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.
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 4-500 IU chorionic gonadotropin (Chorulon, Intervet Laboratory, 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 NaHCO3; 1 mM MgSO4; 0.5 mM Na2HPO4 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 MgCl2; 0.3 mM CaCl2), 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 MgCl2; 2 mM β-mercaptoethanol) containing 10 µg ml⁻¹ cytochalasin B (Sigma Chemical Co.). Eggs were spun crushed at 9000 rpm for 10 min in a SW60Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. The resultant supernatant was made 5% with respect to glycerol and frozen as 16-µ1 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 spermine; 0.15 mM spermidine) and 4 x 10⁶ cells ml⁻¹. Purity of erythrocytes was >95% as judged by light microscopy. Buffer N containing 2 mg ml⁻¹ lysoseltin (Sigma Chemical Co.) was added to give a final concentration of 200 µg ml⁻¹ cells. Cells were held at 23°C until 50% 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 1000 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.). Cytochrome oxidase (Sigma Chemical Co.) was added to a final concentration of 100 µg ml⁻¹. Erythrocyte nuclei were added at ~5 µg ml⁻¹ extract and labeled with 100 µCi ml⁻¹ [α²P]dATP (6800 Ci mmol⁻¹, Amersham Corp., Arlington Heights, IL) or 30-40 µM 5-biotin-1-deoxynucleotide triphosphate (biotin-1-UTP; Calbiochem-Behring Corp., San Diego, CA) or 5-biotin-1-deoxyuridine triphosphate (biotin-1-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 ~5.5 µg ml⁻¹ were incubated for 12 h in egg extract containing 0.25 mM bromodeoxyuridine triphosphate (BrdUTP) (Sigma Chemical Co.) and 100 µCi ml⁻¹ [α²P]dATP. Substituted DNA was separated on a CsCl equilibrium gradient centrifuged in a Ti50 rotor (Beckman Instruments) for >60 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 of *Xenopus* erythrocyte nuclei in egg extract.

**Determination of [α²P]dATP Incorporation**

Incorporation of [α²P]dATP was measured 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 a scintillation counter (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 nuclei 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/dimethyldioctadecylammonium chloride was diluted in buffer A (60 mM NaCl; 15 mM Hepes-KOH, pH 7.4; 1 mM β-mercaptoethanol; 0.5 mM spermine; 0.15 mM spermidine), 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 NaCl; 15 mM Hepes-KOH, pH 7.4; 1 mM β-mercaptoethanol) and fixed with ethylene-glycolic (succinimidylsulfonate) (EGS) (Blow and Watson, 1987). Fixed samples were labeled over 30% sucrease in buffer A and spun at 3900 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 Episcopic-fluorescence attachment EF-D or an MRZ 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 [α²P]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. 1A are representative of the results obtained from several extracts. During the first 2 h...
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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 [α³²P]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 [α³²P]dATP. Substituted DNA was then separated by centrifugation on a cesium chloride equilibrium gradient, as shown in Fig. 1B. 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. 1B (heavy/heavy DNA; HH). In this experiment, 97% of the input template was replicated according to [α³²P]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 [³²P]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 (100x) 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 horseshoe-like 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.
Formation offing- or horseshoe-like 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 assembled.
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.

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).

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.

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 reerupt 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 multinucleated 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 multinucleate 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 multinucleate 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.

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