Authors’ correction

Assembly of A- and B-type lamins studied in vivo with the baculovirus system

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In this paper we reported that Drosophila lamin C assembled into paracrystals in the cytoplasmic and nuclear compartment of Sf9 cells but was not found in association with the nuclear lamina.

In the process of generating Drosophila lamin C mutants we discovered that the specific lamin C cDNA we had used for this study was missing nucleotide G1781 of the published lamin C cDNA sequence (Bossie and Sanders, 1993, J. Cell Sci. 104, 1263-1272).

This single nucleotide deletion resulted in a frame shift which completely changed the sequence of the carboxy-terminal 69 amino acids. As a further consequence, the mutant protein was 9 amino acids shorter than the wild-type protein.

When the correct Drosophila lamin C was expressed in Sf9 cells we observed the following difference in its behavior in comparison to the frame shift mutant. Nuclearily localized paracrystals assembled by the wild-type protein were associated with the lamina. This result indicates that Drosophila lamin C contains in its carboxy-terminal tail a domain that mediates its targeting to the lamina. The targeting behavior of Drosophila lamin C and mutants derived thereof will be described in detail elsewhere by G. Krohne, N. Stuurman and A. Kempf.
Assembly of A- and B-type lamins studied in vivo with the baculovirus system

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SUMMARY

We have expressed an A-type lamin (Xenopus lamina A), a probable A-type lamin (Drosophila lamina C), two B-type lamins (Xenopus lamina II, Drosophila lamina Dmo), and two mutants of Xenopus lamina A in S9 cells. All proteins were synthesized at high levels resulting in formation of paracrystals with an axial repeat of 18.5-20.0 nm by A-type lamins; in contrast B-type lamins assembled into aggregates with a fibrillar ultrasticture. Of the four wild-type proteins analyzed only lamin Dmo was found in the nuclear compartment of S9 cells in association with the lamina whereas the three other lamins assembled into polymers localized in the cytoplasm as well as the nucleoplasm.

INTRODUCTION

Lamins are members of the multigene family of intermediate sized filaments, which form a filamentous meshwork lining the nucleoplasmic surface of the inner nuclear membrane (Aebi et al., 1986; Scheer et al., 1976; McKeon et al., 1986; for a recent review see Moir et al., 1995). In vertebrates two major isotypes can be distinguished according to their behavior in mitotic cells. B-type lamins remain associated with membranes throughout the cell cycle, whereas A-type lamins depolymerize with the onset of mitosis into distinct soluble oligomers (Gerace and Blobel, 1980; Stick et al., 1988; for reviews see Nigg, 1992; Moir et al., 1995). In Drosophila lamin behavior is more difficult to categorize (see Smith and Fisher, 1989; Ashery-Padan et al., 1997). Lamin binding to the nuclear envelope is at least partly mediated by interaction with integral membrane proteins of the inner nuclear membrane (for reviews see Georgatos et al., 1994; Gerace and Foisner, 1994).

Domains have been identified on lamin molecules that are required for: (1) nuclear transport (Loewingier and McKeon, 1988; Hennekes et al., 1993; Chelsky et al., 1989; Haas and Jost, 1993); (2) membrane association (Hennekes and Nigg, 1994; Holtz et al., 1989; Kitten and Nigg, 1991; Krohne et al., 1989; Schmidt et al., 1994; Schmidt and Krohne, 1995); (3) in vitro assembly (Gieffers and Krohne, 1991; Heitlinger et al., 1992; Moir et al., 1991; Stuurman et al., 1996); (4) mitotic disassembly (Haas and Jost, 1993; Heald and McKeon, 1990; Peter et al., 1990; Stuurman et al., 1996); (5) in vitro chromatin binding (Glass et al., 1993; Höger et al., 1991a); and (6) lamin degradation during apoptosis (Rao et al., 1996).

The lamin assembly in vitro revealed that parallel aligned dimers assemble into longitudinal ‘head-to-tail’ aggregates by the association of the highly conserved amino-terminal end domain of the alphahelical rood of one dimer with the carboxy-terminal end domain of the rod of a second dimer (Aebi et al., 1986; Gieffers and Krohne, 1991; Heitlinger et al., 1991, 1992; Moir et al., 1991; Stuurman et al., 1996). Under optimal buffer conditions ‘head-to-tail’ aggregates of A-type as well as B-type lamins associate laterally and form paracrystals with a periodicity of 24-25 nm (Aebi et al., 1986; Gieffers and Krohne, 1991; Heitlinger et al., 1992; Moir et al., 1991; Stuurman et al., 1996). Mutations in the end domains of the alphahelical rod disturb the ordered polymerization of lamins in vitro (Heitlinger et al., 1992; Stuurman et al., 1996; Schmidt and Krohne, 1995).

So far the formation of lamin polymers has only been analyzed in vitro at the electron microscopical level for one mutant of human lamin A containing an insertion in the alphahelical domain (Holtz et al., 1989). No distinct substructures were visible in the membrane associated aggregates of lamin A.

To acquire more information on the in vivo assembly of nuclear intermediate filament proteins we have expressed Xenopus lamins A and LI (Krohne et al., 1987) and Drosophila lamins Dmo (Gruenbaum et al., 1988) and C (Bossie and Sanders, 1993; Riemer et al., 1995) at high levels in insect cells...
by the use of the baculovirus system (for review see O'Reilly et al., 1994). This system has been successfully used for structural analysis concerning the interactions of microtubules with microtubule associated proteins (Chen et al., 1992), the assembly of neuronal intermediate filament proteins (Nakagawa et al., 1995), and the behavior of an integral membrane protein of tight junctions (Furuse et al., 1996).

We report here that polymers assembled in vivo by A-type and B-type lamins are clearly different. Xenopus lamin A and Drosophila lamin C formed paracrystals in the nucleoplasm as well as in the cytoplasm whereas the B-type lamins LI and Dmo polymerized into fibrillar aggregates without obvious periodic substructures. The assembly properties of the two Xenopus lamin A mutants were as predicted by their behavior in vitro.

MATERIALS AND METHODS

Cell culture

Sf9 cells of the butterfly Spodoptera frugiperda were grown in the absence of CO2 at 27°C in TC100 medium (Gibco, Eggenstein/Germany) supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine. Cells were routinely grown in 50 ml Falcon flasks (Labor Schubert, Schwandorf/Germany) containing 5 ml medium. All transfections were performed in flasks of this size.

Antibodies

Xenopus lamins LI, A, and lamin A mutant LA386 were detected with the murine monoclonal antibody X67, and the lamin A mutant LAN28 with murine monoclonal antibody X94 (Schmidt and Krohne, 1995; Höger et al., 1991a). The B-type lamin of Sf9 cells was detected with polyclonal antibodies raised in guinea pigs against the Drosophila lamin Dmo (Schmidt et al., 1995). Murine monoclonal antibody ADL195 is specific for the Drosophila lamin Dmo, and monoclonal antibody LC28 exclusively reacts with Drosophila lamin C. Both monoclonal antibodies were raised against the full length Drosophila proteins using standard methods (Stuurman et al., 1995; Riemer et al., 1995). Antibodies ADL195 and LC28 do not react with endogenous lamins or any other proteins of Sf9 cells. The epitope for antibody X67 is localized in the non-helical amino-terminal head domain of vertebrate lamin isotypes B1 and A, and the epitope for antibody ADL195 is localized between amino acids 542 and 622 of lamin Dmo.

Lamin constructs

All Xenopus lamin cDNAs contained a bacterial ribosomal binding site cloned two nucleotides in front of the initiating ATG (Gieffers and Krohne, 1991; Schmidt et al., 1995; Schmidt and Krohne, 1995). The generation of the lamin A mutants lacking the complete carboxy-terminal tail (LA386; Gieffers and Krohne, 1991) or the amino-terminal 28 amino acids (LAN28; Schmidt et al., 1995) has been described. The cDNA for Drosophila lamin Dmo (Gruenbaum et al., 1988) had been previously subcloned into the bacterial expression vector pET3A (Studer et al., 1990; Stuurman et al., 1996; Riemer et al., 1995). A NdeI-HpaI fragment from this plasmid was transferred into pET17bx cut with NdeI and EcoRV (Novagen, Madison, WI). Drosophila lamin C DNA (Bosse and Sanders, 1993) was inserted into pET17bx as described previously (Riemer et al., 1995). By digestion of both plasmids (lamin Dmo: pET17LFL; lamin C: pET17LC) with XbaI and NotI both cDNAs were excised together with the bacterial ribosome binding site of the pET-vector and cloned into the multiple cloning site of the transfer plasmid pVL1393. Standard protocols (Ausubel et al., 1992) were used for the subcloning of the Xenopus cDNAs into the multiple cloning site of the transfer plasmids pVL 1392 and pAcG2 (Pharmingen, Hamburg/Germany). For transfection experiments the plasmid DNA was purified using QIAGEN Plasmid Midi Kit (QIAGEN, Hilden/Germany). The purified DNA was resuspended in distilled H2O, sterile filtered (Millipore filters, pore size 0.22 µm; Hartenstein, Würzburg/Germany), and stored at −20°C. The quotient of the optical density of the DNA at 260 nm/280 nm was in general 1.7-2.0, and its concentration 0.3-1 µg/µl H2O.

Transfection of Sf9 cells

The transfection solutions were prepared in polystyrol Falcon centrifuge tubes (Sarstedt, Nümbrecht/Germany) as follows. One tube contained 1-2 µg of the transfer plasmid and 100-500 ng of linearized BaculoloGold™-DNA (Pharmingen) in a final volume of 12 µl H2O. A second tube contained 6 µl Lipofectin (Gibco, Eggenstein/Germany) and 6 µl H2O. The solutions of both tubes were combined and gently mixed, avoiding the shearing of the viral DNA, and subsequently incubated for 15 minutes at room temperature.

2×10^6 Sf9 cells growing at a logarithmic rate were resuspended in 5 ml of complete TC 100 medium and cultured for approximately 2 hours until they had attached to the bottom of the flask. All attached cells were washed two times each with 5 ml of incomplete TC100 medium (medium without fetal calf serum, glutamine and antibiotics), avoiding their resuspension. 2.5 ml of incomplete TC100 medium were added per flask, followed by 24 µl of the transfection solution. The Sf9 cells were then incubated for 2 hours at 27°C, after which 2.5 ml of complete TC100 medium were added to each flask, and the cells were cultured for 7 days. We did not observe significant differences in the transfection efficiency among the transfer vectors. Recombinant baculoviruses contained in the culture medium were amplified by growing 2×10^6 Sf9 cells for 7 days in 3 ml of complete TC100 medium supplemented with 2 ml of culture medium containing the recombinant baculoviruses. The amplification was repeated a second time to ensure a high virus titer. Each virus containing culture medium was cleaned of cell debris by centrifugation (2,000 g; 10 minutes) and stored at either 4°C or −196°C in liquid nitrogen. Prior to freezing, 9 parts of the virus containing culture medium were mixed with 1 part of fetal calf serum.

Cell fractionation

Approximately 2×10^6 Sf9 cells were infected with the recombinant viruses, harvested 2-3 days later by centrifugation (1,000 g, 5 minutes), washed twice with incomplete TC100 medium, and then once with phosphate buffer (100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaHPO4; 1 mg/100 ml trypsin inhibitor, 0.2 mM PMSF, final pH 7.2). After each washing step cells were pelleted as described above. All further steps were performed at 4°C. Cells were resuspended by pipetting in phosphate buffer containing 0.5% Triton X-100, incubated for 15 minutes, and then pelleted by centrifugation (13,000 g, 10 minutes). The supernatant containing solubilized proteins was removed and frozen immediately in liquid nitrogen, and stored at −70°C. The pellet was washed once with phosphate buffer and then stored frozen.

Gel electrophoresis and immunoblotting

Proteins contained in the supernatant of fractionated cells were precipitated with chloroform/methanol (Schmidt et al., 1994). Proteins were separated by SDS-PAGE (10% polyacrylamide; Laemmli, 1970) and transferred to nitrocellulose filters (Schleicher and Schüll, Dassel/Germany) using the semidry method of Khysse-Andersen (1984). Culture medium containing monoclonal antibodies against Drosophila lamins was diluted 1:100 (ADL195) or 1:10,000 (LC28) with TBST buffer (140 mM NaCl, 0.3% Tween-20, 10 mM Tris-HCl, pH 8.0) containing 5% nonfat dry milk. Blocking of the filters, dilution of antibodies against Xenopus lamins, incubation with antibodies and washing of the filters were performed as described (Schmidt et al., 1994). Bound antibodies were detected using an...
Microscopic procedures

For immunofluorescence microscopy infected Sf9 cells were pelleted (1,000 g, 5 minutes), resuspended in incomplete TC100 medium, and pelleted again (see above). The cells were washed one more time, resuspended in incomplete TC100 medium and pelleted with a cyto spin 2 (Shandan, Frankfurt/Germany) onto coverslips (1,000 rpm, 10 minutes). Prior to use, the coverslips, which had been washed in ethanol and dried, were coated for 12 hours with polylysine (100 μg/ml H2O2), briefly washed in distilled water and air dried. Coverslips with attached cells were washed in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, 7 mM Na2HPO4; pH 7.4), fixed at −20°C for 10 minutes in methanol, then with acetone at −20°C for 2-5 minutes, and then air dried. Alternatively, Sf9 cells were grown directly on coverslips for 36-48 hours and fixed as described above.

Cells expressing Drosophila lamins Dmo or C were pelleted with a cyto spin onto coverslips and washed with PBS as described above. Coverslips with attached cells were then fixed for 10 minutes with 2% formaldehyde (freshly prepared from paraformaldehyde) in PBS, followed by an incubation in PBS containing 0.2% Triton X-100 for 5-10 minutes.

All coverslips were incubated for 5 minutes in PBS containing 0.3% bovine serum albumin (BSA), and then with antibodies X67 or X94 at a dilution of 1:200 in PBS/0.3% BSA. Culture medium containing monoclonal antibodies against Drosophila lamins was used non-diluted (ADL195) or diluted 1:100 (LC28) in PBS/0.3% BSA. Incubation with fluorescently labelled secondary antibodies and mounting of the specimens were performed as described previously (Schmidt et al., 1994, 1995).

Cells treated with phosphate buffer containing 0.5% Triton X-100 (see Cell fractionation) were pelleted (3,000 g, 10 minutes), resuspended in phosphate buffer and pelleted again (3,000 g, 10 minutes). Extracted cells were resuspended in phosphate buffer and centrifuged with the cyto spin onto coverslips as described above. Triton extracted cells expressing Xenopus or Drosophila lamins were processed without further fixations for immunofluorescence microscopy as described above. Micrographs were taken with a Zeiss Axiophot (Zeiss, Jena/Germany).

For electron microscopy native Sf9 cells were either pelleted (1,000 g, 5 minutes), fixed and embedded in Epon as described (Gieffers and Krohne, 1991) or were treated as follows prior to fixation. Sf9 cells were washed with incomplete TC100 medium, pelleted (see above), resuspended at 4°C in PBS containing 3% formaldehyde (freshly prepared from paraformaldehyde), and fixed for 15 minutes at 4°C. Fixed cells were pelleted (1,000 g, 10 minutes), washed once with phosphate buffer (see Cell fractionation) and extracted for 15 minutes at 4°C with phosphate buffer containing 0.5% Triton X-100. The Triton resistant structures were pelleted (1,000 g, 10 minutes), fixed, embedded in Epon and processed for ultrathin sectioning (Gieffers and Krohne, 1991).

For electron microscopical immunolocalizations native cells were first extracted with phosphate buffer containing 0.5% Triton X-100 (see above), and attached to coverslips by centrifugation as described for immunofluorescence microscopy. Attached cells were first incubated for 5 minutes with PBS, 0.3% BSA, and then with lamin antibodies X67, X94 (dilution 1:200 in PBS, 0.3% BSA), ADL195 (non-diluted culture medium) or LC28 (dilution 1:100 in PBS, 0.3% BSA) for 45 minutes. Coverslips were washed twice each for 5 minutes in PBS and were then incubated (dilution 1:10 in PBS, 0.3% BSA) for 2 hours at 4°C with secondary antibodies conjugated to 6 nm colloidal gold (rabbit anti-mouse; Dianova, Hamburg/Germany). To control for non-specific binding, cells were incubated with the gold-conjugated antibodies alone. Cells on coverslips were washed in PBS, fixed (Gieffers and Krohne, 1991), and flat embedded in Epon. An overnight staining of the fixed samples with aqueous uranylacetate was omitted.

Alternatively, native cells attached to coverslips by centrifugation were permeabilized by fixation for 20 minutes at −20°C in acetone, directly transferred in PBS for 10 minutes, and then processed for the immunolocalization as described for the Triton extracted cells.

RESULTS

The comparison by SDS-PAGE of total cellular proteins of non-infected Sf9 cells (Fig. 1A, lane 5; Fig. 2A, lane 4) with Sf9 cells expressing Xenopus (Fig. 1A) or Drosophila (Fig. 2A) lamins revealed that Xenopus lamins A, LI, the lamin A mutant LA386 lacking the complete carboxy-terminal tail (Fig. 1A, lanes 2-4) and the Drosophila lamins Dmo and C (Fig. 2A, lanes 2 and 3) were expressed at a very high level, enabling their visualization on Coomassie blue stained gels. Identical results were obtained for the lamin A mutant LAN28 (data not shown).

To determine whether the Xenopus and Drosophila lamins formed polymeric structures in Sf9 cells or whether they were present as soluble oligomers, we extracted the insect cells with buffers containing physiological salt concentrations and the detergent Triton X-100 (Figs 1B and 2B). The insoluble cell residues were pelleted, and proportional amounts of the

Fig. 1. (A) Coomassie blue stained SDS-PAGE (10% polyacrylamide) of total proteins of non-infected Sf9 cells (lane 5) and of cells expressing Xenopus lamins A (lane 2), LI (lane 3), LA386 (lane 4). Molecular masses of reference proteins (lane 1) are from top to bottom (in kDa) 205, 116, 97, 66, 45, 29.

(B) Subfractionation of Sf9 cells expressing Xenopus lamins A (LA, lanes 1-3), LI (lanes 4-6), and lamin A mutants LA386 (lanes 7-9) and LAN28 (lanes 10-12) by extraction with Triton X-100. Proteins were separated by SDS-PAGE, and lamins detected by immunoblotting with antibodies X67 (lanes 1-9) and X94 (lanes 10-12). T, total cellular proteins; P, pellet fraction; S, Triton supernatant. Molecular masses of reference proteins are marked by bars (in kDa, from top to bottom: 205, 116, 97, 66, 45, 29).
proteins recovered in the pellet fraction and in the supernatant were analyzed by immunoblotting with anti-lamin antibodies. We observed that Xenopus lamins A (Fig. 1B, lanes 2 and 3), LI (Fig. 1B, lanes 5 and 6), LA386 (Fig. 1B, lanes 8 and 9) and Drosophila lamins Dmo (Fig. 2B, lanes 2 and 3) and C (Fig. 2B, lanes 5 and 6) remained insoluble under these experimental conditions and were recovered in the pellet fraction. In contrast, when Sf9 cells which expressed the Xenopus lamin A mutant lacking the 28 amino-terminal amino acids (LAN28) were treated with Triton X-100, at least 50% of this lamin was released when nearly physiological buffers have been used for extraction of soluble cellular components that could mask some of the cells exhibiting a high expression of the recombinant protein it is obvious that the Xenopus lamin is contained in the nuclear compartment of the infected cells (Fig. 3A, arrowhead); however, the majority of lamin aggregates seem to be localized in the cytoplasm. In contrast to the two Xenopus wild-type lamins the lamin A mutant LA386 polymerized into long threadlike structures (Fig. 3C) that were already visible 36 hours p.i. These lamin aggregates increased with the period of infection in length and thickness (Fig. 3C, compare the two lamin ‘threads’ marked by arrows). In some cells these threads seem to occupy most of the cytoplasmic space (Fig. 3C, arrowhead). The cell marked by the arrowhead also shows that lamin LA386 is not enriched in the nucleus.

When we analyzed Sf9 cells expressing the lamin A mutant LAN28 (Fig. 3D) we did not observe lamin aggregates, instead the infected cells were uniformly stained and the protein seemed to be enriched in the nuclei of several cells (Fig. 3D, arrows). This staining pattern was only observed when we used cells that had been fixed with acetone or 2-3% formaldehyde, whereas cells that had been permeabilized with Triton X-100 showed a drastically reduced reaction with antibodies against Xenopus lamins (data not shown). When we performed identical experiments with Sf9 cells infected with the lamins described in Fig. 3A-C and the Drosophila lamins (see below), we never observed fixation-dependent changes in the staining pattern and intensity. This indicates that these lamins had formed polymeric aggregates, whereas most of the lamin LAN28 molecules were present in vivo in a soluble form.

Sf9 cells expressing the Drosophila lamins C (Fig. 4A) or Dmo (Fig. 4B) were easy to distinguish from each other by immunofluorescence microscopy. Numerous granular and streak-like structures were decorated by antibodies in cells (Fig. 4A) which synthesized lamin C, whereas the expression of lamin Dmo resulted in most infected cells (Fig. 4B) in a rim-like staining reminiscent of a nuclear lamina staining in Sf9 cells (see inset in Fig. 3B).

For electron microscopical analysis, Sf9 cell cultures that synthesized a specific Xenopus or Drosophila lamin were processed in parallel in three different ways. First, cells were directly fixed with glutaraldehyde and osmiumtetroxide to guarantee an optimal preservation of all cellular components. Second, cells were fixed with 3% buffered formaldehyde and then incubated with the detergent Triton X-100 to allow the extraction of soluble cellular components that could mask cytoskeletal structures (for details see Bartnik et al., 1985; Nakagawa et al., 1995). Third, by electron microscopical immunolocalization on permeabilized cells we demonstrated that the identified structures represented lamin polymers.

The comparative analysis of the four wild-type lamins revealed that Xenopus lamin A and Drosophila lamin C assembled into similar structures and that polymers of lamins LI and Dmo exhibited similar morphologies. Therefore the results concerning lamins A and C, respectively, and the B-type lamins LI and Dmo will be presented together.

The inspection of ultrathin sections through Sf9 cells that
expressed *Xenopus* lamin A (Fig. 5) or *Drosophila* lamin C (Fig. 6) demonstrated that electron dense aggregates of variable dimensions were detectable in the cytoplasm (Figs 5A,B, 6A, left half of the micrograph) as well as in the nucleoplasm (Fig. 6A, right half of the micrograph). These aggregates exhibited in some areas a paracrystalline morphology with an axial repeat of 18.5-20 nm, whereas other areas had an apparently amorphous substructure. The periodic substructure of the

**Fig. 3.** Indirect immunofluorescence (left) and phase contrast (right) microscopy of Sf9 cells expressing *Xenopus* lamins A (A,A’), LI (B,B’), LA386 (C,C’), and LAN28 (D,D’) after staining with antibodies specific for *Xenopus* lamins (antibody X67: A-C; antibody X94: D). The inset (B,B’) shows an Sf9 cell of a culture expressing lamin LI that has been stained with antibodies specific for the endogenous B-type lamin of insect cells (guinea pig antibodies against lamin Dmo: B,B’, insets). Micrographs show cells under ultraviolet light (A-D, inset in B) and under phase contrast optics (A–D, inset in B`). Sf9 cells were processed for immunofluorescence 48 hours (A) or 60 hours (B-D, inset in B’) post infection. (A’,B’) Arrows mark cells at early stages of lamin expression containing small dot-like lamin aggregates and the arrowhead in A’ denotes a cell exhibiting a strong nuclear lamin staining. (C) Arrows mark lamin threads of different size and the arrowhead in C denotes a cell of which the whole cytoplasmic compartment is stained. (D,D’) Arrows denote cells where lamin LAN28 is enriched in the nuclei. Bar, 20 μm.
protein aggregates was better visible on sections through cells that had been fixed with formaldehyde and extracted with Triton (Figs 5C and 6B). In these preparations, the thinnest paracrystals that could be identified unambiguously had diameters of 15-20 nm (Fig. 5C, arrows).

Electron microscopical immunolocalizations on Triton permeabilized Sf9 cells revealed that the protein polymers identified in Sf9 cells expressing *Xenopus* lamin A (Fig. 5A-C) or *Drosophila* lamin C (Fig. 6A and B) were labelled by the antibodies specific for *Xenopus* lamins A and LI (Fig. 5D and E) or the *Drosophila* lamin C (Fig. 6C). Numerous gold particles decorated the surface of the paracrystals (Figs 5D and 6C) and the amorphous aggregates (Fig. 5E). No labelling of these structures was observed when permeabilized cells were incubated with secondary antibodies alone (data not shown) demonstrating the specificity of the anti-lamin antibodies and of the gold-conjugated secondary antibodies (see Figs 5D,E and 6C). The immunolocalizations demonstrate that amorphous as well as paracrystalline areas of the protein polymers did contain lamins A or C. It is important to note that only when they were sectioned parallel to their longitudinal axes could large and small paracrystals be identified in any of the preparations (Figs 5 and 6). We are convinced that several lamin polymers with periodic substructure are hidden in areas described as amorphous aggregates and that the majority of dimers of lamins A and C have assembled at least into thin paracrystals.

We noted that the lamin A and C paracrystals had a slightly different morphology. In some lamin C paracrystals every second interband appeared more electron dense than the interbands in polymers of lamin A (compare Figs 5-7). To determine whether the paracrystals of the two species had similar axial repeats we selected electron micrographs showing paracrystals that had been sectioned parallel to their longitudinal axis (Fig. 7). The side by side alignment of the *Xenopus* lamin A (Fig. 7A) and *Drosophila* lamin C (Fig. 7B) polymers revealed that the length of the axial repeats of both polymers were very similar. In the marked region we counted 22 dark bands in the lamin A paracrystal and 21 dark bands in the aligned lamin C polymer. This corresponds to an average axial repeat of 18.5 nm for lamin A and 19.5 nm for lamin C. The dimensions of axial repeats of paracrystals had not been altered by the fixation and/or extraction procedure used for the processing of Sf9 cells.

In vitro reconstitution experiments of *Xenopus* lamin A and the mutant LA386 (Gieffers and Krohne, 1991) have demonstrated that the carboxy-terminal tail is responsible for the alternating light and dark stained bands in lamin A paracrystals. To determine whether this holds true for in vivo assembled lamin A polymers, we analyzed Sf9 cells that expressed the tailless lamin A mutant (Fig. 8).

The immunofluorescence microscopy has shown that in contrast to the wild-type protein the lamin A mutant LA386 polymerized in the cytoplasm of Sf9 cells into long threads (see Fig. 3C). The electron microscopical inspection revealed that each thread had a thickness of at least 0.4 μm and was composed of numerous filaments that were less tightly packed in the periphery of the bundle (Fig. 8A). The filament bundle shown in Fig. 8A had a diameter of at least 1.7 μm. By electron microscopical immunolocalizations these filament bundles could be easily identified as lamin polymers by their intense reaction with lamin antibodies (Fig. 8B and C). In peripheral areas of lamin filament bundles, filaments were less tightly associated, allowing the labelling of individual fibrils by the lamin antibodies. Using the 6 nm gold particles as a size marker we estimated that these fibrils had diameters of less than 6 nm (Fig. 8C, arrows).

We have never seen periodic substructures in the filament
bundles comparable in morphology to paracrystals assembled by wild-type proteins, indicating that in vivo the carboxy-terminal tail of lamin A contributes to the morphology of the polymer (see Figs 5 and 6).

The filament bundles were often found in close contact with the cytoplasmic surface of the nuclear envelope, but we never detected polymers of this lamin mutant in the nuclear compartment. The morphology of the polymers formed by lamin LA386 was indistinguishable in all specimens regardless of the fixation procedure.

Fig. 5. Electron micrographs of ultrathin sections through the cytoplasm of Sf9 cells showing Xenopus lamin A assembled into paracrystals (A-E) and in apparently amorphous structures (B,C,E). (A and B) Cells fixed with glutaraldehyde/OsO₄. The fibrillar structures (vp) visible in A and B represent aggregates of a viral protein. (C) Lamin A polymers of a cell that had been fixed with formaldehyde and then extracted with Triton X-100. The arrow marks the thinnest visible polymers with periodic repeats. (D,E) Electron microscopical immunolocalizations with lamin antibody X67 and secondary antibodies coupled to 6 nm colloidal gold on Sf9 cells that had been permeabilized with Triton. Areas containing lamin A paracrystals (D) and polymers consisting of paracrystals and apparently amorphous aggregates (E) are shown. Arrowheads in C and E mark viral capsids. Bars: 100 nm (D,E); 200 nm (A-C).
The ultrastructure of polymeric B-type lamins LI (Fig. 9) and Dmo (Fig. 10) was different from that of A-type lamins but similar to each other. The two B-type lamins assembled in the cytoplasm (Figs 9A,B, 10A) and in the nucleus (Fig. 10A,B; data not shown for lamin LI) into aggregates with a fibrillar ultrastructure. The thinnest fibrils (Figs 9B, 10B) had diameters of approximately 5 nm. The electron microscopical immunolocalizations (Figs 9C,D, 10C) confirmed that these protein aggregates contained lamins. After processing of the cells for immunolocalization the thin lamin fibrils were less frequently visible (compare Figs 9B and D, 10B and C). Often, the lamin LI polymers were associated with the cytoplasmic membranes.

Fig. 6. Electron micrographs of ultrathin sections through SF9 cells showing *Drosophila* lamin C polymers with paracrystalline and amorphous morphology. (A) Cell fixed with glutaraldehyde/OsO₄. Lamin polymers with periodic repeats are present in the cytoplasm and the nucleus (arrows). (B) Lamin C polymers of a cell that had been fixed with formaldehyde and then extracted with Triton X-100. (C) Electron microscopical immunolocalizations with lamin antibody LC28 and secondary antibodies coupled to 6 nm colloidal gold on SF9 cells that had been permeabilized with Triton. An area containing a cytoplasmic localized lamin C paracrystal is shown. ne, nuclear envelope; cy, cytoplasm; nu, nucleoplasm; v, viral capsids. Bars: 100 nm (C); 200 nm (A,B).
Lamin assembly studied in vivo

When we compared the electron microscopical immunolocalizations (Figs 5, 6, 8-10) we noticed that the electron dense polymers of lamins A, C and Dmo were less intensely labelled than the polymers of lamins LI and the lamin A mutant that are composed of loosely packed fibrils. We suggest that especially the secondary antibodies conjugated to colloidal gold cannot penetrate dense structures such as paracrystals or a thick

side of the nuclear envelope but we have never detected lamin LI in the nuclear lamina of infected Sf9 cells (Fig. 9C).

Lamin Dmo was expressed at a very high level in more than 80% of the cells of a virus infected culture and assembled in the cytoplasm (Fig. 10A, left cell) and the nucleus (Fig. 10A, cell on the right side) into prominent aggregates which could occupy large areas. Interestingly the Dmo polymers were more electron dense than the lamin LI aggregates (compare Figs 9A and 10A). In contrast to the other three wild-type lamins, lamin Dmo in the nuclear compartment was always associated with the inner nuclear membrane (arrows in the left cell shown in Fig. 10A). In the case that large amounts of lamin Dmo had been transported into the nucleus, a thick continuous lamina was formed (Fig. 10A, brackets in the cell on the right side) that could extend locally deep into the nucleoplasm (Fig. 10A, cell nucleus on the right side).

Fig. 7. Alignment of paracrystals of *Xenopus* lamin A (A) and *Drosophila* lamin C (B) to allow the comparison of the axial repeats. The areas of both paracrystals marked by arrows contain 22 (A) and 21 (B) repeats. (A) Paracrystal of a cell fixed with glutaraldehyde/OsO₄. (B) Lamin C paracrystal of a cell that had been fixed with formaldehyde and then extracted with Triton X-100. Bars, 100 nm.

Fig. 8. Electron micrographs of ultrathin sections through the cytoplasm of Sf9 cells expressing lamin LA386. (A) Cell fixed with glutaraldehyde/OsO₄. A cross section through a filament bundle is shown. Lamin filaments are less tightly packed in the periphery of the bundle. (B, C) Cells were permeabilized with Triton X-100 and processed for electron microscopical immunolocalizations with lamin antibody X67 and secondary antibodies coupled to 6 nm colloidal gold. (B, C) A filament bundle intensely labelled by lamin antibodies is shown. (C) the periphery of a filament bundle is shown at higher magnification. Thin lamin fibrils labelled by 6 nm gold particles are visible (C, arrows). Bars: 100 nm (C); 200 nm (A, B).
nuclear lamina. The intense labelling of the surface of the polymers of the *Xenopus* lamin A mutant also suggests that the carboxy-terminal tail domain might partially mask the epitope of the antibody in polymers formed by the wild-type molecule (compare Figs 8B and C, 5D and E).

**DISCUSSION**

We have demonstrated clearly that the polymeric structures formed in vivo by A- and B-type lamins in Sf9 cells can be distinguished at the electron microscopical level. Because of their conserved carboxy-terminal CxxM-motives, their acidic isoelectric points, and their association with membranes in mitotic cells, the lamins LI of *Xenopus* (Krohne et al., 1987; Lourim et al., 1996) and Dmo of *Drosophila* (Gruenbaum et al., 1988; Ulitzur et al., 1992) have been classified as B-type lamins.

Both B-type lamins assembled in vivo into polymers with a fibrillar substructure. It can be speculated that the thin irregularly arranged fibrils in the polymers of lamins LI and Dmo may represent single longitudinal ‘head-to-tail’ aggregates of lamin dimers or lateral assemblies of a few longitudinal aggregates. The absence of an obvious periodicity in the polymers indicates that the ordered lateral aggregation of B-type lamins is prevented in vivo, whereas B-type lamins including lamin Dmo have been assembled in vitro into paracrystals (Heitlinger et al., 1991, 1992; Stuurman et al., 1996).

To our surprise lamins A of *Xenopus* and C of *Drosophila* assembled in vivo into polymers with a paracrystalline morphology very similar to polymers assembled in vitro from vertebrate A-type lamins (Aebi et al., 1986; Moir et al., 1991; Gieffers and Krohne, 1991; Heitlinger et al., 1992). The absence of the CxxM-motives in the sequences of *Drosophila* lamin C and the vertebrate lamin C have led to the proposition that this insect protein may be an A-type lamin (Bossie and Sanders, 1993). Two observations favour the classification of *Drosophila* lamin C as an A-type lamin. These are its developmentally regulated expression (Riemer et al., 1995) and its assembly behavior in vivo. However, it should be noted that vertebrate A-type lamins and *Drosophila* lamin C have a significantly lower degree of homology (29% sequence identity with human lamin C; alphahelical rod domain: 35% sequence identity with human lamin C).
Fig. 10. Electron micrographs of ultrathin sections through Sf9 cells showing *Drosophila* lamin Dmo polymers in the cytoplasm (A, left cell) and in the nucleus (A-C). (A,B) Cells fixed with glutaraldehyde/OsO₄. Arrowheads in A denote the cytoplasmic localized lamin polymers. Lamin Dmo associated with the inner nuclear membrane is marked by arrows and brackets. (B) Higher magnification of the thick 'Dmo-lamina' showing its fibrillar substructure. (C) Electron microscopical immunolocalizations with lamin antibody ADL195 and secondary antibodies coupled to 6 nm colloidal gold on Sf9 cells that had been permeabilized with Triton. An area of a nuclear localized lamin polymer is shown that was specifically decorated by antibody ADL195 on the surface. cy, cytoplasm; nu, nucleoplasm; vp, aggregates of a viral protein. Bars: 100 nm (B,C); 1 μm (A).
identity) than the *Drosophila* lamins Dmo and C (46.5% sequence identity; alphahelical rod domain: 61.9% sequence identity; see Fig. 8 in Bossie and Sanders, 1993).

Paracrystals formed by A-type lamins in the cytoplasmic as well as nuclear compartment of the insect cells showed transverse light-dark bands with a distance of 18.5-20.0 nm. In the case of *Drosophila* lamin C paracrystals, a broad light band often alternated with a smaller light band (Figs 6B,C, 7B), therefore, the true axial repeat in these paracrystals is 37-40 nm. The 18.5-20.0 nm repeat found in *Xenopus* lamin A is slightly smaller than the 22.0 nm repeat of in vitro formed *Xenopus* lamin A paracrystals that were embedded in Epon (Gieffers and Krohne, 1991). Because of shrinkage of biological material during the embedding procedure, these values are smaller than the axial repeat lengths of 22.5-25 nm measured for negatively stained paracrystals of A-type lamins (Moir et al., 1990; Aebi et al., 1986; Gieffers and Krohne, 1991; Heitlinger et al., 1992). In negatively stained paracrystals full-length nuclear lamins displayed a ‘simple pattern’ of evenly spaced alternating transverse light and dark bands (Moir et al., 1990; Aebi et al., 1986; Gieffers and Krohne, 1991; Heitlinger et al., 1991) with a repeat length of 22.5-25 nm, whereas headless and tailless chicken lamin B2 yielded paracrystals with alternating smaller and thicker light and dark bands, reflecting an axial repeat length of 45-50 nm (Heitlinger et al., 1992). In either case, it is likely that the repeat length reflects the length of the lamin dimer (52 nm) minus the length of a short overlap of the end segments of the lamin rod domains as found in lamin head-to-tail polymers (Heitlinger et al., 1992; for review see Heins and Aebi, 1994). The morphology of paracrystals formed by *Xenopus* lamin A in vivo (this study) and in vitro (Gieffers and Krohne, 1991) are virtually identical indicating that the packing of lamin dimers within these structures is the same. We therefore assume that, similar to in vitro assembly, A-type lamins assemble in vivo in a longitudinal fashion to yield long head-to-tail polymers which subsequently associate laterally into paracrystalline structures.

To our knowledge, no paracrystals formed by A-type lamins have previously been localized within cells. However, paracrystalline structures with a transverse light dark banding pattern with a repeat of 22 nm were described in certain murine epithelial cells (Kallman and Wessels, 1967). Those particular paracrystals were most likely formed by keratins. Apparently, the tendency of both *Xenopus* lamin A and *Drosophila* lamin C to form paracrystalline fibers when expressed at high levels in vivo reflects the tendency of these proteins to assemble into well ordered structures. When these proteins are expressed at more physiological levels and are normally localized (at the nuclear envelope and sometimes in internal nuclear structures), this order is not readily apparent using current imaging techniques. We observed that two A-type lamins in vivo form structures that are more highly ordered than those formed by two B-type lamins. If this holds true as a distinction between A-type and B-type lamins in general, then the ordered assembly of A-type lamins must harbor insights into A-type lamin specific functions.

The tailless lamin A mutant assembled in the cytoplasm of SF9 cells into thin filaments that are remarkably similar to the thin filaments found in vitro (Gieffers and Krohne, 1991). The filament bundles formed by this mutant protein in vivo were much longer than the paracrystals formed by the wild-type protein. Similar observations were previously made during in vitro reconstitution experiments with human lamin C (Moir et al., 1991) and chicken lamin B2 (Heitlinger et al., 1992). The tailless B-type lamin formed long ‘head-to-tail’ aggregates that assembled ‘wavy’ filament bundles (Heitlinger et al., 1992), and the lamin C mutant assembled into 15-30 nm thick filament bundles. Mammalian cells transfected with the human lamin A mutant lacking the complete tail assembled in the cytoplasm ‘tubular lamin structures’ (Loewinger and McKeon, 1988) of remarkable size, which were at the light microscopical level indistinguishable from the filament bundles assembled by mutant LA386 in SF9 cells. Taking these data together, one may speculate that the length of the lamin polymer is modulated in vivo and in vitro by the carboxy-terminal tail domain of the lamin molecule.

We have the following explanation for the assembly of the four wild-type lamins in the cytoplasm. It is obvious from our experiments and previously published data (Loewinger and McKeon, 1988) that the cytoplasm of insect and mammalian cells does not inhibit the polymerization of lamin molecules. Therefore it is most likely that newly synthesized lamins present in high local concentrations in the cytoplasm can assemble immediately into oligomeric structures that are too large to allow their free diffusion and transport into the nucleus. In vitro experiments have shown that lamin concentrations of 5 μg/ml and more are sufficient to enable their rapid polymerization (Glass and Gerace, 1990); therefore one would expect lamins that are concentrated substantially higher in the SF9 cells will assemble immediately at the place of their synthesis. Previously, *Drosophila* lamin Dmo behaved similarly (i.e. polymerization in the cytoplasm) after high level expression, controlled by the GAL1 promoter, in Saccharomyces cerevisiae (B. Benton and P. A. Fisher, unpublished). The behavior of the lamin mutant LAN28 in infected SF9 cells demonstrates that lamins are efficiently transported into the nucleus when their polymerization is inhibited (for the false localization of other proteins expressed in SF9 cells see Furuse et al., 1996).

Only lamin Dmo did interact with the nucleoplasmic side of the nuclear envelope of SF9 cells and assembled in several cells into a 0.5-2 μm thick lamina. We know that the B-type lamin of SF9 cells is indistinguishable in its isoelectric point and mobility on SDS-PAGE from *Drosophila* lamin Dmo (Kraus and Krohne, unpublished data), and that it is immunologically related to *Drosophila* lamin Dmo (see Fig. 3B, inset; N. Stuurman unpublished data). Therefore it seems likely that this SF9 lamin mediated the association of lamin Dmo with the nuclear lamina of the butterfly cells.

The ‘Dmo-lamina’ of infected SF9 cells and the 50-70 nm thick nuclear lamina of human synovial cells (Höger et al., 1991b) have some structural similarities. Both laminae have a fibrillar texture that will change into a more granular substructure in extracted cells.

In contrast to lamin Dmo, molecules of the three other wild-type lamins that had been transported into the nucleus formed polymeric structures in the nucleoplasm. The intranuclear assembly of *Drosophila* lamin C in SF9 cells can be explained by the absence of the CxxM-motif in this lamin and the arrest of baculovirus infected cells in interphase. It has been shown that cells expressing human lamin C require progression through the cell cycle for the assembly of this protein into the nuclear lamina (Horton et al., 1992) and that a mutant of this
human protein containing the CxxM-motif is efficiently incorporated into the nuclear lamina of amphibian oocytes, cells that are naturally arrested in the prophase of meiosis (Krohne et al., 1989).

The lack of interaction of Xenopus lamins with the nuclear lamina can be explained by the low degree of homology between insect and vertebrate lamins. The Drosophila lamin Dmo possesses less than 30% sequence identity with Xenopus B-type lamins (see Fig. 5 of Riemer et al., 1993). These data are corroborated by microinjection experiments which show that lamin Dmo is incompletely incorporated into the nuclear lamina of Xenopus oocytes (Schmidt et al., 1995).

We anticipate that in future experiments the baculovirus system will allow study of the in vivo interactions between different proteins of the nuclear envelope by their simultaneous expression in SF9 cells.

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