Lamin Activity Is Essential for Nuclear Envelope Assembly in a *Drosophila* Embryo Cell-free Extract

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Abstract. The role of the *Drosophila* lamin protein in nuclear envelope assembly was studied using a *Drosophila* in vitro assembly system that reconstitutes nuclei from added sperm chromatin or naked DNA. Upon incubation of the embryonic assembly extract with anti-*Drosophila* lamin antibodies, the attachment of nuclear membrane vesicles to chromatin surface and nuclear envelope formation did not occur. Lamina assembly and nuclear membrane vesicles attachment to the chromatin were inhibited only when the activity of the 75-kD lamin isoform was inhibited in both soluble and membrane-vesicles fractions. Incubation of decondensed sperm chromatin with an extract that was depleted of nuclear membranes revealed the presence of lamin molecules on the chromatin periphery. In addition, high concentrations of bacterially expressed lamin molecules added to the extract, were able to associate with the chromatin periphery, and did not inhibit nuclear envelope assembly. After nuclear reconstitution, a fraction of the lamin pool was converted into the typical 74- and 76-kD isoforms. Together, these data strongly support an essential role of the lamina in nuclear envelope assembly.

ADDITION of naked DNA to extracts of *Xenopus* eggs or *Drosophila* embryos is sufficient to trigger the assembly of unpolymerized components to form a structure that resembles the cell nucleus in all features except its genetic content (fore review, see Laskey and Leno, 1990). Such cell-free systems were established using extracts derived from *Xenopus* eggs (Lohka and Masui, 1983, 1984; Newport, 1987; Mills et al., 1989; Finlay and Forbes, 1990), mitotic mammalian cells (Burke and Gerace, 1986) and mitotic avian cells (Nakagawa et al., 1989). A *Drosophila* cell-free cytoplasmic extract derived from 0–6-h-old embryos that reconstitutes nuclear structure around added rooster-sperm or lambda DNA has also been described (Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990; Crevel and Cotterill, 1991).

Xenopus or *Drosophila* cell-free extracts are prepared from early embryonic cells, whose cytoplasm contains sufficient nuclear precursors to allow the formation of thousands of nuclei during the rapid cell divisions of the early embryo. These cell-free extracts have been used to analyze chromatin organization (Newport, 1987; Ulitzur and Gruenbaum, 1989), nuclear transport (Finlay and Forbes, 1990), DNA replication (Blow and Laskey, 1986; Mills et al., 1989; Crevel and Cotterill, 1991) and chromatin-membrane interaction (Murrey and Kirschner, 1989*a*,*b*; Pfaller et al., 1991). The assembly of chromatin from naked DNA or demembranated sperm occurs through distinct intermediate steps of chromatin decondensation and nuclear envelope formation (Newport, 1987; Ulitzur and Gruenbaum, 1989). The first step in the assembly is the interaction of histones with added DNA or sperm chromatin. Although the assembly of nucleosomes with the DNA has been intensively studied, beside topoisomerase II (Newport, 1987; Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990 and N. Ulitzur, unpublished observations), very little is known about the function of other proteins in the process. Candidates for such proteins include other nuclear and nucleolar proteins as well as nuclear envelope proteins (Dilworth et al., 1987; Philpott et al., 1991).

The last step in the assembly of the nucleus is the formation of nuclear lamina and nuclear membranes around the decondensed chromatin (Newport, 1987; Ulitzur and Gruenbaum, 1989). It was recently demonstrated that the nuclear membrane assembly requires specific, yet unidentified, proteins which are present in the nuclear membrane fraction and on the chromatin surface, whose activity is regulated by phosphorylation (Wilson and Newport, 1988; Pfaller et al., 1991).

The role of the nuclear lamina in the nuclear envelope assembly process was shown to be essential in the case of mammalian cells (Burke and Gerace, 1986) and is still not clear in the case of *Xenopus* extracts, where the nuclear lamina is probably composed of only the embryonic L_{III} lamin. While in one report, depletion of lamin L_{III} with anti-L_{III} lamin antibodies inhibited nuclear envelope formation (Dabauvalle et al., 1991). In other reports, depletion of lamin L_{III} from the extract did not obstruct nuclear envelope reconstitution. However, the assembled nuclei were fragile and unable to undergo DNA replication (Newport et al., 1990; Meier et al., 1991).

The possible interaction of the lamina with chromatin is still not well defined. Experiments with mammalian mitotic chromosomes have shown affinity of lamin type A/C to chromosomes (Burke, 1990; Glass and Gerace, 1990). Three-dimensional light and EM experiments have indicated that in *Drosophila*, as in mammalian cells, the nuclear lamina is composed of 200-nm-thick fibers and that only a small portion of the chromatin is suficiently close to these lamin fibers to allow an interaction between lamina and chromatin (Paddy et al., 1990).

Drosophila melanogaster is an organism well suited to the study of nuclear envelope structure and function. Homologues of many nuclear envelope proteins have been identified (Fisher, 1988) including the well-characterized lamin. The *Drosophila* nuclear lamina is probably composed of a single polypeptide which is encoded by a single gene (Gruenbaum et al., 1988). The single primary translation product of lamin is modified to give rise to several isoforms. During early embryogenesis and in mitosis, lamin is present as a single 75-kD isoform. Upon assembly into the nuclear envelope, two isoforms of 74 and 76 kD are present in nearly equimolar ratios (Smith et al., 1987; Gruenbaum et al., 1988; Smith and Fisher, 1989). Thus, one advantage of the *Drosophila* cell-free system over *Xenopus* cell-free system is that some of the proteins of interest, including the otefin protein (Padan et al., 1990), are not embryo specific.

To analyze whether the formation of the nuclear lamina is required for nuclear envelope formation, the *Drosophila* embryonic extract was incubated with anti-*Drosophila* lamin antibodies and the nuclear assembly process was followed. Analysis of the assembly process in extracts depleted of lamin activity, both by electron and fluorescence microscopy, revealed that, although chromatin decondensation proceeded as normal, membrane vesicles did not bind to the surface of the chromatin. Thus, our results suggest that, in *Drosophila* as in mammalian cells (Burke and Gerace, 1986), lamin is required for the proper attachment and assembly of the nuclear membrane vesicles around decondensed chromatin. Upon fractionation of the extract, lamin was found both in the membrane-pellet and the membrane-free fractions. In the absence of nuclear membranes, the endogenous lamin molecules showed affinity to chromatin surfaces. Addition of bacterially expressed lamin to the extract resulted in the assembly of these molecules on chromatin surfaces and did not inhibit membrane vesicle attachment. Taken together, these results support the essential role of the lamins in nuclear envelope assembly.

Materials and Methods

Nuclear Assembly Extracts and In Vitro Conditions

Sperm chromatin was prepared as described in Ulitzur and Gruenbaum (1989). Lambda DNA was prepared as described in Newport (1987). The preparation of *Drosophila* embryo extracts was as followed: 0–6-h-old *D. melanogaster* (Canton S) embryos were collected on feeding plates, washed with ST solution (0.7% NaCl, 0.1% Triton X-100), dechorionated for 90 s in 1.25% sodium hypochlorite, washed again in ST and PBS. The dechorionated embryos were rinsed three times with 3 vols of N buffer (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 100 mg/ml cycloheximide, 5 μg/ml cytochalasin B, 1 mM DTT, 10 μM chymostatin, 10 μM antipain, 10 μM pepstatin, 10 μM bestatin and 10 μM leupeptin). The embryos were then packed by 30 s centrifugation at 500 *g* and squashed in an eppendorf tube. The homogenized embryos were centrifuged (14,000 *g* for 5 min at 4°C) and the supernatant was transferred into a 0.6-ml tube and centrifuged again under the same conditions. The cytoplasm was removed with a syringe (crude extract) and used for nuclear assembly assays.

Subfractionation of the extract was achieved by a 150,000 *g* centrifugation at 4°C in a TLA-100 Beckman rotor (Beckman Instruments, Inc., Palo Alto, CA). The cytoplasmic fraction was supplemented with an ATP regenerating system (Newport, 1987; Ulitzur and Gruenbaum, 1989) and either used immediately or rapidly frozen in liquid nitrogen, and stored at −70°C. The membrane fraction was diluted 10-fold with N buffer spun at 10,000 *g*, and resuspended in the same buffer at a 10-fold concentration, compared with the original extract. Aliquots were frozen in liquid nitrogen and stored at −70°C. The nuclear assembly reaction was carried out as described (Ulitzur and Gruenbaum, 1989).

Binding of Nuclear Membranes to Chromatin

The binding of membranes to chromatin was carried out essentially as described by Pfaller et al. (1991). A standard binding assay consisted of 20 μl of N buffer containing 0.5 mg/ml polyglutamic acid (PGA)[1] (average molecular mass = 51 kD) (Sigma Chemical Co., St. Louis, MO), 5 μl of membranes isolated from interphase extracts, 1 μl demembranated rooster sperm (1,000 sperm cells/μl extract) and 1 μl of 100 mM ATP. Binding of membranes to the surface of the chromatin was followed under the fluorescence microscope using bisbenzimide to stain the DNA, and 3,3'-Dihexyloxacarbocyanine iodide (DECC) to stain the membranes.

Lamin Over-Expression

The expression and purification of the *Drosophila* lamin from *E. coli* and its assembly properties in vitro will be described in details elsewhere. The over-expression was achieved through the pT7 double plasmid system developed by Tabor and Richardson (1985). The *Drosophila* lamin cDNA was cloned into the target plasmid pT7-7 (pT7Dm0) and a NdeI restriction site containing the ATG start codon was introduced via polymerase chain reaction (PCR) directed mutagenesis (Higuchi, 1989). The entire segment amplified by PCR (380 bp) was confirmed by dideoxy sequencing (Sanger et al., 1977) from both strands and the final construct was verified by restriction digests. *E. coli* XL1-Blue cells carrying the pGP1-2 and pT7Dm0 plasmids were grown at 30°C to an OD_{600} of ~1.2. Expression was induced by a rapid shift to 42°C, for 20 min and continued growth at 37°C for 1–2 h. The lamin protein was isolated and purified to near homogeneity by conventional chromatography techniques. The purified protein exhibited the characteristic salt-dependent polymerization/depolymerization reported for interphase lamins isolated from *Drosophila* embryos (Lin and Fisher, 1990).

Antibody Production

After verification of the overproduced lamin with anti-*Drosophila* lamin mAb 611A3A6 (Harel et al., 1989) and with affinity purified anti-*Drosophila* lamin polyclonal antibody (gift of P. Fisher, SUNY at Stony Brook, Stony Brook, NY), 500 μg of purified protein were produced and injected to two rabbits. Highly specific anti-*Drosophila* lamin polyclonal antibodies, as judged by Western and immunofluorescence analyses, were obtained in both rabbits. The IgG fraction of the antibodies was obtained by passing the serum through a Protein A-column. For the inactivation experiments the antibodies were concentrated to a 100 μg/μl stock solution and 1–3 μl were used for each experiment. Preimmune sera were prepared in a similar manner. Rabbit IgG fraction was purchased from Sigma Chemical Co.

Inactivation of Lamin

To inhibit lamin activity in the embryonic extract, anti-*Drosophila* lamin antibodies (IgG fraction) from the two antibody preparations were separately preincubated for 60–90 min on ice with extracts supplemented with an ATP regenerating system with occasional mixing. A typical assay included 20 μl of either lamin-inactivated crude extract or 150,000 *g* cytoplasm, 3 μl of the ATP regenerating system and 1 μl of sperm chromatin (1,000 cells). The reaction mix was then incubated for additional 2–3 h at 22°C.

To assess the extent of lamin binding to the antibodies, 50 μl of packed formaldehyde-fixed *Staphylococcus aureus* (Sigma Chemical Co.) were

1. *Abbreviations used in this paper*: DECC, 3,3'-Dihexyloxacarbo-cyanine iodide; PGA, polyglutamic acid.

added to the extract for a further 60-min incubation at room temperature. The mixture was centrifuged and the antibody-bound pellet was washed several times with PBS. Aliquots of the remaining extract and untreated extracts (supernatant and pellet) were analyzed for the presence of proteins by Western blotting.

Immunofluorescent Staining of Nuclei

Nuclei and chromatin were stained for the presence of lamin by placing them on poly-L-lysine-coated coverslips and fixed for 10 min in 0.4% paraformaldehyde in PBS. The nuclei were washed 3 times in PBS containing 0.1% Triton X-100 (PBST) and incubated for 60 min with either polyclonal, or monoclonal 611A3A6 anti-*Drosophila* lamin antibodies. After three washes with PBST, either goat anti-rabbit or rabbit anti-mouse fluorescently conjugated IgG (Fab' fragment) (Jackson Immuno Research Laboratories, Inc., West Grove, PA) were added and incubation proceeded for an additional 45 min. After several washes with PBST, the nuclei were stained with bisbenzimide and the lipophilic dye DECC. Coverslips were mounted on glass slides in 2% n-propyl gallate, and viewed under a Leitz microscope equipped with epifluorescence (E. Leitz, Inc., Rockleigh, NJ). Photographs were taken using ASA 400 film (AGFA Corp., Orangeburg, NY).

Electron Microscopy

Samples were prepared for electron microscope analysis essentially as described (Dabauvalle et al., 1991). Aliquots were fixed by fivefold dilution in cold 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.5, for 30 min. After 10 min centrifugation at 3,000 *g*, the pellet was resuspended in 15 μl of 2% low melting agarose (Gibco-BRL, Bethesda, MD) prepared in 0.2 M cacodylate buffer at 35°C. After solidifying the agarose by chilling, the sample was fixed again for 5 min in 2.5% glutaraldehyde. After 1 h fixation in 1% OsO_4 and 30 min staining with 2% uranyl acetate, the samples were washed and dehydrated through a series of graded ethanol solutions. After embedding the samples in EPON, ultrathin sections were cut, stained according to standard procedures, and viewed under a Phillips 300 transmission electron microscope at 60 kV (Phillips Electronic Instruments Co., Mahwah, NJ).

In Vitro Lamin Assembly with Chromatin

Lamin assembly was carried out by incubating 3–10 μg of purified lamin with 10 μl of extract or 150,000 *g* cytoplasmic fraction and 1 μl sperm chromatin (1,000 sperm/μl extract) at 22°C for 30–120 min. Samples were taken at various time points, washed extensively with 10% FCS in PBS, and processed for immunofluorescence.

ATP Depletion from the Extract

To deplete the endogenous ATP, 20 μl of extract were incubated with 2 mM glucose, 10 mM $MgCl_2$, and 5 μg hexokinase (Sigma Chemical Co.), for 15 min at 22°C. The ATP-depleted extract was then used for the nuclear assembly assay.

Results

Characterization of Nuclear Assembly Process in Drosophila Extracts

Crude extracts of *Drosophila* embryos were used to reconstitute nuclei from naked lambda DNA (Fig. 1) or sperm chromatin (Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990). We found no difference in assembly efficiency with cell-free extracts prepared from 0–2-h-old embryos which were subjected to a 1-h cold treatment before homogenization (Crevel and Cotterill, 1991), as compared with a cell-free extract prepared from 0–6-h-old embryos. To further investigate the assembly process, the kinetics of sperm chromatin assembly was analyzed by EM (Fig. 2). As the chromatin begins to decondense, small membrane vesicles are seen attached to the chromatin periphery (Fig. 2, *A* and *B*). These vesicles presumably flatten and fuse to form a complete nuclear envelope perforated with nuclear pores, while the chromatin is seen associated with the inner nuclear membrane (Fig. 2, *C* and *D*).

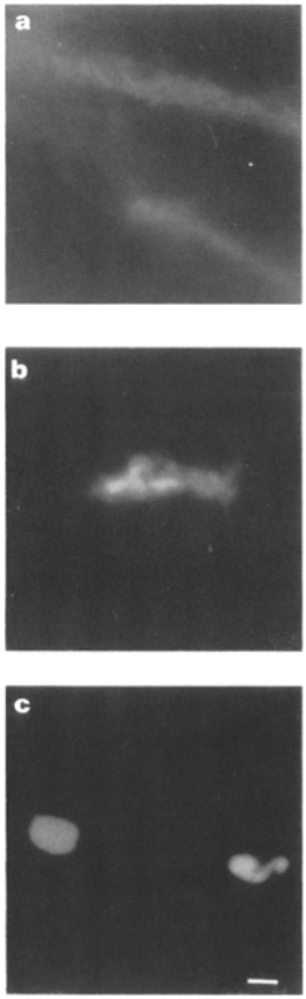

Figure 1. Assembly of Lambda DNA in *Drosophila* embryonic extracts. 1–2 μg of lambda DNA were incubated at 22°C in 20 μl of nuclear-assembly extract. Aliquots were taken at different time points: 0 (*a*), 60 (*b*), and 150 (*c*) min, stained with bisbenzimide and viewed under fluorescence microscope. Bar, 5 μm.

It was previously shown that the *Xenopus* assembly extract can be subdivided into a membrane-pellet fraction and a membrane-free cytoplasm (Lohka and Masui, 1984; Wilson and Newport, 1988). Similarly, the *Drosophila* extract can be subdivided into these two components by a 150,000 *g* centrifugation. The presence of both fractions in the assay was obligatory for completion of nuclear assembly (see Fig. 5 *B*, *k*). In addition, Pfaller et al. (1991) have recently reported that if sperm chromatin was first allowed to swell in the presence of PGA, incubation, in the absence of ATP, of the PGA-treated chromatin with the membrane pellet fraction, resulted in the binding of *Xenopus* nuclear membrane vesicles to the perichromatin. Thus, it was interesting to note that in the *Drosophila* extract, while chromatin decondensation is ATP dependent (Ulitzur and Gruenbaum, 1989), incubation of PGA-treated chromatin in the absence of an ATP regenerating system, resulted in complete nuclear reconstitution (data not shown).

Lamin Isoforms During Nuclear Assembly

Smith and Fisher (1989) have shown that during the first 7.5 h of *Drosophila* embryonic development most of the solu-

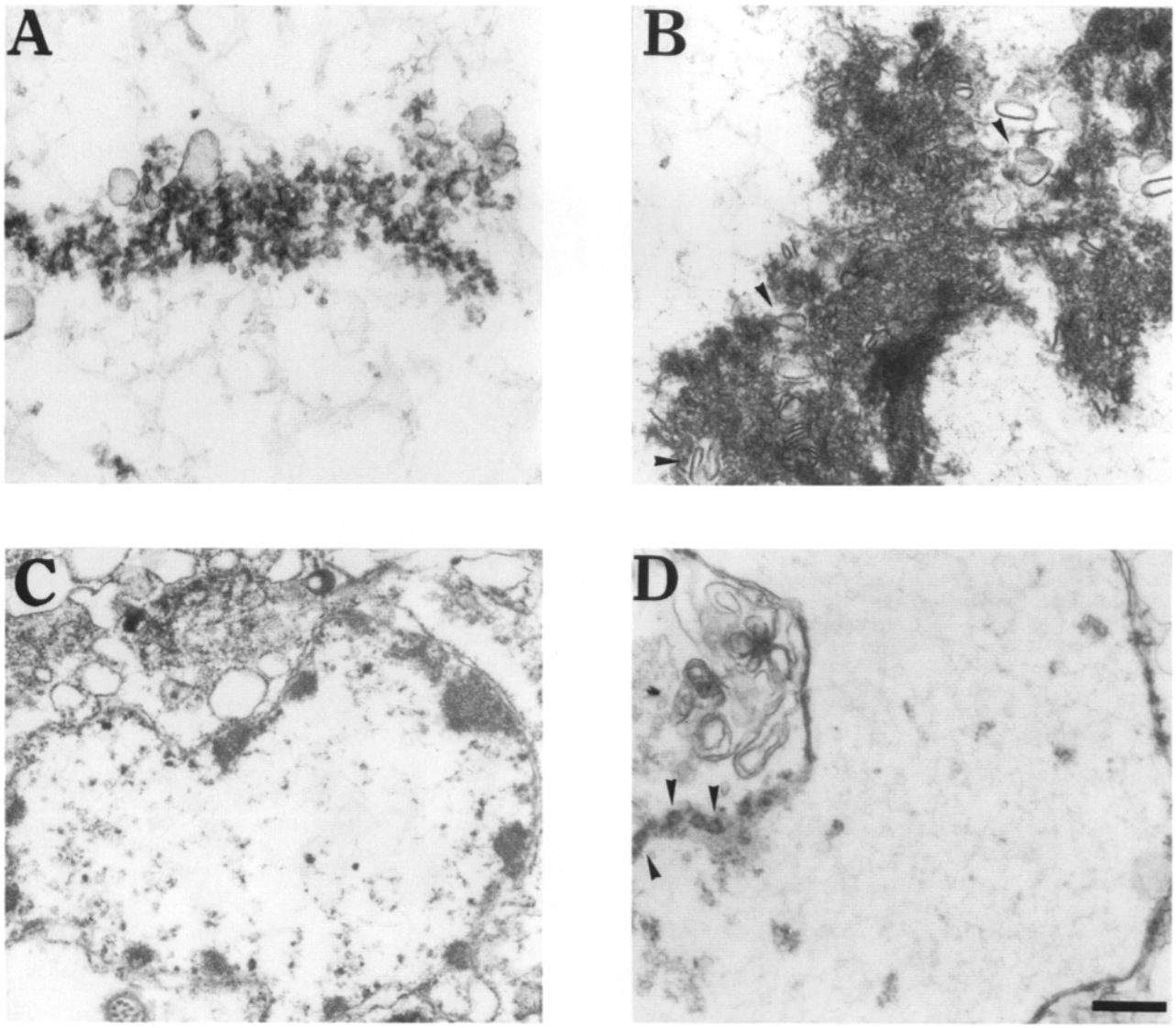

Figure 2. EM analysis of nuclear assembly in vitro. The embryonic extract was incubated with sperm chromatin for 2 h. At different time points, aliquots were taken from the reaction mixture and prepared for EM. During the first 5 min the chromatin was found in a condensed form (*A*). Within 15 min the chromatin began to decondense and membrane vesicles were detected attached to its surface (*arrows* in *B*). In the following 30–60 min the vesicles flattened and fused to form a double membrane enveloping the decondensed chromatin. The membrane surrounding the reconstituted nuclei appeared continuous and consisted of two membrane bilayers separated by a cisternal space (*C*). The double membrane was periodically interrupted by gaps that morphologically resembled nuclear pores (*arrows* in *D*). Bar, 1 μm.

ble lamin is present as a 75-kD isoform, while in interphase nuclei it appears as 74- and 76-kD isoforms. Protein blot examination of lamin in the two 150,000 *g* fractions revealed a single 75-kD isoform, present in nearly equimolar quantities in the membrane and the soluble fractions (Fig. 3, *b* and *c*). To determine which isoform is found attached to the chromatin, we allowed nuclear reconstitution to proceed for 2 h at 22°C. The chromatin was then isolated by 3,000 *g* centrifugation, washed with N buffer and subjected to protein blot analysis, using the 611A3A6 mAb. As shown in Fig. 3 *e*, after nuclear assembly, most or all of the lamin was converted into the 74- and the 76-kD isoforms.

Lamin Is Essential for Nuclear Envelope Formation

To analyze whether assembly of the nuclear lamina is required for nuclear envelope formation, we first preincubated the complete reconstitution extract with 100–300 μg polyclonal anti-*Drosophila* lamin antibodies (IgG fraction). Since immunodepletion of lamin from the membrane-pellet fraction resulted in coprecipitation of the nuclear membranes (data not shown), we chose to inactivate the protein instead of depleting it altogether. Incubation of the extract under the same conditions with 100–300 μg of preimmune rabbit sera (IgG fraction) served as controls. Quite dramatically, no membrane assembly was observed when lamin activity was inhibited. Electron and light microscope analyses revealed that while chromatin went through the characteristic decondensation process, membrane vesicles did not attach to its surface, and nuclear envelope did not assemble around it (Fig. 4 *A*). Two different preparations of antilamin polyclonal antibodies were used independently and gave

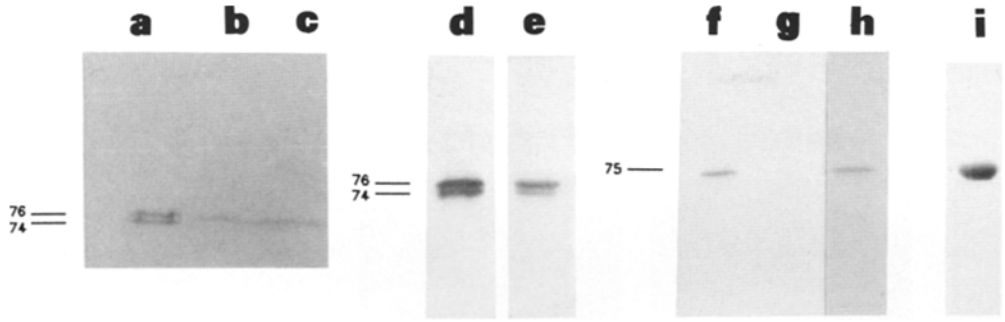

Figure 3. Protein blot analysis of lamin in: (*a* and *d*) *Drosophila* nuclei derived from 0–6 h-old-embryos; (*b* and *f*) the cytosolic fraction of the embryonic extract (membrane free) after 150,000 *g* centrifugation; and (*c*) the membrane-pellet fraction. Lamin associated with the chromatin was analyzed after incubation of chromatin with the complete extract (*e*). Lamin absence in the membrane-free fraction after depletion with antilamin antibodies is shown in *g* and the association of the depleted lamin with *S. aureus* is shown in *h*. Lane *i* shows a Coomassie blue staining of the purified bacterially over-expressed lamin. Proteins were separated on a 7.5% polyacrylamide-SDS gel and electrotransferred to nitrocellulose filters. The filters were incubated with 611A3A6 antilamin mAb followed and immunodecorated by alkaline phosphatase conjugated anti-mouse IgG and staining with bromochlorindolyl phosphate and nitro blue tetrazolium. The chromatin in *e* was separated from the extract by 3,000 *g* centrifugation and washed with N buffer. The position of the 74-, 75-, and 76-kD lamin isoforms is indicated on the left side of each panel.

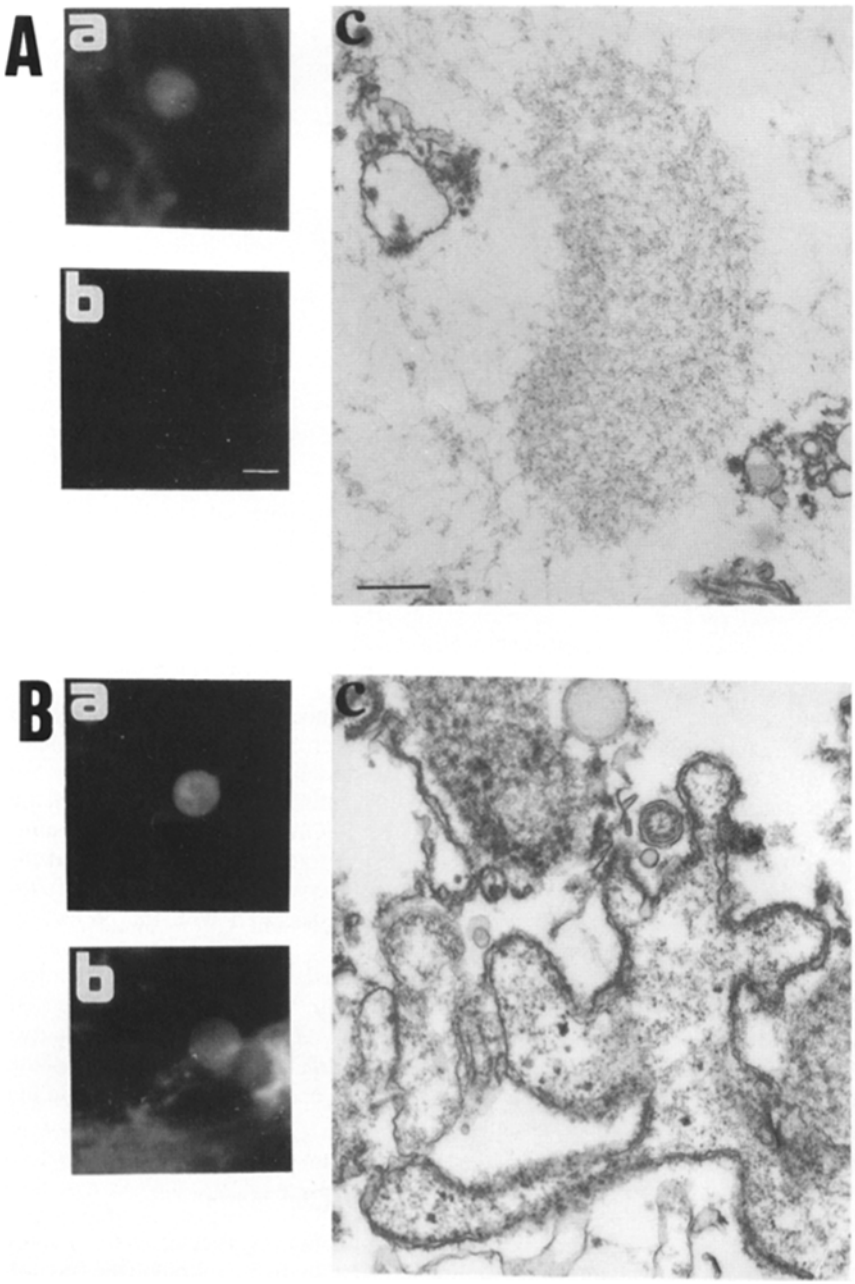

Figure 4. Inhibition of lamin activity prevents the in vitro nuclear envelope assembly in embryonic extracts. 20 μl of embryonic extracts were preincubated for 90 min with either 300 μg polyclonal antilamin antibodies (IgG fraction) (*A*), or 300 μg of preimmune serum antibodies (IgG fraction) (*B*). Sperm chromatin was added and the incubation proceeded for additional 90 min. Samples from the complete extract were removed and stained with the DNA dye bisbenzimide (*a*) and the membrane dye DECC (*b*). In contrast to the control experiments, when lamin activity was inhibited by antibodies, attachment of nuclear membrane vesicles to chromatin could not be detected (*A,b* vs *B,b*). Samples from the two experimental systems were also viewed under the electron microscope (*c*). Decondensed chromatin was enveloped with nuclear membranes in preimmune antibodies-treated extracts (*B,c*) but not in antilamin antibodies-treated extracts (*A,c*). Bars: (*a* and *b*) 10 μm; (*c*) 1 μm.

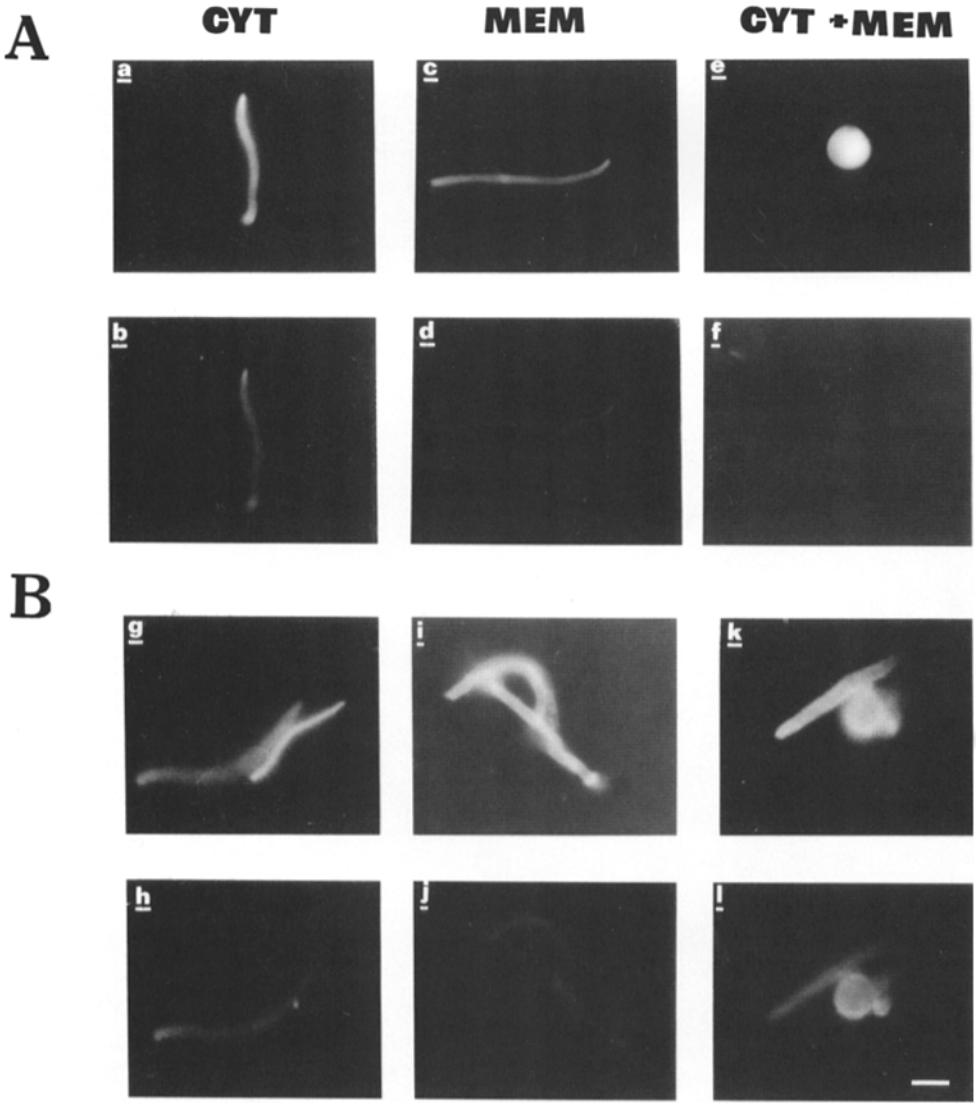

Figure 5. Lamin assembly on chromatin surface during in vitro nuclear assembly. The crude extract was fractionated by 150,000 *g* centrifugation as described in Fig. 3. Either the membrane-free (*CYT*) or the membrane-pellet fraction (*MEM*), or both (*CYT + MEM*), were incubated with antilamin antibodies (*A*). Preimmune serum served as a control (*B*). Incubation was for 90 min, in the presence of an ATP regenerating system. The membrane-free fraction was incubated for a further 60 min with packed formaldehyde-fixed *S. aureus* bacteria. The membrane-pellet fraction was washed of excess antibodies and each fraction was complemented by its untreated (*a–d* and *g–j*) or treated counterpart (*e* and *f*, and *k* and *l*). Sperm chromatin was added and incubation proceeded for 2 h at room temperature. Samples were stained for DNA with bisbenzimide (*a,c,e,g,i*, and *k*) and with antilamin antibodies (*b,d,f,h,j*, and *l*). The fraction that was treated with the antibody is indicated on top of each panel. Only when both fractions were treated with antibodies, an inhibition of nuclear lamin and nuclear membrane assembly could be observed (*e* and *f* vs. *k* and *l*). In *b* and *d* the exposure time of the film was three times longer. Staining with DECC gave similar results (data not shown). Bar, 10 μm.

similar results. Incubation of the extract with either of the two preimmune sera, (IgG fraction), or with commercially purchased normal rabbit IgG fraction had no effect on nuclear assembly and the presence of membranes and lamin around the chromatin was observed (Fig. 4 *B*, *c*).

To reveal whether lamin activity is mainly present in the membrane fraction or in the membrane-free fraction we inhibited the lamin activity in either or both fractions. In the first set of experiments, the 150,000 *g* cytoplasmic fraction was lamin-depleted by incubation with anti-*Drosophila* lamin polyclonal antibodies (IgG fraction) and immunoprecipitation of the immune complex with *S. aureus* protein blot analysis revealed that most or all the lamin was depleted from the extract (Fig. 3, *g* and *h*). When this lamin-depleted fraction was incubated with PGA-treated chromatin, lamin staining with 611A3A6 antibody was not observed. A similar assay with preimmune sera resulted in a weak but specific signal. This result indicated that there was indeed an interaction between soluble lamin and the chromatin which could be blocked by antilamin antibodies.

As mentioned above, immunodepletion of lamin from the membrane-pellet fraction is problematic since the vesicles tend to sediment with the immune complex. Therefore, we incubated this fraction with antilamin antibodies, washed the pellet and incubated it with PGA-treated sperm chromatin. When both antibody-treated fractions were joined and incubated with PGA-treated chromatin, nuclear membranes and lamin were not found associated with the chromatin (compare Fig. 5, *A*, *f* and *B*, *l*). However, when only one of the fractions was preincubated with antilamin antibodies, nuclear membranes and lamin reconstitution commenced normally (Fig. 5 *A*, *b* and *d*). Hence, it seems that the two lamin forms which sediment differentially, are not only of the same size but also are able to complement each other in the nuclear assembly process.

Association of Lamin with Chromatin

As mentioned above, immunofluorescence analysis of chromatin which was incubated with the membrane-free fraction

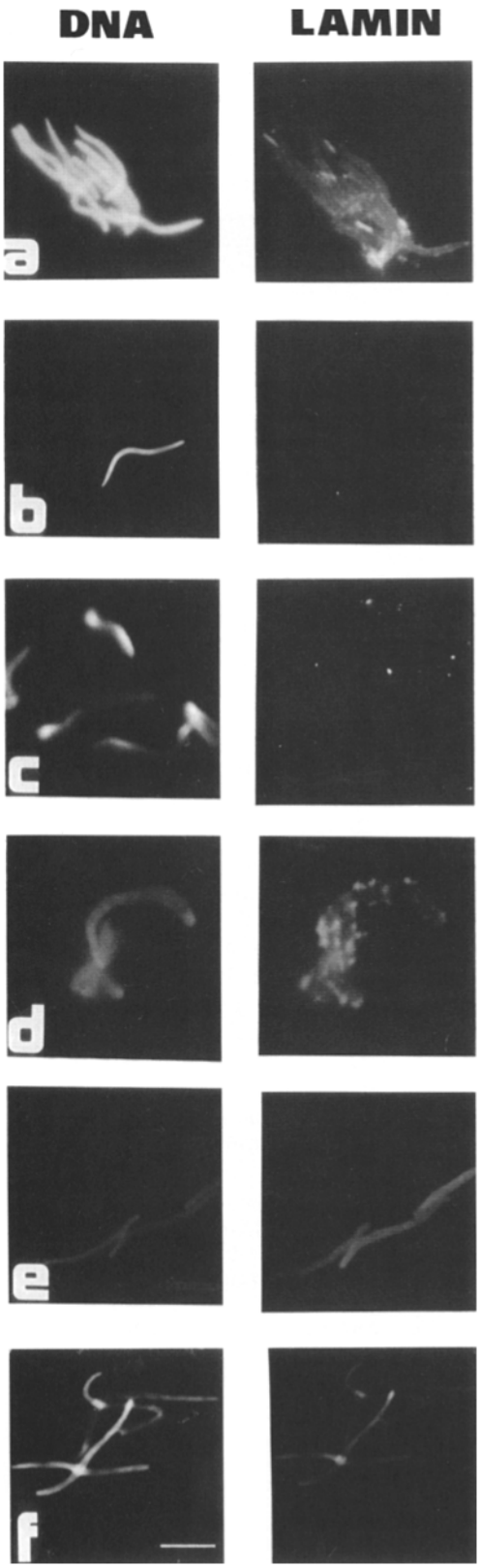

Figure 6. Association of lamin, expressed in *E. coli*, with chromatin in vitro. Purified lamin protein was incubated in the presence of 100 μg BSA in the following manner: (*a*) 5 μg of lamin in 15 μl of complete extract containing 1 μl of ATP regenerating system, and 1 μl of demembranated sperm; (*b*) 5 μg of lamin and 1 μl of demembranated sperm in N buffer; (*c*) 5 μg of lamin and 1 μl of PGA-treated demembranated sperm in N buffer; (*d*) 3 μg of lamin in 15 μl of membrane-free fraction of the extract and 1 μl of demembranated sperm. (*e*) similar to *d* but in the presence of 10 μg of lamin. *f* shows the association of endogenous lamin with the chromatin. After 60 min incubation the chromatin was separated by 3,000 *g* centrifugation and washed with PBS containing 10% FCS. DNA was stained with bisbenzimide, left; and lamin was stained with antilamin 611A3A6 antibody, right. In *f* the exposure time of the film was three times longer. Bar, 10 μm.

of the assembly extract resulted in weak but specific lamin staining (Fig. 6 *f*). To further study the association of lamin with chromatin in the absence of membranes, we performed an in vitro assay in which lamin expressed in bacteria was purified (Fig. 3 *i*) and incubated with PGA-treated chromatin, in the presence of either the 150,000 *g* membrane-free fraction or the complete extract. Association of lamin with chromatin was followed by immunofluorescence, using the 611A3A6 antilamin mAb (Fig. 6). A significant increase in lamin staining on the surface of the decondensed chromatin was detected when the crude extract or the cytoplasmic fraction were present (compare Fig. 6, *a* and *f*). In contrast, association of lamin with sperm chromatin, either treated or untreated with PGA, was not observed in the absence of the extract (Fig. 6, *b* and *c*). Thus, it seemed that the presence of the embryonic extract was essential for lamin association with the chromatin. It was interesting to find that while addition of 3 μg of purified lamin protein produced a patchy staining on the surface of the chromatin (Fig. 6 *d*), incubation of 10 μg of lamin resulted in a more homogenous pattern of staining (Fig. 6 *e*).

To analyze the effect of increased amounts of lamin in the extract on nuclear envelope reconstitution, 3–10 μg of *Drosophila* lamin expressed in *E. coli* were added to the nuclear assembly reaction. The efficiency of nuclear assembly was similar to that in regular extracts (Ulitzur and Gruenbaum, 1989). Immunofluorescence analysis, using the 611A3A6 antibody and the DECC dye revealed a typical peripheral staining (Fig. 7). In another set of experiments the chromatin was assembled in the 150,000 *g* membrane-free fraction in the presence of the *E. coli* expressed lamin. Addition of the membrane-pellet fraction to the reaction resulted in the formation of nuclear membranes (data not shown). Thus, the increased amount of lamin associated with the chromatin, did not have a visible effect on the nuclear assembly process.

Discussion

The present study indicates that in *Drosophila* embryonic extracts nuclear membrane vesicles attachment to chromatin can be abolished by inhibiting lamin activity. Immunofluorescence and EM analyses revealed that extracts in which lamin activity was inhibited, were able to support chromatin assembly but not membrane vesicles attachment to the surface of chromatin. In these experiments lamin activity was inhibited with specific lamin antibodies. The specific inactivation of lamin in these experiments was judged by several criteria. (*a*) Protein blots and immunofluorescence analyses revealed a specific interaction between these antibodies and the lamin protein present in the nuclear periphery. This reaction was indistinguishable from that with the anti-*Drosophila* lamin 611A3A6 mAb. (*b*) Preimmune sera or nonspecific

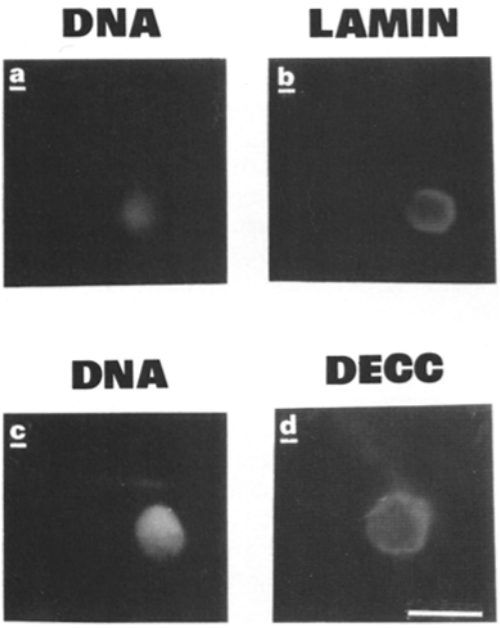

Figure 7. Nuclear membrane assembly in the presence of added lamin. 10 μg of lamin expressed in *E. coli* were added to the extract, and nuclear assembly of demembranated sperm chromatin into nuclei was allowed to proceed. After a 90-min incubation samples were taken and subjected to immunofluorescence analysis. Using bisbenzimide (*a* and *c*), antilamin 611A3A6 antibody (*b*), or DECC (*d*). Bar, 10 μm.

anti-rabbit antibodies had no effect on nuclear envelope reconstitution. (*c*) Immunodepletion experiments of the soluble lamin from the 150,000 *g* membrane-free cytosolic fraction revealed that most or all the lamin could be immunoprecipitated from the solution. Lamin binding to chromatin was not detected with either the immunodepleted lamin fraction or with crude extracts which were incubated with these antibodies. (*d*) Independent anti-*Drosophila* lamin antibody preparations gave similar results.

Similar experiments in the *Xenopus* system have also indicated an essential role of the nuclear lamina in proper nuclear assembly. However, depletion of lamin L_{III} from *Xenopus* extracts did not inhibit nuclear membrane vesicles attachment to chromatin (Newport et al., 1990; Meier et al., 1991). In comparing the nuclear assembly process in *Xenopus* and *Drosophila*, one should bear in mind that the single lamin L_{III} of *Xenopus* is oocyte specific and upon nuclear envelope disassembly is apparently not membrane associated (Benavente et al., 1985; Newport et al., 1990). In *Drosophila*, on the other hand, the same lamin isoforms are found throughout development (Smith and Fisher, 1989) and during mitosis in early embryos, a fraction of the lamin remains with the membranes in an envelope-like structure (Harel et al., 1989). Thus, the *Drosophila* lamin isoforms may have an additional role as compared to the *Xenopus* L_{III} lamin. The essential role of the nuclear lamina in the nuclear envelope formation as observed here in *Drosophila* extracts, agrees well with the in vivo (Benevente and Krohne; 1987) and in vitro (Burke and Gerace; 1986) observations in mammalian cells.

Roughly half of the lamin pool in *Drosophila* embryos was found to be present in the membrane-pellet fraction. Indeed, when lamin activity in the membrane fraction was inhibited, the cytoplasmic soluble lamin could assemble on chromatin surface and support nuclear envelope assembly. The membrane-associated lamin seemed to have the same activity as compared with the soluble lamin, since its interaction with antibodies did not abolish nuclear envelope assembly.

The present study also provides evidence that lamin molecules can specifically associate with chromatin. The soluble lamin in the extract was found to be associated with the chromatin surface. This association did not require the presence of the nuclear membrane vesicles. However, when the membrane fraction was added back to the extract, more lamin was found associated with the chromatin, as judged by immunofluorescence analysis. This increase probably resulted from lamin associated with the membranes. Furthermore, addition of the bacterially expressed lamin to the extract resulted in the association of these molecules with the chromatin. This association was specific since it was detected even in the presence of 100 μg BSA, or normal serum proteins, which were added to the extract. Washing the chromatin with PBS containing 10% FCS did not affect this association either. The specific interaction between lamin and chromatin is in agreement with other reports (Glass and Gerace, 1990; Hoger et al., 1991; Burke, 1990; Yuan et al., 1991), which demonstrated that in vertebrate cells, the chromosome-directed lamin assembly may be a cooperative process mediated by both lamin–lamin and lamin–chromatin interactions. Thus, it is likely that one of the initial steps in nuclear envelope assembly requires the interaction between lamin and chromatin.

The addition of increased amounts of lamin to the nuclear assembly reaction did not have a visible effect on the attachment of nuclear membranes to the chromatin. The interaction between the nuclear lamina and the nuclear membranes is probably mediated by proteins present in the nuclear membrane vesicles (Wilson and Newport, 1988). Candidates for such proteins are the lamin B receptor (Worman et al., 1988), otefin (Padan et al., 1990), or as yet unidentified proteins. Further studies making use of this in vitro assay may allow us to determine and characterize the binding sites of lamin to the chromatin, and to elucidate the importance of lamin–chromatin interaction for chromatin structure.

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