The Nuclear Membrane Determines the Timing of DNA Replication in *Xenopus* Egg Extracts

Gregory H. Leno and Ronald A. Laskey

Cancer Research Campaign Molecular Embryology Group, Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, England

Abstract. We have exploited a property of chicken erythrocyte nuclei to analyze the regulation of DNA replication in a cell-free system from *Xenopus* eggs. Many individual demembranated nuclei added to the extract often became enclosed within a common nuclear membrane. Nuclei within such a "multinuclear aggregate" lacked individual membranes but shared the perimeter membrane of the aggregate. Individual nuclei that were excluded from the aggregates initiated DNA synthesis at different times over a 10–12-h period, as judged by incorporation of biotinylated dUTP into discrete replication foci at early times, followed by uniformly intense incorporation at later times. Replication forks were clustered in spots, rings, and horseshoe-shaped structures similar to those described in cultured cells. In contrast to the asynchronous replication seen between individual nuclei, replication within multinuclear aggregates was synchronous. There was a uniform distribution and similar fluorescent intensity of the replication foci throughout all the nuclei enclosed within the same membrane. However, different multinuclear aggregates replicated out of synchrony with each other indicating that each membrane-bound aggregate acts as an individual unit of replication. These data indicate that the nuclear membrane defines the unit of DNA replication and determines the timing of DNA synthesis in egg extract resulting in highly coordinated triggering of DNA replication on the DNA it encloses.

INITIATION of DNA replication occurs at thousands of sites throughout the eukaryotic genome, yet initiations are coordinated so that the entire genome is replicated in a discrete time. The regulation of entry into S-phase and the initiation of DNA synthesis at these sites is not well understood; however, there is growing evidence indicating that nuclear structure is involved (for review, see Laskey et al., 1989).

The regulation of DNA replication and its relationship to nuclear structure can be studied in extracts derived from eggs of the frog *Xenopus laevis*. Egg extracts prepared by variants of the method of Lohka and Masui (1983) initiate and complete semiconservative replication on a variety of DNA templates under cell cycle control. (Blow and Laskey, 1986; Newport, 1987; Hutchison et al., 1987; Coppock et al., 1989). Using demembranated sperm nuclei or purified DNA, numerous authors have reported a correlation between the efficiency of nuclear assembly and DNA replication in this cell-free system (Lohka and Masui, 1984; Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990). In addition, if formation of the nuclear membrane is prevented, DNA replication does not occur (Newport, 1987; Sheehan et al., 1988). Furthermore, *Xenopus* sperm nuclei act as independent and integrated units of replication (Blow and Watson, 1987), indicating that some feature of nuclear structure defines the unit of DNA replication in the egg extract. One feature of nuclear structure, the nuclear membrane, has been implicated in regulation of DNA replication. Blow and Laskey (1988) showed that integrity of the nuclear membrane is necessary for the mechanism that prevents reinitiation of DNA replication within a single cell cycle.

Here we show that the nuclear membrane is also the feature of nuclear structure that defines the unit of replication allowing coordinate and near synchronous replication of all the DNA it contains. To approach this question, we have exploited features of chicken erythrocyte nuclei. First, like sperm nuclei, they provide a template that is inactive in replication, allowing a low background to the assay. Second, they can be reactivated for DNA replication by fusion with (for review, see Harris, 1974) or transplantation into (Ege et al., 1975; Lipsich et al., 1978) replicating cells. Third, we find that multiple demembranated erythrocyte nuclei often become enclosed within a common nuclear membrane after addition to the egg extract.

We show that individual free erythrocyte nuclei resemble *Xenopus* sperm in replicating asynchronously and behaving as independent and integrated units of replication. However, in striking contrast to the behavior of free individual nuclei, the nuclei enclosed within each membrane-bound "multinuclear aggregate" replicate synchronously with one another. Nevertheless, each multinuclear aggregate replicates

© The Rockefeller University Press, 0021-9525/91/02/557/10 $2.00

out of synchrony with its neighbors. These observations indicate that the nuclear membrane is the feature of nuclear structure that defines the unit of DNA replication and that determines the time of initiation of DNA replication in the *Xenopus* cell-free system.

Materials and Methods

Preparation of Egg Extracts

Extracts from activated eggs of *Xenopus laevis* were prepared essentially according to Blow and Laskey (1986). Female frogs were injected with 400–500 IU chorionic gonadotropin (Chorulon, Intervet Laboratories, Cambridge, England) into the dorsal lymph sac. Eggs were collected in High Salt Barth (110 mM NaCl; 15 mM Tris·HCl, pH 7.4; 2 mM KCl; 2 mM $NaHCO_3$; 1 mM $MgSO_4$; 0.5 mM Na_2HPO_4 and dejellied in 2% cysteine hydrochloride (pH 7.8). After several washes in Barth (88 mM NaCl; 15 mM Tris·HCl, pH 7.6; 2 mM KCl; 1 mM $MgCl_2$, 0.5 mM $CaCl_2$), eggs were activated in Barth containing 0.5 μg ml^{-1} calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) for up to 5 min. Activated eggs were washed in Barth and then ice-cold extraction buffer (50 mM Hepes·KOH, pH 7.4; 50 mM KCl; 5 mM $MgCl_2$; 2 mM β-mercaptoethanol) containing 10 μg ml^{-1} cytochalasin B (Sigma Chemical Co.). Eggs were spin crushed at 9,000 rpm for 10 min in a SW60Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. The cytoplasmic layer was removed and recentrifuged at 15,000 rpm for 10 min in an SW60Ti rotor at 4°C. The resultant supernatant was made 5% with respect to glycerol and frozen as 16-μl beads in liquid nitrogen.

Preparation of Erythrocyte Nuclei

Erythrocytes from adult chickens were isolated according to Bates et al. (1981). Frozen erythrocytes were thawed at 37°C into buffer N (340 mM sucrose; 60 mM KCl; 15 mM NaCl; 15 mM Hepes·KOH, pH 7.5; 15 mM β-mercaptoethanol; 0.5 mM spermidine; 0.15 mM spermine) at 4×10^8 cells ml^{-1}. Purity of erythrocytes was >99% as judged by light microscopy. Buffer N containing 2 mg ml^{-1} lysolecithin (Sigma Chemical Co.) was added to give a final concentration of 200 μg ml^{-1}. Cells were held at 23°C until >99% were permeabilized as determined by uptake of trypan blue. Further permeabilization was stopped by addition of 3% BSA in buffer N. Nuclei were pelleted by centrifugation at 1,000 *g* for 5 min, washed three times in buffer N, and resuspended in buffer N containing 50% glycerol. Aliquots were stored at −80°C. The concentration of DNA was determined by measuring absorbance at 260 nm and by counting nuclei in a hemacytometer. Both the plasma membrane and nuclear membrane were permeabilized by this protocol as judged by electron microscopy conducted as described by Sheehan et al. (1988) (data not shown).

In Vitro Replication of Nuclei in Egg Extract

Frozen extract was thawed and supplemented with an ATP regenerating system to a final concentration of 60 mM phosphocreatine (Sigma Chemical Co.) and 150 μg ml^{-1} creatine phosphokinase (Sigma Chemical Co.). Cycloheximide (Sigma Chemical Co.) was added to a final concentration of 100 μg ml^{-1}. Erythrocyte nuclei were added at ~5 ng μl^{-1} extract and labeled with 100 μCi ml^{-1} [α^{32}P]dATP (~800 Ci mmol^{-1}, Amersham Corp., Arlington Heights, IL), or 30–40 μM 5-biotin-19-deoxyuridine triphosphate (biotin-19-dUTP; Calbiochem-Behring Corp., San Diego, CA) or 5-biotin-11-deoxyuridine triphosphate (biotin-11-dUTP; Enzo Biochem Inc., New York, NY). Samples were incubated at 23°C for the appropriate time as indicated in each experiment.

Density substitution was performed as described by Blow and Laskey (1986). Specifically, erythrocyte nuclei at ~5 ng μl^{-1} were incubated for 12 h in egg extract containing 0.25 mM bromodeoxyuridine triphosphate (BrdUTP)[1] (Sigma Chemical Co.) and 100 μCi ml^{-1} [α^{32}P]dATP. Substituted DNA was separated on a CsCl equilibrium gradient centrifuged in a Ti50 rotor (Beckman Instruments) for >40 h at 20°C.

Coppock et al. (1989) have reported that a pretreatment with trypsin was required for nuclear decondensation and DNA replication of *Xenopus* erythrocyte nuclei in egg extract. Trypsin pretreatment was not required for nuclear decondensation and DNA replication in our extracts. Although we cannot completely rule out limited proteolytic activity during preparation of our nuclei or in our extracts, we consider the former possibility highly unlikely because examination of total protein from these nuclei and whole erythrocytes by SDS-PAGE showed distinct, undegraded histone and nonhistone protein profiles in each (data not shown). In addition, the observations we describe that individual erythrocyte nuclei replicate synchronously within multinuclear aggregates indicates that any template heterogeneity, either innate or generated during preparation, can be overcome by enclosure within a common nuclear membrane. Accordingly, it would be interesting to know what effect the trypsin treatment of Coppock et al. (1989) has on nuclear membrane integrity and reassembly.

Determination of [α^{32}P]dATP Incorporation

Incubations containing [α^{32}P]dATP were stopped by addition of 190 μl stop mix C (0.5% SDS, 20 mM EDTA, 20 mM Tris·HCl, pH 8.0). 10 μl proteinase K (10 mg ml^{-1} in 10 mM Hepes; 50% glycerol) was added and the mixture was incubated for 1 h at 37°C. The DNA was extracted with phenol and phenol chloroform. Incorporation into acid-insoluble material was determined by spotting samples onto glass fiber filters (GF/C; Whatman Inc., Clifton, NJ) followed by TCA precipitation, washing with ethanol, drying and counting in Optiscint (LKB Instruments, Inc., Gaithersburg, MD). Quantitation of DNA replication was based on a dATP pool size of 50 μM (Blow and Laskey, 1986) and expressed as nanograms of DNA synthesized μl^{-1} extract.

Microscopy

Nuclear membranes were identified by staining unfixed erythrocyte and *Xenopus* sperm nuclei assembled in the egg extract with Hoechst 33258 (100 μg ml^{-1}) and the lipid dye, Nile red (0.1–1.0 μg ml^{-1}) (Eastman Kodak Co., Rochester, NY). Specifically, templates were incubated in egg extract for 4–6 h at 23°C. After incubation, a stock solution of Nile red/dimethylsulfoxide was diluted in buffer A (60 mM KCl; 15 mM NaCl; 15 mM Tris·HCl, pH 7.4; 1 mM β-mercaptoethanol; 0.5 mM spermidine; 0.15 mM spermine), and an aliquot was added directly to each sample. Samples were incubated an additional 30 min before addition of Hoechst 33258. Aliquots were placed on slides and Nile red fluorescence was viewed with the red fluorescence channel on either a conventional or a confocal scanning microscope.

To detect incorporated biotin-dUTP, nuclei were fixed and spun onto polylysine-coated coverslips as described by Mills et al. (1989). Samples were diluted with fixation buffer (60 mM KCl; 15 mM NaCl; 15 mM Hepes·KOH, pH 7.4; 1 mM β-mercaptoethanol) and fixed with ethyleneglycolbis (succinimidylsuccinate) (EGS) (Blow and Watson, 1987). Fixed samples were layered over 30% sucrose in buffer A and spun at 3,000 rpm for 10 min in a Sorval HB4 rotor onto underlying polylysine-coated coverslips. Coverslips were washed in buffer A and nuclei were stained with 5 μl Texas red- or fluorescein-conjugated streptavidin (Amersham Corp.) and 5 μl Hoechst 33258 (100 μg ml^{-1}) in 500 μl buffer A containing 50 μg ml^{-1} RNase A (Sigma Chemical Co.) for 10 min. Then coverslips were washed with buffer A and deionized water before mounting in 90% glycerol, 1% DABCO in PBS. Nuclear structures were viewed with an Optiphot microscope (Nikon) with Episcopis-fluorescence attachment EF-D or an MRC 500/600 confocal microscope (Bio-Rad Laboratories, Cambridge, MA).

Results

Erythrocyte Nuclei Replicate Asynchronously and Show Clustered Sites of DNA Synthesis

Erythrocyte nuclei were incubated in egg extract containing 100 μCi ml^{-1} [α^{32}P]dATP for up to 12 h. At 2-h intervals, incubations were stopped, and the extent of incorporation of labeled precursor into TCA-insoluble material was determined as described in Materials and Methods. Extracts varied considerably in their ability to replicate erythrocyte nuclei ranging from ~30–100% of the input template. Only highly efficient extracts were selected for further experiments. The data presented in Fig. 1 *A* are representative of the results obtained from several extracts. During the first 2 h

1. *Abbreviations used in this paper*: BrdUTP, bromodeoxyuridine triphosphate; EGS, ethyleneglycolbis (succinimidylsuccinate); HH, heavy/heavy DNA; HL, heavy/light DNA.

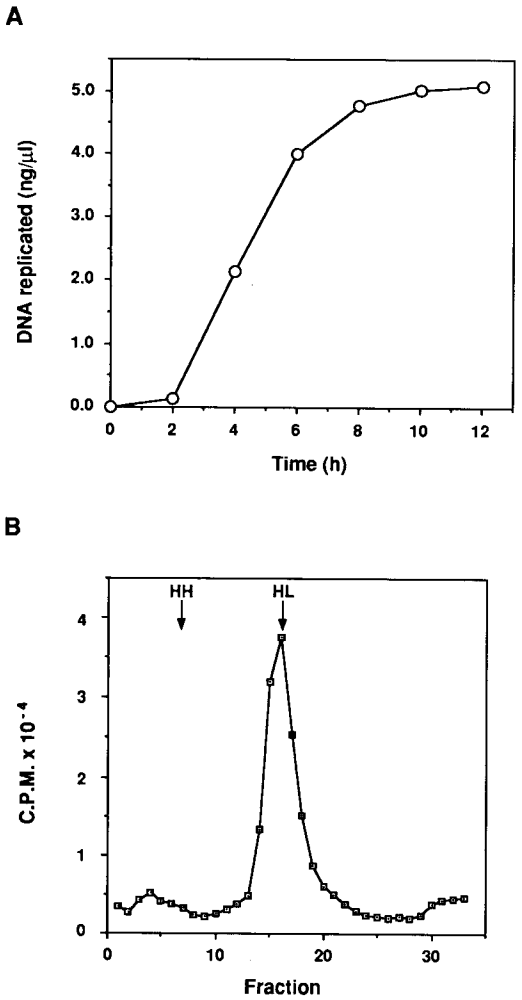


Figure 1. Replication of demembranated chicken erythrocyte nuclei in *Xenopus* egg extract. (*A*) Lysolecithin-permeabilized nuclei at ~5 ng μl^{-1} were incubated in egg extract containing [α^{32}P]dATP. Reactions were stopped at the times indicated and the DNA was precipitated with TCA. DNA replication is expressed as ng of DNA synthesized μl^{-1} of extract. (*B*) Erythrocyte nuclei were incubated in egg extract containing BrdUTP and [α^{32}P]dATP for 12 h at 23°C. The substituted DNA was separated by centrifugation on a CsCl equilibrium gradient. *HL* indicates density of heavy/light DNA and *HH* the expected density of heavy/heavy DNA.

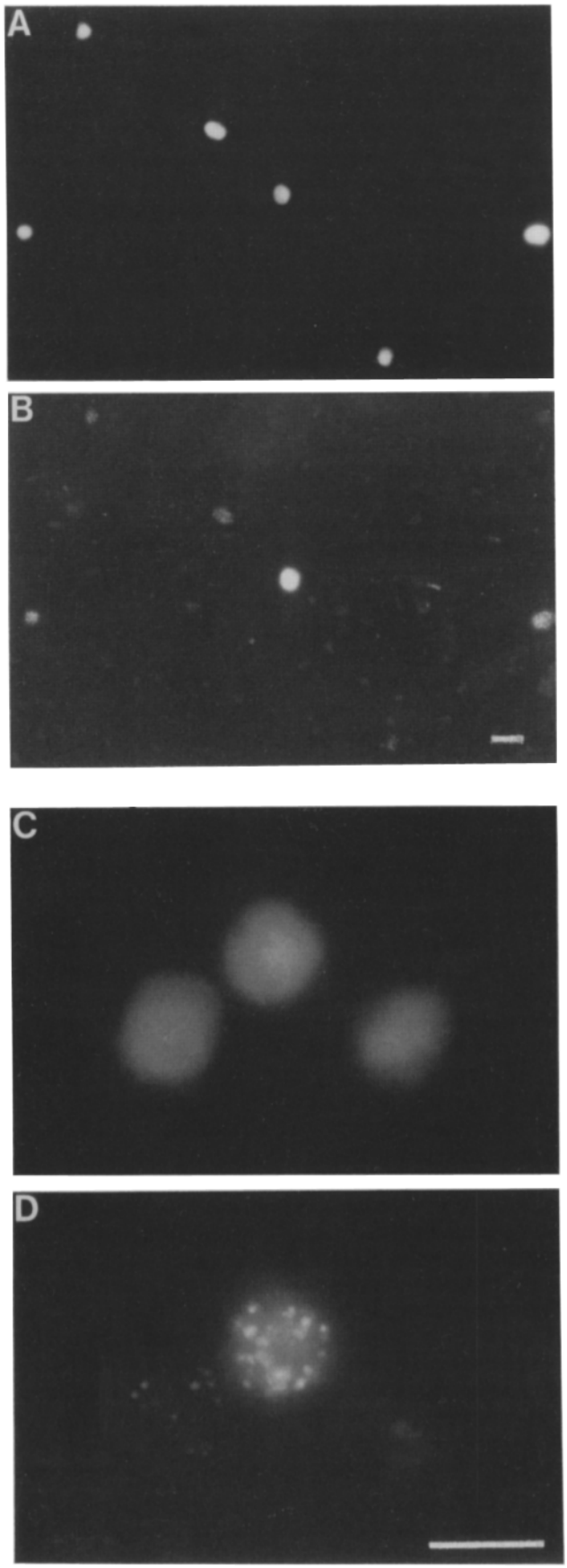
of incubation very little incorporation is observed. After this lag, synthesis increases progressively reaching levels equivalent to the concentration of the input DNA by 10–12 h, indicating complete replication of the template. Replication was consistently reduced >90% by addition of aphidicolin (10 μg ml^{-1}), an inhibitor of DNA polymerases α and δ.

To determine whether the incorporation of [α^{32}P]dATP was the result of a single round of semiconservative replication, we conducted density substitution experiments using the dense precursor BrdUTP essentially as described by Blow and Laskey (1986). Erythrocyte nuclei were incubated for 12 h in egg extract supplemented with 0.25 mM BrdUTP and 100 μCi ml^{-1} [α^{32}P]dATP. Substituted DNA was then separated by centrifugation on a cesium chloride equilibrium gradient, as shown in Fig. 1 *B*. The majority of labeled material banded at a CsCl density of 1.762 g cm^{-3} indicating a single complete round of semiconservative replication (heavy/light DNA; HL). Neither incomplete strand synthesis, nor significant rereplication of erythrocyte DNA were observed in our experiments. Rereplicated DNA would be expected to band at a density of ~1.78 g cm^{-3} as indicated in Fig. 1 *B* (heavy/heavy DNA; HH). In this experiment, ~87% of the input template was replicated according to [α^{32}P]dATP incorporation.

To determine the timing of replication between individual erythrocyte nuclei, we incubated nuclei in extract containing biotin-dUTP for up to 12 h. Biotin-dUTP, an analogue of thymidine triphosphate, is readily incorporated into nascent DNA (Langer et al., 1981) and can be visualized by staining with fluorescent streptavidin. Blow and Watson (1987) showed that biotin-dUTP incorporation increased linearly with the content of DNA in sperm nuclei replicating in egg extract, therefore accurately indicating the extent of replication.

During the first 2 h of incubation, nuclei swelled from ~2–5 μm to a diameter of ~10–15 μm and most showed considerable chromatin decondensation. By 4 h, streptavidin fluorescence was detected within most nuclei. Fig. 2 shows two fields of nuclei incubated for 4 h and stained with Hoechst 33258 (*A* and *C*) and Texas red streptavidin (*B* and *D*). Intensity of streptavidin staining and hence extent of replication is distinctly variable between individual nuclei (*B* and *D*); while some nuclei were uniformly bright, others were unstained. However, the vast majority of nuclei at 4 h showed a punctate pattern of incorporation (*D*) that was not reflected in the total DNA (*C*) but resembled the pattern that Mills et al. (1989) showed to be intermediates in *Xenopus* sperm replication in this system. Fig. 3 shows that the punctate pattern is also an intermediate in replication of erythrocyte nuclei and that initiation of replication is highly asynchronous. Nuclei from each time point in our time course experiments were classified as "bright" (intense uniform fluorescence), "punctate" (possessing distinct fluorescent foci), or "negative" (no fluorescence). The proportion of nuclei incorporating biotin increased from 23% at 2 h, through 63% at 3 h to 94% at 6 h indicating highly asynchronous initiation (Fig. 3). 79% of the nuclei at 4 h showed a punctate fluorescence; however, by 12 h, 95% of all nuclei were uniformly bright demonstrating that the punctate erythrocyte nuclei seen at early time points are precursors to the uniformly bright nuclei seen later in replication. Within any individual punctate nucleus, fluorescent foci were of relatively similar intensity suggesting a similar extent of DNA synthesis; however, the intensity of foci was distinctly heterogeneous between different nuclei (Fig. 2, *B* and *D*). Although we classified all nuclei showing any clear fluorescent foci as punctate (Fig. 3), we observed that overall, fluorescent intensity of foci within individual nuclei increased with time of incubation. Therefore, we were able to compare visually the extent of replication between individual nuclei. As with the incorporation of [^{32}P]dATP, streptavidin fluorescence and hence biotin-dUTP incorporation was reduced to near background levels by treatment with aphidicolin (30 μg ml^{-1}) (data not shown).

Mills et al. (1989) showed that the replication foci in replicating sperm nuclei each represented clusters of at least 300–1,000 replication forks, apparently remaining clustered throughout the period of DNA synthesis. Pulse labeling erythrocyte nuclei with biotin-dUTP late in replication (i.e., 8 h) resulted in a fine punctate pattern of fluorescence in



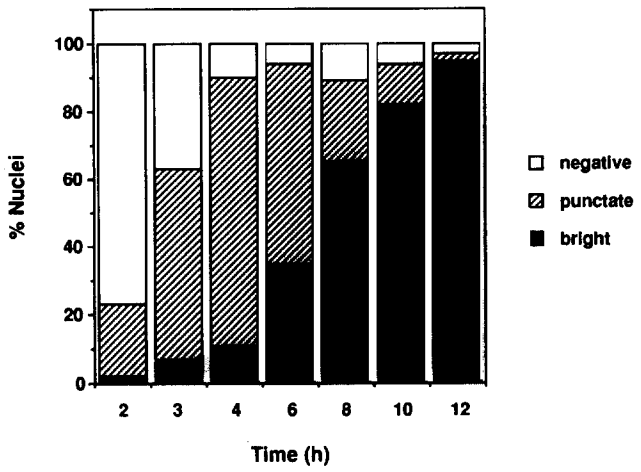


Figure 3. Replication initiates asynchronously and elongates via punctate intermediates. Nuclei were incubated in egg extract containing biotin-dUTP for the times indicated and fixed as described in Materials and Methods. Replication was visualized by staining with fluorescein-streptavidin and total DNA was stained with Hoechst 33258. 100 nuclei from each time point were selected at random and viewed under oil immersion optics (100×) using a fluorescein filter. Nuclei were classified as bright (intense uniform fluorescence); punctate (possessing distinct fluorescent foci); or negative (no fluorescence or very pale background fluorescence). Only single nuclei were included in these data.

most nuclei (our unpublished observation) suggesting that replication occurs at similar clustered sites throughout the period of DNA replication as with sperm nuclei.

In addition, replicating erythrocyte nuclei often showed ring or horseshoelike patterns of biotin incorporation, similar to those described in cultured cells replicating in vivo (Nakamura et al., 1986; Nakayasu and Berezney, 1989). Fig. 4 is a confocal micrograph of a single nucleus incubated in extract with biotin-dUTP for 4 h and stained with fluorescein-streptavidin. Replication rings and horseshoes can be seen throughout this nucleus. It will be of considerable interest to determine how these structures are generated during replication and if they represent the temporal organization of replicon domains during S-phase as has been suggested (Nakamura et al., 1986).

Erythrocyte Nuclei form "Multinuclear Aggregates" in Egg Extract

During analysis of the replication patterns of erythrocyte nuclei, a pronounced heterogeneity in nuclear size was observed. Although the majority of nuclei were between

Figure 2. Erythrocyte nuclei replicate asynchronously and show clustered sites of DNA synthesis. Nuclei were incubated in egg extract for 4 h with biotin-dUTP and fixed as described in Materials and Methods. Replication was visualized by staining with Texas red streptavidin (*B* and *D*), and total DNA was stained with Hoechst 33258 (*A* and *C*). Intensity of streptavidin fluorescence is distinctly heterogeneous between individual nuclei (*B*). Discrete fluorescent foci of uniform intensity can be seen within two nuclei (*D*). Bars: (*A* and *B*) 20 μm; (*C* and *D*) 10 μm.

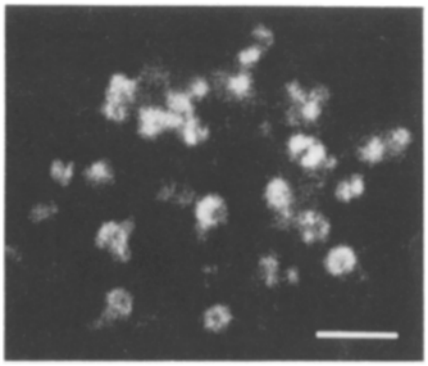


Figure 4. Formation of ring- or horseshoelike structures during replication seen in a confocal micrograph of a single nucleus. Nuclei were incubated in egg extract for 4 h with biotin-dUTP, fixed as described in Materials and Methods and stained with Hoechst 33258 (not shown) and fluorescein streptavidin as seen here. Bar, 2 μm.

~10–15 μm in diameter (Fig. 2), many ranged from 20 to 100 μm across. Closer inspection revealed that these large "nuclei" were composites of up to an estimated 50 or more individual nuclei apparently enclosed within a common membrane. This extreme variation in size is illustrated in Fig. 5 *A* which shows a single nucleus (*arrow*) and a multinuclear aggregate viewed by phase-contrast microscopy. The heterogeneous "raspberry-like" appearance of the bulk DNA, stained with Hoechst 33258, illustrates the presence of many partially decondensed individual nuclei (Fig. 5 *B*). Extensive decondensation of erythrocyte chromatin was observed in many multinuclear aggregates resulting in a near homogeneous distribution of bulk DNA (see Fig. 8, *A* and *C*). To determine whether these multinuclear aggregates were in fact bounded by a common membrane, we stained unfixed samples with the lipid dye Nile red (Greenspan et al., 1985). Cox and Leno (1990) showed that this dye binds to the nuclear membrane of *Xenopus* sperm nuclei assem-

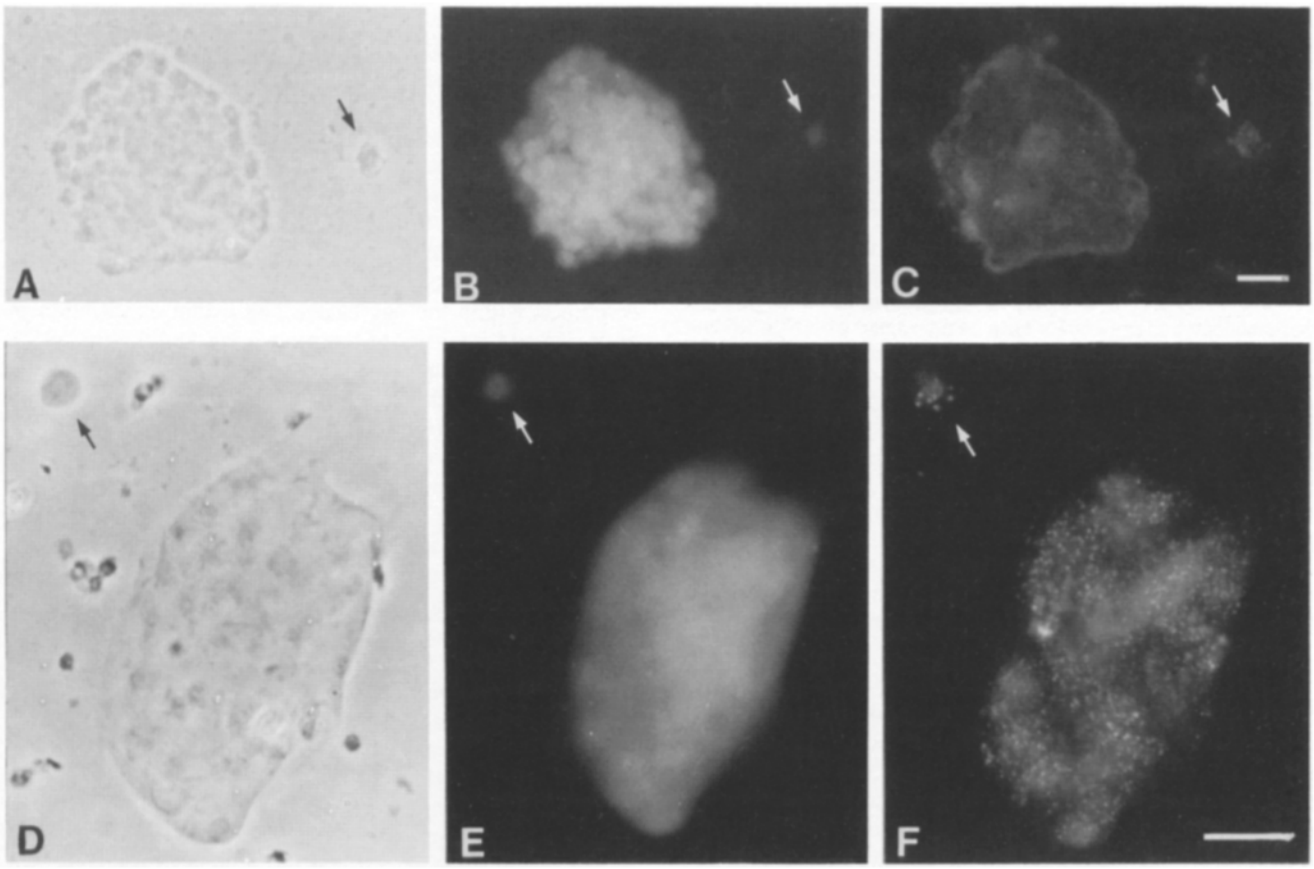


Figure 5. Multinuclear aggregates are bounded by a common nuclear membrane and contain synchronously replicating nuclei. Erythrocyte nuclei were incubated in egg extract for 4 h. Unfixed samples (*A–C*) were stained with Hoechst 33258 and the lipid dye, Nile red. A multinuclear aggregate and a single nucleus (*arrow*) are shown by phase-contrast (*A*) and by Hoechst 33258 (*B*) and Nile red (*C*) fluorescence. Distinct Nile red fluorescence appears at the perimeter of the aggregate and around the single nucleus; however, no fluorescence was observed around the individual nuclei within the aggregate (*C*). The multiple individual nuclei can be seen within this aggregate in *A* and *B*. For analysis of DNA replication (*D–F*), nuclei were incubated in egg extract with biotin-dUTP for 4 h, fixed with EGS and stained with Texas red streptavidin as described in Materials and Methods. Fixation of nuclear structures was required for consistent fluorescent streptavidin staining in both erythrocyte and sperm nuclei. However, this fixation protocol disrupted membrane structure, preventing analysis of replication and membrane integrity within the same nuclear structures. A single nucleus (*arrow*) and a multinuclear aggregate are visualized by phase-contrast (*D*), Hoechst 33258 (*E*), and Texas red streptavidin (*F*). A distinctly punctate pattern of streptavidin fluorescence can be seen both within the individual nucleus (*arrow*) and within the multinuclear aggregate (*F*). These fluorescent foci are of similar size and intensity within the aggregate. Bars, 20 μm.

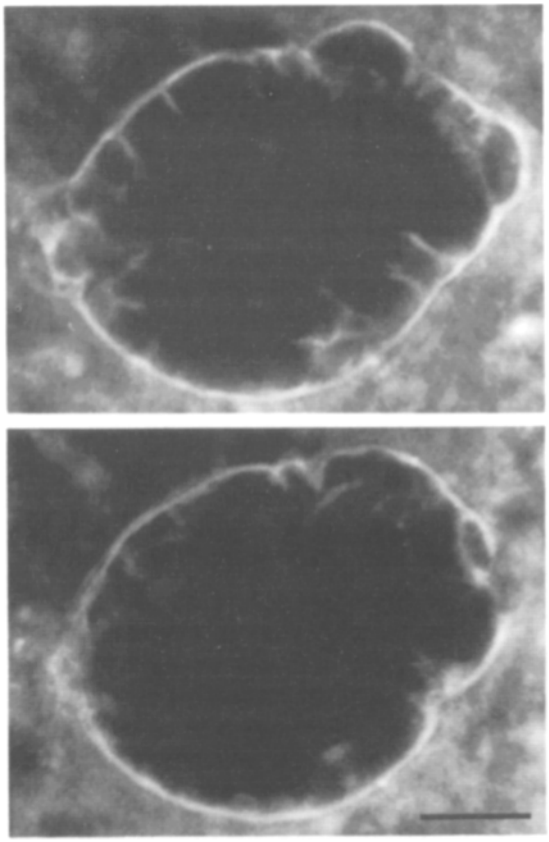


Figure 6. Multinuclear aggregates possess a continuous peripheral nuclear membrane but nuclei contained within the aggregates do not possess their own membranes. Erythrocyte nuclei (5 ng μl^{-1}) were incubated in egg extract for 6 h and stained with Nile red and Hoechst 33258 (not shown). Unfixed samples were viewed with the red fluorescence channel on a confocal scanning microscope. Shown are two noncontiguous optical sections through one multinuclear aggregate stained with Nile red. A continuous Nile red fluorescence can be seen around the perimeter of the aggregate; however, very little fluorescence is observed within the aggregate itself demonstrating that individual nuclei contained within the aggregate do not possess their own nuclear membranes. The fluorescence seen within the aggregate may represent invaginations of the common perimeter membrane and/or remnant membrane fragments from the individual nuclei. Bar, 10 μm.

bled in egg extract resulting in a continuous peripheral staining. Fig. 5 *C* shows the same nuclear structures in Fig. 5, *A* and *B* stained with Nile red. A distinct and continuous fluorescence was observed around the perimeter of the aggregates of nuclei as well as around the free single nuclei (*arrow*); however, no fluorescence was observed around the perimeter of individual nuclei within the aggregate itself confirming the lack of membranes in phase-contrast images. Fig. 6 shows higher resolution images of the perimeter membrane and lack of internal membranes in confocal optical sections through a large multinuclear aggregate stained with Nile red. We conclude that multinuclear aggregates are bounded by a common membrane and that the individual nuclei contained within do not possess their own membranes.

To determine whether nuclear pore complexes were associated with the common membrane enclosing the multinuclear aggregates, we incubated the nuclear structures assembled in egg extract with fluorescein-conjugated wheat germ agglutinin. Finlay et al. (1987) demonstrated that wheat germ agglutinin binds directly to the nuclear pore and inhibits nuclear protein transport. We detected fluorescence along the outer membrane and within the aggregates themselves (data not shown), suggesting that nuclear pore complexes are present in association with the limiting membrane and retained by the individual nuclei within the multinuclear aggregate. LaFond and Woodcock (1983) showed that adult chicken erythrocyte nuclei lack an internal nuclear matrix inside the fibrous pore complex-lamina. Thus, the residual nuclear architecture often seen within multinuclear aggregates may be remnants of the pore complex-lamina. These putative remnant structures appear as dark oval objects within the nuclear aggregate when viewed by phase-contrast microscopy (Fig. 5, *A* and *D*).

Replication Occurs Synchronously within Multinuclear Aggregates

In contrast to individual erythrocyte nuclei, which replicate asynchronously in egg extract (Fig. 2), those nuclei enclosed within a common nuclear membrane replicate synchronously with respect to one another. Fig. 5 (*D–F*) shows a single nucleus (*arrow*) and a multinuclear aggregate incubated in egg extract with biotin-dUTP for 4 h, fixed, and stained with Texas red streptavidin. In many of these multinuclear aggregates, a punctate pattern of streptavidin fluorescence was observed (Fig. 5 *F*) similar to that seen in individual nuclei (Fig. 5, *arrow*, and Fig. 2). The majority of replication foci are of similar size and fluorescent intensity indicating DNA replication is proceeding at the same rate within all nuclei contained within the aggregate. The extent of variation in both size and intensity of the replication foci within >95% of all multinuclear aggregates was no greater than the extent of variation seen within an individual nucleus. The somewhat heterogeneous distribution of foci observed in the largest aggregates (Fig. 5 *F*) correlates with the heterogeneous distribution of the individual nuclei making up the bulk DNA (Fig. 5 *E*) which can be seen more clearly in paler Hoechst exposures. Synchronous replication was observed within ~150 multinuclear aggregates, irrespective of aggregate size and the time of incubation. A slight heterogeneity of streptavidin fluorescence that could not be accounted for by the distribution of the bulk DNA was seen in ~2–3% of the multinuclear aggregates. However, in these cases, the extent of variation between replication foci was far less than would be expected from many individual nuclei replicating independently (see Fig. 2, *B* and *D* and also Fig. 8). One possibility is that this apparent slight asynchrony may be the result of occasional inclusion in the aggregates of intact (unpermeabilized) erythrocytes or aberrant nuclei that are unable to replicate. An alternative permeabilization protocol using 0.5% NP-40 resulted in less aggregation of erythrocyte nuclei but qualitatively identical results. Replication rings and horseshoes were also seen within multinuclear aggregates (data not shown). As with individual nuclei, nearly all multinuclear aggregates were uniformly bright when stained with fluorescent streptavidin after 12 h in egg extract (data not shown).

Replication Foci Are Distributed Uniformly throughout Multinuclear Aggregates

To confirm that replication was synchronous within mul-

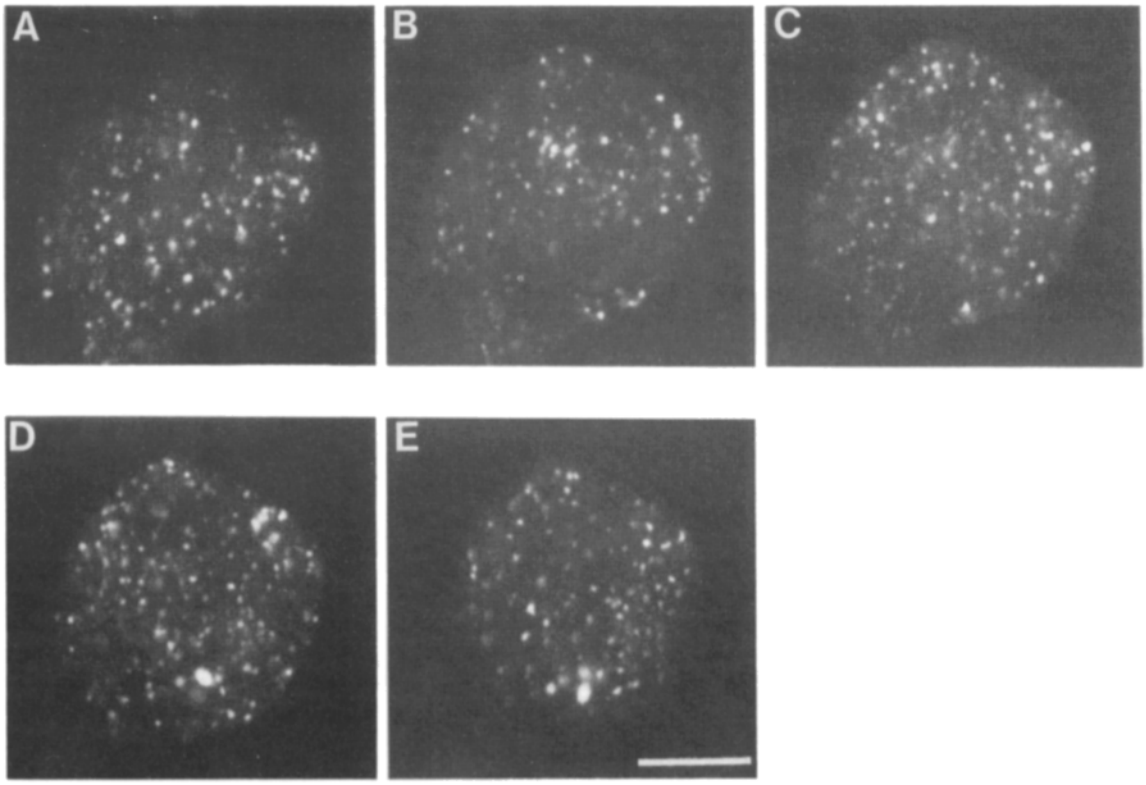


Figure 7. Clustered sites of replication occur uniformly throughout multinuclear aggregates. Nuclei were incubated in egg extract for 4 h with biotin-dUTP and fixed as described in Materials and Methods. A confocal series of optical sections taken at 2-μm intervals through a multinuclear aggregate stained with Texas red streptavidin (*A–E*) is shown. This aggregate contained completely decondensed, uniformly distributed chromatin and contains a ~27-fold greater volume than the individual nuclei shown in Fig. 2 *C*. The foci of streptavidin fluorescence appear uniformly distributed and of similar intensity throughout the aggregate. The extent of variation in size and intensity of the replication foci seen in these optical sections is no greater than that seen within individual nuclei and far less than would be expected if many individual nuclei were replicating independently within the common nuclear membrane. Bar, 10 μm.

tinuclear aggregates and to determine whether replication foci were evenly distributed throughout these structures, aggregates were examined using confocal microscopy. Fig. 7 shows a confocal series of optical sections taken at 2-μm intervals through a multinuclear aggregate stained with Texas red streptavidin. This aggregate contained completely decondensed uniformly distributed chromatin and, although relatively small (~25 μm diam), this structure contains a ~27-fold greater volume than the individual nuclei shown in Figure 2 *C*. In this aggregate, replication foci are of similar size and fluorescent intensity in all sections (*A–E*) and their distribution appears uniform throughout this structure. Note that sites of DNA replication are not preferentially associated with the nuclear membrane. As with the aggregates viewed by conventional light microscopy, the variation in size and intensity of the replication foci, as seen in these optical sections, is no greater than that observed in an individual nucleus and far less than would be expected from many individual nuclei replicating independently within each aggregate (see Fig. 8).

Multinuclear Aggregates Replicate out of Synchrony with Each Other

Within each multinuclear aggregate DNA replication was synchronous; however, the extent of replication between aggregates differed markedly. Fig. 8 shows two fields of nuclear structures containing both multinuclear aggregates (*arrows*) and individual nuclei incubated in extract for 4 h with biotin dUTP and stained with Hoechst 33258 (Fig. 8, *A* and *C*) and fluorescent streptavidin (Fig. 8, *B* and *D*). The field shown in (Fig. 8, *C* and *D*) represents a ~1.5-fold greater magnification than that seen in (Fig. 8, *A* and *B*). As shown in Fig. 8, *B* and *D*, the replication foci in all the multinuclear aggregates appear to be uniformly distributed and of similar fluorescent intensity within the perimeter membrane (see also Fig. 5 *F* and Fig. 7). However, the fluorescent intensity of foci was distinctly different when comparing different aggregates, even aggregates of similar size. For example, in Fig. 8 *B* the aggregate on the right has just begun to replicate while synthesis in the adjacent aggregate is extensive. In addition, we observed that some larger aggregates had replicated to a similar or even greater extent than their smaller counterparts within the same incubation (e.g., compare the multinuclear aggregate in Fig. 5 *F* with the right-hand aggregate in Fig. 8 *B*). Examination of the bulk DNA in Fig. 8 *A* reveals considerable heterogeneity in Hoechst staining between multinuclear aggregates of similar size. This most probably reflects different concentrations of nuclei contained within the aggregate. It may be that the extent of decondensation of chromatin is limited in some aggregates. However, the extent of replication did not correlate with the degree of chromatin decondensation or the amount of DNA contained within the aggregates; i.e., different Hoechst "bright" aggregates showed either more or less streptavidin fluorescence than their paler counterparts.

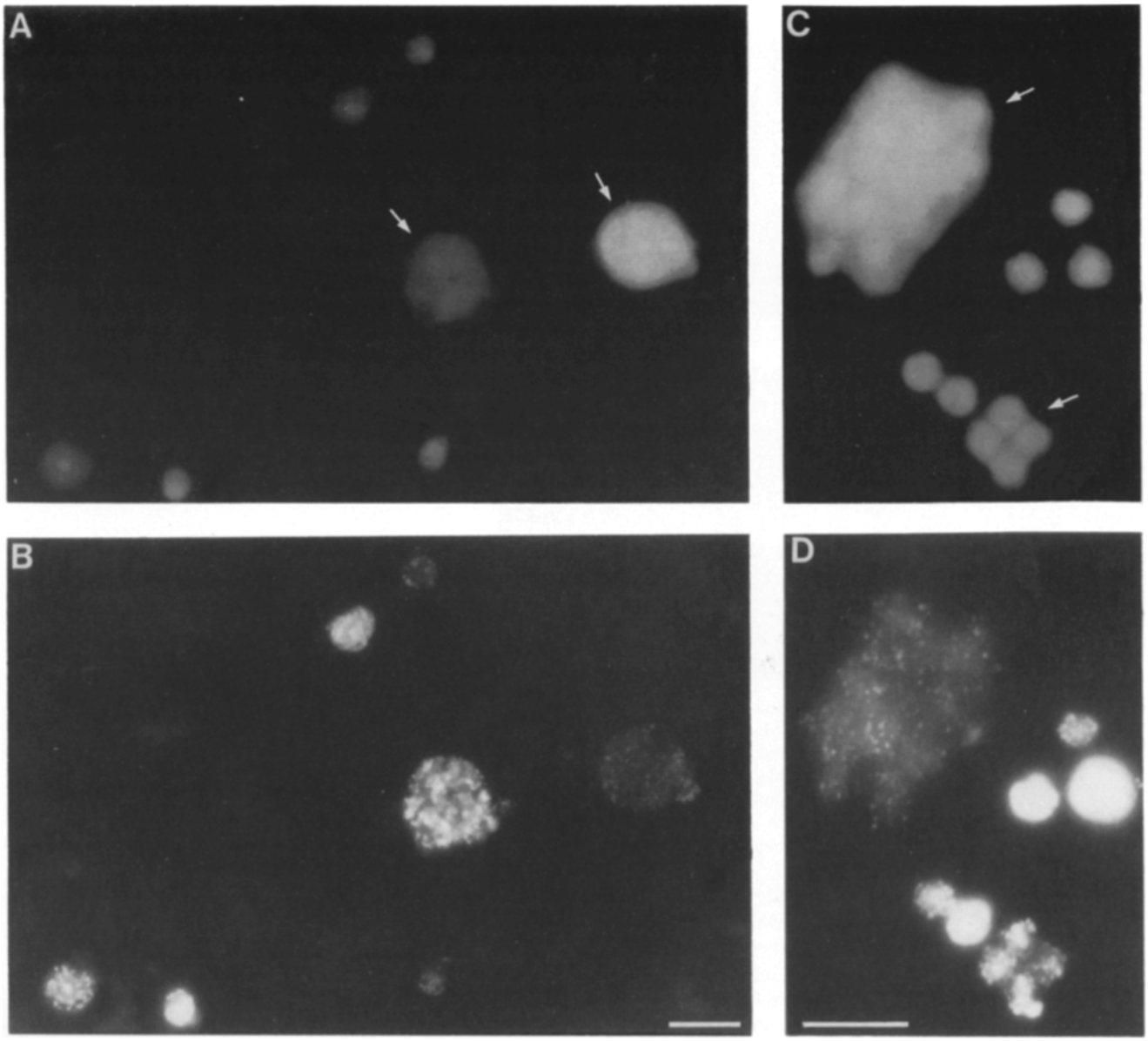


Figure 8. Although nuclei within multinuclear aggregates replicate synchronously, aggregates replicate out of synchrony with each other. Erythrocyte nuclei were incubated in egg extract for 4 h with biotin-dUTP and stained with Hoechst 33258 (*A* and *C*) and Texas red (*B*) or fluorescein (*D*) streptavidin. Two fields of nuclear structures containing multinuclear aggregates (*arrows*) and many individual nuclei are shown. Within each multinuclear aggregate streptavidin fluorescence is of similar intensity and relatively uniformly distributed; however, when comparing different aggregates, the extent of streptavidin fluorescence is distinctly heterogeneous. The variation in extent of fluorescence between aggregates did not appear to be dependent upon the size of the aggregate (*B* and *D*). At 4 h, certain aggregates had replicated to a greater extent than some individual nuclei (*B*). Note that nuclear size should only be compared with Hoechst stain (*A* and *C*) as out of focus flare exaggerates the size of those nuclei that have incorporated most biotin (*D*). Bars, 20 μm.

The data in Fig. 8 illustrate two important points. First, the asynchronous replication seen between different multinuclear aggregates is analogous to that seen between single nuclei (Fig. 8, *B* and *D*; and also Fig. 2, *B* and *D*) and, second, the extent of replication observed within multinuclear aggregates did not appear to depend upon aggregate size. In fact, the extent of replication in multinuclear aggregates was often equal to (Fig. 5 *F*) or greater than (Fig. 8 *B*) that observed in many individual nuclei further supporting the notion that extent of replication was not dependent on the size of the nuclear structure.

Finally, an extraordinary feature of the multinuclear aggregates is the coordination between many hundreds of replication foci each of which must represent hundreds of replication forks (see Mills et al., 1989). The uniformity of the punctate pattern even at early times of labeling indicates a highly cooperative initiation mechanism on all the DNA enclosed within each nuclear membrane whether it encloses one or many nuclei.

Discussion

Single Erythrocyte Nuclei Replicate Asynchronously

When demembranated nuclei are added to the *Xenopus* egg extract, they are induced to enter S-phase asynchronously

over a 10–12-h period. These data are consistent with the results of Blow and Watson (1987) for *Xenopus* sperm nuclei though most erythrocyte nuclei require considerably longer to complete replication than the ~30–60 min required for the majority of sperm nuclei in egg extract. The timing of initiation of replication in erythrocyte nuclei was not simply related to the extent of chromatin decondensation and nuclear swelling. Although all replicating nuclei showed some degree of decondensation, nuclei of similar size replicated asynchronously (Figs. 2 and 8).

The discrete replication foci observed in erythrocyte nuclei by streptavidin fluorescence probably represent the replicon clusters or domains previously described in cultured cells in vivo (Nakamura et al., 1986; Bravo and McDonald-Bravo, 1987; de Bruyn Kops and Knipe, 1988; Nakayasu and Berezney, 1989) and in *Xenopus* sperm nuclei replicating in vitro (Mills et al., 1989). The intensity of fluorescence of these foci increases with time confirming Blow and Watson's (1987) flow cytometry observations that biotin incorporation is directly proportional to DNA synthesis.

Enclosure within a Common Nuclear Membrane Imposes Synchronous Replication

When aggregates of nuclei become enclosed within a common nuclear membrane, they replicate synchronously (Figs. 5, 7, and 8). This has been observed in over 150 aggregates ranging in number of included nuclei from 4 (Fig. 8) up to an estimated 50 or more (Fig. 5). The fact that nuclei contained within multinuclear aggregates replicate synchronously suggests that the timing of DNA synthesis is determined at the level of the nuclear membrane. One role for the nuclear membrane in the cell-cycle regulation of DNA replication has been suggested by Blow and Laskey (1988) based on the observation that permeabilizing the membrane of a replicated nucleus was sufficient to allow it to rereplicate without passing through mitosis. This observation supports a model by which an essential replication factor is unable to enter the nucleus but binds to the DNA only at mitosis when the nuclear membrane is broken down. In this way it licenses the DNA to replicate once and only once after nuclear reassembly. The observation that all the nuclei within a common giant nuclear membrane replicate synchronously as an integrated unit is consistent with this model. Furthermore, it indicates that the nuclear membrane is also the feature of nuclear structure that defines the nucleus as an integrated and independent unit of DNA replication in the egg extract.

The experiments described here also suggest the way in which the nuclear membrane defines the unit of replication. In theory it could either provide an essential structural framework to which DNA is attached for replication, or it could serve as a concentrating device to accumulate threshold levels of nuclear proteins. Two observations argue against the nuclear membrane providing a direct structural framework for replication. First, the fluorescent foci that represent sites of replication are not preferentially associated with the nuclear membrane. Instead they are distributed throughout the interior of the aggregate (Fig. 7). Second, individual nuclei that lie in the center of the multinuclear aggregate have no obvious physical contact with the perimeter membrane, yet they still replicate in synchrony with the nuclei which contact the membrane. Therefore, we favor the alternative possibility, namely that the nuclear membrane defines a unit of replication by selectively concentrating nuclear proteins within it. In this way each nucleus or multinuclear aggregate could only initiate DNA replication when it reaches a critical threshold level of nuclear proteins. Nevertheless, it is remarkable that initiation should be so sudden and so complete throughout either a single nucleus or a large multinuclear aggregate suggesting a highly cooperative triggering event that can extend between individual chromatin masses. The way in which enclosure within a single membrane causes a cooperative event throughout the chromatin masses is not clear yet. Possibilities might include assembly of an integrated scaffold or matrix system or alternatively a threshold for activation of a soluble component with widespread secondary consequences. Whichever way the nuclear membrane defines the unit of replication, the results described here focus further attention on the importance of the nuclear membrane in regulating the control of DNA replication in eukaryotic cells.

We wish to thank A. D. Mills for his invaluable assistance with the confocal microscopy and for numerous useful discussions throughout the course of this work. We also thank Jean Thomas for providing us with chicken erythrocytes. We appreciate the useful comments on the manuscript made by Michael Taylor, Micaela Fairman, Lynne Cox, and Anna Philpott.

This work was funded by the Cancer Research Campaign.

Received for publication 8 June 1990 and in revised form 10 November 1990.

References

Bates, D. L., P. J. G. Butler, E. C. Pearson, and J. O. Thomas. 1981. Stability of the higher-order structure of chicken-erythrocyte chromatin in solution. *Eur. J. Biochem.* 119:469–476.

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

Blow, J. J., and A. M. Sleeman. 1990. Replication of purified DNA in Xenopus egg extract is dependent on nuclear assembly. *J. Cell Sci.* 95:383–391.

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

Bravo, R., and H. McDonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.

Coppock, D. L., R. A. Lue, and L. J. Wangh. 1989. Replication of Xenopus erythrocyte nuclei in a homologous egg extract requires prior proteolytic treatment. *Dev. Biol.* 131:102–110.

Cox, L. S., and G. H. Leno. 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.

de Bruyn Kops, A., and D. M. Knipe. 1988. Formation of DNA replication structures in Herpes virus-infected cells requires a viral DNA binding protein. *Cell.* 55:857–868.

Ege, T., J. Zeuthen, and N. R. Ringertz. 1975. Reactivation of chick erythrocyte nuclei after fusion with enucleated cells. *Somatic Cell genet.* 1:65–80.

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

Greenspan, P., E. P. Mayer, and S. D. Fowler. 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100:965–973.

Harris, H. 1974. Nucleus and Cytoplasm. Clarendon Press Oxford. 186 pp.

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

LaFond, R. E., and L. F. Woodcock. 1983. Status of the nuclear matrix in mature and embryonic chick erythrocyte nuclei. *Exp. Cell Res.* 147:31–39.

Langer, P. R., A. A. Waldrop, and D. C. Ward. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA.* 78:6633–6637.

Laskey, R. A., M. P. Fairman, and J. J. Blow. 1989. S phase of the cell cycle. *Science (Wash. DC).* 246:609–614.

Lipsich, L. A., J. J. Lucas, and J. R. Kates. 1978. Cell cycle dependence of

the reactivation of chick erythrocyte nuclei after transplantation into mouse L929 cell cytoplasts. *J. Cell Physiol.* 97:199–208.

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

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

Mills, A. D., J. J. Blow, J. G. White, W. B. Amos, D. Wilcock, and R. A. Laskey. 1989. Replication occurs at discrete foci spaced throughout nuclei replicating in vitro. *J. Cell Sci.* 94:471–477.

Nakamura, H., T. Morita, and C. Sato. 1986. Structural organizations of replicon domains during DNA synthetic phase in mammalian nucleus. *Exp. Cell Res.* 165:291–297.

Nakayasu, H., R. Berezney. 1989. Mapping replicational sites in the eucaryotic cell nucleus. *J. Cell Biol.* 108:1–11.

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

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