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A role for nuclear lamins in nuclear envelope assembly

Reynold I. Lopez-Soler,[1] Robert D. Moir,[1] Timothy P. Spann,[1] Reimer Stick,[2] and Robert D. Goldman[1]

[1]Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611
[2]Institut für Zellbiologie, Universität Bremen, D-28334 Bremen, Germany

The molecular interactions responsible for nuclear envelope assembly after mitosis are not well understood. In this study, we demonstrate that a peptide consisting of the COOH-terminal domain of *Xenopus* lamin B3 (LB3T) prevents nuclear envelope assembly in *Xenopus* interphase extracts. Specifically, LB3T inhibits chromatin decondensation and blocks the formation of both the nuclear lamina–pore complex and nuclear membranes. Under these conditions, some vesicles bind to the peripheral regions of the chromatin. These "nonfusogenic" vesicles lack lamin B3 (LB3) and do not bind LB3T; however, "fusogenic" vesicles containing LB3 can bind LB3T, which blocks their association with chromatin and, subsequently, nuclear membrane assembly. LB3T also binds to chromatin in the absence of interphase extract, but only in the presence of purified LB3. Additionally, we show that LB3T inhibits normal lamin polymerization in vitro. These findings suggest that lamin polymerization is required for both chromatin decondensation and the binding of nuclear membrane precursors during the early stages of normal nuclear envelope assembly.

Introduction

The nuclear envelope of higher eukaryotic cells consists of a double membrane, pores, and the lamina, which functions to separate the cytoplasm from chromatin and other nucleoplasmic components (Moir et al., 1995). Through the selective transport of molecules, this envelope establishes conditions suitable for the regulation of DNA replication, transcription, and RNA processing. During mitosis, the nuclear envelope breaks down through the vesicularization of the nuclear membranes and the disassembly of the lamina and nuclear pore complexes as the mitotic spindle forms (Gerace and Blobel, 1980; Gant and Wilson, 1997). The speed with which the nucleus reforms when chromosomes reach the spindle poles has made it difficult to determine the order of the molecular interactions required for envelope reassembly in daughter cells. In particular, the role of nuclear lamins (the major constituents of the lamina) in this process remains unresolved. Some morphological studies indicate that lamins bind to chromosomes early during envelope assembly, whereas others suggest that lamins do not associate with chromosomes and are imported into the nucleus only after the nuclear membrane and pores reform (Yang et al., 1997). However, recent studies of green fluorescent protein–tagged human lamin B1 in mitotic cells have shown that lamins begin associating with the peripheral regions of chromosomes during late anaphase to mid telophase (Moir et al., 2000). Interestingly, a mutation in the *Drosophila* lamin Dm0 gene that reduces lamin expression produces a phenotype characterized by abnormal nuclear pore organization and a lack of nuclear membranes in some cells of early developing embryos (Lenz-Bohme et al., 1997). These two studies suggest that lamins could be involved in the early phases of nuclear envelope assembly in daughter cells.

Experiments attempting to directly determine if lamins are involved in nuclear envelope assembly have also yielded conflicting results. In some studies, the immunodepletion of lamins from *Xenopus* nuclear assembly extracts does not inhibit nuclear envelope assembly (Newport et al., 1990; Meier et al., 1991; Hutchison et al., 1994), whereas other studies using *Drosophila*, mammalian, and *Xenopus* nuclear assembly extracts indicate that the immunoadsorption of lamins does inhibit nuclear envelope assembly (Burke and Gerace, 1986; Dabauvalle et al., 1991; Ulitzur et al., 1992). The different results and conclusions drawn from these studies may be due to difficulties inherent in attempts to completely remove lamins by immunodepletion (Lourim and Krohne, 1994; Moir et al., 1995). For example, small amounts of lamin can be detected in nuclei assembled in lamin-depleted *Xenopus* extracts (Jenkins et al., 1993). It is possible that in the cases in which envelope assembly is not inhibited, the amount of lamin remaining after immunodepletion, while insufficient

Address correspondence to Robert D. Goldman, Department of Cell and Molecular Biology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611. Tel.: (312) 503-4215. Fax: (312) 503-0954. E-mail: r-goldman@northwestern.edu

R.D. Moir and T.P. Spann contributed equally to this work.

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to assemble a lamina, may be adequate for normal nuclear envelope formation (Lourim and Krohne, 1993).

The lamins, type V intermediate filament (IF)* proteins, are divided into two isoforms (A and B) and like all IFs are comprised of variable NH_2 and COOH termini flanking a conserved central α-helical rod domain (Aebi et al., 1986). During interphase, lamin polymerization appears to drive the formation of the lamina, a proteinacous electron-dense layer underlying the nucleoplasmic face of the inner nuclear membrane (Fawcett, 1966; Gerace et al., 1978; Krohne et al., 1978). Although the rod domain is essential for lamin polymerization (McKeon, 1987; Zhou et al., 1988), both the NH_2- and COOH-terminal domains contain unique sequences that may also influence proper lamin assembly (Moir et al., 1991). In addition, the COOH terminus contains a nuclear localization signal, a chromatin binding site, and sites involved in isoprenylation, proteolytic cleavage, and interactions with a number of lamin-associated proteins (LAPs), some of which may be involved in nuclear envelope assembly (Moir et al., 1995; Ellis et al., 1997; Gant and Wilson, 1997; Spann et al., 1997).

The *Xenopus* cell-free nuclear assembly system is ideal for determining the steps involved in the formation of the nuclear envelope. The addition of sperm chromatin to interphase extracts results in chromatin decondensation and the formation of nuclear envelopes consisting of a double membrane, functional pores, and a lamina. Furthermore, these extracts can be separated into cytosolic and membrane fractions that have specific roles in nuclear envelope assembly (Lohka and Masui, 1984; Newport, 1987; Vigers and Lohka, 1991, 1992; Newport and Dunphy, 1992; Lourim and Krohne, 1993). The membrane fractions can be separated into two types of vesicles, "nonfusogenic" and "fusogenic." The former can bind to chromatin, but cannot fuse to form the double membrane of the nuclear envelope (Vigers and Lohka, 1991; Walter et al., 1998; Drummond et al., 1999). The latter contain lamin B3 (LB3) and can bind to chromatin only in the presence of the nonfusogenic vesicles. The binding of these two types of vesicles induces a fusion process requiring GTP hydrolysis to form the double nuclear membrane (Boman et al., 1992; Newport and Dunphy, 1992; Sullivan et al., 1993; Macaulay and Forbes, 1996; Walter et al., 1998; Drummond et al., 1999). In addition, inhibitors of membrane fusion block nuclear pore complex assembly, indicating that pore assembly is dependent on normal membrane formation (Boman et al., 1992; Macaulay and Forbes, 1996). Protease treatment of these vesicle fractions has been shown to block chromatin binding, suggesting that the vesicles possess surface components that interact with chromatin very early during nuclear envelope assembly (Wilson and Newport, 1988).

In this study we describe results supporting an important role for nuclear lamins in the early stages of nuclear envelope assembly. To avoid the complications and uncertainties inherent in immunoadsorption protocols, we have used a lamin fragment consisting of the entire COOH-terminal domain of *Xenopus* LB3 (LB3T) in an attempt to block normal lamin function. We find that the addition of this lamin fragment to *Xenopus* interphase extracts containing sperm chromatin prevents the formation of the nuclear lamina, membrane, and pore complexes.

*Abbreviations used in this paper: $DiOC_6$, dihexyloxacarbocyanine; GST, glutathione *S*-transferase; HSS, high-speed supernatant; IF, intermediate filament; LAB, lamin assembly buffer; LAP, lamin-associated protein; LB3, lamin B3; LB3T, COOH-terminal domain of *Xenopus* LB3; LBR, lamin B receptor; MWB, membrane wash buffer; NWB, nuclear wash buffer; PB, protein buffer; VIM-C, COOH-terminal vimentin protein.

Results

The mutant lamin, LB3T, prevents chromatin decondensation, nuclear growth, and envelope assembly

The role of nuclear lamins in nuclear envelope assembly was investigated using LB3T, a lamin fragment consisting of the COOH-terminal nonhelical domain of *Xenopus* LB3. The effects of LB3T were assayed by adding demembranated sperm chromatin (1,000 sperm heads/μl) to *Xenopus* interphase extracts containing different concentrations of LB3T (see Materials and methods). In control reactions, equivalent amounts of wild-type LB3 or an equal volume of protein buffer (PB) (see Materials and methods) was added. The morphological features of the resulting nuclei were examined 2 h later. We observed a concentration-dependent effect of LB3T on nuclear size, and determined that the minimal effective concentration of LB3T was 10 μM (equivalent to approximately 10-fold molar concentration of the endogenous LB3). At this concentration, sperm chromatin remained small and highly condensed (Fig. 1, A and E). Higher concentrations of LB3T had no additional effects on nuclear size; therefore, this concentration of LB3T was used throughout the study.

To define the effects of LB3T on nuclear formation, we assayed for nuclear functions known to be related to normal nuclear envelope assembly, DNA replication, and nuclear transport (see Materials and methods; Spann et al., 1997). Transport-competent nuclei were identified by the nucleoplasmic accumulation of TRITC-labeled human serum albumin containing the SV-40 nuclear localization signal (Newmeyer and Forbes, 1988). The addition of LB3T to interphase extracts prevented the accumulation of this transport substrate when compared with controls (unpublished data). DNA replication was assayed by measuring ^{32}P-labeled dATP incorporation (Spann et al., 1997). The presence of LB3T reduced ^{32}P-dATP incorporation by >95% relative to nuclei assembled in control extracts (unpublished data). These results demonstrate that LB3T inhibits nuclear transport and DNA replication, indicating defective nuclear envelope assembly.

LB3T blocks the assembly of the major components of the nuclear envelope

Due to the LB3T-mediated inhibition of chromatin decondensation, DNA replication, and nuclear transport, the effect of LB3T on the assembly of the nuclear lamins, membranes, and pore complexes was also assessed (see Materials and methods). 2 h after the addition of chromatin to interphase extracts containing LB3T, samples were fixed and stained with either an mAb directed against LB3 (Stick, 1988), which does not react with LB3T on Western blots (unpublished data), or a nucleoporin antibody (Davis and

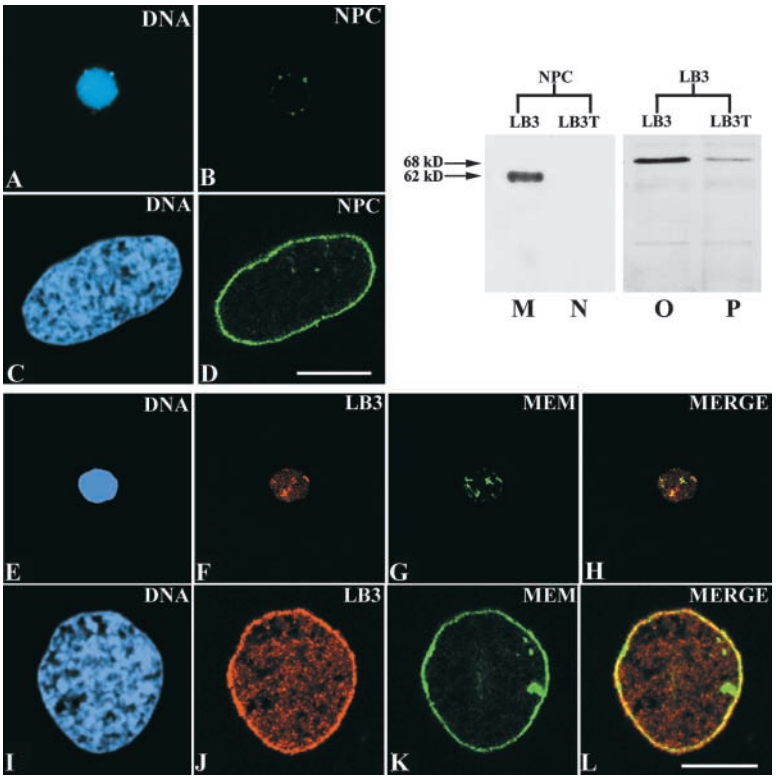

Figure 1. **Sperm chromatin was incubated in extracts containing LB3T (A, B, and E–H) and as a control, purified LB3 (C, D, and I–L).** These preparations were stained with the DNA dye TOTO (A, C, E, and I), the mAb 414 directed against nucleoporins (B and D; NPC), an mAb directed against LB3 (F and J), and the lipophillic dye, $DiOC_6$ (G and K; MEM). Chromatin in extracts containing LB3T remained highly condensed (A and E), in some cases assuming an elongated appearance, and remained surrounded by patches of fluorescence for all three envelope markers (B, F, and G). In controls, the chromatin was decondensed (C and I) and surrounded by rims of nuclear pore complex, lamin, and membrane fluorescence (D and J–L). All images are from confocal sections taken through the midregions of nuclei. Immunoblot analyses of chromatin confirmed the fluorescence studies. As compared with the control samples, the addition of LB3T resulted in a significant reduction in the amount of p62, the major nucleoporin recognized by mAb 414 (compare lanes M and N). In a 10-fold longer exposure to this antibody, traces of p62 could be detected in the LB3T-treated preparations (unpublished data). In controls, other 414-reactive bands were detected following longer exposures, and were barely detectable in the presence of LB3T (unpublished data). In the presence of LB3T there was also a large reduction in the amount of LB3 (lane P) associated with chromatin, as compared with controls (lane O). Bars (A–L), 10 μm.

Blobel, 1986). Instead of the typical rim staining patterns, only patches of weak fluorescence were detected around condensed chromatin with both antibodies (Fig. 1, A, B, E, and F). Similar results were obtained with the membrane dye dihexyloxacarbocyanine ($DiOC_6$) (Fig. 1 G). In control preparations (see Materials and methods), nuclei were normal in size, containing decondensed chromatin surrounded by rims of lamins, pores, and membranes (Fig. 1, C, D, and I–L; Spann et al., 1997). Lamin staining was also detected in the nucleoplasm of control nuclei as described previously (Fig. 1 J; Spann et al., 1997). These observations suggest that the normal assembly of the major components of the nuclear envelope is inhibited by LB3T.

Immunoblotting analyses confirmed that LB3T inhibited the association of nucleoporins with chromatin. Whereas a prominent 62-kD band was detected with the mAb 414 in the control samples (Fig. 1 M), p62 was barely detected in chromatin isolated from extract containing LB3T (Fig. 1 N and see Materials and methods). Similarly, the amount of LB3 associated with chromatin incubated in extracts containing LB3T was greatly diminished when compared with controls (Fig. 1, O and P). The large reductions in the amounts of lamin and nucleoporins associated with chromatin in the presence of LB3T support the immunofluorescence observations, and further demonstrate that nuclear envelope assembly is blocked by LB3T.

Electron microscopic analyses of 50 sperm heads incubated in extracts containing LB3T (see Materials and methods) revealed the absence of double membrane/pore complexes that typify normal nuclear envelopes (Fig. 2 A). Instead, only a small number of membrane vesicles appeared in the peripheral region of the condensed chromatin in LB3T-treated preparations (compare Fig. 2, A and B). These results also confirm that LB3T blocks the formation of double membranes and pore complexes around chromatin.

LB3T blocks the binding of fusogenic vesicles to chromatin

It has been shown that during the early stages of nuclear envelope assembly, nonfusogenic vesicles bind to the surface of chromatin. The nonfusogenic vesicles are unable to fuse to form the nuclear membrane without a second type of vesicle known as the fusogenic vesicle (Vigers and Lohka, 1991; Drummond et al., 1999). In the presence of LB3T, relatively few vesicles were seen associated with condensed chromatin (Fig. 2 A), as compared with the many vesicles seen during the early steps of normal nuclear membrane assembly (Macaulay and Forbes, 1996; Wiese et al., 1997). Based on these observations, we determined whether LB3T prevented either the fusogenic or nonfusogenic vesicles from binding to chromatin. Equivalent amounts of chromatin were added to control and LB3T-containing extracts. After 2 h the chromatin was pelleted, washed, and analyzed by immunoblotting with antibodies against the 58-kD lamin B receptor (LBR), a marker for fusogenic vesicles, and a 78-kD protein associated with nonfusogenic vesicles (Drummond et al., 1999; see Materials and methods). Under these conditions, LBR was not detected in the LB3T-treated preparations (Fig. 3, compare A and B). In contrast, p78 was detected in both control and LB3T-treated preparations (Fig. 3, C and D). These results suggest that LB3T inhibits the binding of only fusogenic vesicles to chromatin.

LB3 interacts with chromatin during the initial stages of normal nuclear envelope assembly

Based on our results, it appeared that LB3T was preventing an early step in nuclear envelope assembly. However, previ-

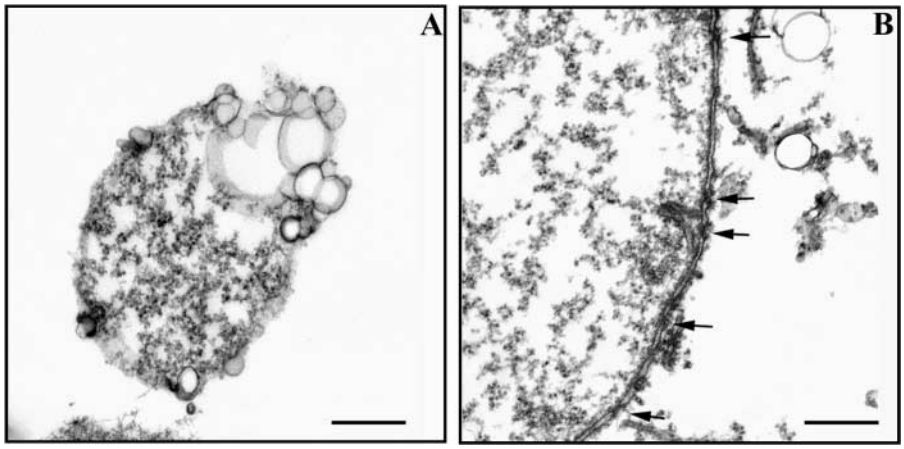

Figure 2. **Electron microscopy analysis of LB3T-treated and control nuclei.** In the presence of LB3T, only membrane vesicles are found along the surface of condensed chromatin (A). In controls, decondensed chromatin masses surrounded by typical double membranes with nuclear pore complexes are seen (B, arrows). Bars, 2 μm.

ous results using *Xenopus* interphase extracts suggested that lamins become associated with chromatin only after nuclear envelope assembly is completed (Newport et al., 1990; Meier et al., 1991; Hutchison et al., 1994). In light of these conflicting results, we reexamined the earliest stages of normal envelope assembly using confocal microscopy. The assembly process was monitored by immunofluorescence at various times after adding chromatin to interphase extracts (see Materials and methods). Within 5 min, LB3 appeared to stain chromatin diffusely with a few brighter foci (Fig. 4 A). After 10–20 min, increasing amounts of lamin were observed at the periphery of the chromatin until an obvious rim of fluorescence formed at 40 min (Fig. 4, B–D). In a similar fashion, discontinuous patches of membrane fluorescence were detected with $DiOC_6$ during the first 5 min, and by 40 min the decondensing chromatin was surrounded by a rim of membrane fluorescence (Fig. 4, E–H). In contrast, very little fluorescence was detected with the 414 nucleoporin antibody until 10 min after initiating nuclear envelope assembly, when a patchy fluorescent pattern coincident with membrane staining appeared (Fig. 4, compare J and F).

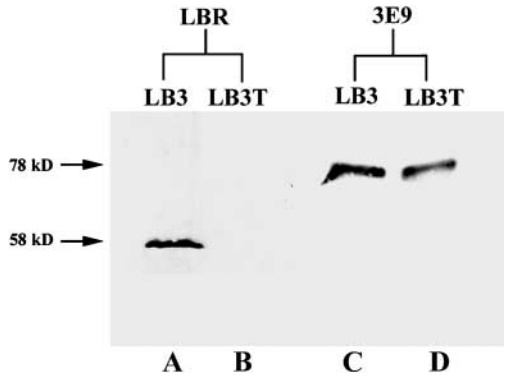

Figure 3. **Sperm chromatin was incubated in normal interphase extracts (A and C) or in extracts containing LB3T (B and D).** After incubation, the chromatin-associated proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting with the LBR antibody (58 kD; A and B), a fusogenic vesicle marker. LBR could not be detected in preparations containing LB3T (B). Samples were also blotted with an antibody directed against p78 (3E9), a marker for the nonfusogenic vesicles (C and D). This protein was present in both control and LB3T-treated chromatin samples.

After 40 min, the pore staining appeared as a rim along the surface of chromatin (Fig. 4, K and L, compare with Fig. 6, G and H). These results indicate that both lamins and membranes associate with chromatin at very early time points in nuclear envelope assembly.

LB3T was also added at time intervals between 5 and 20 min after initiating nuclear assembly in normal interphase extracts (see Materials and methods). At these times, only condensed chromatin was surrounded by patches of membrane, lamin, and nucleoporins similar to those seen when LB3T is added at the initiation of assembly, as detected by fluorescent staining (unpublished data; Fig. 1). When LB3T was added 40 min after the initiation of nuclear assembly, normal envelopes were detected (Fig. 4, D, H, and L). It should be noted that LB3T tagged with glutathione *S*-transferase (GST) can be transported into nuclei following membrane enclosure, ~40 min and after (unpublished data). These results suggest that LB3T blocks nuclear envelope assembly before membrane enclosure, ~30 min after initiation of assembly (Gant and Wilson, 1997), but does not disrupt assembled envelopes.

Experiments were also carried out to determine the reversibility of the LB3T effects. Chromatin incubated for 2 h in extracts containing LB3T was transferred to normal extracts for 2 h (see Materials and methods). Under these conditions, chromatin remains condensed and the nuclear membrane/pores fail to assemble normally (unpublished data), demonstrating that the inhibition of nuclear envelope assembly by LB3T is irreversible.

LB3T interacts with chromatin

To determine whether LB3T binds to chromatin, GST–LB3T was added to interphase extracts (see Materials and methods). The addition of GST-LB3T blocked chromatin decondensation and nuclear envelope assembly (Fig. 5 A). In addition, only weakly fluorescent patches of GST–LB3T were seen in the peripheral regions of chromatin (Fig. 5 B). Further microscopic analyses of these preparations showed that the distribution of lamins, membranes, and pores was abnormal and appeared to be identical to LB3T-treated chromatin (Fig. 1, A, B, and E–H). As a control, when equimolar amounts of purified GST were added to interphase extracts, there were no effects on nuclear envelope assembly, and no

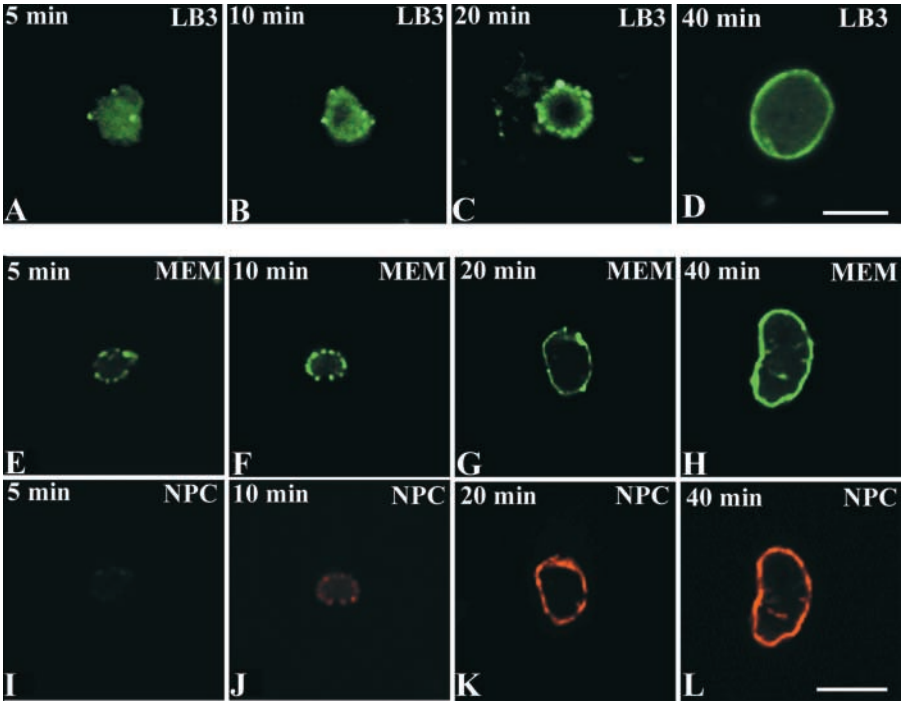

Figure 4. **Chromatin was added to interphase extracts and samples were fixed 5 (A, E, and I), 10 (B, F, and J), 20 (C, G, and K), and 40 min (D, H, and L) after initiating assembly.** Nuclei were then stained with either the LB3 mAb (A–D) or the membrane dye $DiOC_6$ (E–H) and the 414 nucleoporin antibody (I–L). At 5 min, lamin appeared to coat the chromatin with a few foci of brighter fluorescence observed at the edges (A). At this same time point, patches of membrane fluorescence were associated with the surface of the chromatin (E), although very little nucleoporin staining was seen at this stage (I). After 10 min, patches of fluorescence for all three markers were detected around chromatin (B, F, and J). At this time, membrane and nuclear pore fluorescence were mainly coaligned (F and J). Normal rim staining patterns were observed at 40 min for each envelope marker (D, H, and L). Bars, 10 μm.

fluorescent staining of chromatin could be detected with the mAb against GST (Fig. 5, C and D). These results suggest that LB3T binds to chromatin in interphase extracts.

The LB3T interaction with chromatin is independent of the sperm-specific lamin

It was important to determine whether the effects of LB3T on nuclear assembly were mediated by interactions with LB4, the sperm-specific lamin. Therefore, we tested whether LB3T blocked nuclear envelope formation around bacteriophage λDNA. Purified λDNA was added to extracts containing LB3T, and after 6 h samples were prepared for microscopy (see Materials and methods). Normal nuclear envelopes did not assemble around λDNA incubated in extracts containing LB3T, as demonstrated by patches of membrane fluorescence, discontinuous spots of LB3, and little or no nuclear pore staining (Fig. 5, E–I); however, normal nuclear assembly did take place in controls (Fig. 5, J–N). These results show that the inhibitory effects of LB3T are not mediated by the sperm-specific lamin, LB4.

LB3T interacts with LB3 to bind to chromatin and block nuclear assembly

To begin to determine the nature of the factors involved in LB3T's inhibition of nuclear envelope assembly, interphase extracts were fractionated by differential centrifugation into membrane vesicle–rich pellets (fusogenic vesicle fraction), and a supernatant fraction (Ultra-S). The Ultra-S fraction was divided into a membrane-depleted high-speed supernatant (HSS) and a second membrane vesicle–rich pellet (nonfusogenic vesicle fraction) (Vigers and Lohka, 1991; Drummond et al., 1999; see Materials and methods).

Sperm chromatin was incubated in HSS containing 10 μM LB3T for 20 min at 22°C. The chromatin was pelleted by centrifugation, washed, and the chromatin-associated proteins were separated by SDS-PAGE followed by transfer to nitrocellulose for immunoblotting with affinity-purified LB3 antibody (see Materials and methods). Bands corresponding to LB3T and LB3 were detected (Fig. 6 B). Nuclear envelope assembly was blocked when the pretreated chromatin was subsequently transferred to normal extracts for 2 h at 22°C (see Materials and methods). Under these conditions, discontinuous patches of membrane (Fig. 6 D), lamin, and nuclear pore complex components (unpublished data) were seen at the surface of condensed chromatin. However, chromatin incubated in HSS in the absence of LB3T under the same conditions displayed only one band, corresponding to LB3 (Fig. 6 A), and nuclear assembly was normal (Fig. 6, E and F).

Because LB3 is a component of HSS, we tested whether LB3 alone could mediate the binding of LB3T to chromatin in order to block envelope formation. To this end, sperm chromatin in nuclear wash buffer (NWB) was pretreated with 10 μM LB3T and 1 μM LB3 (see Materials and methods) for 20 min at 22°C. The chromatin was pelleted by centrifugation, washed, and then added to normal extracts (see Materials and methods). Under these conditions, chromatin decondensation and nuclear envelope assembly were inhibited. Discontinuous LB3 staining was observed at the periphery of the condensed chromatin (Fig. 6, G and H). As a control, pretreatment of sperm chromatin in NWB containing only 10 μM LB3T or 1 μM LB3 assembled normally (Fig. 6, I and J, and unpublished data). Immunoblotting of these preparations revealed that LB3T only bound to chromatin in the presence of LB3 (unpublished data). These results imply that LB3T requires LB3 in order to bind to chromatin and block nuclear envelope assembly.

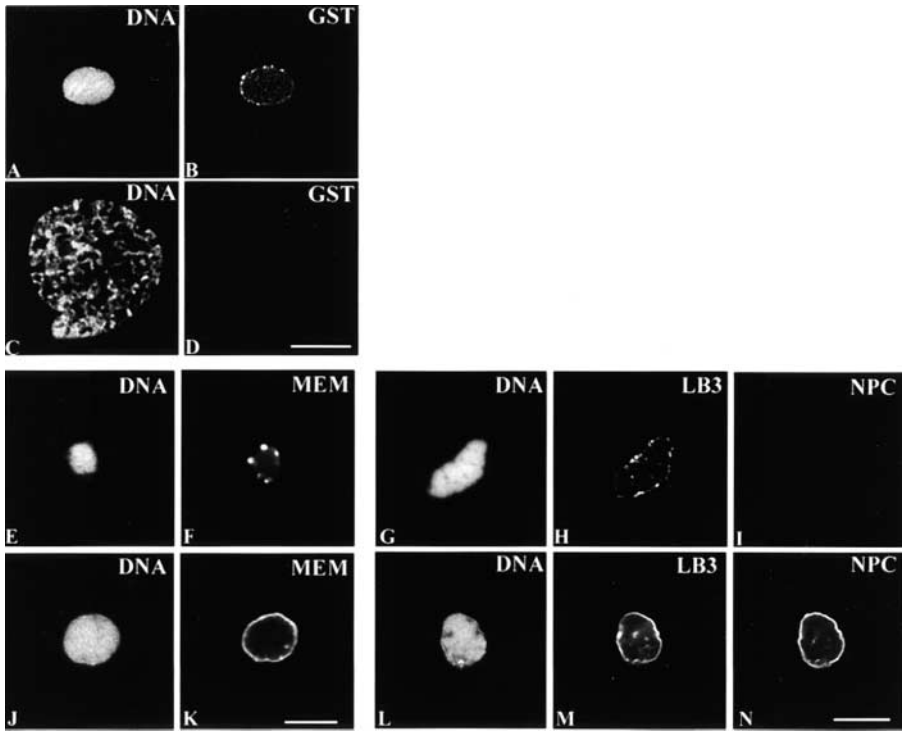

Figure 5. **Sperm chromatin incubated in extracts containing GST–LB3T (A and B) or GST as a control (C and D) was visualized with TOTO (A and C) and a monoclonal GST antibody (B and D).** GST–LB3T was found in bright patches at the surface of chromatin, with less intense staining throughout the chromatin (B). GST alone had no apparent effect on chromatin decondensation (C), and was not detected in association with chromatin (D). λDNA was added to normal extracts (J–N) or LB3T-treated extracts (E–I). After 6 h, samples were fixed and stained with $DiOC_6$, TOTO, and the LB3 and nucleoporin antibodies. In extracts containing LB3T, small patches of membrane (MEM) and LB3 fluorescence were seen at the edge of λDNA (E–H), but nucleoporin staining was difficult to detect (I). In controls, bright rims of fluorescence were observed for all three envelope markers (J–N). Bars, 10 μm.

LB3T also interacts with a factor(s) in the fusogenic vesicle fraction to block nuclear envelope assembly

The inhibition of nuclear envelope assembly by LB3T could also be mediated by factors associated with membrane vesicles. To test this possibility, either fusogenic or nonfusogenic vesicles were pretreated with LB3T in membrane wash buffer (MWB) for 20 min at 22°C, washed, and then transferred to HSS containing chromatin and the complementary vesicles required for nuclear envelope assembly (see Materials and methods). After 3 h, samples were stained with TOTO, $DiOC_6$, and the mAb against LB3 (see Materials and methods). Pretreatment of the fusogenic vesicles with LB3T blocked chromatin decondensation and the formation of both the nuclear membrane and lamina (Fig. 7, A–C). Nuclear pore assembly was also inhibited (unpublished data). In contrast, when nonfusogenic vesicles were pretreated with LB3T, normal membrane, lamin, and nuclear pore staining patterns were detected, indicating that normal assembly had taken place (Fig. 7, D–F, and unpublished data). Since LB3 is found only in the fusogenic vesicle fraction, these results suggest that LB3T may be interacting with LB3 to prevent vesicle binding to chromatin. Alternatively, LB3T may be interacting with LAPs such as LBR and LAP2β to block their association with chromatin and to prevent vesicle binding to chromatin. To test this possibility, membrane fractions were treated with detergent using the method of Steen et al. (2000) (see Materials and methods) in order to release LBR and LAP2β from the membranes. These detergent-treated preparations were incubated with sperm chromatin with or without LB3T. Immunoblotting analyses of these preparations showed that similar amounts of LBR and LAP2β bound to chromatin both in the presence and absence of LB3T (unpublished data). Therefore, LB3T does not appear to interact with LBR or LAP2β to block vesicle binding to chromatin.

LB3T blocks normal lamin polymerization in vitro

The findings that LB3T can bind to chromatin in the presence of LB3 and to fusogenic vesicles, which contain LB3, suggest that this mutant protein may inhibit a lamin–lamin interaction required for lamin assembly into higher order structures. These structures may be required for the binding of fusogenic vesicles to chromatin. For example, LB3T may act to inhibit the head-to-tail interactions of lamin dimers, which have been proposed to be involved in the early steps in the lamin polymerization process in vitro (Heitlinger et al., 1991, 1992; Stuurman et al., 1996). To begin to determine whether LB3T can inhibit lamin assembly, in vitro lamin polymerization assays were carried out using bacterially expressed LB3 and LB3T (Moir et al., 1991; see Materials and methods). When LB3 polymerization was induced by dilution into lamin assembly buffer (LAB) (see Materials and methods), it became insoluble and ~95% of the protein was recovered in pellets following centrifugation (Fig. 8, A and B). In contrast, when LB3T was added to LB3 in a 3:1 molar ratio, most of the LB3 remained in the supernatant following high-speed centrifugation, strongly suggesting that LB3T inhibited normal lamin assembly (Fig. 8, C and D). In controls, LB3T alone remained soluble when mixed with LAB (Fig. 8, E and F). As a further control, the COOH-terminal vimentin protein (VIM-C), a cytoplasmic IF protein, was added to solutions of LB3 at a 5:1 molar ratio as described above. Under these conditions, no effects on the solubility and assembly of LB3 could be detected (Fig. 8, G–J). These results imply that the normal assembly of LB3 is significantly inhibited by LB3T in vitro. This observation may represent the major mechanism underlying the inhibition of nuclear envelope assembly seen in the *Xenopus* interphase extracts containing LB3T.

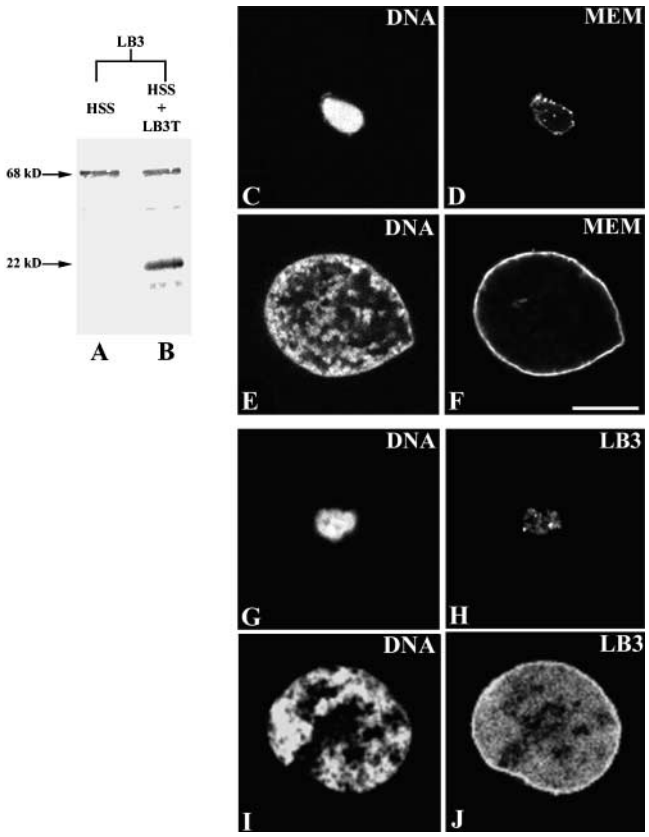

Figure 6. **Chromatin was incubated in HSS or HSS containing LB3T, washed, pelleted by centrifugation, and the associated proteins were separated by SDS-PAGE.** Immunoblotting with the LB3 polyclonal antibody revealed both LB3T (22 kD) and LB3 (68 kD) in the presence of LB3T (B), whereas only LB3 bound to chromatin in the absence of LB3T (A). Chromatin pretreated in HSS with LB3T or HSS alone was added to interphase extracts containing no exogenous proteins. After 2 h, samples were fixed and stained with TOTO (C and E) and $DiOC_6$ (D and F). The pretreated chromatin remained condensed (C) and contained only patches of membrane fluorescence (D). Chromatin incubated in HSS alone displayed normal decondensation and was surrounded by a rim of membrane fluorescence (E and F). Sperm chromatin preincubated in NWB containing LB3 and LB3T did not assemble nuclear envelopes when transferred to normal extracts (G and H; Materials and methods). In contrast, sperm chromatin pretreated with only LB3T assembled normally (I and J). Bar, 10 μm.

Discussion

In this study, the involvement of nuclear lamins in nuclear envelope assembly was examined using LB3T. When added to *Xenopus* nuclear assembly extracts, LB3T prevented the formation of nuclear envelopes around chromatin as shown by a lack of nuclear membranes, pores, and lamina. The morphological features of sperm chromatin incubated in interphase extracts containing LB3T for up to 2 h appeared to be nearly identical to chromatin incubated in normal extract for only 5–10 min (compare Figs. 1 and 4), supporting an early role for lamins in the process of nuclear assembly. We also found that LB3T prevented the vesicles required for membrane fusion from associating with chromatin. Consistent with these findings, ultrastructural analyses of the LB3T-treated preparations revealed unfused membrane vesicles dispersed along the surface of condensed chromatin, instead of the normal continuous membrane and lamina studded with nuclear pores.

The involvement of lamins in chromatin decondensation and nuclear envelope assembly is strongly supported by the findings that LB3T binding to chromatin is dependent on the presence of LB3, and chromatin pretreated with LB3T and LB3 cannot assemble a nuclear envelope when transferred to complete interphase extracts. In contrast, pretreatment of chromatin with LB3T alone does not inhibit subsequent envelope formation. The role of lamins in membrane formation is also supported by the findings that pretreatment of fusogenic vesicles with LB3T prevents envelope assembly. In contrast, pretreatment of nonfusogenic vesicles with LB3T has no effect on nuclear assembly. Since LB3 is present only in fusogenic vesicle preparations, LB3T binding to these vesicles may also be mediated by the interactions of LB3T with LB3 (Drummond et al., 1999).

Taken together, the results of this study suggest that interactions between lamins bound to chromatin and fusogenic vesicles may be involved in the initial steps leading to membrane binding and fusion. If this is the case, LB3T may block envelope formation by preventing lamin–lamin interactions required for the association of the fusogenic vesicles with chromatin. Lamin polymerization appears to involve a number of discrete steps. For example, in vitro, the first step involves the formation of dimers, followed by the head-to-tail association of these dimers to form long-chain intermediate structures (Heitlinger et al., 1991, 1992). The formation of these intermediate structures may be competitively inhibited by LB3T. This is supported by in vitro assembly assays in which LB3T significantly alters the assembly properties of bacterially expressed LB3 (Fig. 8).

However, it must be emphasized that the assembly of a normal peripheral nuclear lamina is clearly not required for nuclear membrane formation. This was first demonstrated by studies of *Xenopus* extracts depleted of the majority of LB3 (Newport et al., 1990; Meier et al., 1991; Hutchison et al., 1994). Similarly, lamin mutants lacking only their NH_2-terminal domains inhibit the formation of a normal lamina but do not prevent normal membrane and pore formation (Ellis et al., 1997; Spann et al., 1997). In contrast, LB3T consists of only the COOH terminus and lacks the rod domain. Consequently, the interaction of LB3T with LB3 most likely occurs at an early stage of lamin assembly, analogous to the head-to-tail interactions of lamin dimers (Heitlinger et al., 1991, 1992; Stuurman et al., 1996). Consistent with this model, we find that LB3 associates with chromatin before vesicles fuse to form a continuous nuclear membrane (Fig. 4). These results are consistent with the findings that LB3 interacts with *Xenopus* chromatin in the presence of inhibitors of vesicle fusion, and that lamins become associated with chromatin during the early stages of envelope assembly in mammalian cells (Gerace et al., 1984). Finally, observations of live mammalian cells have revealed that green fluorescent protein–tagged lamin B1 interacts with the surface of chromosomes before they reach the spindle poles during mitosis, further suggesting a role for lamins in the earliest stages of nuclear envelope assembly (Moir et al., 2000).

The inhibition of normal lamin assembly by LB3T may also block the interaction of lamins with other proteins in-

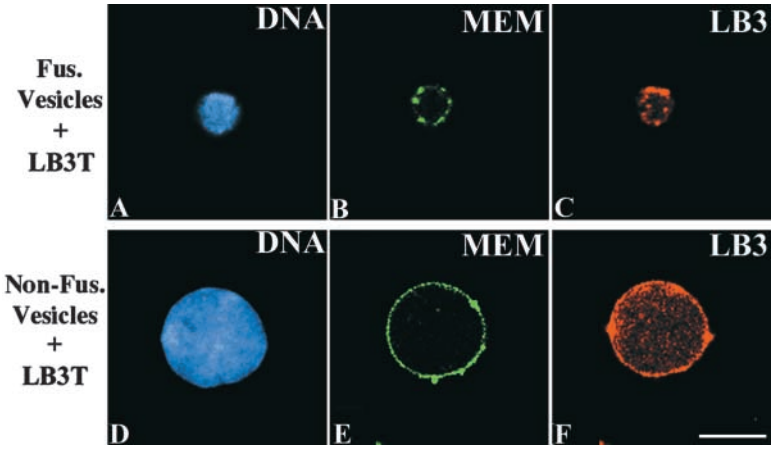

Figure 7. **Isolated fusogenic and nonfusogenic membrane vesicles from *Xenopus* extracts were incubated in MWB containing LB3T.** The pretreated membrane vesicles were washed, isolated, and combined with chromatin, HSS, and the complementary membrane vesicle. Samples were fixed and stained with $DiOC_6$, TOTO, and the LB3 mAb. Pretreatment of the fusogenic membrane vesicles with LB3T yielded condensed chromatin structures (A) with patches of membrane (MEM) and LB3 fluorescence (B and C). In contrast, pretreatment of nonfusogenic membrane vesicles with LB3T resulted in normal nuclear membrane and lamin rim staining patterns surrounding decondensed chromatin (D–F). Bar, 10 μm.

volved in the targeting of membrane vesicles to chromatin. In support of this, there is evidence that lamins bind to a number of chromatin-associated proteins including histones, the *Drosophila* young arrest protein, the MAN antigens, the barrier to autointegration factor, and LAP2α (Glass and Gerace, 1990; Taniura et al., 1995; Paulin-Levasseur et al., 1996; Goldberg et al., 1998, 1999; Furukawa, 1999; Gant et al., 1999). Similarly, it has been reported that lamins interact with nuclear membrane–bound proteins such as LAP2β, Emerin, LBR, and Otefin (Schuler et al., 1994; Furukawa and Kondo, 1998; Goldberg et al., 1998; Clements et al., 2000). In addition, a number of these proteins appears to be involved in nuclear envelope assembly (Gant and Wilson, 1997; Wilson et al., 2001). Based on these considerations, lamin structures formed during the early stages of nuclear envelope assembly could also mediate associations with chromatin- and membrane-associated LAPs. Further experiments will be required to define the sequence of lamin–lamin and lamin–LAP interactions required for envelope assembly.

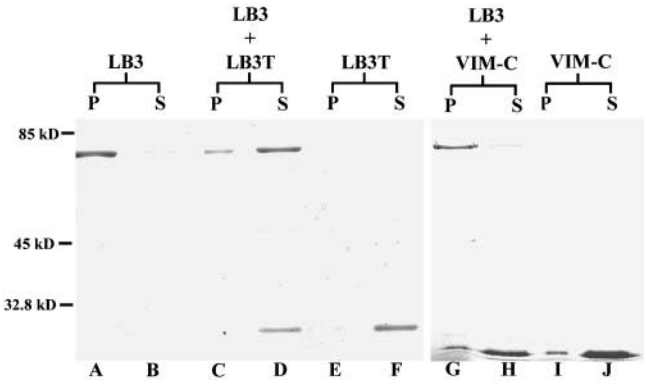

Figure 8. **Bacterially expressed LB3 (A and B), LB3T (E and F), or a combination of LB3 and LB3T at a 1:3 molar ratio (C and D, respectively) were diluted into LAB, pelleted, and analyzed by SDS-PAGE.** Under these conditions, ~95% of the LB3 was detected in the pellet (A and B). However, when LB3 and LB3T were combined, most of the LB3 was detected in the supernatant (C and D). Under identical assembly conditions, most LB3T remained in the supernatant fraction (E and F). As a control, mixtures of LB3 and VIM-C in a 1:5 molar ratio, respectively, did not alter the assembly properties of LB3 (G–J). VIM-C runs to the bottom of the gel due to its low molecular weight (8.5 kD). Size markers are indicated on the left.

In conclusion, the LB3T-mediated inhibition of nuclear membrane assembly demonstrates that lamins are required for the formation of the nuclear envelope. More importantly, data from our study suggest that lamin polymerization may be required for the binding and/or stabilization of nuclear membrane precursors to chromatin during the initial stages of nuclear assembly.

Materials and methods

Bacterial expression and purification of wild-type and mutant nuclear lamins

LB3T (amino acids 383–583) was generated using PCR and the sequence confirmed. LB3T and LB3 were cloned into pET-derived vectors and expressed in *Escherichia coli* as described previously (Spann et al., 1997). Bacteria expressing LB3T were lysed in 8 M urea, 20 mM Tris, pH 8.0, 1 mM EDTA, and 1 mM DTT, and sonicated for 45 s. Ammonium sulfate was added to the lysates to 20%. After 1 h at 22°C, the solution was centrifuged at 20,000 *g* for 30 min at 4°C in a fixed angle rotor and the pellet discarded. Ammonium sulfate was added to the supernatant for an 80% solution. After 4 h at 4°C, the sample was centrifuged for 30 min at 20,000 *g* at 4°C. The pellet was resuspended in 6 M urea, 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, and dialyzed against 1,000× excess of this buffer. LB3T and LB3 were purified using Mono Q resin (0–1 M NaCl elution gradient; Pharmacia) (Spann et al., 1997). VIM-C (87 amino acids, a gift from Dr. Ying Hao Chou, Northwestern University, Chicago, IL) was also purified using Mono Q resin. Fractions containing LB3T, VIM-C, or LB3 were identified by SDS-PAGE, dialyzed into PB (300 mM NaCl, 20 mM Tris, pH 9.0, 1 mM DTT), and stored at −80°C in 20 μl aliquots.

A GST–LB3T fusion protein was expressed by subcloning LB3T (see above) into a pGEX-4T GST fusion vector (Amersham Pharmacia Biotech). Protein expression was carried out as described above. Protein purification was carried out in 6 M urea according to the manufacturer's instructions (Amersham Pharmacia Biotech). The GST–LB3T samples were dialyzed against 1,000× vol of PB and stored at −80°C.

In vitro assembly of nuclei in *Xenopus* egg interphase extracts

Xenopus egg interphase extracts were prepared as described previously (Spann et al., 1997). Demembranated/deflagellated sperm chromatin was prepared from two *Xenopus* testes by resuspending isolated sperm in 4 ml of 1% Triton X-100, 200 mM sucrose, 7 mM $MgCl_2$, and buffer A (80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM Pipes-NaOH, pH 7.4). After 4 min at 22°C, 3 ml of buffer A containing 3% BSA, 200 mM sucrose, and 7 mM $MgCl_2$ was added, and the suspension was layered over buffer A containing 2 M sucrose and 7 mM $MgCl_2$. The chromatin was isolated by centrifugation for 10 min at 20,000 *g* at 4°C in a swinging bucket rotor. Chromatin was resuspended in buffer A, 200 mM sucrose, and 7 mM $MgCl_2$ (100,000/μl), and aliquots (2 μl) stored at −80°C.

To assemble nuclei, 2 μl of chromatin was added to 5 μl of a nucleoplasmin-enriched preparation (Philpott et al., 1991). After 10 min at 22°C, chromatin (~1,000/μl) was added to interphase extracts containing LB3T

(10 μM), LB3 (10 μM), or an equivalent volume of PB. In some cases, LB3T was added after nuclear envelope assembly was initiated. Nuclear assembly proceeded for 120 min at 22°C.

When λDNA (5 μg/ml; New England Biolabs) was used as an assembly template, nuclear formation was assessed after 6 h (Newport, 1987).

Treatment of chromatin, fusogenic, and nonfusogenic membrane fractions of *Xenopus* extracts with LB3T

Xenopus egg interphase extract was separated into three major fractions by differential centrifugation to yield two membrane vesicle fractions, fusogenic and nonfusogenic, and a membrane-depleted HSS (Vigers and Lohka, 1991; Drummond et al., 1999). Aliquots of the fusogenic (10 μl), nonfusogenic (10 μl), and membrane-depleted fractions (HSS; 25 μl) were frozen in $N_{2(l)}$ and stored at −80°C (Vigers and Lohka, 1991; Drummond et al., 1999).

Sperm chromatin (1,000/μl) was incubated in 30 μl of HSS containing LB3T or LB3 (10 μM) for 20 min at 22°C, and washed in 10 vol of NWB (50 mM KCl, 250 mM sucrose, 2.5 mM $MgCl_2$, 1 mM DTT, and 12.5 mM Hepes, pH 7.4). Chromatin was pelleted by centrifugation at 3,000 *g* for 10 min at 4°C in a swinging bucket rotor. Under these conditions, unbound LB3T did not pellet. The chromatin was resuspended in complete extract, and after 2 h nuclear envelope assembly was assessed as described below.

Alternatively, sperm chromatin (1,000/μl) was pretreated with LB3T (10 μM), or LB3 (1 μM), or a combination of both for 30 min at 22°C in 50 μl of NWB. NWB (500 μl) was added and the preparations were layered over a 1.8 M sucrose NWB. Chromatin was isolated by centrifugation at 3,000 *g* for 20 min at 4°C in a swinging bucket rotor and the pellets suspended in interphase extracts. After 2 h at 22°C, nuclear envelope assembly was assessed. For immunoblotting, samples were resuspended in 1.5 ml of NWB, and after a 2-min centrifugation in a microcentrifuge (Eppendorf), resuspended in sample buffer and subjected to SDS-PAGE (see below).

In other experiments, 75 μl of MWB (50 mM KCl, 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM Hepes-NaOH, pH 8.0, 1 mM DTT, 1 mM ATP, and 1 μg/ml leupeptin and aprotinin [Sigma-Aldrich]) were added to aliquots of fusogenic or nonfusogenic membrane vesicles, and either LB3 or LB3T (10 μM) was added to each fraction. After 25 min at 22°C, the resulting four vesicle preparations (fusogenic and LB3, nonfusogenic and LB3, fusogenic and LB3T, and nonfusogenic and LB3T) were diluted in 10 vol of MWB, isolated by centrifugation at 20,000 *g* for 20 min at 4°C in a swinging bucket rotor, and resuspended in 10 μl of MWB. An aliquot (3 μl) from each treated fraction was added to 30 μl of HSS containing sperm chromatin (1,000/μl). Subsequently, 3 μl of the untreated complementary membrane vesicles was added (i.e., treated fusogenic with untreated nonfusogenic and untreated fusogenic with treated nonfusogenic), and nuclear assembly assessed after 3 h at 22°C as described below.

Immunofluorescence techniques

Nuclei assembled in *Xenopus* extracts were fixed and processed for immunofluorescence as described previously (Spann et al., 1997). Nuclear lamins were visualized with L6-5D5, an mAb directed against *Xenopus* LB3 (Stick, 1988). This antibody did not react with the sperm-specific lamin, LB4 (Stick, 1988), or with LB3T (unpublished data). Nuclear pores were detected with the mAb 414 (Davis and Blobel, 1986; BABCO). GST fusion proteins were detected with an mAb directed against GST (Amersham Pharmacia Biotech). Secondary antibodies included FITC- and lissamine rhodamine–labeled donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories). Membranes were stained with the lipophilic dye $DiOC_6$ (2.5 μg/ml) (Melchior et al., 1995). DNA was stained with Hoechst dye (1 μg/ml) for conventional epifluorescence or with TOTO-3 iodide (1 μM) for confocal studies (Molecular Probes) (Spann et al., 1997). Nuclei were examined with a Zeiss Axiophot equipped with a Photometrics SenSys Pro cooled CCD camera using the Metamorph Imaging Program (Universal Imaging Corp.), or an LSM 510 confocal microscope (Carl Zeiss, Inc.) equipped with argon/krypton and helium/neon lasers.

Electron microscopy

Nuclei assembled in vitro were prepared for electron microscopy as described elsewhere (Macaulay and Forbes, 1996). Before fixation, demembranated sperm heads (1,000/μl) were incubated in 300 μl of extract containing LB3T or LB3 for 2 h as described above. Samples were embedded and sectioned as previously described (Yang et al., 1985). Thin sections were examined and micrographs taken on a JEM-1200 EX electron microscope at 60 kV.

Gel electrophoresis and immunoblotting

125 μl assembly reactions +/− LB3T were diluted by adding 600 μl of NWB and then layering over a 1.6 M sucrose solution. The nuclei/chromatin were pelleted by centrifugation at 12,000 *g* in a swinging bucket rotor for 15 min at 4°C. The pellets were resuspended in 1 ml of NWB and centrifuged again for 5 min at 20,000 *g* in an Eppendorf 5417c centrifuge. The resulting pellets were subjected to SDS-PAGE (Laemmli, 1970) and immunoblotting as described below.

In other experiments, sperm chromatin (1, 000/μl) was incubated in 50 μl of HSS containing LB3T for 30 min at 22°C. After addition of 1 ml of NWB, the samples were layered over a 2 M sucrose NWB, and the chromatin was isolated by centrifugation at 20,000 *g* for 15 min in a swinging bucket rotor. The pellet was resuspended in 1 ml of NWB, centrifuged again at 20,000 *g* for 5 min at 22°C, and subjected to SDS-PAGE and immunoblotting as described below.

In another experiment, isolated *Xenopus* membranes were solubilized in 0.5% NP-40 as described previously (Steen et al., 2000), and incubated with chromatin in MWB +/− LB3T (10 μM). After 20 min at 22°C, the samples were layered over 1 M sucrose in MWB, pelleted by centrifugation at 3,000 *g* for 10 min in a swinging bucket rotor, and subjected to SDS-PAGE and immunoblotting as described below.

Immunoblotting with primary antibodies was carried out as described previously (Spann et al., 1997). Antibodies used were: L6-5D5 mAb (1:100), 414 mAb (1:1,000), 3E9 mAb (1:1,000, provided by Chris Hutchison, and Carl Smythe, University of California, San Diego, La Jolla, CA; Drummond et al., 1999), a guinea pig antibody (1:2,000) directed against the *Xenopus* LBR (p58; Gajewski and Krohne, 1999), an affinity-purified pAB raised against bacterially expressed LB3, and an mAb directed against *Xenopus* LAP2β (a gift from Dr. Katherine Wilson, Johns Hopkins School of Medicine, Johns Hopkins University, Baltimore, MD). The appropriate secondary horseradish peroxidase-labeled IgGs (1:5,000; Molecular Probes) were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech) using radiographic film (Amersham Pharmacia Biotech).

Lamin solubility assays

Lamin assembly was assessed by determining the solubility of purified lamins under assembly conditions as described (Moir et al., 1991). LB3 (600 nM) was combined with either LB3T (1:3 molar ratio) or VIM-C (1:5 molar ratio) in 400 μl of LAB (100 mM NaCl, 1 mM DTT, 25 mM MES, pH 6.6). Samples were incubated for 30 min at 22°C, followed by centrifugation at 20,000 *g* in a microcentrifuge for 20 min at 22°C. The supernatants were removed and the pellets were washed three times by the addition of 1 ml LAB followed by centrifugation. All samples (pellets and supernatants) were subjected to SDS-PAGE (10.5% for LB3T and LB3 preparations, and 16% for VIM-C preparations).

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