepared for gel electrophoresis on 0-9 % agarose gels and autoradiographed, again as previously described (Hutchison et al. 1987).

Density substitution and CsCl gradient analysis

40 µl samples of egg extracts were incubated with 2 µCi of ³²P-dCTP, 2 µl of 20 mm-bromodeoxyuridine triphosphate (BrdUTP) and 0-4 X 10⁵ sperm heads. At 22°C. Reactions were terminated by adding 80 µl of a solution containing 50 mm-Tris—HCl (pH 8-0) and 5 mm-EDTA (T.E.) and placing the samples on dry ice/ethanol. Samples were prepared for CsCl gradient analysis as follows: 80 µg of calf thymus DNA was added to each sample. The samples were then incubated with proteinase K (0-5 mg ml⁻¹ final concentration) at 37°C for 2 h. Following this the samples were precipitated with 2 vol. of EtOH (2 h at −20°C) and the precipitate resuspended in T.E. Following treatment with RNase A (21 u ml⁻¹ for 1 h at 37°C) the samples were loaded onto CsCl gradients (6 ml gradients; final density 1-74 g ml⁻¹) and centrifuged for 60 h at 44 000 revs min⁻¹ in a Beckman Ti50 rotor. Fractions were collected and analysed as described by Ford & Woodland (1975). For single-strand analyses, samples were extracted as above and resuspended in 200 µl of T.E. with 80 µg of calf thymus DNA. Each sample was incubated with 4-7 µl of 5 m-NaOH for 5 min, then neutralized with 4-7 µl of 5 m-HCl. Denatured samples were separated on CsCl gradients and fractionated as described above.

Protein synthesis assays

5 µCi of [³H]methionine (Amersham International; spec. act. 70–85 Ci mmol⁻¹) was dried under vacuum and redisolved in 100 µl of cytoplasm containing 10⁵ sperm heads. Incubations were carried out at 22°C and 7 µl samples were taken at 20 min intervals. Aliquots were placed on squares of Whatman no. 1 filter paper and precipitated in 10% TCA at 4°C and then dried by rinsing in ethanol followed by acetone. Dry filters were immersed in 2 ml of scintillant (Beckman ready protein+) and counted on a Beckman LS 1801 scintillation counter. 4 µl samples of the incubation mixture were taken to measure total radioactivity.

Biotin-11-dUTP labelling and immunofluorescence

10 µl samples of extract containing 10⁵ sperm heads were incubated at 22°C. At 10 min intervals from time zero, 1 µl of 40 µM-biotin-11-dUTP (this was a generous gift from Gibco/BRL) was added to separate aliquots and incubated...
Buffer containing 1 mM-ethylene glycol bis(succinic acid N-hydroxysuccinimide ester) (EGS) and incubated for 30 min at 37°C. Fixed nuclei were prepared for fluorescence microscopy by spinning them through a 25% glycerol cushion onto glass coverslips, in an MSE-mistral 2L centrifuge (2000 revs min⁻¹ for 15 min at 4°C). The coverslips were air dried and then incubated with monoclonal antibody (L6 8A7 – this was a generous gift from Dr R. Stick, Tubingen, W. Germany; Stick & Hausen, 1985) overnight at 4°C. The coverslips were then washed in phosphate-buffered saline (PBS) and simultaneously incubated for a further 4 h with fluorescein-labelled rabbit anti-mouse Ig (DAKO-PATT dil.1/20 in PBS) and Texas Red Streptavidin (Amersham International dil. 1/50 in PBS) at 37°C. After washing again in PBS, the coverslips were mounted on glass slides (in 50% glycerol/PBS containing 5 mM-EDTA, 15 mM-NaCl, 10/igmP1 DAPI 3-3% formaldehyde) on a glass slide. The sample was then covered with a glass coverslip, sealed with nail varnish and examined under a Zeiss photomicroscope III.

**Phase-contrast microscopy**

Samples were prepared for phase-contrast microscopy as previously described (Hutchison et al. 1987). Briefly, 4 µl samples of extract containing pronuclei were pipetted into 4 µl of DAPI mountant (15 mm-Pipes (pH 7-2), 80 mm-KCl, 5 mm-EDTA, 15 mm-NaCl, 10 µg ml⁻¹ DAPI 3-3% formaldehyde) on a glass slide. The sample was then covered with a glass coverslip, sealed with nail varnish and examined under a Zeiss photomicroscope III.

**Results**

**Periodic DNA synthesis in vitro**

In a previous report (Hutchison et al. 1987), we had demonstrated that extracts prepared from *Xenopus* eggs supported periodic DNA replication on sperm pronuclei. In addition, the periodic properties of the cytoplasm were dependent on protein synthesis, implying that they reflected a basic cell-cycle mechanism. However, we found no evidence of mitoses during periods in which DNA synthesis was not occurring, even though nuclei formed in the extracts responded to exogenous maturation-promoting factor (MPF) by undergoing nuclear envelope breakdown (NEBD) and chromosome formation (K. Campbell and C.C.F., unpublished data). The preparation of extracts was thus modified in three ways.

First, eggs were obtained from mature female frogs which were used on a strict three month rotation; second, extracts were centrifuged at 2°C rather than 4°C; third PMSF was omitted from the extraction buffer, but aprotonin was added to the final extract.

Pulse-labelling studies using ³²P-dCTP indicated that the modified extracts supported periodic DNA synthesis on sperm pronuclei for up to 5 h. Incorporation of radionucleotide into DNA started after 20 min and proceeded in bursts. Each burst lasted approximately 40 min and was followed by a period of 20–40 min during which no incorporation occurred (Fig. 1A). Some variation in this pattern was observed, mainly involving the duration of the gap between the first burst of DNA synthesis and the second burst of DNA synthesis, which could be as long as 60 min. In extracts containing 10 µg ml⁻¹ cycloheximide (CHM) only a single burst of incorporation occurred which started after 20 min and lasted 40–50 min (Fig. 1B). (In two out of eight extracts a second burst of synthesis was observed after prolonged incubation i.e. 5 h. However, this synthesis represented less than 1% of the available DNA.)

Calculations of the amount of DNA synthesized after five bursts of replication over a 5 h period, based on incorporation of ³²P-dCTP into DNA, indicated that during this time over 200% of the starting material had been synthesized (Fig. 2B). However, during the first burst of DNA synthesis, even in the presence of CHM, only 25–35% of the input DNA had been replicated (Fig. 2C). These results imply that in any one burst of replication either all nuclei initiate synthesis but few complete S-phase, or that

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**Fig. 1.** Pulse labelling of egg extracts containing sperm pronuclei (10⁵ per 100 µl) with ³²P-dCTP, both in the presence (B) and absence (A) of 10 µg ml⁻¹ CHM. Each lane contains 15 µl of extract which had been treated with proteinase K (0-5 mg ml⁻¹) for 1 h at 37°C. Label (2 µCi ³²P-dCTP) was added to 40 µl samples of extract at the times indicated and incubated at 22°C. After 20 min the reaction was terminated by the addition of a solution containing 5% SDS, 10% ficoll and 0-5% bromophenol blue in 80 mm-Tris–HCl (pH 8.0), 8 mm-EDTA, 0-13% phosphoric acid. Samples were separated by electrophoresis on 0-9% agarose gels. After extensive washing, gels were dried and exposed to X-ray film for 18 h at −70°C. 23 is the position to which a 23 kb λHindIII molecular weight marker migrated on each gel, while origin marks the position of the pockets. The bands were sensitive to digestion with DNase I but insensitive to treatment with proteinase K.
replication is initiated and completed in only a fraction of the available nuclei. In order to distinguish between these two possibilities, extracts were pulse labelled with biotin-11-dUTP and nuclei prepared from such extracts for fluorescence microscopy with Texas red streptavidin. Using a similar cytoplasmic extract, Blow & Watson (1987) have shown that biotin-11-dUTP is incorporated into the DNA of replicating nuclei by a DNA-polymerase-α-dependent mechanism. Our results indicate that during a single burst of DNA replication, both in the presence and absence of CHM, greater than 90% of the nuclei recovered from the extracts were labelled with biotin-11-dUTP (Fig. 2B,C). This suggested that DNA replication occurred in most nuclei during each burst of synthesis but a doubling of DNA content did not. While this result seemed predictable for extracts undergoing periodic synthesis, it was more surprising that nuclei in CHM-treated extracts should not complete replication, as it has been shown that CHM-arrested Xenopus embryos retain the capacity to replicate DNA for at least 5 h (Harland & Laskey, 1980). Our result could be explained if, in the presence of CHM, sufficient protein synthesis was occurring to maintain some of the cell-cycle events. To test this, protein synthesis was measured in extracts by [3H]methionine incorporation into acid-insoluble material. The results, illustrated in Fig. 2A, indicated that extensive protein synthesis had occurred throughout the labelling period in this extract. In other extracts, however, incorporation was linear only for the first 2 h, after which it slowed rapidly. In the presence of 10 μg ml⁻¹ CHM, the incorporation of [3H]methionine was inhibited by 94%. Increasing the dose of CHM by a factor of ten did not increase the inhibition. Thus, it seems unlikely that this residual incorporation could account for the premature termination of DNA synthesis.

If DNA replication was initiated during the first burst of synthesis but was not completed, then the replication occurring during the second burst might involve synthesis of chromatin not replicated during the first burst. In order to investigate this point, density substitution analyses using BrdUTP and ³²P-dCTP were carried out. Extracts containing sperm heads were labelled with BrdUTP and ³²P-dCTP for 80, 140 and 200 min, either in the presence or absence of CHM. Following CsCl density gradient analysis of the reaction products, it was found that during the first period of DNA synthesis in control extracts all of the labelled material migrated to the heavy-light (HL) position indicating fully substituted DNA which had been replicated once (Fig. 3A). During the second period of DNA synthesis, in some extracts, incorporation into the HL peak increased but no material migrating at the heavy-heavy (HH) position was observed, implying that material that had not been synthesized during the first burst was synthesized during the second burst of replication (Fig. 3B). In other extracts, small amounts of material (3% of total incorporation) were recovered at the HH position, indicating that some re-replication
had occurred. If there is a random probability of material replicated during the first burst of DNA synthesis, being re-replicated during the second burst of synthesis, we calculate that in all extracts studied at least 10% of the acid-insoluble radioactivity should have migrated at the HH position in gradients of S1-plus S2-labelled material. In the third period of DNA synthesis, the amount of labelled material migrating with the HL peak decreased, but a large peak migrating at the HH position had appeared (Fig. 3C).

This implied that after three bursts of DNA synthesis efficient reinitiation events were occurring. In the presence of CHM, accumulation of label into HL but not HH material occurred (Fig. 3D), indicating that re-replication was dependent upon protein synthesis inducing multiple bursts of DNA synthesis. Furthermore, the amount of material recovered from CHM-treated extracts after 200 min was only slightly more than the amount of material recovered from control extracts after 80 min, indicating that, in the absence of protein synthesis, replication stops at the same time as in S1 of control extracts.

As only limited reinitiation was detected during S2, in most extracts, the replication products of both S1 and S2 were compared by single-strand analysis in order to determine whether any initiation was occurring during S2. Extracts were pulse labelled for 40 min periods with BrdU TP and 32P-dCTP through either S1 or S2. The reaction products were then alkaline denatured and analysed on CsCl density gradients. Material labelled during S1 migrated as a single sharp peak centred at the single-stranded heavy position, indicating the synthesis of large molecules of DNA which were fully substituted with BrdUTP (Fig. 4A). In contrast, material labelled during S2 peaked at the single-stranded heavy position, but a substantial shoulder of labelled material (43% of the total) extended to the light position (Fig. 4B). This implied that while the majority of material replicated during S2 was large and fully substituted, a significant proportion of the replicated material was only partially substituted. These results suggested that while some synthesis (57%) can be accounted for by new initiation events, a proportion (43%) of the synthesis appears to result from extension of existing strands, presumably material that was incomplete during S1. If the replication resulting from new initiation events during S2 accounted for only 57% of the products, then the amount of re-replicated material expected if initiation events occur randomly would be 5–7%. This is in fact only double the amount of re-replicated material detected in a majority of the experiments performed. As the recovery of unlabelled material from each gradient varied between 60–75%, then the low level of re-replicated material could be accounted for by unequal loss.

**The mitotic capacity of egg extracts**

Changes in chromatin and nuclear morphology during incubations have been followed by phase-contrast and fluorescence microscopy. Following the addition of sperm heads to egg extracts, DNA decondensed rapidly and acquired a nuclear lamina, so that after 10 min *in vitro* 90% of nuclei prepared for fluorescence microscopy had uniformly dispersed DNA (DAPI stain) surrounded by continuous, though...
weak, lamin staining (Fig. 5A). Pulse-labelling studies using biotin-11-dUTP indicated that these nuclei had not begun to synthesize DNA during the previous 10 min period (Fig. 5A). Parallel studies in which nuclei were fixed in formaldehyde and examined by phase-contrast microscopy, revealed that at this stage chromatin was surrounded by a continuous nuclear membrane, characterized as a phase-dark ring (Fig. 6A), which expanded over the next 10 min as the DNA continued to decondense (Fig. 6B). As nuclear membranes expanded the intensity of lamin staining increased and many nuclei incorporated biotin, indicating that DNA synthesis had been initiated (Fig. 5B). It is interesting that at this time nuclei were relatively small and did not increase dramatically in size until after biotin incorporation had ceased. This implied that the extensive swelling associated with embryonic nuclei may not be critical for the process of initiation. Biotin incorporation into nuclei continued for the next 30 min so that after 40 min in vitro >90% of nuclei were apparently in S-phase. Biotin incorporation into most (94%) nuclei terminated abruptly after 50 min in vitro; at this time the nuclei began to swell rapidly and the DNA could be seen to have condensed into distinct thread-like strands which were uniformly distributed within a continuous nuclear lamina (Fig. 5C). Such nuclei were described as early prophase. After the first period of DNA synthesis nuclei remained in a prophase-like state for up to 50 min (the precise duration of this varied from extract to extract, the minimum time being 20 min). During this time, the nuclei continued to swell and the DNA continued to condense into dense strands. By 90 min in vitro these strands were highly condensed and no longer appeared to be anchored to the nuclear lamina (Fig. 5D).

Nuclear membrane breakdown and lamin dispersal occurred abruptly at times varying between 80 and 100 min in vitro. During this first mitotic period, little evidence of chromosome formation was observed in any of the extracts. Instead the DNA appeared as amorphous clumps with no associated lamina (Fig. 7A) or membrane structures (not shown). In most extracts, this mitotic period lasted 15–20 min so that after 120 min in vitro decondensing DNA could be seen to be stained with anti-lamin antibodies presumably reflecting the reacquisition of a nuclear lamina (Fig. 7B). At this early stage of nuclear reformation, within one mass of chromatin, defined by DAPI staining, small regions of bright lamin fluorescence were observed. The appearance of ‘islands’ of lamin stain paralleled the appearance of membrane vesicles surrounding parts of a single mass of chromatin (Fig. 8A). With continued incubation, the membrane vesicles expanded, presumably by fusion with neighbouring vesicles, until the chromatin mass was completely surrounded by a continuous membrane (Fig. 8B). Incorporation of biotin could be detected within the ‘islands’ of lamin-staining material. Not all the lamin ‘islands’ had incorporated biotin, but biotin stain was never detected in regions of chromatin that were not lamin-positive. This suggested that, during nuclear reformation from chromosomes, DNA replication restarts in different local regions of chromatin since lamin polymerization, presumably within membrane vesicles, increases but before the whole mass of chromatin is surrounded by a continuous lamina. Biotin incorporation continued as the ‘lamin islands’ appeared to fuse to produce nuclei with a continuous lamina and membrane (Figs 7C, 8C) and lasted for up to 30 min. Fig. 9 illustrates three masses of chromatin at various stages of nuclear reformation. One mass has only two small areas of lamin fluorescence, only one of these areas appears to be incorporating biotin. A second mass of chromatin shows extensive lamin fluorescence but some areas are unstained; biotin incor-

![Fig. 4. CsCl gradient analysis of the replication products of S1 and S2. 40 μl samples of egg extracts containing 0.4×10^5 sperm heads were pulse labelled with 2 μCi of 32P-dCTP and 1 mM BrdU for 40 min periods during either (A) S1 (20–60 min) or (B) S2 (100–140 min). DNA was extracted and denatured by treatment with 5 M-NaOH and the reaction products analysed on CsCl density gradients. R.I., the refractive index measured across the gradient reflects the density of CsCl. Open circles (O) represent the position of the unsubstituted carrier DNA, determined by optical density. Closed circles (●) represent the acid-insoluble radioactivity in each gradient fraction.](image-url)
Fig. 5. Fluorescence micrographs showing nuclear formation and DNA synthesis in egg extracts during the first period of DNA synthesis. 10 µl samples of egg extract were incubated at 22°C with 10⁴ sperm heads. At 10 min intervals separate aliquots were labelled with 1 µl of 40 µM biotin-11-dUTP and fixed 10 min later by incubating with 200 µl of EGS (1 mM) at 37°C. Fixed nuclei were prepared for fluorescence microscopy by spinning onto coverslips and staining first with monoclonal mouse anti-lamin antibodies, followed by fluorescein-labelled rabbit anti-mouse Ig and Texas red streptavidin. Slides were mounted in 50% glycerol/PBS containing 1 µg ml⁻¹ 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) and examined using a Zeiss photomicroscope III. The left-hand column illustrates nuclei viewed using the DAPI filter and shows the distribution of DNA. The central column illustrates nuclei viewed using the FITC filter and shows the distribution of anti-lamin antibodies. The right-hand column illustrates nuclei viewed using the RhITC/Texas red filter and shows nuclei that have incorporated biotin-11-dUTP. The nuclei in this figure were pulse labelled for 10 min immediately prior to fixation at 10 min (A); 20 min (B); 50 min (C); 90 min (D). Bar, 5 µm.
Fig. 6. Phase-contrast micrographs of sperm pronuclei formed in egg extracts. Sperm heads were incubated in egg extracts and prepared for phase-contrast and fluorescence microscopy by fixing 3 μl of 15 mM-Pipes (pH 7.2), 80 mM-KCl, 5 mM-EDTA, 15 mM-NaCl, 10 μg/ml DAPI, 3-3% formaldehyde on glass coverslips. (A) A pronucleus fixed after 10 min incubation in extract and viewed by phase-contrast optics; (B) the same pronucleus viewed by fluorescence optics; (C) a pronucleus fixed after 20 min incubation in extracts and viewed by phase contrast optics and (D) the same nucleus viewed by fluorescence optics. Bar, 5 μm.

Poration corresponds only to the areas of lamin fluorescence and differs in intensity as the extent of lamin fluorescence varies. The third chromatin mass appears to have a continuous lamina and is uniformly stained with biotin. At the end of 'second' S-phase there was no discrete gap before 'second mitosis' so that at 140 min in vitro S-phase nuclei, prophase nuclei (Fig. 7D) and mitotic chromosomes (Fig. 7E) could all be seen on the same slide. In vivo, the first two nuclear division cycles of Xenopus embryos last approximately 90 min (at 21°C). First S-phase takes 15–20 min to complete and is followed by a G2 period before first mitosis, which occurs at about 60 min after fertilization. Second S-phase immediately follows first mitosis and lasts 15 min and is followed by second mitosis (Graham & Morgan, 1966; Miake-Lye et al. 1983). In the cell-free system described here, a remarkably similar pattern is observed. All the processes take longer to complete (150 min for the first two cycles in most extracts) but the sequence of events are the same, implying that the mechanism operating the cycle is retained.

Protein synthesis and entry into mitosis
A feature of the extract is its capacity to support extensive protein synthesis, characterized by the rapid incorporation of [3H]methionine into acid-insoluble material (Fig. 2A). CHM used at concentrations of 10, 1 and 0.5 μg/ml inhibit this incorporation by an average of 96%, 50% and 14%, respectively. In order to determine the role of protein synthesis in DNA replication and mitotic events, we have examined nuclear morphology and 32P-dCTP incorporation in extracts in which protein synthesis had been inhibited. As described in Fig. 1B, inhibition of protein synthesis by >94% does not alter the time at which DNA replication starts in the first round or the time at which it is terminated. This was in contrast to our previous results, where we had reported that the duration of the single period of DNA synthesis in CHM-arrested extracts was longer than any of the periods of DNA synthesis in control extracts (Hutchison et al. 1987). In our earlier report, however, we had not observed the dramatic changes in chromosome condensation described here. Therefore it was possible that at least a part of this process might be independent of protein synthesis and consequently result in the termination of DNA replication in CHM-arrested extracts. Fluorescence microscopy revealed that, as in controls, sperm heads decondensed rapidly in extracts containing 10 μg/ml CHM and within 10 min had acquired a nuclear envelope and lamina (Fig. 10A). After 20 min small round nuclei, which were extensively stained with anti-lamin antibodies, had started to incorporate biotin (Fig. 10B). This incorporation continued for the next 20–30 min but terminated at the appearance of large nuclei with thread-like chromatin (Fig. 10C). The appearance of these early prophase-like nuclei was seen in all the extracts examined and with increased incubation of nuclei with extracts the chromatin continued to condense, so that after 80 min in vitro nuclei containing highly condensed DNA were observed (Fig. 10D). However, even after 3 h, these nuclei had not undergone lamin dissolution, nuclear membrane breakdown or mitotic chromosome formation.

In Fig. 11, the timing of DNA synthesis is compared to the major nuclear events of swelling, prophase and mitosis, in extracts containing 0, 0.5, 1.0 or 10 μg/ml CHM. In all the extracts, DNA synthesis, pronuclear formation and the appearance of prophase-like nuclei occur at the same time during the first cycle. However, with increased concentrations of CHM the mitotic events are delayed or completely inhibited. Thus, by inhibiting protein synthesis by 14%, mitosis is delayed by 5–10 min (Fig. 11B); inhibition of protein synthesis by 50% led to a delay in mitosis of 20 min (Fig. 11C) and inhibition of protein synthesis by 94% led to the complete inhibition of mitosis (Fig. 11D). In all the extracts, DNA synthesis restarted during nuclear reformation after
DNA Lamin
Control of DNA replication in vitro

Fig. 7. Fluorescence micrographs showing lamin dispersal, chromosome formation and lamin reassembly during the first and second 'mitoses' and second 'S-phase' in egg extracts. 10 µl samples of extract containing 10⁶ sperm heads were labelled with biotin-11-dUTP and prepared for fluorescence microscopy as described in Fig. 5. The left-hand column shows the distribution of DNA, the central column the distribution of anti-lamin antibodies, and the right-hand column the distribution of biotin-11-dUTP. The nuclei in this figure were pulse labelled for 10min prior to fixation at 110 min (A); 120 min (B); 130 min (C); 150 min (D); and 150 min (E). Bar, 5 µm.
mitosis. Hence, these results imply that entry into mitosis is a rate-limited step which is dependent upon the accumulation of protein(s) to a threshold level, so that reductions in the overall rate of protein synthesis delays this accumulation. As termination of first S-phase and the process of chromatin condensation in post-S-phase nuclei are not delayed by inhibiting protein synthesis, we assume that these events are not dependent on protein synthesis.

**Discussion**

Cytoplasmic extracts from *Xenopus* eggs have been prepared, which, on addition of lysolecithin-treated sperm heads, recapitulates the temporal sequence of cell-cycle events of early embryos. DNA synthesis in these extracts occurs periodically. Essentially all nuclei synthesize DNA in each burst, though a doubling in DNA content is not observed in any one burst, as judged by estimation of the amount of precursor incorporated. Extensive initiation and reinitiation occurs over a 5 h period resulting in some 200% of the input DNA being replicated. Only one burst of replication occurs in the absence of protein synthesis and in this situation no reinitiation is detected.

Cytological analysis indicates that between bursts of DNA synthesis nuclear envelope breakdown (NEBD) occurs, reflected in the loss of lamin staining and disappearance of the phase-dark ring of the nuclear membrane. Chromatin condensation also occurs concurrently with NEBD, though distinct chromosomes are only seen in the second and subsequent mitoses.

**DNA replication in vitro**

The temporal order of cell cycle events in the cell-free extract is the same as observed in intact embryos, though the duration of the individual phases *in vitro* is somewhat slower. However, the amount of DNA synthesized in the first S phase (*S*_1) is only 25–35% of the input DNA. Density analysis of newly synthesized DNA in a single-stranded state (Fig. 4) indicates that synthesis in second S phase (*S*_2) differs from that in

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**Fig. 8.** Phase-contrast micrograph showing nuclear reformation from chromosomes. Extracts containing sperm pronuclei were fixed at various times after first mitosis, as described in Fig. 6, and examined using both phase-contrast and fluorescence optics. (A,B) Phase-contrast and fluorescence micrographs, respectively, of pronuclei fixed after 120 min incubation in egg extract. The arrows indicate the relative positions in both micrographs of membrane vesicles. (C,D) Phase-contrast and fluorescence micrographs, respectively, of pronuclei incubated in egg extracts for 130 min. Bar, 5 μm.

**Fig. 9.** Fluorescence micrograph of nuclei at different stages of reformation following first mitosis. Pronuclei were incubated in egg extracts for 120 min then fixed after labelling with biotin-11-dUTP as described in Fig. 5. The left-hand column illustrates nuclei viewed using the DAPI filter and shows the distribution of DNA. The central column illustrates nuclei viewed using the FITC filter and shows the distribution of anti-lamin antibodies. The right-hand column illustrates nuclei viewed using the RhITC/Texas red filter and shows biotin-11-dUTP incorporation. The arrows indicate the relative positions in each micrograph of lamin-staining vesicles. Bar, 5 μm.
S₁, in that during S₂ both initiation of new strands and continuation of preexisting strands occurs, while in S₁, as expected, only new initiations are detected. The possibility that the material banding at intermediate densities in Fig. 4 represents increasing repair synthesis at later incubation times is unlikely for two reasons. First, random repair would be expected to generate a range of molecules with densities close to that of unsubstituted DNA, as is occasionally observed (see Hutchison et al. 1987 fig. 7). Second, and more important, continuous labelling through S₁ and S₂ indicates that all synthesis is semiconservative.

![Fig. 10. Fluorescence micrographs of nuclei formed in egg extracts containing 10 μg ml⁻¹ CHM. 10 μl samples of egg extract containing 10⁴ sperm heads and 10 μg ml⁻¹ CHM were pulse labelled with biotin-11-dUTP and prepared for fluorescence microscopy as previously described. The left-hand column shows the distribution of DNA, the central column shows the distribution of nuclear lamins and the right-hand column shows the distribution of biotin. The nuclei in this figure were pulse labelled for 10 min immediately prior to fixation at 10 min (A); 20 min (B); 50 min (C); and 110 min (D). Bar, 5 μm.](image-url)
and no repair synthesis is detected even after labelling through S₂ (Fig. 3).

Pronuclear assembly from sperm heads occurs rapidly in the first cycle such that a weakly staining, but continuous lamina is seen surrounding each decondensed sperm head at 10 min. DNA synthesis is detected in these small pronuclei by 20 min. Nuclear reformation after first mitosis (and after second mitosis) is notably different to nuclear formation from sperm heads in that, within one mass of chromatin, lamina 'islands' and membrane vesicles are observed covering only part of the chromatin mass. Many of the lamina islands have incorporated biotin, though in other regions of the same mass of chromatin neither lamins nor DNA synthesis is detected. In this situation, DNA synthesis is never seen in the absence of lamina staining. Since chromatin masses are observed with different degrees of lamina staining from small 'islands' to large lamina structures surrounding a chromatin mass, we interpret these observations as indicating that lamina islands (and membrane vesicles) fuse during the formation of a single nucleus. The corollary of this is that DNA synthesis starts asynchronously in different parts of a chromatin mass during the formation of a single nucleus. It is possible that the DNA synthesis observed in some lamina islands involves continuation rather than initiation during S₂. However, since replication also starts in lamina islands during S₃ when much of the replication involves reinitiation (Fig. 3C), it seems probable that initiation of DNA synthesis occurs in lamina islands during nuclear reformation following mitosis.

Lamin islands are not detected during pronuclear formation from sperm heads and DNA synthesis is only observed following the appearance of lamina staining surrounding each decondensing sperm. This is consistent with the observation of Blow & Watson (1987) who concluded that pronuclei enter S phase with a burst of (essentially) synchronous initiations. While our results support the conclusion that initiation is critically dependent upon nuclear structure, by observing nuclear reassembly following mitosis rather than nuclear assembly from sperm heads we conclude that initiations can occur asynchronously as chromatin acquires nuclear structure. This does not exclude the specific proposal made by Blow & Watson (1987) that initiation follows the accumulation of regulatory elements to a critical threshold level. Our results imply an asynchrony of initiation events within populations of 'mini nuclei', as not all lamina islands are incorporating biotin at the time of fixation. The major difference would thus be that initiations occur as regulatory proteins accumulate to the same threshold concentration within mini nuclei, but fail to spread rapidly because the mini nuclei are temporarily isolated units.

Other authors have commented on the differences in the pathway of nuclear assembly on different templates, concluding that it is dependent upon modifications to the DNA (Newport, 1987) such as the accumulation of prespore complexes (Sheehan et al. 1988). Sheehan et al. (1988) point out that struc-
tures similar to prespore complexes were not described in the analysis of nuclear reassembly in mitotic CHO cell extracts analysed by Burke & Gerace (1986). However, these latter authors did not comment on DNA synthesis in relation to nuclear reformation. The fusion of membrane vesicles during nuclear formation have been described (Burke & Gerace, 1986; Sheehan et al. 1988). However, the membrane vesicles we observed by phase contrast are much larger than the vesicles observed by electron microscopy (Burke & Gerace, 1986; Sheehan et al. 1988), already surround chromatin and, presumably, reflect a later stage in nuclear formation.

The \(G_2\)-state and the termination of DNA replication

DNA replication during the first cycle in vitro terminates between 20 and 50 min before first mitosis. This finding is reminiscent of the first cell cycle in vivo, which is the only cell cycle to have a detectable \(G_2\) period (Graham & Morgan, 1966). Incorporation of biotin into nuclei (and \(^{32}\)P-dCTP into DNA) stops at the end of \(S_1\), with the appearance of rapidly swelling nuclei, having condensed chromatin. These condensation and swelling processes are apparently independent of the rate of protein synthesis. Newport & Kirschner (1984) have studied the regulation of DNA synthesis and mitosis in Xenopus eggs. These authors describe a \(G_2\) state, induced by inhibiting protein synthesis, and show pictures of \(G_2\) nuclei (Newport & Kirschner, 1984, Fig. 1) which resemble the post-S-phase nuclei described here. During \(S_1\), replication is apparently not completed in the majority of nuclei either in the presence or absence of CHM. It is possible that entry into the \(G_2\) state coincides with signals that specifically terminate DNA replication. If so, such signals would have to be independent of the mitotic cycle. It has already been demonstrated that nuclei contain important information that determines when they will replicate during the first cell cycle and also prevents reinitiation (Blow & Laskey, 1986; Blow & Watson, 1987). In addition, we have observed that entry into the \(G_2\) state in vitro is accompanied by loss of important nuclear antigens such as PCNA (C. J. Hutchison and I. Kill, unpublished data) again implying some regulation of the nucleus. How the timing of such events is determined remains unclear but this could be related to the timing of initiations. In such a situation, the completion of replication upon entry into the \(G_2\) state would critically depend upon the efficiency of the initiation process. As the precise requirements for initiation are unclear it is not possible to measure the efficiency of initiation during \(S_1\) in our system. However, nuclear assembly measured by chromosome decondensation, accumulation of a nuclear membrane and lamina are both rapid and extensive. Despite this, initiation may not occur at a sufficient number of sites to ensure completion of replication during \(S_1\). It may be that chromatin decondensation is less than maximal through the retention of some MPF activity at the time of isolation of the extract. It will be valuable to measure MPF levels during the cell cycle in vitro.

The role of protein synthesis in cell cycle control in vitro

The cell-free extracts that we have described support protein synthesis. This incorporation is sensitive to protein synthesis inhibitors and has been shown to involve new initiation events (T. Patrick, C. Lewer and V. Payne, personal communication). In the absence of protein synthesis nuclear assembly around sperm DNA occurs, and replication of the chromatin is initiated. In addition, DNA synthesis is terminated at the same time as the chromatin becomes condensed into threadlike strands. However, in the absence of protein synthesis, nuclear membrane breakdown, lamina dispersal and mitotic chromosome formation do not occur; instead the chromatin threads continue to condense into a single thick fibre which lies centrally within the nucleus. Indeed, if protein synthesis is depressed rather than inhibited, then it can be shown that the timing of mitotic events is dependent on the rate of protein synthesis, but that the chromatin condensation occurring at the end of first S-phase is an independent event. Hence, the first cell cycle in this system could be thought of as a three-phase process involving initiation of DNA synthesis, termination of DNA synthesis and induction of mitosis. Of these events only the induction of mitosis appears to require protein synthesis. At its simplest, entry into mitosis could be regulated by the accumulation of a single protein throughout interphase, which reaches a threshold level, leading to the induction of mitosis. During mitosis this protein would either be destroyed or inactivated and interphase nuclei could then reform. This type of mechanism has been proposed for clam, sea urchins and starfish embryos, in which proteins known as cyclins are synthesized throughout the cell cycle but disappear at mitosis (Rosenthal et al. 1980; Evans et al. 1983; Standart et al. 1987). Synthetic mRNA to clam cyclin A, has subsequently been shown to induce meiotic events when injected into Xenopus oocytes (Swenson et al. 1986), and a cDNA with sequence homology to cyclin A has now been isolated from Xenopus embryos (Pines & Hunt, 1987). Thus it seems likely that cyclins have a role in mitotic control in Xenopus cell cycles.

Conclusions

We have described a cell-free extract of Xenopus
eggs, which undergoes multiple rounds of DNA replication and mitosis. This basic cell cycle is dependent on efficient protein synthesis and displays several interesting features which might be important in the initiation and termination of DNA replication. This system should allow us to study, not only the role of protein synthesis in cell cycle control, but also the organization of the replicase during S-phase and the cytoplasmic signals responsible for its assembly and disassembly.

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References


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