

A Lamin-independent Pathway for Nuclear Envelope Assembly

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Abstract. The nuclear envelope is composed of membranes, nuclear pores, and a nuclear lamina. Using a cell-free nuclear assembly extract derived from *Xenopus* eggs, we have investigated how these three components interact during nuclear assembly. We find that the *Xenopus* embryonic lamin protein L_{III} cannot bind directly to chromatin or membranes when each is present alone, but is readily incorporated into nuclei when both of the components are present together in an assembly extract. We find that depleting lamin L_{III} from an extract does not prevent formation of an envelope consisting of membranes and nuclear pores.

However, these lamin-depleted envelopes are extremely fragile and fail to grow beyond a limited extent. This suggests that lamin assembly is not required during the initial steps of nuclear envelope formation, but is required for later growth and for maintaining the structural integrity of the envelope. We also present results showing that lamins may only be incorporated into nuclei after DNA has been encapsulated within an envelope and nuclear transport has been activated. With respect to nuclear function, our results show that the presence of a nuclear lamina is required for DNA synthesis to occur within assembled nuclei.

THE outer boundary of the nucleus is defined by the nuclear envelope. This complex structure consists of three major elements: the nuclear membrane, the nuclear pores, and the nuclear lamina (for review see Newport and Forbes, 1987; Gerace and Burke, 1988). The nuclear membrane is composed of two separate lipid bilayers which act as passive barriers against diffusion of molecules into the nucleus, whereas the nuclear pores act as selective barriers regulating molecular traffic both into and out of the nucleus. These two elements together create a biochemical environment within the nucleus that is distinct in composition from that present in the cytoplasm. The nuclear lamina is a polymeric meshwork underlying the inner nuclear membrane (Aebi et al., 1986). The lamina is formed from one or more members of a family of proteins (the lamins; for review, see Franke, 1987) which share strong homology with intermediate filaments (Fisher et al., 1986; McKeon et al., 1986). How these three elements (membrane, pores, and lamina) interact to regulate the assembly of the nuclear envelope is not understood at the molecular level.

In cells that undergo an open mitosis the unique biochemical environment within the nucleus breaks down due to the disassembly of the nuclear envelope. At this time in the cell cycle the nuclear membranes are vesicularized, the nuclear pores are disassembled, and the lamina is depolymerized. Although little is known about the molecular elements causing membrane vesicularization and pore disassembly, there is good evidence that lamina depolymerization into lamin dimer or tetramers is driven by hyperphosphorylation of the lamin proteins at specific sites (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985).

In order for the nuclear envelope to reassemble at the end of mitosis, the membranes, pores, and lamins must be targeted back to the surface of chromatin. Cell-free systems that can assemble nuclei around a variety of DNA and chromatin substrates have provided much insight into how this assembly process might occur (Lohka and Masui, 1983; Lohka and Masui, 1984; Burke and Gerace, 1986; Newport, 1987; Spann and Newport, 1987; Wilson and Newport, 1988). Using a cell-free nuclear assembly extract derived from *Xenopus* eggs, we have shown that the association of nuclear membrane vesicles and chromatin is mediated by a trypsin-sensitive integral membrane receptor present on vesicles (Wilson and Newport, 1988). This membrane receptor probably recognizes a specific chromatin-bound protein. Using the same cell-free system, Sheehan et al. (1988) have presented evidence that pore formation is initiated by chromatin-bound pre-pore complexes. It is currently not clear whether these pre-pore complexes can form in the absence of membrane fraction or whether interaction with membrane vesicles is needed. In CHO cells lamina is composed of three different lamins, A, B, and C (Gerace and Blobel, 1980). During mitosis lamins A and C are present in a soluble form in the cytoplasm, whereas the bulk of lamin B appears to be associated with a membrane fraction. Using a cell-free system derived from CHO cells, Burke and Gerace (1986) have demonstrated that Lamins A and C can bind directly to metaphase chromosomes independent of membrane assembly. Furthermore, they showed that immunodepletion of lamins A and C from the cell-free extract both prevented binding of lamin B to chromosomes and strongly inhibited growth of the nuclear envelope. These results lead to the proposal that targeting of membrane vesicles back to

the surface of chromatin at the end of mitosis was the result of association between chromatin-bound lamins A and C with membrane vesicle-bound lamin B. In contradiction to this model, Benevente and Krohne (1986) have shown that injection of anti-lamin antibodies into cells during mitosis, although blocking growth of the nuclear envelope, did not prevent an envelope from forming directly on the surface of metaphase chromosomes. This latter result supports a role for lamins in envelope growth but does not necessarily support a role for lamins in targeting membrane to the surface of chromatin.

For lamins to be directly involved in the targeting of membrane vesicles to the surface of chromatin requires that a minimum of two lamin types be present within the cell: a vesicle-bound type and a chromatin-bound type. However, in recent years it has become apparent that during the early stages of development in many organisms only a single lamin is present (for review, see Krohne and Benavente, 1986). For example, in *Xenopus* both the large nucleus of the oocyte and the nuclei of the first 4,000 embryonic cells contain a single lamin of 68 kD, called L_{III} , which remains soluble during mitosis (Krohne et al., 1981; Benavente et al., 1985; Stick and Hansen, 1985; Stick, 1988). Breakdown of the oocyte nuclear lamina at the completion of meiosis generates a large pool of L_{III} , which is recycled during early development to form the lamina of the first 4,000 embryonic nuclei. Similarly, during the early cleavages of both mouse and surf clam embryos only a single lamin of the B type has been identified (Schatten et al., 1985; Stewart and Burke, 1987). Furthermore, stable cell lines derived from embryonic tissue contain a single lamin of the B type and lack both lamins A and C (Lebel et al., 1987; Stewart and Burke, 1987). That a single lamin appears sufficient to construct the nuclei in an entire organism is indicated by the fact that the lamins present in *Drosophila* are encoded by a single gene (Gruenbaum et al., 1988). Since the nuclear envelope of all of the above actively dividing cell types can reform at the end of mitosis, the initial targeting mechanism directing vesicles to the surface of the chromosome may not depend exclusively on the association of different lamin types.

To examine how the nuclear envelope assembles and to assess the role of the lamin protein L_{III} during assembly, we have used a fractionated *Xenopus* cell-free nuclear assembly extract. We find that quantitative immunodepletion of the *Xenopus* lamin L_{III} from the nuclear assembly extract does not block the assembly of either the nuclear membrane or the nuclear pores around a chromatin substrate. Lamin depletion does, however, appear to inhibit subsequent growth of this newly formed envelope and blocks DNA replication. We also find that L_{III} cannot associate directly with either chromatin or membrane vesicles alone, but that assembly of an intact polymeric L_{III} within the nucleus is linked to and dependent on nuclear transport. Our results support a model whereby nuclear assembly at the end of mitosis occurs first by formation of an envelope capable of transport and then growth of this initial envelope occurs due to the assembly of a lamina.

Materials and Methods

Nuclear Assembly Extracts

Fractionated nuclear assembly extracts were prepared from *Xenopus* eggs

essentially as described in Wilson and Newport (1988). Briefly, unfertilized dejellied eggs were rinsed in buffer, packed at 500 g for 30 s in a clinical centrifuge to remove excess buffer, and then broken by centrifugation at 10,000 rpm in a HB-4 rotor for 10 min. After this the crude assembly extract was fractionated into membranous and soluble fractions by centrifugation at 55,000 rpm in a TL-100 centrifuge using a TLS-55 rotor. The light membranous fraction was collected and either fractionated as described earlier (Wilson and Newport, 1988) on block sucrose gradients or washed and used without further fractionation. To wash the membrane fraction the membranes were resuspended in 10 vol of buffer B and then layered over a cushion of 2 M sucrose in buffer B and pelleted at 20,000 rpm for 20 min in a T100 centrifuge using a TLS-55 rotor. The membrane pellet was then rinsed and resuspended at a concentration equal to one-tenth of the volume of the soluble fraction and then frozen in liquid nitrogen and stored at -70°C until use. The soluble fraction was centrifuged a second time at 55,000 rpm for 20 min to remove the minor amount of membrane material remaining before it was frozen in nitrogen and stored at -70°C . Buffer B, 0.5 M sucrose, 5 mM KCl, 2 mM MgCl_2 , 10 mM Hepes, pH 7.5, and 1 mM DTT.

Depletion of L_{III} from Extracts

To deplete L_{III} from the soluble fraction of a nuclear assembly extract 40 μl of packed Staph A in PBS was incubated with 200 μg of affinity purified rabbit anti-mouse IgG (Cappel Laboratories, Malvern, PA) for 4 h with gentle rotation. The unbound rabbit anti-mouse IgG was then removed by repeated washing of the Staph A with PBS containing 0.1% TX100 followed by centrifugation. The Staph A rabbit anti-mouse IgG complex was then resuspended in 400 μl of ascites containing anti- L_{III} antibody (L46F7 provided by G. Krohne; see Benavente et al., 1985) and this mixture was then incubated for 4 h at 4°C with gentle rocking, followed by several washes in PBS. The final Staph A-bound anti- L_{III} complex was resuspended in 500 μl of the soluble nuclear assembly fraction containing an ATP regenerating system and incubated with gentle mixing for 4 h at 4°C . After this the L_{III} bound to the protein A antibody complex was removed from the soluble fraction by two 5-min centrifugations in an Eppendorf centrifuge. To determine the extent of the depletion of L_{III} from the soluble fraction by this method, aliquots of depleted and untreated extracts were analyzed for the presence of L_{III} via Western blotting.

Immunofluorescent Staining of Nuclei

Nuclei were stained for the presence of L_{III} by fixing the nuclei for 5 min with 3% formaldehyde in PBS, washing the nuclei in PBS containing 1% glycine several times, staining nuclei with anti- L_{III} for 30 min, washing two times with PBS containing 0.1% Triton X-100 staining with rhodamine-labeled rabbit anti-mouse IgG for 30 min, and then washing three times with PBS containing 0.1% Triton X-100. To wash nuclei during the staining the nuclei were pelleted at 1,000 g for 5 min. Chromatin was stained for L_{III} binding directly by observing binding of rhodamine-labeled anti- L_{III} . Rhodamine was covalently linked to anti- L_{III} as described in Newmeyer et al., 1986b.

Inhibition of Nuclear Transport

Transport of lamins into nuclei was blocked by adding 1 mg/ml wheat germ agglutinin (WGA)¹ to assembly extracts containing membranes and sperm nuclei (1,000 sperm/ μl of extract). To reverse transport inhibition N,N,N' -triacetylchitotriose was added to 1 mM.

Sedimentation of Lamin Proteins

The sedimentation value of interphase extracts and activated eggs was determined using 5–30% sucrose gradients centrifuged for 19 h at 35,000 rpm in a SW40 rotor as described by Benavente et al. (1985). Eggs were activated by pricking with a needle or fertilizing with sperm and were then allowed to incubate for 6 h in media containing 100 $\mu\text{g}/\text{ml}$ of cycloheximide before lysis. The location of L_{III} within the gradient was determined by Western blotting of fractions collected from the gradient using anti- L_{III} antibody.

Assays for DNA Replication

DNA replication in complete and L_{III} -depleted extracts was measured by monitoring the rate of incorporation of radiolabeled $\alpha^{32}\text{P}$ dCTP into

1. Abbreviation used in this paper: WGA, wheat germ agglutinin.

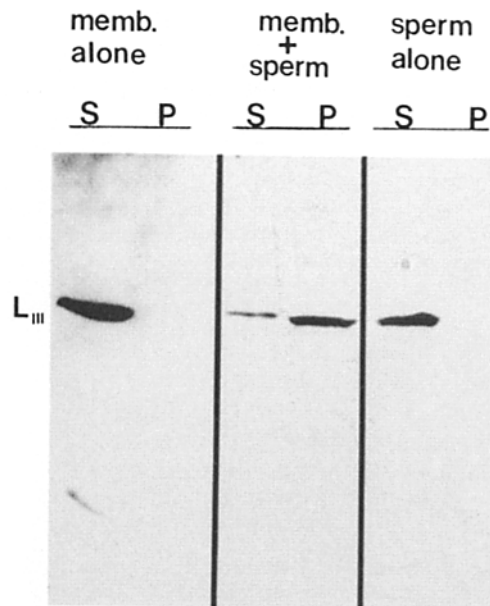
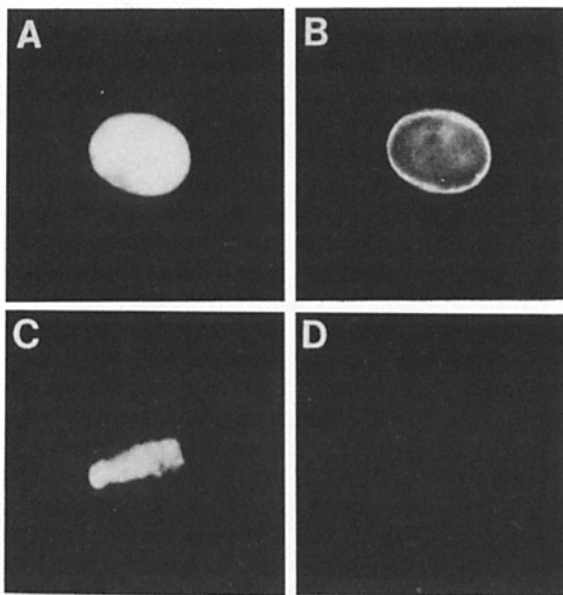
A**B**

Figure 1. Binding of lamin L_{III} to chromatin and membranes probed with immunoblotting and immunofluorescence. (A) The soluble fraction of an assembly extract was mixed with either membrane components alone (left), membrane components and sperm chromatin (middle; 1,500 sperm/ μ l), or sperm chromatin alone (right; 1,500 sperm/ μ l). Each reaction was incubated for 3 h at 22°C and then the membranes, assembled nuclei, or decondensed sperm chromatin were separated from the soluble fraction by centrifugation. The soluble (S) and pellet (P) fractions from each sample were then analyzed for the presence of L_{III} via immunoblotting. In the presence of either membranes (left) or chromatin alone (right), L_{III} remained soluble, whereas when both chromatin and membranes were present L_{III} became pelletable (middle). (B) The soluble fraction of the assembly extract was mixed with either chromatin alone (1,500 sperm/ μ l) or chromatin and membranes. After a 3-h incubation the reaction was made 0.1% in Triton X-100 and then rhodamine-labeled anti-L_{III} antibody was added. After 20 min samples were stained with the fluorescent DNA dye Hoechst

DNA assembled into nuclei. Extracts containing 1,000 sperm/ μ l of extract were incubated with α [³²P]dCTP (10 μ Ci/100 μ l of extract) for 3 h at room temperature. After this the DNA was isolated and run on agarose gels as described previously (Newport, 1987).

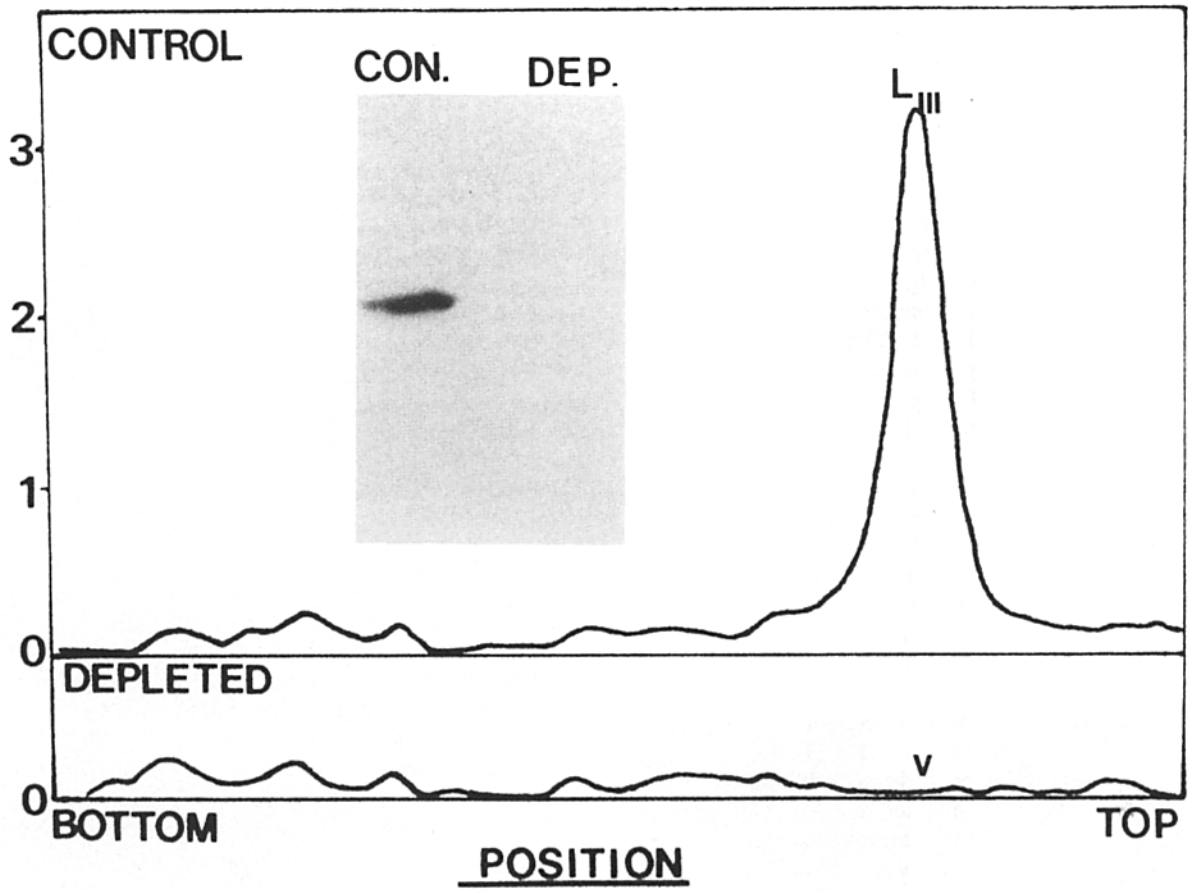
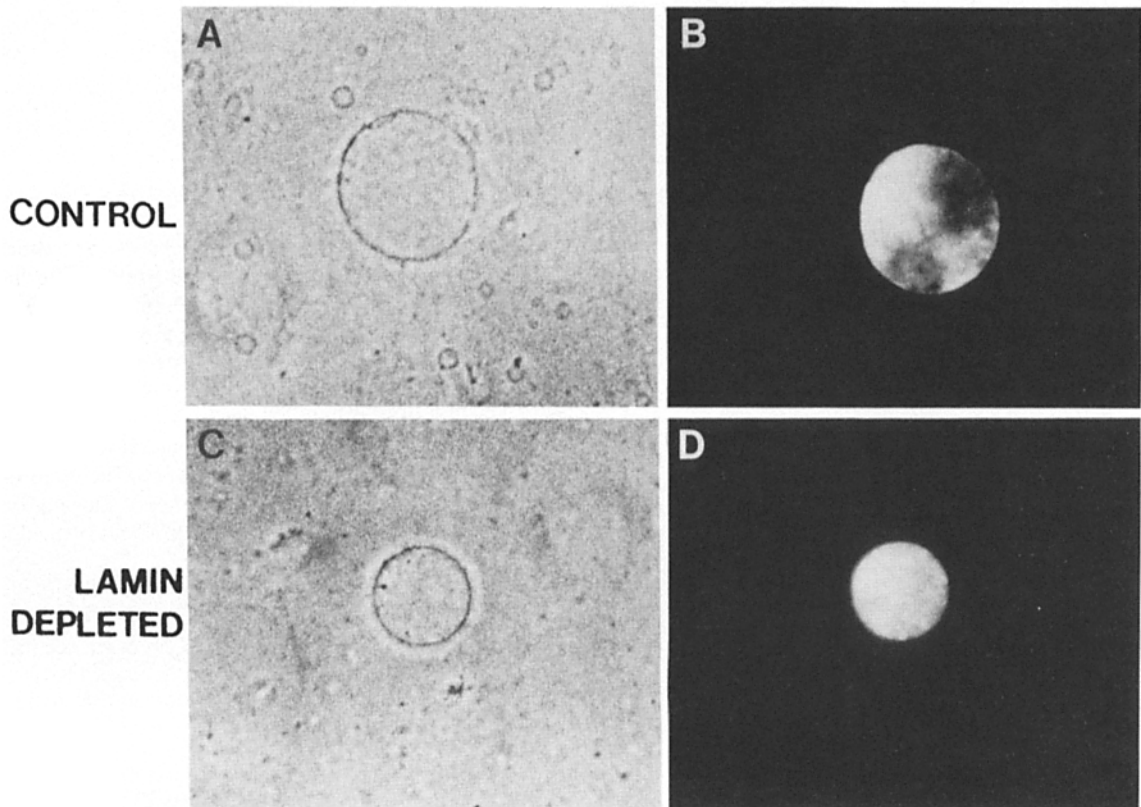
Results

Crude extracts of *Xenopus* eggs can be used to reconstitute intact nuclei around sperm chromatin, metaphase chromosomes, or DNA (Lohka and Masui, 1983, 1984; Newport, 1987). Fractionation of the crude extracts via centrifugation (see Materials and Methods) can be used to separate soluble components from total membrane components (Lohka and Masui, 1984; Wilson and Newport, 1988). The total membrane components can be further separated into light and heavy fractions by sedimentation through sucrose block gradients (Wilson and Newport, 1988). When the partially purified light membrane fraction is mixed with the soluble fraction containing sperm chromatin, metaphase chromosome, or DNA, intact nuclear envelopes form around the DNA templates (see Fig. 1 B, panels A and B). To determine whether the soluble egg lamin L_{III} is incorporated into the envelope of assembling nuclei in vitro, we asked whether L_{III} became converted into a pelletable form with nuclear assembly (Stick and Hansen, 1985). For this, we mixed purified membranes, soluble assembly components, and sperm chromatin and allowed nuclei to form for 2 h. The intact nuclei and unincorporated membranes were then separated from the soluble fraction by low speed centrifugation. The amount of L_{III} in the pellet and soluble fractions was determined from Western blots using an anti-L_{III} primary antibody and an iodinated rabbit anti-mouse secondary antibody. As shown in Fig. 1 A (middle), most (80%) of the L_{III} was found in the pellet under these conditions. Similar experiments demonstrated that the amount of L_{III} present in the pellet was directly proportional to the number of sperm used to initiate nuclear formation in an assembly mixture. All of the L_{III} (>95%) was found in the pellet at sperm concentrations >3,000 sperm/ μ l of soluble fraction (not shown). These results demonstrate both that L_{III} is incorporated into the nuclei forming in our reconstitution system, and that all of the L_{III} initially present in the soluble fraction is competent to participate in nuclear assembly (see also Stick and Hansen, 1985).

Lamin L_{III} Does Not Bind to Chromatin Directly

In CHO cells lamins A and C, like L_{III}, are present in a soluble form during mitosis. Using a cell-free nuclear assembly system derived from CHO cells, Burke and Gerace (1986) have demonstrated that these two lamins can bind directly to the surface of metaphase chromosomes in the absence of envelope formation. To examine whether L_{III} behaves in a similar manner, sperm chromatin (1,500 sperm/ μ l) and the soluble components of the nuclear assembly system were mixed together and allowed to incubate for 3 h in the absence of the membrane components. During this period the sperm chromatin decondensed \sim 50-fold based on mea-

and observed under fluorescent optics. Chromatin and membranes (panel A, DNA staining; panel B, L_{III} staining); chromatin alone (panel C, DNA staining; panel D, L_{III} staining). Note the absence of L_{III} staining in panel D.

A**B**

measurements of the increase in DNA volume. After this incubation the chromatin was separated from the soluble fraction by centrifugation and both the pellet and soluble fractions were analyzed for the presence of L_{III}. As shown in Fig. 1 *A* (right), under these conditions all of the L_{III} remained in the supernatant, indicating that L_{III} is unable to bind directly to sperm chromatin in the absence of membranes. An alternative method for addressing this question was to determine whether L_{III} could bind to chromatin in the absence of membrane components by staining the chromatin directly with an anti-L_{III} antibody carrying a rhodamine label. Results from this experiment (Fig. 1 *B*) demonstrate that in the presence of membrane components the nuclei that formed displayed intense staining (Fig. 1 *B*, panel *B*), while in the absence of membrane components decondensed chromatin showed no staining (panel *D*). Together these results indicate that under the conditions tested lamin L_{III} cannot bind directly to chromatin in the absence of membrane components.

In CHO cells lamin B, unlike lamin A, remains largely associated with membrane components during mitosis (Gerace and Blobel, 1980). To test whether the *Xenopus* lamin L_{III} could associate directly with membranes in the absence of a chromatin substrate we mixed both the soluble and membrane fractions of the nuclear assembly extract together and incubated this mixture for 3 h at 22°C. Afterward membranes were separated from the soluble components by centrifugation and each fraction was assayed for the presence of L_{III} by immunoblotting. As shown in Fig. 1 *A* (left), lamin L_{III} was quantitatively retained in the soluble fraction under these conditions. This demonstrates that L_{III}, unlike lamin B, does not associate with membranes alone. Furthermore, this observation in conjunction with the previous results demonstrates that stable interaction of the lamin L_{III} with either membranes or chromatin requires that both of these elements be present.

Depletion of L_{III} Does Not Prevent Nuclear Envelope Formation

Because L_{III} associates only with nuclei and not with the individual components making up the nucleus (membranes or chromatin), this raises the possibility that L_{III} is incorporated into nuclei after these two components have associated with each other. In this case, lamina formation might not be a primary or necessary event for envelope formation. If true, one would predict that the nuclear envelope would be able to form under conditions in which L_{III} was absent. To test this prediction L_{III} was depleted from the soluble *Xenopus* extract by first coupling an L_{III} monoclonal antibody (generous gift of G. Krohne) to Staph A and then incubating the Staph A-anti-L_{III} with the soluble fraction for 5 h at 4°C. The Staph A- α -L_{III} complex containing bound L_{III} was separated from the soluble components by centrifugation.

This method provided a means of quantitatively removing all (>99.75%) of the L_{III} from the soluble fraction as judged by Western analysis of the depleted fraction (Fig. 2 *A*).

Quite surprisingly, when the L_{III}-depleted soluble fraction was mixed with washed membranes and sperm chromatin (1,500 sperm/ μ l of extract), nuclei with apparently normal envelopes formed around the sperm chromatin (Fig. 2 *B*, panels *C* and *D*). Measurements of the diameter of nuclei during the first 30 min of assembly showed that initial envelope growth around sperm chromatin in depleted extracts was equal to that of control extracts. At later time periods (60 min) the envelope of control extracts continued to grow, while growth in depleted extracts slowed dramatically. By 2 h the DNA enclosed within L_{III}-depleted nuclei had decondensed \sim 60-fold in volume and the envelope surrounding this DNA was on average 15–20 times larger than the surface area of the sperm chromatin immediately after addition to the extract. Although the envelopes that formed around sperm chromatin added to L_{III}-depleted extracts appeared normal at the light microscope level, it was clear that these nuclei were structurally much more fragile than control nuclei. Thus, when aliquots of nuclei grown in control extracts were placed under a coverslip for observation, few of the control nuclei were disrupted by this procedure. In contrast, 30–50% of the nuclei grown in the depleted extract were fractured, as indicated by the presence of a discontinuous line at the perimeter of the nuclei when observed with phase optics. This fracturing could be prevented if the nuclei were first fixed in 3% glutaraldehyde, demonstrating that the envelope was largely intact, before the nuclei were subjected to the shear forces of spreading. That the nuclei which formed did indeed lack a nuclear lamina was confirmed by indirect immunofluorescent staining of the nuclei present in control and lamin-depleted extracts. As shown in Fig. 3 *C*, while control nuclei displayed an intense peripheral staining pattern typical of an intact lamina, such staining was completely absent from nuclei assembled in lamin-depleted extracts (Fig. 3 *F*). These results indicate that formation of a lamina during the initial stages of nuclear envelope formation is not necessary for membrane vesicles to associate with chromatin and fuse together to form an intact envelope. The results do, however, suggest that further envelope growth beyond this initial stage is strongly inhibited by the absence of the lamin L_{III}.

As shown above, sperm chromatin can act as a substrate for nuclear envelope formation in extracts in which the lamin L_{III} is absent. However, isolated sperm contain an extremely small amount of a spermatocyte-specific lamin L_{IV} (Benavente and Krohne, 1985) which might be used to form nuclei in the absence of L_{III}. We demonstrated that this was not the case by using two DNA substrates that did not contain lamins, metaphase chromosomes and bacteriophage

Figure 2. Nuclear reassembly in lamin-depleted extracts. (*A*) Immunoblots of extracts depleted with anti-L_{III} antibody (*DEP*) or mock-depleted (*CON*). The autoradiogram shown was obtained after a 24-h exposure. After a 10-d exposure, the amount of L_{III} in each sample was quantitated via densitometric scanning. Although a large peak was obtained for the mock-depleted sample, no signal was observed in the depleted extract (arrow). (*B*) Demembranated *Xenopus* sperm chromatin was added at a concentration of 1,500 sperm/ μ l to control or lamin-depleted soluble components in the presence of membranes. Assembly continued for 3 h. Shown is a typical nucleus assembled in a control extract visualized either with phase optics (panel *A*) or DNA staining (panel *B*). A typical nucleus assembled in L_{III}-depleted extracts is shown visualized with phase optics (panel *C*) or DNA staining (panel *D*).

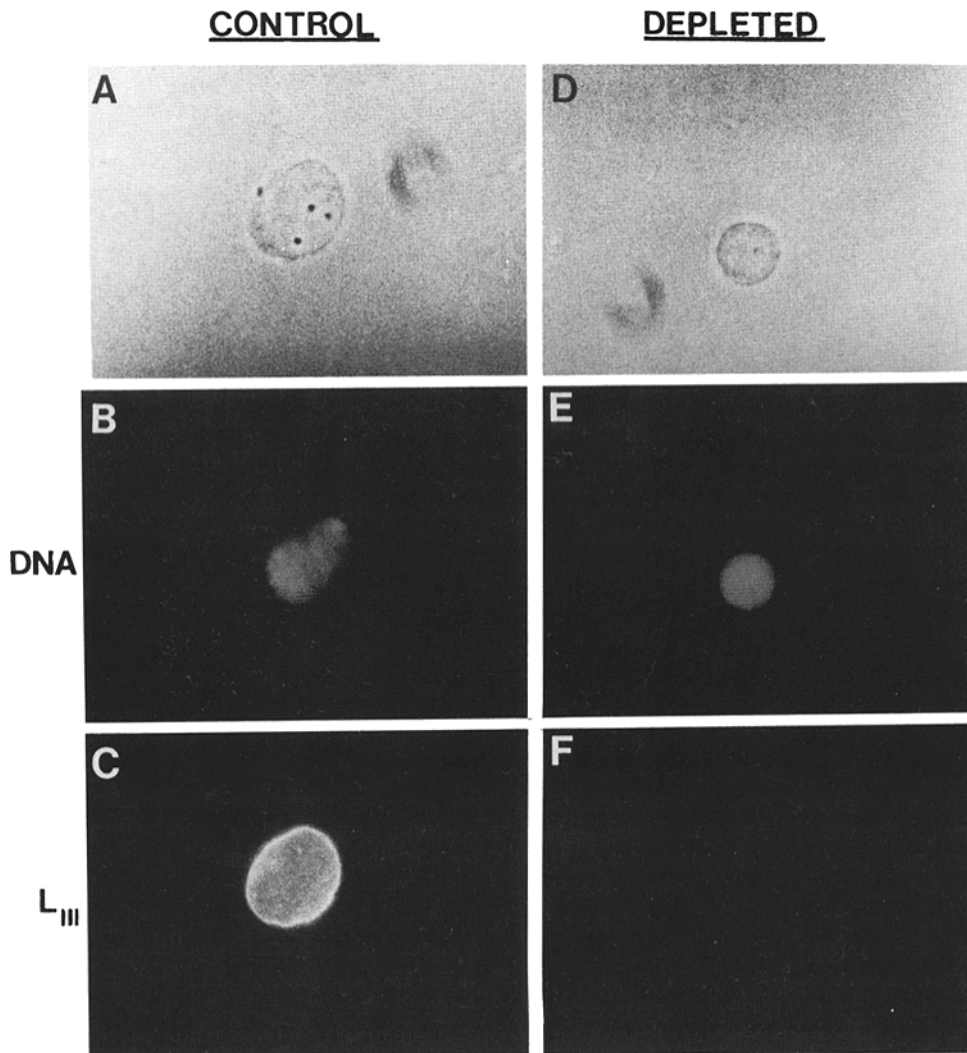


Figure 3. Absence of L_{III} staining in nuclei formed in lamin-depleted extracts. Membranes and sperm chromatin were added to mock-depleted and lamin-depleted soluble fractions at a concentration of 1,500 sperm/ μ l. After 3 h of assembly the nuclei were fixed and stained with an anti- L_{III} monoclonal antibody, followed by staining with a rhodamine-labeled rabbit anti-mouse antibody. Control (A–C) and lamin-depleted (D–F) nuclei are shown. The nuclei were observed with phase optics to visualize the nuclear envelope (A–D), stained for DNA with Hoechst (B, E), and stained with anti- L_{III} to visualize the nuclear lamina (C, F). Note the strong L_{III} staining in panel C of the control nucleus and the absence of L_{III} staining in panel F of depleted nucleus.

λ DNA, as substrates for nuclear regrowth in lamin-depleted extracts. When metaphase chromosomes, isolated from mitotically arrested CHO cells, were added to a lamin-depleted soluble fraction containing membranes, these chromosomes acted as a template for nuclear envelope assembly (Fig. 4, C and D). The envelopes formed were identical in size and shape to nuclei formed around metaphase chromosomes added to control extracts containing lamins (not shown). We have previously shown that bacteriophage λ DNA also serves as a template for assembly of morphologically normal nuclei when added to extracts containing both soluble and membrane fractions (Forbes et al., 1983; Newport, 1987). We now find that when λ DNA is added to lamin-depleted extracts containing membranes, it serves as a template for formation of a nuclear envelope indistinguishable from that formed in control extracts (Fig. 4, A and B). These observations demonstrate that DNA substrates lacking lamin components can interact with membrane vesicles to form a nuclear envelope. This indicates that the initial interaction between nuclear envelope membrane vesicles and chromatin can occur in the absence of L_{III} .

Lamin-depleted Nuclei Contain Nuclear Pores

It is clear from the results presented above that an envelope

can form around chromatin in the absence of a lamina. To determine if this lamin-deficient envelope contained nuclear pores, nuclei assembled in lamin-depleted extracts were fixed, sectioned, and examined by electron microscopy. As shown in Fig. 5 A, the envelopes surrounding chromatin in the absence of a lamina are continuous and typical of those surrounding normal nuclei in that they are composed of two membrane bilayers and contain nuclear pores. However, the envelopes are unlike normal nuclear envelopes in that the density of pores is two to three times higher than normal (compare Fig. 5, B and C). This may be due to the fact that in the absence of a lamina growth of the membrane component of the envelope is restricted, while pore assembly continues at a normal rate.

Abolition of Nuclear Transport Blocks Lamin Incorporation

The results presented above demonstrate that L_{III} does not associate with either membrane vesicles or sperm chromatin when either is present in a reconstitution extract by itself. We have furthermore demonstrated that an intact nuclear envelope can form in the absence of L_{III} . These results together suggest that the formation of a nuclear envelope at the end of mitosis might occur in two phases. In the first phase nu-

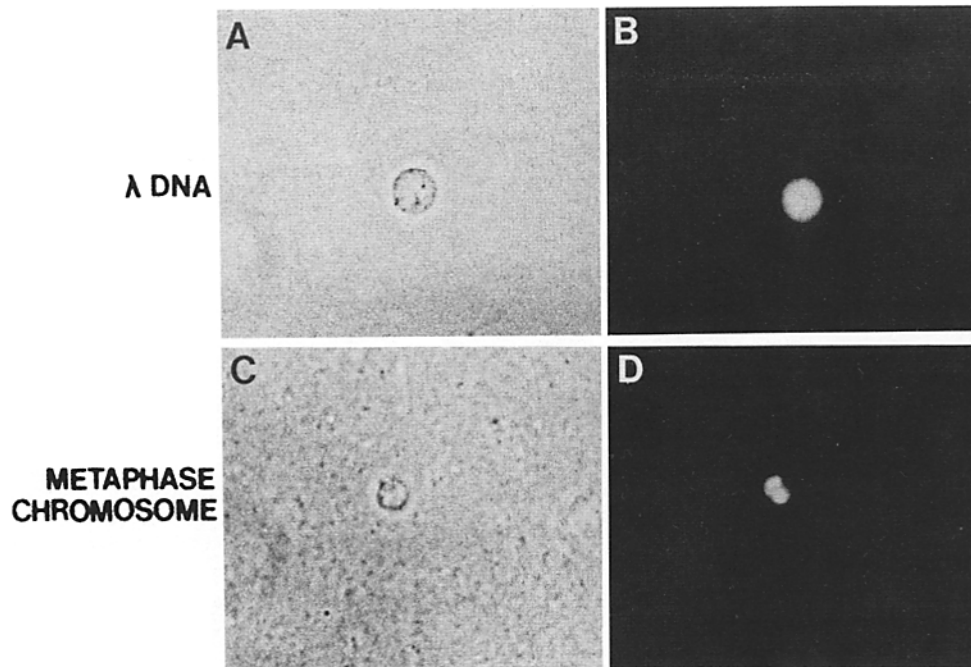


Figure 4. Nuclei assembled around λ DNA or metaphase chromosomes in L_{III} -depleted extracts. An L_{III} -depleted extract was used to assemble nuclei around either λ DNA (A, phase optics; B, DNA staining) or single isolated metaphase chromosomes (C, phase optics; D, DNA staining). λ DNA was added at a concentration of 20 ng/ μ l of extract. Metaphase chromosomes were added at a concentration of $\sim 2,000$ chromosomes/ μ l. Phase dense envelopes can be seen around both the λ DNA and metaphase chromosome. Identically sized nuclei were formed in control extracts in which L_{III} was present.

clear membranes and pores would bind to chromatin and fuse to form an envelope. The second phase would begin when the envelope had formed sufficiently to begin selectively transporting the lamins into the nucleus. In this two-step process lamin assembly would occur only after the components needed for selective transport had assembled. A simple prediction of this model is that by inhibiting nuclear transport during nuclear assembly we would not block envelope formation but would block formation of a nuclear lamina.

Recently, Finlay et al. (1987) have demonstrated that the lectin WGA binds to the nuclear pore and blocks transport of proteins into the nucleus. Furthermore, this block can be reversed by addition of N,N,N' -triacetylchitotriose. To test the affect of blocking nuclear transport on lamin formation, sperm chromatin was added to an assembly extract containing 1 mg/ml WGA. Under these conditions the sperm chromatin expanded 20–30-fold in volume and became encapsulated within an envelope (Fig. 6 B). Growth of the envelope under these conditions was limited relative to a control extract that contained WGA and triacetylchitotriose (Fig. 6 D), probably indicating that selective transport is necessary for continued growth beyond this initial stage. When the nuclei from control and the transport-inhibited reaction were stained for the presence of L_{III} via indirect immunofluorescence, control nuclei showed strong staining, while the transport-inhibited nuclei displayed no staining above background (Fig. 6, C and A, respectively). To determine if the inhibition of lamin formation due to blocking transport was reversible nuclei were first assembled in the presence of WGA and then the inhibitory effect of WGA on transport was removed by adding triacetylchitotriose to the extract. Under these conditions we found that when transport was restored the initially small nuclear envelopes began to grow based on visual measurements of nuclei. Furthermore, coincident with this growth an intact lamina began forming within the nuclei based on immunofluorescent observations using fluo-

rescently labeled anti- L_{III} antibody (not shown). These observations support a model in which lamin assembly occurs only after a nuclear envelope has formed around chromatin and the conditions for selective nuclear transport are operational (see Discussion).

Lamins Do Not Polymerize in the Absence of Nuclei

During interphase lamins A and C in CHO cells and lamin L_{III} in *Xenopus* embryos are localized at the periphery of the nucleus in a polymeric state, whereas during mitosis they are present in a soluble form within the cytoplasm. Our results indicate that incorporation of the lamins into a polymeric form within the nucleus at the end of mitosis requires an intact envelope and active transport. Such a restriction on polymerization would insure that the lamins formed polymers only in the correct location (i.e., within the nucleus and not in the cytoplasm). A strong prediction of this model is that lamins would remain in a soluble form in cells that were in interphase but lacked nuclei. The *Xenopus* egg provides an ideal system for testing this hypothesis. The *Xenopus* egg after fertilization contains one fully formed diploid nucleus and enough L_{III} to potentially assemble 4,000 diploid nuclei (Stick and Hansen, 1985; this report). A further advantage of the egg system is that immediately after fertilization the egg cell cycle can be arrested in interphase by blocking protein synthesis with cycloheximide (Harland and Laskey, 1980; Miake-Lye et al, 1983; Newport and Kirschner, 1984). Therefore, a fertilized egg blocked in interphase represents a situation in which almost all of the lamin protein will be present in the cytoplasm rather than in the nucleus. To test whether L_{III} remains in a nonpolymeric form in interphase cells lacking nuclei, *Xenopus* eggs were fertilized, immediately blocked in interphase by the addition of cycloheximide, and then incubated for 6 h. After this incubation the eggs were lysed and the components were fractionated on a 5–30% sucrose gradient. The sedimentation value of L_{III} from

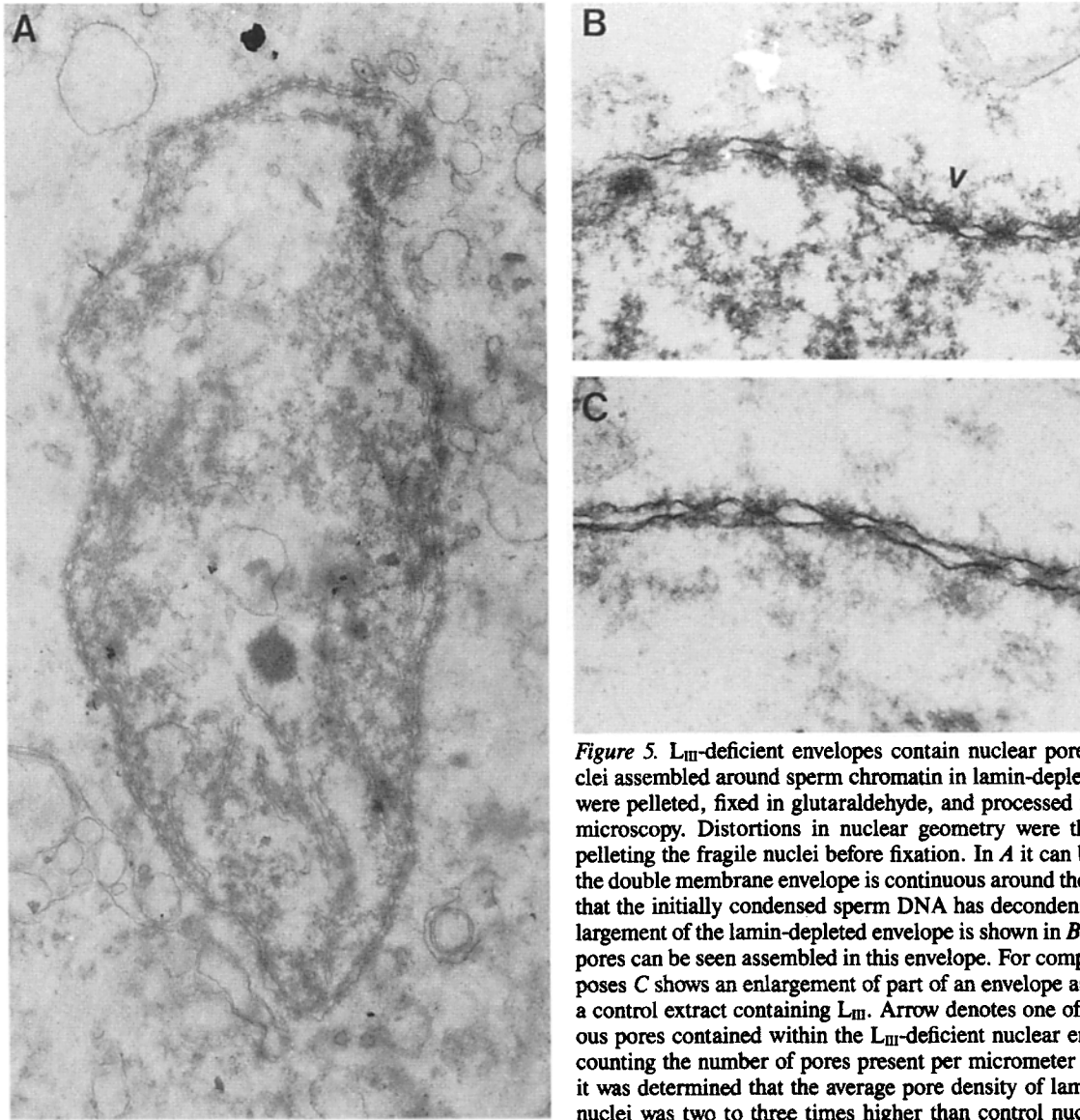


Figure 5. L_{III} -deficient envelopes contain nuclear pores. (A) Nuclei assembled around sperm chromatin in lamin-depleted extracts were pelleted, fixed in glutaraldehyde, and processed for electron microscopy. Distortions in nuclear geometry were the result of pelleting the fragile nuclei before fixation. In A it can be seen that the double membrane envelope is continuous around the sperm and that the initially condensed sperm DNA has decondensed. An enlargement of the lamin-depleted envelope is shown in B. Numerous pores can be seen assembled in this envelope. For comparison purposes C shows an enlargement of part of an envelope assembled in a control extract containing L_{III} . Arrow denotes one of the numerous pores contained within the L_{III} -deficient nuclear envelope. By counting the number of pores present per micrometer of envelope it was determined that the average pore density of lamin-depleted nuclei was two to three times higher than control nuclei.

these eggs was found to be 9 S, by assaying fractions from the sucrose gradient for the presence of L_{III} with immunoblotting (not shown). This is approximately the S value of a lamin dimer or tetramer (Benavente et al., 1985; Aebi et al., 1986). This experiment demonstrates that the cytoplasmically located L_{III} does not polymerize in eggs arrested in interphase unless they can be assembled into nuclei.

To further test this *in vivo* result *in vitro*, the soluble fraction of an assembly extract was incubated for 4 h at room temperature and then the S value of the L_{III} in the extract was determined. Again we found that the S value of L_{III} did not change relative to a control sample not incubated for 4 h (Fig. 7). The S value of L_{III} in all cases both *in vivo* and *in vitro* remained 9 S (dimer or tetramer; see Aebi et al., 1986). This is precisely the value measured for L_{III} derived from eggs in mitosis (Benavente et al., 1985). Identical results were obtained when mitotic extracts from CHO cells were incubated in *Xenopus* extracts and then the S values of lamins A and C were determined (not shown). These obser-

vations demonstrate that *Xenopus* lamin L_{III} and CHO lamins A and C remain in a soluble 9 S form in interphase cytoplasm when they cannot be assembled into the nuclear envelope. These results, in conjunction with those presented above, indicate that the initiation of polymerization of the soluble lamin proteins is an event that may be mediated by components present in an intact nuclear envelope.

Lamin-depleted Nuclei Do Not Synthesize DNA

Previously we have demonstrated that efficient replication of sperm chromatin added to *Xenopus* cell-free extracts was dependent on nuclear envelope formation (Newport, 1987). In the experiments described above we have shown that an envelope consisting of two membrane bilayers and nuclear pores can form around the sperm chromatin in the absence of the lamin L_{III} . To determine if this lamin-depleted nuclear envelope was sufficient for activating DNA replication, we measured uptake of radioactive nucleotides into sperm chro-

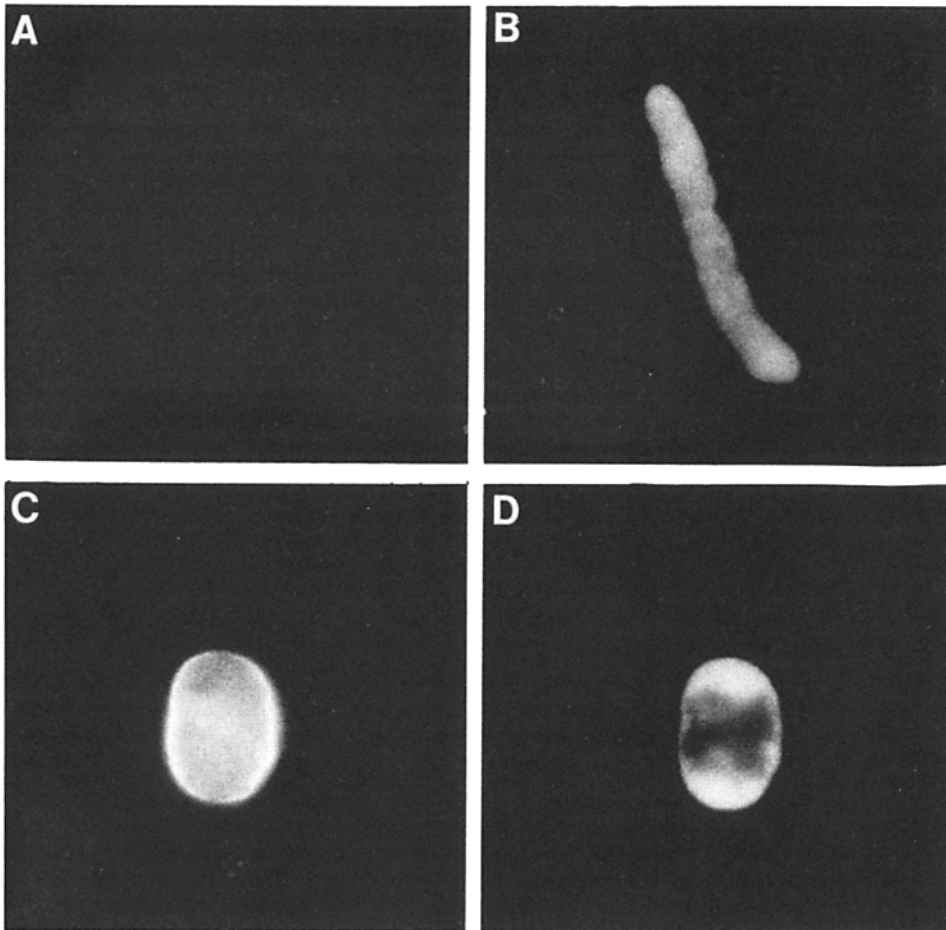


Figure 6. Inhibition of nuclear transport blocks formation of the nuclear lamina. Sperm chromatin (500 sperm/ μ l of extract) were added to complete assembly extracts containing 1 mg/ml WGA. Under these conditions sperm chromatin decondensed and became enclosed within an intact nuclear envelope. Envelope formation could be seen by phase optics or staining with the membrane-specific fluorescent dye DECC. Once enclosed, the sperm nucleus usually remained elongated in shape (*B*). When these nuclei were stained for the presence of a lamina containing L_{III} , no L_{III} was found in the envelope (*A*). When 1 mM *N,N,N',N'*-triacetylchitotriose was added to the extract to bind WGA and restore transport, nuclear envelopes resumed growth (*D*) and lamins were incorporated into the envelope as indicated by indirect immunofluorescent staining with anti- L_{III} antibodies (*C*). *A* and *C*, indirect immunofluorescence with α - L_{III} antibody; *B* and *D*, staining for nuclear DNA with Hoechst.

matin incubated in lamin-depleted extracts. To do this, sperm chromatin was added to control and lamin-depleted extracts containing membranes. DNA synthesis in these two extracts was measured by following incorporation of α [32 P]-dCTP into sperm DNA. From autoradiographs of gels containing the labeled sperm DNA it is clear that DNA synthesis occurred efficiently in control extracts (Fig. 8, lane *A*), while in lamin-depleted extracts little if any DNA synthesis was observed (Fig. 8, lane *B*). Densitometric scans of long exposures of autoradiographs showed that the rate of DNA synthesis in lamin-depleted extracts was <1% of the rate of DNA synthesis measured in extracts containing lamin L_{III} . This experiment demonstrates that the limited nuclear envelope that forms around sperm chromatin in lamin-depleted extracts is not sufficient to activate DNA replication.

Discussion

In this report we have presented evidence that in extracts made from *Xenopus* eggs the initial stages of nuclear envelope assembly can occur by a process that is independent of the presence of the sole embryonic lamin protein, L_{III} . Specifically, we have shown that quantitative removal of L_{III} from a nuclear assembly extract by immunodepletion does not slow the initial binding and fusion of membrane vesicles around chromatin, nor does the absence of a lamina inhibit the assembly of nuclear pores. Our results strongly support

a role for the nuclear lamina as an essential element for nuclear envelope growth beyond the initial stage of envelope formation and as a necessary element for DNA replication as well as a critical role for the lamina in providing a structural support for the envelope. Thus, although an intact envelope-pore complex will form around chromatin in the absence of lamin, the envelope grows at a rate that is one-third to one-fourth as rapid as an envelope growing around chromatin in the presence of lamin L_{III} . Furthermore, the enve-

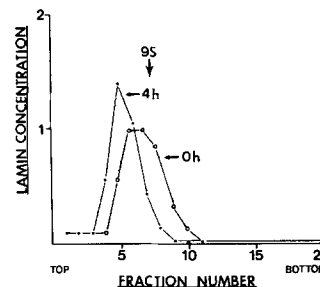


Figure 7. Sedimentation properties of L_{III} . Soluble components were incubated for 0 or 4 h at 22°C. After this, the samples were layered onto a 5–30% sucrose gradient and centrifuged for 19 h at 35,000 rpm. 0.5-ml fractions were collected and the L_{III} content was determined by immunoblotting. The total amount of L_{III} recovered in all fractions combined was identical for the 0- and 4-h time points. Differences in sedimentation rate between samples (0 and 4 h) were negligible. Based on these experiments, it appears that soluble L_{III} remains as a complex smaller than 9 S, indicating that it is either a dimer or tetramer.

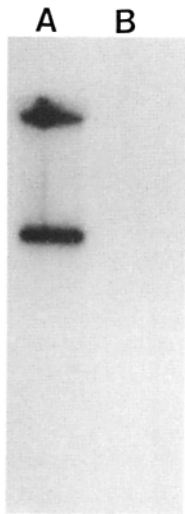


Figure 8. DNA replication is inhibited in L_{III} -depleted nuclei. Sperm nuclei (1,000 nuclei/ μ l of extract), membranes, and radiolabeled dCTP were added to L_{III} -depleted (lane B) or control nuclear assembly extracts (lane A). After a 3-h incubation at room temperature DNA was isolated from the samples and run on agarose gels. Autoradiographs of the gels demonstrated that DNA synthesis was inhibited >99% in L_{III} -depleted extracts relative to control extracts containing L_{III} .

lope that forms around chromatin in the absence of L_{III} is exceedingly fragile. Based on our results, we conclude that the initial interaction between chromatin and membrane vesicles that will form the nuclear envelope of embryonic nuclei can be mediated by a molecular targeting mechanism that does not involve the nuclear lamin protein L_{III} .

Our results further indicate that incorporation of lamin proteins into the nuclear lamina may not occur until an intact, actively transporting envelope has formed around the condensed chromatin. This conclusion is supported by the observation that L_{III} does not appear to interact with either chromatin or membrane vesicles when either is present in an assembly extract alone, but is readily incorporated into nuclei when both these components are present. This observation indicates that chromatin and membranes must first interact with each other to form a complex able to initiate incorporation of L_{III} into the lamina. That active transport through nuclear pores is probably an important component of the process regulating assembly of the lamina is indicated by the observation that when assembly occurs in the presence of the transport inhibitor WGA, an intact envelope forms around chromatin, but this envelope lacks a nuclear lamina. The lamina forms only when the nuclear transport block is removed via addition of N,N,N' -triacetylchitotriose. Together these results predict that lamin polymerization is dependent on both the formation of an envelope and the initiation of selective nuclear transport. Consistent with this prediction, we find that L_{III} remains in an unpolymerized form in interphase eggs and extracts when growing nuclei are absent. Thus, the mechanism that controls initiation of lamin polymerization appears to be localized within the nucleus, probably at the surface of the inner nuclear envelope.

Taken together, our observations indicate that assembly of the nuclear envelope at the end of mitosis occurs in at least three steps. A schematic diagram illustrating these proposed steps is shown in Fig. 9. In this model the first step of envelope assembly involves small membrane vesicles targeted to the surface of individual condensed chromosomes via the interaction between a chromatin-bound protein and a vesicle-bound protein (Wilson and Newport, 1988). Our results indicate that this initial interaction can occur in the absence of L_{III} . Therefore, the proteins involved in this interaction have

yet to be definitively identified, although several excellent candidates have been reported. These include the proposed lamin B receptor (Worman et al., 1988) as well as several proteins identified as integral membrane proteins associated with the inner nuclear membrane (Fields and Shaper, 1988; Senior and Gerace, 1988; Padan et al., 1990). In the second step of assembly these vesicles fuse to form patches of intact double membrane bound to chromatin. Subsequently the patches fuse to each other to form an intact double membrane surrounding the entire condensed chromosome. Nuclear pores would be predicted to form in membranes as soon as the first double membrane patch is formed. The end result of these two steps would be to surround the condensed chromatin with a double membrane envelope containing nuclear pores. Therefore, this complex would become capable for the first time of selective nuclear transport. Once completely enclosed within the envelope, further growth of the nuclear membrane would have to occur by transport of proteins through the pore and fusion of membrane vesicles to the outer nuclear envelope, rather than by direct binding to the no longer accessible chromatin. Our results indicate that this is the first time that formation of the nuclear lamina can be observed. We propose that the third step of envelope formation represents the selective transport of lamin proteins into the nucleus, where they interact with components of the inner nuclear membrane to form the nuclear lamina.

The lamina, like other cytoskeletal elements, is not uniformly distributed within the nucleus or cell. Rather, it is assembled at a precise location within the nucleus. Generally the spacial distribution of cytoskeletal elements within the cell are controlled by regulating the sites at which initiation of polymerization occurs. According to the model presented above, the formation of an envelope-pore complex around chromatin plays an active role in regulating assembly of the

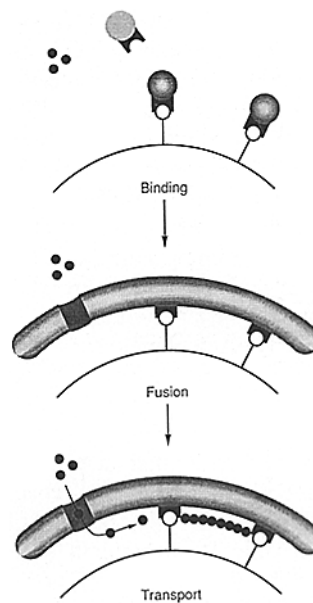


Figure 9. Model for nuclear envelope assembly. Our results suggest that nuclear envelope assembly occurs in three steps. In the first step, membrane vesicles (large shaded spheres) are targeted to the surface of chromatin by the interaction of a nonlamin membrane receptor with a chromatin-associated protein (open circles). In the second step, these vesicles fuse and serve as a substrate for assembly of nuclear pores. Once the chromatin is encapsulated within this pore membrane complex the transport of lamins (small closed circles) into the nucleus occurs through the pores. These transported lamins are then assembled at the surface of the inner nuclear envelope via interaction with unidentified

polymer initiation proteins. Further growth of the envelope would then become dependent on lamin formation and fusion of membrane vesicles to the outer nuclear envelope.

lamina within the nucleus. There are several ways in which this could occur. First, once an intact envelope–nuclear pore complex is established around DNA this complex can begin selectively transporting unassembled (dimeric or tetrameric) lamin into the existing nuclear cavity. Nuclear signal sequence-dependent transport of the lamin proteins into nuclei has been clearly demonstrated by Loewinger and McKeon (1988). As a result of selective transport, the concentration of unassembled lamin within the nucleus would be substantially higher than that present in the cytoplasm. The polymerization properties of cytoskeletal elements such as microtubules and microfilaments are exceedingly sensitive to the concentration of free tubulin and actin, respectively. In each case, below the critical concentration polymerization does not occur, while above it, it does. Recent results indicate that initiation of lamin polymerization may be equally sensitive to the concentration of free lamin present in solution (Georgatos and Blobel, 1987). Thus, the selective transport of free lamin proteins into the nucleus by nuclear pores could play a pivotal role in regulating lamin polymerization by increasing the concentration of free lamin protein within the nucleus relative to the cytosol. Such a concentration-dependent mechanism for polymerization could explain why an intact actively transporting nuclear membrane would be a requirement for lamina formation.

Although transport of L_{III} into the nucleus could play a role in insuring that the lamin would normally polymerize within the nucleus, it cannot explain why such polymerization occurs locally within a specific region of the nucleus, i.e., at the nuclear periphery. Such localized polymerization of cytoskeletal elements is generally regulated by a specific component such as the centriole for microtubules. Polymerization of lamins at the periphery of the nucleus indicates that the initiation sites for polymerization are located in this region. Since L_{III} , unlike mammalian lamins A and C (Burke and Gerace, 1986; Burke, 1990), will not bind to and polymerize on either membranes or chromatin alone, the initiation sites for lamin polymerization may be formed as a result of the interaction of proteins present in these two elements. Such a mechanism would insure that these initiation sites only occurred initially at those areas where chromatin came into contact with the membrane. If L_{III} could interact with and initiate polymerization on membranes in the absence of chromatin, we would expect to find a lamina present on annulate lamellae. This is not the case (Chen and Merisko, 1988). Alternatively, if L_{III} could interact directly with chromatin, a lamina network would form both at the periphery of the nucleus and internally. That the lamina only appears to form when both membranes and chromatin are present indicates that the interaction of these two components creates the sites that control the peripherally localized polymerization of L_{III} .

A third way in which the formation of a transporting nuclear envelope could contribute to the regulation of lamina assembly would be by regulating the dephosphorylation of the lamin proteins at the end of mitosis. A number of excellent studies have demonstrated that the lamin proteins become heavily phosphorylated at the onset of mitosis (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985). Such phosphorylation appears to occur immediately before the lamin proteins are found to be in a soluble form. At the completion of mitosis the lamins

become dephosphorylated. Using an in vitro nuclear assembly system derived from CHO cells, Burke and Gerace (1986) have provided further evidence that lamin dephosphorylation is a prerequisite for lamin assembly. This reversible phosphorylation–dephosphorylation is thought to play a critical role in regulating the depolymerization and repolymerization of the lamina during mitosis. To prevent spontaneous polymerization of lamins within the cytosol at the end of mitosis, lamin dephosphorylation could be restricted to occur only within the nucleus. Such spatially restricted dephosphorylation could result from localizing the lamin phosphatase to the nucleus via selective transport. The best evidence to date that some specific protein phosphatases are targeted to the nucleus comes from work in the fission yeast *S. pombe*. Ohkura et al. (1989) have shown that the gene product of the *dis2* gene in *S. pombe* is 90% homologous to a rabbit protein phosphatase I. Furthermore, these workers have shown by indirect immunofluorescence staining that the *dis2* phosphatase is highly enriched within the nucleus. A nuclear localized phosphatase similar to the *dis2* gene product could be involved in and cause lamin dephosphorylation to occur exclusively within the nucleus. Consistent with this, Stick (1987) has found that in interphase the cytoplasmic pool of L_{III} remains phosphorylated identically to L_{III} isolated from eggs arrested in mitosis. This suggests that in the absence of nuclei the cytoplasmic L_{III} remains in a mitotic state and, as we have shown here, is unable to form polymers.

Our studies indicate that assembly of nuclear pores is not dependent on interaction with lamin proteins. The pores, which formed in lamin-depleted nuclei, appeared morphologically normal at the level of the electron microscope. Clear evidence that nuclear pores can assemble in the absence of interaction with the nuclear lamina is demonstrated by the presence of annulate lamellae within cells. Annulate lamellae are organized membrane stacks containing numerous nuclear pores that are found in many rapidly dividing cells, germ cells, and cells at the onset of differentiation (Kessel, 1983). These membrane stacks contain neither chromatin nor lamin proteins (Chen and Merisko, 1988), demonstrating that nuclear pores can form in the absence of both of these components. Further evidence indicating that an intact membrane lamina complex is not critical to the assembly of nuclear pores was observed by Sheehan and co-workers (1988) who found that nuclear pores can assemble directly onto chromatin in a *Xenopus* nuclear assembly extract. In this instance, evidence was presented that pore assembly was independent of direct association with membranes. Together with the results presented here, these observations indicate that the assembly of nuclear pores is independent of direct association between pore components and the nuclear lamina.

Our results demonstrate that the membrane and pore components of the nuclear envelope can assemble on chromatin in the absence of the lamin L_{III} . However, our results indicate that growth of the envelope beyond the initial encapsulation of DNA requires the incorporation of L_{III} into a polymeric lamina at the inner nuclear envelope. This conclusion is consistent with several published observations. For example, Burke and Gerace (1986) have shown that immunodepletion of lamins A and C from a CHO cell-free nuclear assembly extract inhibits envelope growth by 50%. Similarly, Benavente and Krohne (1986) have injected anti-lamin anti-

bodies into mitotic cells and shown that when the cells enter interphase envelope formation is not blocked but envelope growth is (see also Benavente et al., 1989a,b). These observations in combination with the results presented here suggest that the formation of a nuclear lamina is critical to envelope growth once chromatin has become encapsulated. Precisely how L_{III} contributes to envelope growth at this time is unknown.

Along with a role for L_{III} in envelope growth, our results also demonstrate that polymerization of L_{III} into a lamina is required for DNA synthesis. Previously we have shown that replication of sperm chromatin in *Xenopus* cell-free extracts requires nuclear envelope formation (Newport, 1987). In this report we have shown that in the absence of lamin formation enclosure of sperm chromatin within an envelope is not sufficient for DNA replication to occur. The lamin could play a direct role in DNA synthesis by acting as a solid substrate for the formation of replication complexes. Alternatively, the lamins could be effecting DNA replication indirectly by regulating processes such as DNA decondensation or formation of internal nuclear structures (matrix) which are required for efficient DNA replication. Currently we cannot distinguish between these possibilities.

In summary, our results demonstrate that in *Xenopus* embryonic nuclei an intact nuclear envelope consisting of a double nuclear membrane and nuclear pores can form around chromatin in vitro in the absence of a lamina. However, we have also shown that lamin formation is essential for growth of the nuclear envelope and for DNA replication to occur. Whether envelope assembly can occur in nonembryonic cells by similar means has yet to be demonstrated.

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