Steps in the Assembly of Replication-competent Nuclei in a Cell-free System from *Xenopus* Eggs

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Abstract. We have studied the pathway of nuclear assembly from demembranated sperm chromatin by fractionating a cell-free system from *Xenopus* eggs (Lohka, M. J., and Y. Masui. 1983. *Science (Wash. DC).* 220:719–721). Both the soluble fraction and a washed vesicular fraction are required for formation of normal nuclei that initiate replication in vitro. The soluble fraction alone decondenses chromatin and the vesicular fraction alone surrounds chromatin with membranes. Both fractions are required for formation of nuclear pore complexes. Recombining these two fractions recovers ~100% of the nuclear assembly and DNA replication activities.

Restricting the proportion of the vesicular fraction slows acquisition of the nuclear membrane and allows observation of immature nuclear pores ("prepores"). These form as arrays around and within the chromatin mass before membranes form. Subsequently membrane vesicles bind to these prepores, linking them by a single membrane throughout the chromatin mass. At the periphery this single membrane is surrounded by an outer membrane. In mature nuclei all membranes are at the periphery, the two membranes are linked by pores, and no prepores are seen.

Nuclear assembly and replication are inhibited by preincubating the chromatin with the vesicular fraction. However nuclear assembly is accelerated by preincubating the condensed chromatin with the soluble fraction. This also decreases the lag before DNA replication. Initiation of DNA replication is only observed after normal nuclei have fully reassembled, increasing the evidence that replication depends on nuclear structure. The pathway of nuclear assembly and its relationship to DNA replication are discussed.

Mitosis is accompanied by disassembly of the nucleus in most cells. When mitosis is completed the chromosomes decondense and acquire both a lamina and a double unit envelope studded with pores. Thus nuclear assembly is a common event, occurring once per cell cycle in most cells.

Two separate cell-free systems now exist in which this process can be studied. Burke and Gerace (1986) have described a homogenate of chinese hamster ovary (CHO) cells that supports formation of interphase nuclei from the endogenous chromosomes. Lohka and Masui (1983) demonstrated formation of pronuclei from demembranated sperm chromatin in an in vitro system derived from activated amphibian eggs. Nucleus-like structures are also formed when naked DNA is incubated in such an extract (Newmeyer et al., 1986; Blow and Laskey, 1986; Newport, 1987).

Xenopus eggs are an excellent source for an in vitro system for studying nuclear assembly. After fertilization the *Xenopus* embryo undergoes 11 rounds of division within 7 h without increasing size. This is achieved because each *Xenopus* egg contains a stockpile of materials required for rapid DNA replication and the assembly of both chromatin and nuclei (reviewed by Laskey et al., 1985). *Xenopus* eggs contain very little DNA in comparison to this stockpile, only one genome compared with sufficient components to assemble several thousand nuclei. Therefore exogenous DNA templates can be used to study nuclear assembly in eggs by microinjection (Forbes et al., 1983) or in egg extracts (Lohka and Masui, 1983, 1984: Blow and Laskey, 1986; Newmeyer et al., 1986; Newport, 1987). Another advantage of this system is that the nuclei formed initiate and complete DNA replication (Blow and Laskey, 1986; Blow and Watson, 1987), and can accumulate nuclear proteins (Newmeyer et al., 1986), in a manner totally consistent with that occurring in vivo.

We have manipulated the nuclear assembly system derived from *Xenopus* eggs in order to analyze the pathway of nuclear formation. We have fractionated the extract into soluble and vesicular fractions (Lohka and Masui, 1984; Newport, 1987) and analyzed the structure of demembranated sperm chromatin incubated in each fraction by electron microscopy. Structurally normal nuclei are only formed when the two fractions are recombined and both fractions are needed to initiate and complete DNA replication. By varying the proportions of the fractions the process of nuclear assembly can be modulated.

By manipulating nuclear assembly and studying its time course we have observed a specific pathway of assembly in which immature pore complexes associate with chromatin

before membrane formation. All the structures we have observed by electron microscopy are consistent with the specific pathway that we propose.

We have also studied DNA replication in the manipulated system because initiation in vitro provides an exceptionally stringent criterion of normal nuclear function. There is growing evidence that replication of nonviral DNA in vitro may depend on features of nuclear structure. Three lines of evidence support this view. First, there is a correlation between the ability of extracts and templates to form nuclei in vitro and the efficiency of replication (Lohka and Masui, 1983; Blow and Laskey, 1986; Newport, 1987). Second, nuclear formation precedes the onset of DNA replication in vitro (Blow and Laskey, 1986; Blow and Watson, 1987). Third, different nuclei enter S phase at different times in this extract with a burst of synchronous or near synchronous initiation (Blow and Watson, 1987). Our results reinforce the view that normal nuclear structure is a prerequisite for DNA replication in vitro.

Materials and Methods

Preparation and Partial Fractionation of the Extract

Extracts were prepared from activated eggs of *Xenopus laevis* as previously described (Blow and Laskey, 1986). Before freezing the extract was centrifuged (rotor SW 60Ti; Beckman Instruments, Inc., Palo Alto, CA) at 100,000 *g* for 1 h. If only 1.5 ml were placed in each tube the extract was fractionated into a clear supernatant (the soluble fraction), a loose membranous pellet, and a dense, golden, gellike pellet. The soluble fraction was removed, glycerol added to 7%, and the mixture frozen by dropping 15-μl aliquots into liquid nitrogen. The membranous pellet was washed in 5 ml of the extraction buffer (Blow and Laskey, 1986) containing 5% glycerol and pelleted (rotor SW 60Ti; Beckman Instruments, Inc.) at 100,000 *g* for 10 min. This washed vesicular fraction was frozen in liquid nitrogen in 5-μl aliquots.

In Vitro Nuclear Formation

After thawing the soluble fraction was supplemented with 6 mM phosphocreatine (Sigma Chemical Co., Poole, England) and 150 μg/ml creatine phosphokinase (Sigma Chemical Co.). Demembranated sperm nuclei and thawed vesicular fraction were added in amounts appropriate to the experiment. Optimum nuclear formation occurred at a DNA concentration of 5 ng/μl soluble fraction and a soluble/vesicular fraction ratio of 10:1. This approximates the ratio of the total soluble to the total vesicular fraction volume isolated from a single batch of eggs. Demembranated sperm nuclei were prepared and stored as described in Blow and Laskey (1986). The amount of the soluble fraction used per incubation varied between 15 μl for light microscopy and 50 μl for electron microscopy. Incubations were performed at 23°C.

Microscopy

Nuclei were prepared for phase and fluorescent microscopy as described in Blow and Watson (1987). Samples for electron microscopy were stopped by overlaying with 200 μl of cold fixative (0.25% tannic acid, 0.1% glutaraldehyde, 2% paraformaldehyde, 2% dimethylsulphoxide, 0.1 M cacodylate buffer pH 7.4) and kept on ice for 3 h. A further 800 μl of cold fixative was then added and the samples kept at 4°C overnight. The samples were spun at 600 *g* for 5 min and the pellets washed three times with 0.1 M cacodylate buffer pH 7.4. Pellets were postfixed for 3 h at 4°C using 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4, then washed a further three times in this buffer before block staining overnight in 2% uranyl acetate. The samples were dehydrated through ethanol and embedded using acrylic resin (LR White; London Resin Co. Ltd., London, England). Blocks were sectioned to give silver to pale gold sections that were taken onto collodion- and carbon-coated 600-mesh copper grids before double staining using uranyl acetate and Reynold's lead citrate (5 min each). The sections were viewed using a Phillips EM300 at 60 kV.

In Vitro Replication

The soluble fraction was thawed and supplemented with 60 mM phosphocreatine (Sigma Chemical Co.), 150 μg/ml creatine phosphokinase (Sigma Chemical Co.), and either [α-^{32}P]dATP at 0.1 μCi/μl or 40 μM biotin-11-dUTP (Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland). This was then mixed with the appropriate amounts of the washed vesicular fraction and demembranated sperm nuclei. Incubations were performed at 23°C. Biotin-11-dUTP incorporation was assessed by fluorescent microscopy. The radioactive incubations were stopped with 200 μl Stop C (0.5% SDS, 20 mM Tris-Cl pH 8, 20 mM EDTA), digested with 0.5 μg/μl proteinase K (Sigma Chemical Co.) for 1 h at 37°C, and extracted with phenol chloroform. Incorporation of [α-^{32}P]dATP was assayed by TCA-precipitable counts (Blow and Laskey, 1986).

Endogenous dATP Pool Measurement

The endogenous dATP pool in the soluble fraction was measured by performing parallel incubations using single-stranded M13 for the template with varying concentrations of exogenous dATP and a constant concentration of [α-^{32}P]dATP and [^{3}H]dTTP. Endogenous dATP concentrations were calculated assuming the following relation: ^{32}P incorporated × (exogenous dATP + endogenous dATP) = constant × ^{3}H incorporated. This gave an average dATP pool of 70 μM.

Results

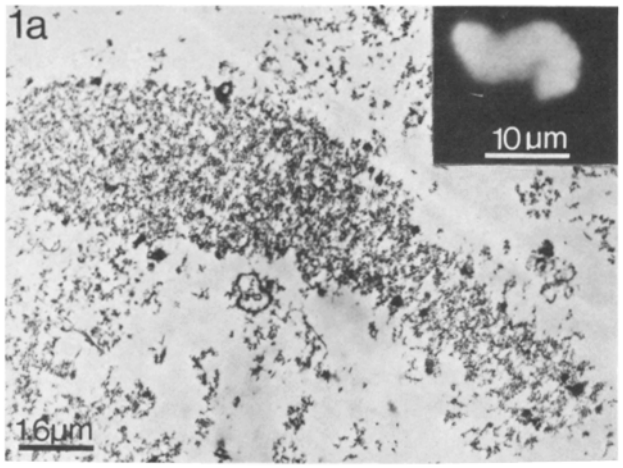
Fractionating the Extract

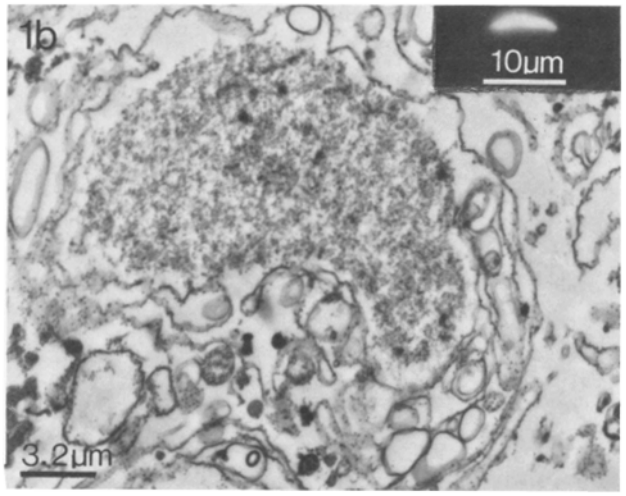
The extract was fractionated as described in Materials and Methods. An extract similar to that of Blow and Laskey (1986) was centrifuged at 100,000 *g* for 1 h. The clear supernatant was removed, glycerol added to 7%, and the mixture frozen in liquid nitrogen. This soluble fraction contains very few structures when examined by electron microscopy. The loose membranous pellet was washed in extraction buffer, spun down, resuspended in a minimum volume of buffer containing 5% glycerol, and also frozen. This vesicular fraction is rich in membrane vesicles when studied using the electron microscope. The golden, gellike pellet beneath the vesicular fraction, consisting mainly of ribosomes, was discarded.

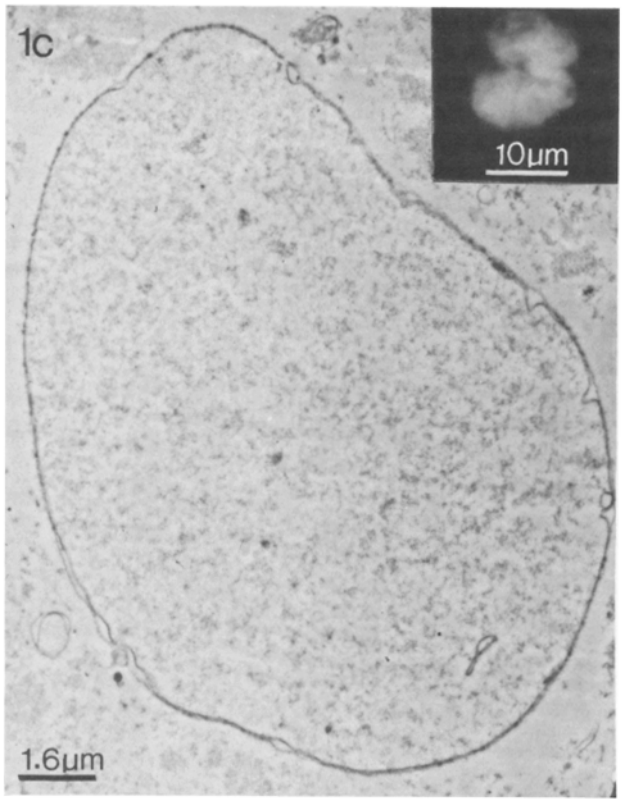
Nuclear Formation in the Reconstituted System

Previous studies have shown that similar in vitro systems support nuclear formation from both condensed sperm chromatin and naked DNA (see Introduction). Fig. 1 shows the morphology of demembranated sperm nuclei after incubation in the different extract fractions. Incorporation of biotin-11-dUTP was used as a measure of in vitro DNA replication (Blow and Watson, 1987). When sperm nuclei are incubated in the soluble fraction the chromatin decondenses but no other structures such as membranes, nuclear pores, or prepores are observed (Fig. 1 *a*). The degree of decondensation varies but is always greater than that with incubation buffer alone (Fig. 1 *a*, *inset*; Barry and Merriam, 1972). No incorporation of biotinylated dUTP is detected, showing that DNA replication has not occurred.

Sperm nuclei incubated in the vesicular fraction alone are decondensed to the same, very limited extent (Fig. 1 *b*, *inset*), comparable to that seen in incubation buffer alone. Under phase contrast optics these structures appear to be surrounded by a membrane. No incorporation of biotinylated dUTP is detected. Electron micrographs of these structures show that the chromatin is surrounded by membranes that are decorated with ribosome-like particles (Fig. 1 *b*); similar







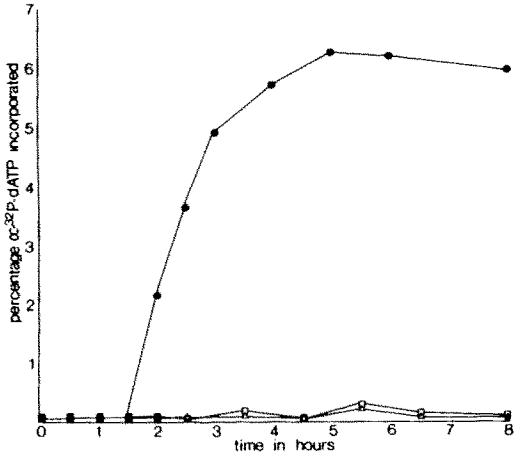


Figure 2. Demembranated sperm nuclei were incubated in: (*a*) the soluble fraction alone (△) at 6 ng DNA per µl; (*b*) the vesicular fraction alone (□) at 6 ng DNA per µl; and (*c*) the soluble and vesicular fractions recombined in a ratio of 10:1 (●) at 6 ng DNA per µl soluble fraction. The soluble fraction was supplemented with 0.1 µCi/µl [α-^{32}P]dATP, 60 mM phosphocreatine, and 150 µg/ml creatine phosphokinase. The vesicular fraction in *b* was also supplemented with these and with 70 µM dATP. 10-µl samples were taken over 8 h, added to 200 µl Stop C (see Materials and Methods), digested with proteinase K, extracted with phenol-chloroform, and the percentage of TCA-precipitatable counts determined.

particles can be seen on outer nuclear membrane (see below). No organized double membrane structure is seen, and no porelike structures are associated with the membrane.

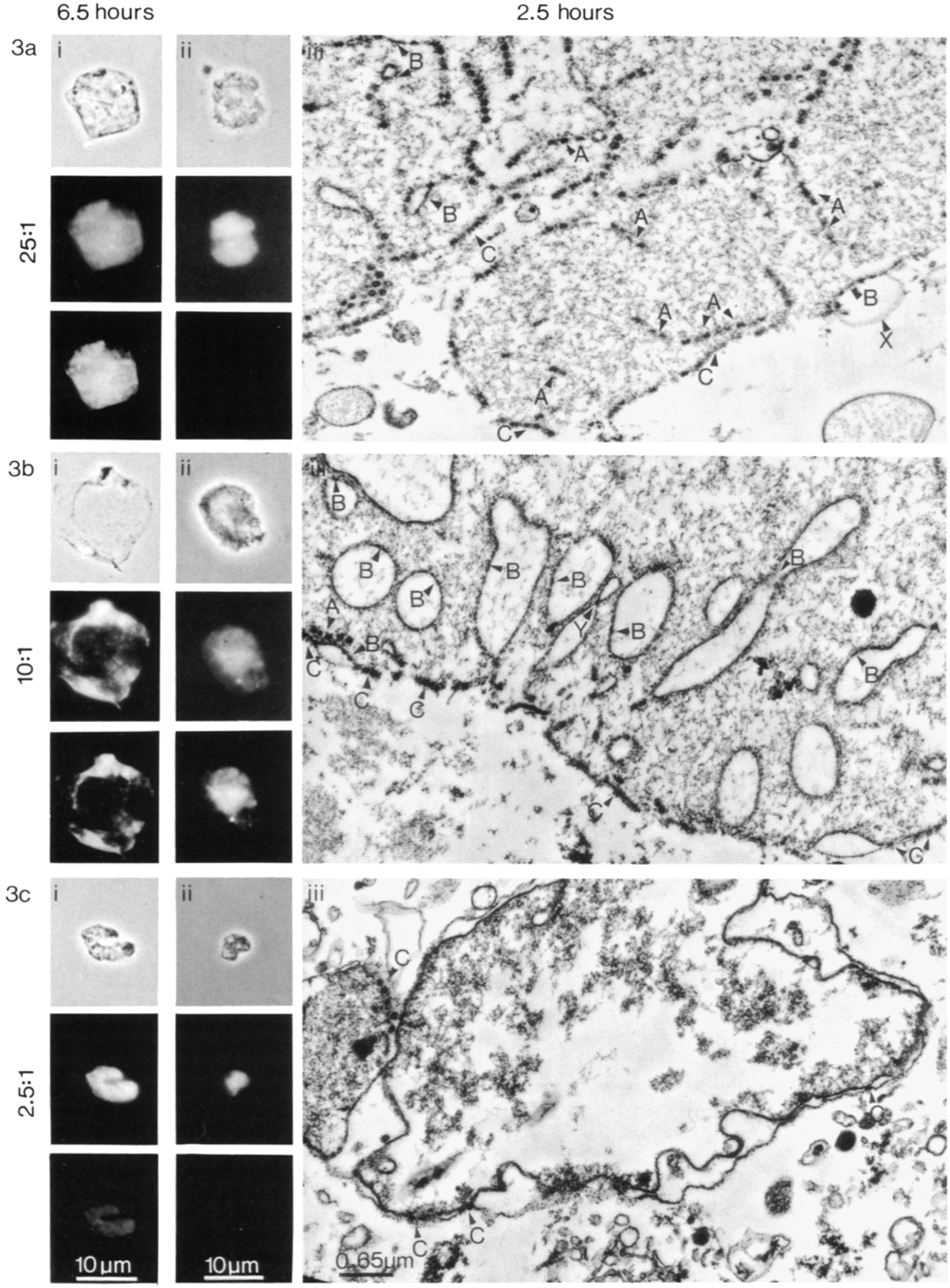
In contrast, nuclei formed when the soluble and the vesicular fractions are mixed resemble those formed in the whole extract. Light microscopy shows that >95% of the input demembranated sperm nuclei have decondensed (Fig. 1 *c*, *inset*) and are surrounded by membrane. These nuclei incorporate biotinylated dUTP, indicating that DNA replication has occurred. When examined by electron microscopy the nuclei are surrounded by a double unit membrane in which pores are embedded.

Thus neither the soluble nor the vesicular fraction alone support the formation of normal nuclei. Although both induce specific structural changes in the demembranated sperm nuclei, no pore structures are seen. However when these two fractions are recombined nuclei are formed. These nuclei are structurally identical to those formed in the unfractionated extract.

DNA Synthesis in the Reconstituted System

When demembranated sperm nuclei are incubated in the unfractionated extract, nuclear formation and DNA replication are highly efficient. A time course of incorporation of [α-

Figure 1. Electron micrographs of demembranated sperm nuclei incubated for 4 h in: (*a*) the soluble fraction alone at 5 ng DNA per µl; (*b*) the vesicular fraction alone at 5 ng DNA per µl; and (*c*) the soluble and vesicular fractions recombined in a ratio of 10:1 at 5 ng DNA per µl soluble fraction. The insets show nuclei from each type of incubation stained with Hoechst 33258 (Calbiochem Brand Biochemicals, Behring Diagnostics, San Diego, CA) to show the DNA.


6.5 hours
2.5 hours
3a
i
ii
iii
25:1
3b
10:1
3c
2.5:1
10 µm
10 µm
0.65µm

^{32}P]dATP shows a lag period in which no incorporation is detected, followed by a steady increase leading to a plateau representing the replication of 70–100% of the input DNA (Blow and Laskey, 1986).

When demembranated sperm nuclei are incubated in either the soluble or vesicular fractions no significant incorporation of [α^{32}P]dATP occurs (Fig. 2). However highly efficient DNA synthesis occurs when the soluble and vesicular fractions are recombined in a ratio of 10:1. This is consistent with the results obtained by others (Newport, 1987) working on similar but nonfrozen in vitro systems.

Fig. 2 shows a typical time course for the replication of demembranated sperm nuclei in the reconstituted in vitro system. This time course resembles that described by Blow and Laskey (1986). From the known pool size of 70 μM (deduced by isotope dilution; see Materials and Methods) the final level of incorporation represents replication of ~90% of the input DNA, confirming that the reconstituted system is as efficient as the complete extract. This incorporation is aphidicolin sensitive, and density substitution analyses give similar profiles to those shown for the unfractionated extract (Blow and Laskey, 1986) (data not shown). This suggests that initiation and completion of DNA replication occurs efficiently in this system.

Therefore both soluble and vesicular fractions are necessary for efficient DNA replication in vitro; however neither fraction alone will support this activity.

Varying the Proportions of the Soluble and Vesicular Fractions

The soluble and vesicular fractions were recombined in three ratios: 25:1, 10:1, and 2.5:1 (soluble/vesicular). We examined the end-point nuclei present in these incubations at 6.5 h at a DNA concentration of 5 ng DNA/μl soluble fraction. The size and shape of the nuclei varies in each incubation, but all appear to be fully membranated. The largest nuclei are seen in the 10:1 incubation (Fig. 3 *b,i*), as they are round and 15–25 μm in diameter. Nuclei in the 25:1 incubation are also round, but only 10–15 μm in diameter (Fig. 3 *a,i*). Nuclei in both the 25:1 and 10:1 incubations incorporate biotin-11-dUTP (Fig. 3, *a,i* and *b,i*, *lower panel*). In the 2.5:1 incubation the majority of the nuclei are elongated, but are only 10–20 μm long and incorporate biotin-11-dUTP poorly (Fig. 3 *c,i*). Nuclei formed in an incubation with a 5:1 ratio show an intermediate morphology, are elongated and 50 μm long, and have a level of incorporation of biotinylated dUTP between that of the 10:1 and 2.5:1 incubations (data not shown).

In the 25:1 and 10:1 incubations (see above) nuclear formation is efficient (>95%). However at 6.5 h the nuclei in the 10:1 incubation are larger than those in the 25:1. As nuclear swelling is a late event in nuclear formation this suggests that nuclear assembly proceeds faster in the 10:1 incubation. This was confirmed by examining the nuclei at earlier time points.

At 2.5 h the nuclei in the two incubations differ considerably. Many nuclei in the 25:1 incubation have no obvious membrane when examined under phase contrast and none of the nuclei had either swollen or incorporated biotinylated dUTP (Fig. 3 *a,ii*). In contrast, all the nuclei in the 10:1 incubation are surrounded by membrane as judged by phase contrast optics (Fig. 3 *b,ii*). Some of these have swollen and incorporated biotinylated dUTP. The two populations are at different stages of nuclear formation, the 10:1 population being further advanced.

Incubations at this 2.5-h time point were also examined by electron microscopy. A common nuclear type in the 25:1 incubation is shown in Fig. 3 *a,iii*. Very little membrane is present. The chromatin is highly decondensed. Porelike structures are present throughout the chromatin mass. Some are embedded in short stretches of double membrane (Fig. 3 *a,iii, arrows marked C*); these resemble mature pores. Other porelike structures are linked by a single membrane (Fig. 3 *a,iii, arrows marked B*) or are isolated on the chromatin (*arrows marked A*). We call these structures "prepores". The distribution of both types of prepores appears to follow the surface of a furrowed chromatin mass.

A common nuclear type in the 10:1 incubation is shown in Fig. 3 *b,iii*. The nucleus is almost completely surrounded by a double membrane, but single membranes are present within the chromatin mass. Prepores present within the chromatin mass are linked by a single membrane (Fig. 3 *b,iii, arrows marked B*). At the periphery of the nucleus there are pores embedded in a double membrane (Fig. 3 *b,iii, arrows marked C*) and prepores embedded in a single membrane (*arrows marked B*). Where prepores linked by a single membrane are present at the periphery there is always an "outer" membrane on its cytoplasmic side with which it is topologically continuous.

In contrast to the nuclei formed in the 10:1 and 25:1 incubations, nuclei assembled in a higher proportion of the vesicular fraction do not resemble those formed in the unfractionated extract. The majority of the nuclei in the 2.5:1 incubation at 6.5 h are small and elongated (Fig. 3 *c,i*). These nuclei also show abnormal morphologies at earlier timepoints. At 2.5 h all the nuclei have membranes as judged by phase optics and are smaller than those in the 25:1 incubation (Fig. 3 *c,ii*). None of the nuclei have incorporated biotinylated dUTP. By electron microscopy all the nuclei in the 2.5:1 incubation are surrounded by a double membrane (Fig. 3 *c,iii*). They have few pores or prepores and there are no internal membranes. A few of the nuclei appear to have no pores at all and resemble the structures formed when sperm

Figure 3. Sperm nuclei were incubated for 6.5 h (*a,i*; *b,i*; and *c,i*) and 2.5 h (*a,ii*; *a,iii*; *b,ii*; *b,iii*, *c,ii*; and *c,iii*) in one of three reconstituted extracts: (*a*, 25:1; *b*, 10:1; and *c*, 2.5:1) at a concentration of 5 ng DNA/μl soluble fraction. (*a,i*; *a,ii*; *b,i*, *b,ii*; *c,i*; and *c,ii*) The incubations were performed in the presence of 40 μM biotin-11 dUTP. The nuclei were prepared for light microscopy. (*Upper panels*) The nuclei under phase, showing the nuclear envelope. (*Middle panels*) The nuclei stained with Hoechst 33258, a fluorescent DNA-binding dye. (*Lower panels*) The nuclei stained with Texas red streptavidin that binds to the biotin-11 dUTP incorporated into the DNA. (*a,iii*; *b,iii*; and *c,iii*) 50 μl of each incubation was fixed and prepared for electron microscopy. The electron micrographs show a common nuclear type from each incubation. Unembedded prepores are indicated by arrows marked *A*, prepores in a single membrane by arrows marked *B*, pores in a double membrane by arrows marked *C*. Arrow marked *X* indicates a vesicle fusing to the prepores and arrow marked *Y* an association between prepores.

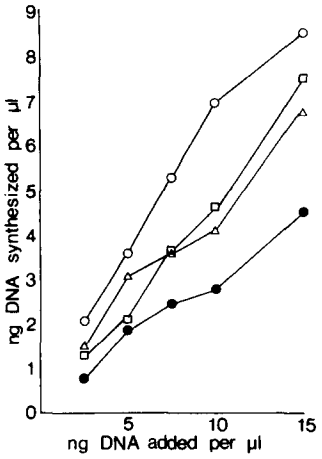


Figure 4. The soluble and vesicular fractions were recombined in a ratio of 25:1 (△), 10:1 (○), 5:1 (□), and 2.5:1 (●). Various concentrations of sperm nuclei (2.5, 5, 7.5, 10, and 15 ng DNA/µl soluble fraction) were incubated for 8 h in each of the incubations. Each incubation included 15 µl of soluble fraction. The reaction was stopped with 200 µl of Stop C (see Materials and Methods), digested with proteinase K, extracted with phenol-chloroform, TCA precipitated, and counted. The percentage of total counts precipitated was converted to nanograms of DNA synthesized using a dATP pool size of 70 µM.

nuclei are incubated in the vesicular fraction alone (Fig. 1 *b*). This suggests that high concentrations of the vesicular fraction inhibit the normal process of nuclear assembly.

The efficiency of DNA replication also varies depending on the proportion of the vesicular fraction present. Fig. 4 shows the efficiency of DNA replication over 8 h for a range of DNA concentrations and proportions of the vesicular fraction used above. The most efficient replication is achieved when the soluble and vesicular fractions are mixed in the ratio of 10:1. Incubations with ratios of soluble/vesicular of 25:1 and 5:1 support less efficient replication for all concentrations of DNA, whilst an incubation with a ratio of 2.5:1 only supports inefficient replication. For the 10:1 incubation, DNA replication is most efficient for DNA concentrations of 10 ng/µl soluble fraction and below. This is comparable with the efficiency observed in the unfractionated system (Blow and Laskey, 1986).

Thus, incubations reconstituted with lower proportions of the vesicular fraction support the formation of morphologically normal nuclei that are functionally active in DNA replication. In contrast, incubations with a high proportion of the vesicular fraction assemble structurally abnormal nuclei that support only inefficient DNA replication, indicating that they are functionally as well as structurally abnormal.

Formation of Pores

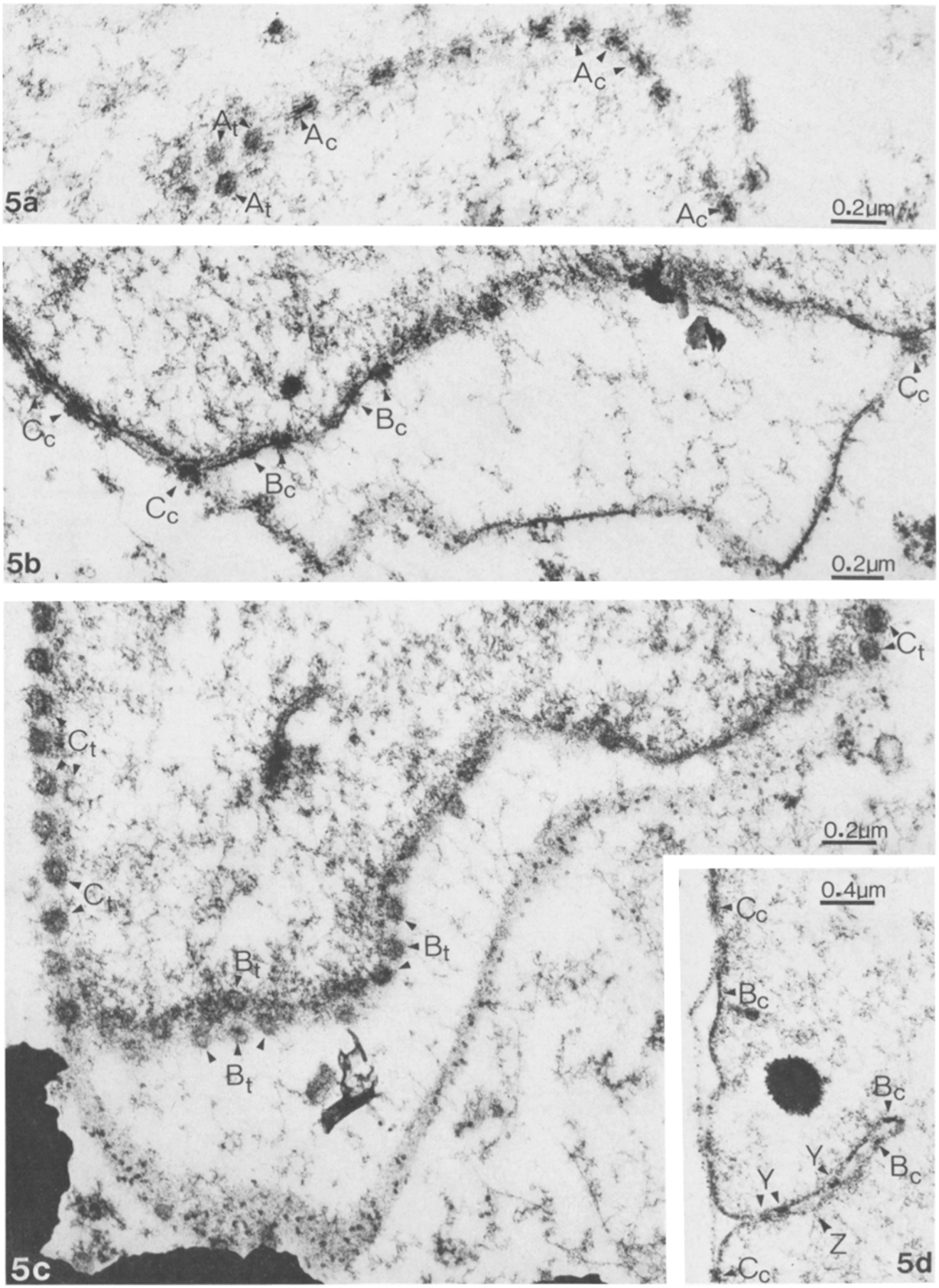
Fig. 5 shows a more detailed examination of the various porelike structures described above. Three types of structures are observed: those found isolated on the chromatin, those linked by a single membrane, and those embedded in a double membrane. When embedded in a double membrane these structures show all the features of mature pore complexes (Fig. 5, *b* and *c*). They are always found at the periphery of the nucleus, they show radial symmetry and a central channel when observed in tangential section (Fig. 5 *c*, *arrows marked* C_t), they show typical porelike structure in cross section (Fig. 5, *arrows marked* C_c), and are of similar dimensions to pore complexes observed in similarly prepared germinal vesicles from oocytes. Fig. 5, *b* and *c* show areas of the nuclear envelope where the two membranes are still separate. In the separate inner membrane we observe prepores. These structures are circular with a central channel and are of the same diameter as the pores (Fig. 5 *c*, *arrows marked* B_t). However they are smaller in cross-sectional depth (Fig. 5 *c*, *arrow* B_c). Structures very similar in cross section to those observed in single membranes are observed that are not linked by any membrane (Fig. 5 *a*, *arrow* A_c). It is likely that the structures marked A_t (Fig. 5 *a*) are these unlinked structures viewed in tangential section. If so they also appear structurally similar to pores, being circular with a central channel and of the correct diameter. The increase in size between prepores and pores corresponds to a structural link between inner and outer membranes.

The inner and outer nuclear membranes are only associated in the typical double unit membrane structure if the two membranes are linked by pore complexes (Fig. 5, *b*, *c*, and *d*). It is unlikely that the unassociated areas are artifactual as they were observed under a number of different fixation conditions and were more common in incubations where immature nuclei were abundant but were rare in incubations rich in mature nuclei that had been processed in parallel. These extracts are asynchronous with respect to nuclear formation; nuclei with a complete double nuclear membrane and those with such separated areas were observed in the same fixed pellet of extract. Prepores can be seen in the inner membrane where the membranes are separated (Figs. 3 *b,iii*, 5, *b* and *c*, *arrow B*). This unassociated inner membrane can extend into the interior of the nucleus (Fig. 5 *d*, *arrow marked Z*). The presence of such "tunnels" suggests that prepores embedded in a single membrane within the chromatin mass may be resolved into mature pores at the periphery (see Discussion).

Effects of Preincubation in Individual Fractions

We investigated the effect of preincubating the demembranated sperm nuclei with one or the other of the two fractions. From the structural study shown in Fig. 1 we know that incubating the sperm nuclei in the soluble phase leads to extensive decondensation and that incubation in the vesicular fraction causes the sperm nuclei to become surrounded by membrane. No replication occurs in either fraction alone (Fig. 2), however, replication is initiated when the second fraction is added to the preincubated mixture. The structure of the endpoint nuclei in each incubation differed. Sperm nuclei preincubated with the soluble fraction form end-point

Figure 5. Electron micrographs showing observed porelike structures. *a* is from a 25:1 incubation at 2.5 h as in Fig. 3 *c,iii* and shows prepores not linked by any membrane. *b*, *c*, and *d* are from a 10:1 incubation as in Fig. 3 *b,iii*. Both *b* and *c* show bubbles where the inner and outer membranes are unassociated and show both prepores and pores. *d* shows a tunnel where the unassociated inner membrane extends into the interior of the nucleus. Unlinked prepores are indicated by arrows marked *A*, prepores in a single membrane by arrows marked *B*, pores in a double membrane by arrows marked *C*. Subscript *t* indicates a tangential section and subscript *c* a cross-sectional section. Arrows marked *Y* show an association between prepores and arrow marked *Z* the position of a "tunnel".


5a
A_t
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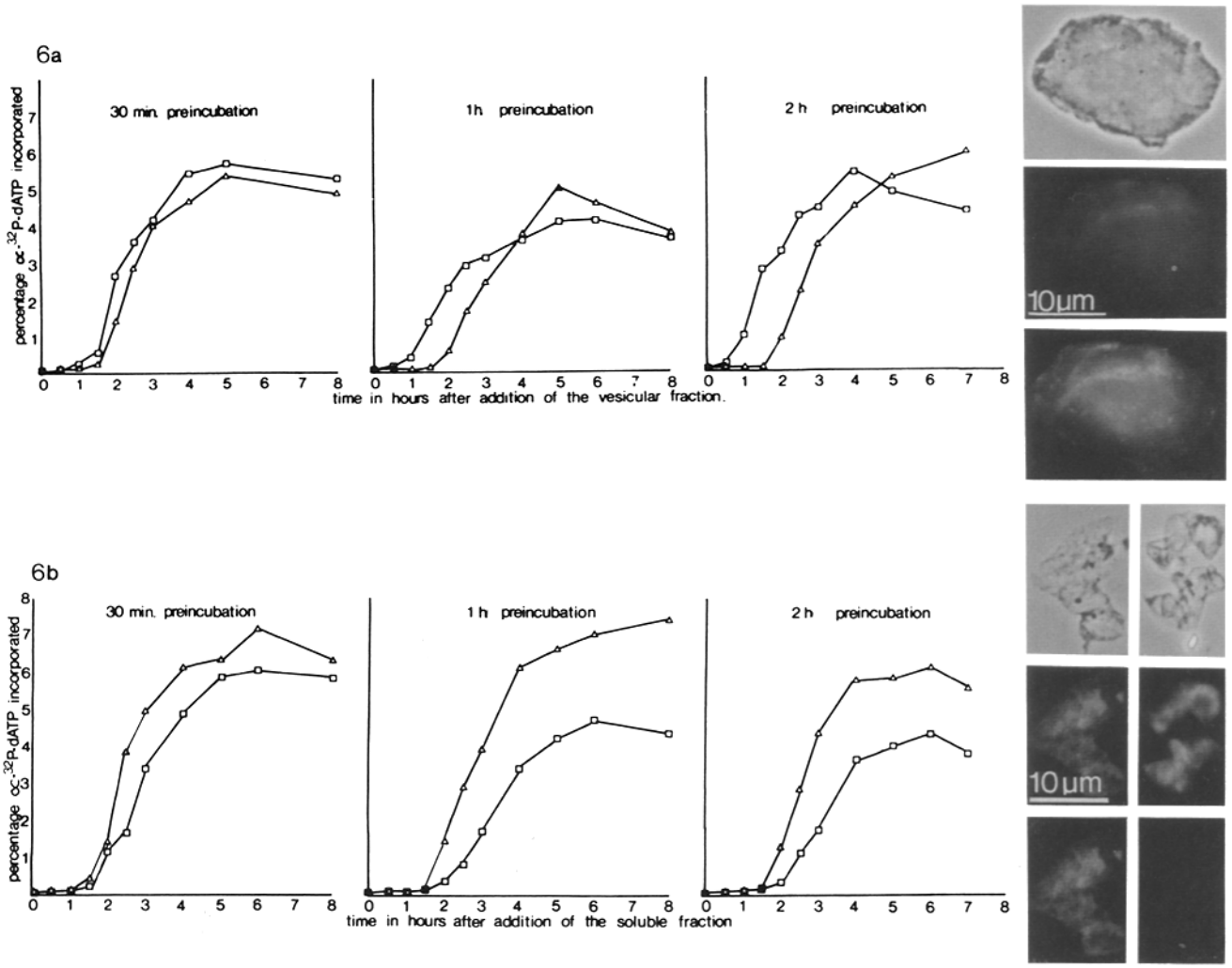

Figure 6. Time courses of DNA replication with (□) and without (△) preincubation. Sperm nuclei at 6 ng/µl soluble fraction (final concentration) were preincubated with either the soluble fraction (*a*) or the vesicular fraction (*b*) for either 30 min (*left-hand panels*), 1 (*middle panels*), or 2 h (*right-hand panels*). [α-^{32}P]dATP was included at 0.1 µCi/µl soluble fraction. The complementary fraction was added at time zero. 10-µl aliquots from each incubation were stopped at the indicated time points with 200 µl Stop C (see Materials and Methods), digested with proteinase K, extracted with phenol-chloroform, precipitated with TCA, and counted. Counts are expressed as a percentage of the total counts present in each incubation. The light micrographs show sperm nuclei that have been preincubated for 2 h in either the soluble fraction (*a*), or the vesicular fraction (*b*), and then for 6 h after the other fraction was added. Biotin-11-dUTP was included at a concentration of 40 µM. The nuclei were prepared for light microscopy. The upper panel(s) of each set show phase contrast optics, the middle panel(s) fluorescent microscopy to detect Hoechst 33528, and the lower panel fluorescent microscopy to detect biotin-11-dUTP using Texas red streptavidin.

nuclei that are structurally normal when examined by light microscopy (Fig. 6 *a*, *micrographs*). However, when the sperm nuclei are preincubated for 2 h with the vesicular fraction ~50% remain small and elongated (<15 µm long). These abnormal nuclei do not incorporate biotinylated dUTP (Fig. 6 *b*, *lower right micrograph*). Even the nuclei that incorporate dUTP are elongated rather than round (Fig. 6 *b*, *left-hand micrographs*).

Fig. 6 *a* shows a time course of replication when sperm nuclei are preincubated in the soluble fraction at 23°C. The lag before the onset of DNA replication is significantly decreased by preincubation of the template with the soluble fraction. The lag is not decreased when the soluble fraction is incubated alone. This implies that a significant proportion of the prereplicative lag in the reconstituted system is required for an interaction between the sperm nuclei and the soluble fraction. With no glycerol present (possible only using a nonfrozen extract) the lag can be completely abolished by preincubation of the sperm nuclei with the soluble fraction; the decondensed chromatin acquires a membrane immediately as judged by phase contrast optics (data not shown). It is possible that the prereplicative lag is required for the decondensation of the chromatin, or for some other interaction between sperm nuclei and the soluble fraction not indicated by our structural study. An interaction between the sperm nuclei and some component of the soluble fraction appears to be an early event in nuclear formation.

Fig. 6 *b* shows the effect of preincubating sperm nuclei with the vesicular fraction. The subsequent replication is progressively inhibited by longer preincubations. This is due

to some interaction between the sperm nuclei and the vesicular fraction, as no such inhibition occurs when the vesicular fraction is preincubated alone. The prereplicative lag is not decreased by preincubation with the vesicular fraction. Therefore, acquisition of a membrane is not the normal initial event, in fact such a membrane appears to inhibit the formation of normal, active nuclei. It is possible that this membrane acts as a barrier that prevents interactions between the chromatin and components of the soluble fraction or the complete extract. Without these interactions the normal primary events in nuclear formation do not occur, e.g., decondensation (requiring the soluble fraction) and the formation of prepores (requiring both fractions). The replication that does occur is possibly due to the dynamic nature of the membrane that surrounds the preincubated sperm nuclei. This membrane is not bound to the chromatin, as no prepores are present (Fig. 1 *b*).

Discussion

We have investigated nuclear formation in a cell-free extract of activated *Xenopus* eggs. This allowed us to construct a series of structural changes that demembranated sperm nuclei undergo before they replicate in vitro. The sperm nuclei are decondensed and then assembled into normal nuclei. By manipulating the proportions of the soluble and vesicular components of the in vitro system we have identified a number of novel events in this process of nuclear formation. Only incubations that proceeded to DNA replication were considered to contain intermediates in the normal pathway of nuclear assembly.

Observed Events in Nuclear Formation

The unfractionated extract is asynchronous with respect to nuclear formation. At time zero all the chromatin is in the form of highly condensed demembranated sperm nuclei. Endpoint nuclei have decondensed chromatin and a double nuclear membrane studded with pores. They also incorporate biotinylated dUTP. However at any time between these stages a range of nuclear types is present.

We fractionated the extract by centrifugation (Lohka and Masui, 1984). Neither of the major fractions, the soluble or vesicular fractions, supports the formation of nuclei from demembranated sperm chromatin. Although the structures formed are structurally and functionally abnormal, studying them allowed us to assign certain activities to the different fractions. Decondensation of the input chromatin is a property of the soluble fraction (Fig. 1 *a*, *inset*). This decondensation, or some other interaction between the soluble fraction and the chromatin, appears to be one of the first events in nuclear assembly as sperm nuclei preincubated in the soluble fraction form nuclei and proceed to replicate faster than untreated sperm chromatin. The membrane of the mature nucleus is derived from the vesicular fraction. The vesicles are able to surround chromatin independent of the soluble fraction (Fig. 1 *b*). Sperm nuclei incubated in either of the fractions alone show no porelike structures.

Recombining the soluble and vesicular fractions reconstitutes the in vitro nuclear assembly system. Structurally normal nuclei are formed that proceeded to replicate their DNA, indicating normal function.

Table I. Proposed Events in Nuclear Formation from Highly Condensed Sperm Nuclei

1. Chromatin decondensation.*
2. Appearance of prepores on the surface of the chromatin.‡
3. Binding of membrane vesicles to prepores.
4. Fusion of vesicles to form a convoluted inner membrane and smooth outer membrane.
5. Joining of inner and outer membrane at pore complexes; formation of mature pores.
6. Fusion of vesicles to outer membrane, resolution of convolutions.
7. Nuclear swelling.§
8. DNA replication.‖

* Requires soluble fraction.
‡ Requires both fractions.
§ Probably due to protein transport.
‖ Initiation occurs in the reconstituted and whole extract. Chain elongation but not initiation can occur in the soluble fraction.

To isolate intermediates in nuclear assembly we limited the amount of the vesicular fraction present in the incubation. This slows the membrane acquisition step but still leads to the efficient formation of structurally normal and functional nuclei, indicating that the structures that we observed at early time points are resolved to form typical mature nuclei. All the structures we have described can be observed at early time points when sperm nuclei are incubated in the unfractionated extract (data not shown) and are also resolved during the assembly process.

This allowed us to identify a prepore structure (Figs. 3 and 5). Although these prepores are observed on the chromatin without any associated membrane (Figs. 3, *a,ii*, 5 *a*, *arrows marked A*) their appearance is dependent on the presence of both the soluble and the vesicular fraction. These prepores are observed in the complete extract at early time points, but they are rare, and are often confined to a limited area within a nucleus. This suggests that they are transient and short-lived during nuclear formation in the unfractionated extract.

These prepores appear to act as receptors for vesicle binding to the decondensed chromatin (Fig. 3 *a,iii*, *arrow marked X*). Initially this vesicle binding gives rise to areas of single membrane in which these prepores are embedded. These single membranes occur both within the nucleus and at the nuclear periphery where they are continuous with the inner membrane of the double membrane structure (Figs. 3 *b*, 5, *b* and *c*, *arrows marked B*). The single membrane structure is structurally similar in both locations, and the two may be continuous (Fig. 5 *d*, *arrow marked Z*). Our study indicated that pores form when a prepore embedded in a single membrane interacts with the outer nuclear membrane (Fig. 5, *b* and *c*).

We increased the proportion of the vesicular fraction present until structurally and functionally abnormal nuclei were formed (Fig. 3 *c*). The structure of such nuclei suggests that the premature acquisition of a nuclear membrane prevents proper chromatin decondensation and prepore formation. A similar phenomenon is observed when the sperm nuclei are preincubated with the vesicular fraction (Figs. 1 *b* and 6 *b*). This indicates that the events of nuclear assembly must occur in a strict temporal order at the level of individual nuclei.

We can construct a series of events that accounts for all the structures that we observe and with the temporal order that our experiments suggest. This is outlined in Table I.

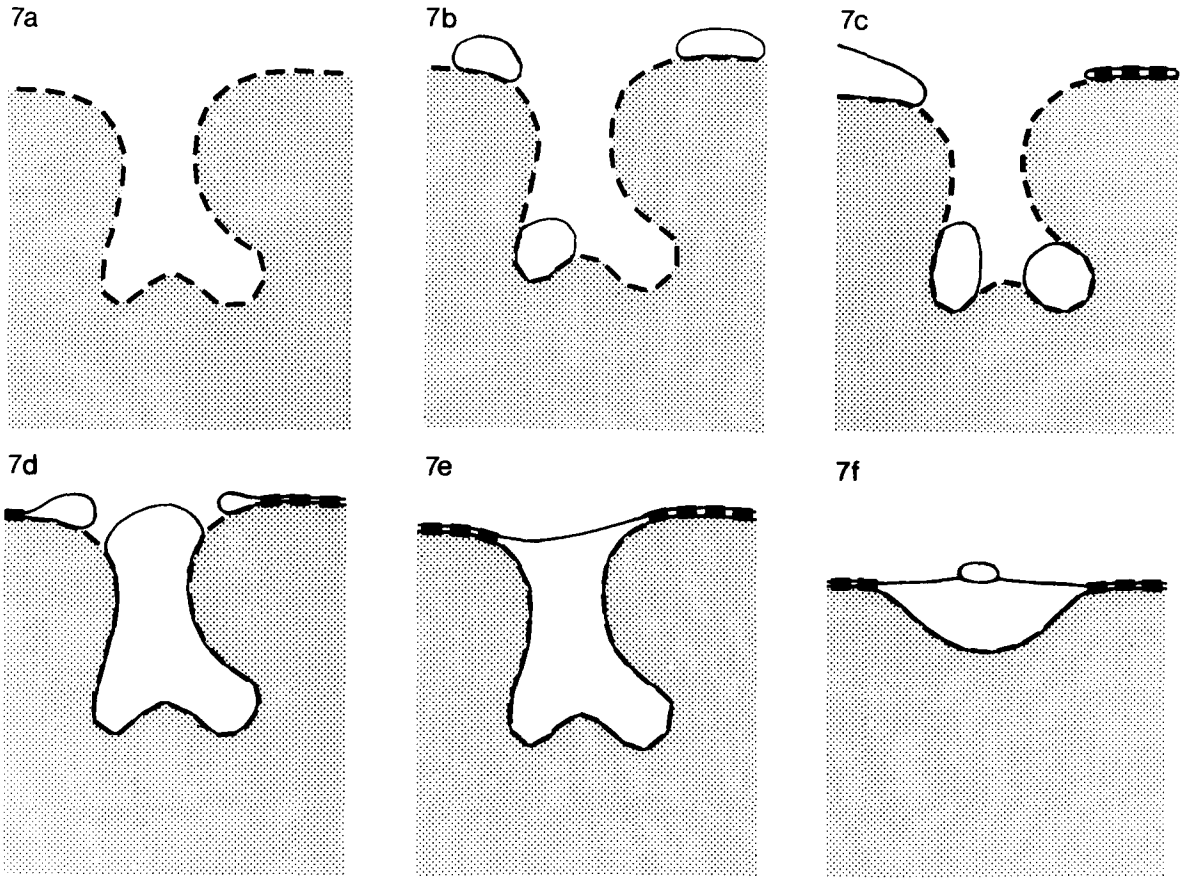

Figure 7. A diagrammatic representation of the assembly of the nuclear envelope. The shaded area represents the chromatin, the thin oblongs prepores, and the thick oblongs mature pores. The thin lines represent membrane.

A Model for Nuclear Formation

We propose a model that accounts for all these observed features of nuclear formation (Table I). Upon decondensation of the chromatin mass, deep furrows appear. Prepores form on the exposed surfaces (Fig. 3 *a,iii*). Vesicles bind to these prepores. Each vesicle binds to as many prepores as is topologically possible.

If a vesicle binds to prepores on the outside of the chromatin mass, up to half the surface of the vesicle could interact with prepores (Fig. 3 *a,iii, arrow marked X*). This could bring the two faces of the vesicle into close proximity and would give rise to a small area of mature nuclear membrane. Such limited areas of double membrane are observed. Mature pores are observed in such structures (Fig. 3 *a,iii, arrows marked C*; Fig. 7 *c*).

However a vesicle binding to prepores on a surface within a furrow would give rise to different structures. Fig. 7 illustrates how prepores on a complex inner surface could give rise to a tunnel lined with a single membrane. Fig. 3 *b,iii* (*arrows marked B*) may represent cross sections of such tunnels. Such a large vesicle lining a tunnel could fuse with a portion of double nuclear membrane at the periphery (Fig. 7 *e*). If so the membrane at the mouth of the tunnel that is exposed to the cytosol will integrate into the outer nuclear membrane. The membrane lining the tunnel, bound to the prepores, could become continuous with the inner nuclear membrane. We think this is likely because the single membrane with embedded prepores in the interior of the nucleus is structurally very similar to that at the periphery. Also tunnel-like structures have been observed in which the lining membrane is continuous with the inner membrane at the periphery (Fig. 5 *d*).

By this process a highly furrowed chromatin mass would acquire a convoluted inner membrane with a higher surface area than its outer membrane (Fig. 3 *b,iii*). This discrepancy must be resolved if the mature nuclear structure is to be achieved (Fig. 1 *c*). One possibility is that additional vesicles bind to the outer nuclear membrane. This would be consistent with the steady increase in size we observe during nuclear formation. Studies in other systems have shown structures that are consistent with such fusion events (Burke and Gerace, 1986). We also have observed structures consistent with this mechanism (data not shown).

Mature pore complexes are formed when the outer membrane contacts the inner membrane at a prepore. The formation of a pore from a prepore is accompanied by an increase in size.

When two sections of inner membrane are brought close together, for example in a tunnel, associations are seen between the prepores (Figs. 3 *b,iii* and 5 *d, arrows marked Y*). These associations must be temporary as they are not seen in mature nuclei.

Once the outer membrane is continuous and pores have formed, proteins can accumulate in the nucleus. This would account for the nuclear swelling, which usually precedes the onset of DNA replication.

Abnormal Nuclear Structures

This model also accounts for the abnormal nuclear structures we have observed. The most common abnormal nuclear structures are "bubbles", where the inner and outer membrane are not attached.

Two types of bubble are observed. The most common has prepores in its inner membrane (Fig. 5). In our model these would arise if the inner and outer membrane had not come into contact at a prepore. Such a bubble could be resolved to form a mature nuclear envelope. The frequency of bubbles is increased with higher proportions of the vesicular fraction, as if there were competition between vesicles for the prepores.

The second type of bubble has no prepores on its inner membrane. These are observed with excessive amounts of the vesicular fraction (Fig. 3 *c,iii*). At such high concentrations vesicles would bind to the first prepores that have formed and would quickly fuse together to form a double membrane. This membrane would then act as a barrier to the formation of more prepores and any further decondensation of the chromatin. Few pores would be formed as there would be few prepores.

When demembranated sperm nuclei are incubated in the vesicular fraction alone they become surrounded by vesicles which fuse to give a continuous membrane barrier with no pores. Such a membrane could be formed by this vesicle to vesicle fusion.

Nuclear Assembly in Other Systems

Burke and Gerace (1986) have described an in vitro nuclear formation system prepared from mitotic CHO cells. An homogenate of these cells forms interphase nuclei from the endogenous mitotic chromosomes. The nuclei they describe show a number of similarities with the ones we have described. They show association of vesicles with the chromatin at early time points following the irregular surface of the chromatin mass. They observe areas where the inner and outer membranes are not associated in the fully formed nucleus. These areas are devoid of nuclear pore complexes and are similar to the bubbles we describe. Burke and Gerace (1986) also described fusion events between vesicles and the outer nuclear membrane. They do not describe any structure similar to the prepore. Their structures resemble nuclei formed in the *Xenopus* extracts with a high vesicle content.

It has been suggested that remnants of the pore complex remain associated with the chromosomes throughout mitosis in some cell types (Comings and Okada, 1970, 1976) and that these structures may be used in the formation of new pores at telophase (Maul, 1977). The prepores that we describe resemble these residual structures.

We have not attempted to study the assembly of the nuclear lamina as this has received detailed attention previously. Previous studies (Gerace and Blobel, 1980; Gerace et al., 1978) have indicated that lamins A and C bind to the chromatin and act as a receptor for lamin B (which is membrane bound) in mammalian cells. Burke and Gerace (1986) studied lamina formation in the cell-free system from CHO cells. Their data indicates that the lamins associate with the chromatin in a temporal pattern that roughly corresponds to the appearance of nuclear envelopes at the chromosome surfaces. In early *Xenopus* embryogenesis only one lamin is present, L_{III}, that is not bound to membrane vesicles during mitosis (Benavente et al., 1985; Stick and Hausen, 1985). Newport (1987) has studied the time course of lamina formation in an unfrozen, unfractionated cell-free system from *Xenopus* eggs and has shown that only decondensed nuclei surrounded by membrane bind antilamin antibodies. No antilamin immunofluorescence is seen on the chromatin before this point.

It is possible that the prepores we describe bind to a lamina that has already formed on the chromatin, but there are two arguments against this possibility. First, Burke and Gerace (1986) and Newport (1987) found similarity between the time course of nuclear lamina formation and nuclear membrane formation, while we observed that the prepores bind to the chromatin before membrane formation and then act as receptors for vesicle binding. Second, if our prepores are indeed similar to the residual pore structure described by Maul (1977) we would expect them to persist through mitosis when the lamina has been disassembled.

DNA Replication Correlates with Nuclear Formation

We provide strong evidence to support the relationship between nuclear structure and DNA replication in this system. There is a direct correlation between extracts that support nuclear formation and those that replicate DNA. Neither the soluble nor the vesicular fractions alone support the formation of normal nuclei (Fig. 1) or DNA replication (Fig. 2). Reconstituted extracts that assemble abnormal nuclei, such as those with an excess of the vesicular fraction (Fig. 4 *c*), show inefficient DNA replication. Preincubation of the demembranated sperm nuclei with the vesicular fraction, which leads to the premature acquisition of a membrane (Fig. 1 *b*), inhibits DNA replication (Fig. 6 *b*). Extracts reconstituted with lower proportions of the vesicular fraction (25:1 and 10:1) show differing rates of nuclear formation. The appearance of the first decondensed and membranated nuclei in these extracts correlated with the appearance of the first nuclei that had incorporated biotinylated dUTP. All the nuclei that had incorporated biotinylated dUTP were both decondensed and membranated. Processes that accelerate nuclear formation, such as the preincubation of the demembranated sperm nuclei with the soluble fraction, decrease the lag that occurs before DNA replication.

Thus in all cases the observed DNA replication was preceded by nuclear formation and inefficient DNA replication correlated with the formation of abnormal nuclei.

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