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Structural organization and biological roles of the nuclear lamina

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Summary

The nuclear lamina is a protein meshwork that lies on the nucleoplasmic side of the nuclear envelope and is associated with the peripheral chromatin. It is involved in several biological activities including: the mitotic disassembly and reassembly of the nuclear envelope, determination of the size and shape of the nucleus, higher order chromatin organization, cell differentiation, and apoptosis. Lamins are the major proteins of the nuclear lamina. They are type V intermediate filaments and, like all intermediate filaments, they form filamentous structures. Lamins can interact in vitro with specific DNA sequences, with chromosomal proteins and with several proteins of the inner nuclear membrane, including otefin, LBR, LAP1 and LAP2. In this paper we show that *Drosophila* lamin Dm_0 and otefin proteins are required for the assembly of the *Drosophila* nuclear envelope. We also demonstrate that the lack of lamin Dm_0 activity causes the dissociation of peripheral chromatin from the nuclear envelope, accumulation of annulate lamellae and lethality. In addition, we show that the carboxy (tail) domain of lamin Dm_0 can interact in vitro with chromosomes and the central (rod) domain of lamin Dm_0 is essential and sufficient for the in vitro assembly of lamin Dm_0 into filamentous structures. These results are discussed in relationship to the biological roles of the nuclear lamina.

I. Introduction

In eukaryotic cells, DNA replication and RNA processing occur in the nucleus, while protein synthesis occurs in the cytoplasm. These activities are physically separated by the nuclear envelope. The nuclear envelope is a complex structure composed of outer and inner lipid bilayer membranes. The two membranes are separated by a 20-40 nm perinuclear space and are connected at the nuclear pore complexes, which are passageways for transport of macromolecules between the nucleoplasm and the cytoplasm (reviewed in Davis, 1995; Gorlich and Mattaj, 1996). Underlying the inner nuclear membrane there is a proteinaceous meshwork of intermediate filaments termed the nuclear lamina (**Fig. 1A**; reviewed in Hutchison et al., 1994; Moir et al., 1995).

A. Proteins of the inner nuclear membrane and nuclear lamina

Several components of the inner nuclear membrane and the lamina have been identified. These include the integral membrane proteins (IMPs): LBR (Worman et al., 1990), LAP1, LAP2 (Furukawa et al., 1995; Harris et al., 1994; Martin et al., 1995), p34 (Simos and Georgatos, 1994) and p18 (Simos et al., 1996), and the peripheral proteins: nuclear lamins (Fisher et al., 1986; McKeon, 1991), otefin (Harel et al., 1989; Padan et al., 1990) and YA (Lopez et al., 1994; Lopez and Wolfner, 1997). The existing experimental data suggests that lamins can interact with LBR, LAP1, LAP2, otefin and YA (Foisner and Gerace, 1993; Goldberg et al., 1997; Worman et al., 1988) . p18 and p34 are associated with LBR and p18 is distributed equally between the inner and the outer nuclear membranes (Simos and Georgatos, 1994). The data on the peripheral proteins indicates that otefin is closely associated with the inner nuclear membrane, lamin can associate with both the inner nuclear membrane and the peripheral chromatin, and YA is associated with the peripheral chromatin (**Fig. 1A**; Goldberg et al., 1997). These proteins are present in the insoluble NMPCL (nu-

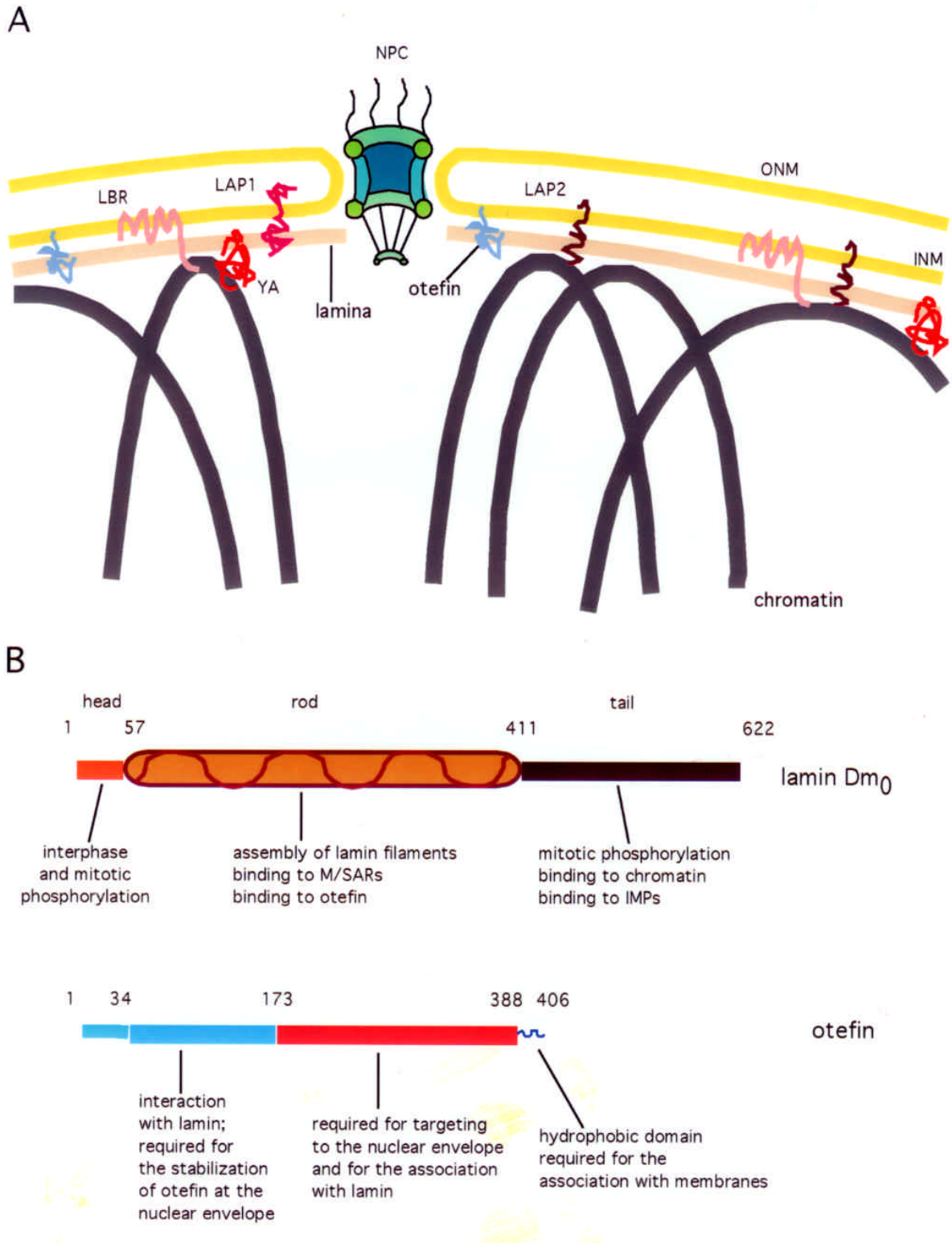

Figure 1. (**A**) Schematic view of the structural organization of nuclear envelope. OMN, outer nuclear membrane; INM, inner nuclear membrane; NPC, nuclear pore complex. (**B**) Schematic view and the putative roles of different regions in lamin Dm_0 and otefin. The numbers are of amino acids positions in these proteins.

clear matrix-pore-complex-lamina) fraction, after salt and Triton X-100 extraction.

LBR (lamin B receptor) was isolated by its ability to bind in a saturable and specific fashion to lamin B. Binding of lamin B to LBR is affected by its phosphorylation. LBR is a 58 kDa protein containing a nucleoplasmic amino-terminal domain of 204 amino acids followed by a hydrophobic domain with eight putative transmembrane segments (Worman et al., 1990). Its sequence shows high homology to the yeast sterol C-14 reductase (Gerace and Foisner, 1994). Both the first transmembrane domain (Smith and Blobel, 1993) and the amino-terminal domain of LBR (Soullam and Worman, 1993; Soullam and Worman, 1995) mediate the targeting of LBR to the inner nuclear membrane. The highly charged amino-terminal domain of LBR can also direct cytosolic proteins to the nucleus and type II integral membrane proteins to the inner nuclear membrane in transfected COS-7 cells (Smith and Blobel, 1993). LBR is phosphorylated in a cell cycle-dependent manner on serine residues in interphase and on serine and threonine residues in mitosis. Its phosphorylation is mediated by p34cdc2-kinase and by an unidentified kinase that resides in the nuclear envelope and associates with LBR in vivo (Nikolakaki et al., 1997; Simos and Georgatos, 1992). LBR can interact with several proteins including p34 and p18 (Simos and Georgatos, 1994), lamin B (Worman et al., 1988) and with the human homologue of the *Drosophila* heterochromatin associated protein HP1 (Ye and Worman, 1996).

LAP1A-C - (Lamina-associated polypeptides 1A-C) is a group of three related integral membrane proteins of the inner nuclear membrane that are recognized by monoclonal antibody RL13. LAP1 proteins can bind both type A and B lamins (Foisner and Gerace, 1993). Cloning of LAP1C revealed that it is a type II integral membrane protein with a single membrane-spanning region and a hydrophilic amino terminal domain that is exposed to the nucleoplasm (Martin et al., 1995). The different LAP1 isotypes are differentially expressed during development and appear to bind lamins with different affinities (Martin et al., 1995).

LAP2 (Lamina-associated polypeptide 2 - also named thymopoietin) is a type II integral membrane protein of the inner nuclear membrane. The LAP2 gene is alternatively spliced to give rise to at least 5 different products (Theodor et al., 1997). The most abundant products: LAP2α, LAP2β, and LAP2γ (75 kDa, 51 kDa and 39 kDa, respectively) are present in most cell types. LAP2α is present diffusely throughout the nucleus, while LAP2β and LAP2γ are confined to the inner nuclear membrane (Harris et al., 1995). LAP2β contains a large hydrophilic domain with several potential cdc2 kinase phosphorylation sites and a single putative membrane-spanning sequence close to its carboxy terminus. The amino-terminal domain of this protein is hydrophilic and is exposed to the nucleoplasm. LAP2 can bind directly to both lamin B and chromosomes and associates with chromosomes at the same time that lamins begin to reassemble around them (Foisner and Gerace, 1993; Yang et al., 1997). The phosphorylation of LAP2 during mitosis inhibits its binding to both lamin B and chromosomes. (Foisner and Gerace, 1993). The mechanism for inner membrane targeting and retention of LAP2 probably involves lateral diffusion in the interconnected membranes of the endoplasmatic reticulum and nuclear envelope, and interaction with components of the nuclear lamina and chromatin (Furukawa et al., 1995).

YA (Young Arrest) is an essential *Drosophila* gene for the transition from meiosis to the initiation of the rapid mitotic divisions by early embryos (Judd and Young, 1973; Lin et al., 1991; Liu et al., 1995). The chromosome condensation state is abnormal in nuclei in YA-deficient eggs and embryos (Liu et al., 1995). The YA protein is present during the first two hours of zygotic development, where it is localized to the nuclear lamina (Lin et al., 1991). Ectopically expressed YA associates with polytene chromosomes in vivo (Lopez and Wolfner, 1997), and YA can associate with both chromosomes and lamin Dm0 (Goldberg et al., 1997; Lopez and Wolfner, 1997).

Otefin is a 45 kDa peripheral nuclear envelope protein with no apparent homology to other known proteins (Padan et al., 1990). It includes a large hydrophilic domain, a single carboxy terminal hydrophobic sequence of 17 amino acids and a high content of serine and threonine residues (**Fig. 1B**). With the exception of sperm cells, otefin is present in the nuclear envelope of all cells examined during the different stages of *Drosophila* development. In eggs and young embryos, otefin is also associated with the maternal fraction of membrane vesicles (Ashery-Padan et al., 1997b). The COOH-terminal, 17-aa hydrophobic sequence of otefin is essential for the targeting of otefin to the nuclear periphery. Other sequences of otefin are required for its efficient targeting to the nuclear envelope and for further stabilizing otefin's interaction with the nuclear envelope (Ashery Padan et al., 1997a). Otefin is a phosphoprotein in vivo and a substrate for in vitro phosphorylation by cdc2 kinase and cAMP-dependent protein kinase.

Lamins are the major proteins of the nuclear envelope. They are classified as type V intermediate filaments and, like all intermediate filaments, they contain an α helical rod domain flanked by amino (head) and carboxy (tail) domains (**Fig. 1B**). Unlike the cytoplasmic intermediate filaments that are 10 nm wide, lamins can make up to 200 nm thick fibers (Belmont et al., 1993; Paddy et al., 1990). The rod domain of lamins is 52 nm long and contains three α helices, each composed of heptad repeats (reviewed in McKeon, 1987). These helices form coiled-coil interactions between lamin monomers. The lamin dimers associate longitudinally to form polar head-to-tail polymers. These polar head-to-tail polymers further associate laterally to form the 10 nm thick filaments (Heitlinger et al., 1991). The 10 nm filaments further associate to form the 50-200 thick

nuclear lamina (this study). The head-to-tail binding sites are at the ends of the rod domain that are highly conserved among all intermediate filament proteins. Point mutants that cause defects in binding were mapped to these conserved regions (Stuurman et al., 1996; Zhao et al., 1996).

Lamins are divided into types A and B. Type A lamins are mainly expressed in differentiated cells, have a neutral isoelectric point and are soluble during mitosis. Type B lamins are expressed constitutively in all somatic cells, have an acidic isoelectric point and remain associated with membrane vesicles during mitosis (reviewed in McKeon, 1991; Nigg, 1992). Different eukaryotes possess between one to six lamin genes. Mammalian lamins A and C are the result of alternative splicing of the same gene. Lamins B1-B3 and C2 are coded by separate genes (Alsheimer and Benavente, 1996). The two major lamins in chicken are lamins A and B2 (Peter et al., 1989). An additional minor species is termed lamin B1. *Xenopus laevis* has at least five different lamin genes (Stick, 1992; Stick, 1994). *Drosophila melanogaster* has two lamin genes, termed lamin Dm_0 and C (Bossie and Sanders, 1993; Gruenbaum et al., 1988). *Caenorhabditis elegans* probably has a single lamin gene, termed CeLam-1 (Riemer et al., 1993).

Lamins undergo specific post translational modifications. All nuclear lamins except lamins C contain CaaX box at their carboxy terminus. The CaaX box undergoes proteolytic cleavage of the last three amino acids, farnesylation of the C-terminal cysteine, and carboxyl methylation. The isoprenylation is essential but not sufficient for the association of lamins with the nuclear envelope (Firmbach and Stick, 1995; Firmbach-Kraft and Stick, 1993; Hennekes and Nigg, 1994). Lamins are phosphorylated by several protein kinases in vivo and in vitro. These include: cdc2 kinase (Dessev et al., 1991; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990), Casein kinase II (Li and Roux, 1992), PKA (Lamb et al., 1991), βII PKC (Fields et al., 1988; Hennekes et al., 1993; Hocevar et al., 1993; Hocevar and Fields, 1991; Kasahara et al., 1991) and MAP kinase (Peter et al., 1992). The phosphorylation state of lamins is cell-cycle regulated (Ottaviano and Gerace, 1985). It is involved in lamin polymerization and disassembly, and in importing lamin molecules into the nucleus. The *Drosophila* lamin Dm_0 undergoes post translational modifications to give rise to at least three distinct isoforms termed, Dm_1, Dm_2 and Dm_{mit} which differ in their phosphorylation pattern. Dm_1 and Dm_2 are present in most types of interphase nuclei as a random mixture of homo- and hetero-dimers (Smith et al., 1987; Stuurman et al., 1995). Dm_{mit} is present in the maternal pool and in mitotic cells (Smith and Fisher, 1989).

3-D *in vivo* studies in *Drosophila* and in mammalian cells revealed that lamin fibers are closely associated with chromatin fibers (Belmont et al., 1993; Paddy et al., 1990). Studies in vitro have shown that lamins can specifically bind chromatin fragments and interphase chromatin (Hoger et al., 1991; Taniura et al., 1995; Yuan et al., 1991), as well as condensed in vitro assembled chromatin (Ulitzur et al., 1992) or mitotic chromosomes (Glass et al., 1993; Glass and Gerace, 1990). Lamins can also bind to specific DNA sequences (Baricheva et al., 1996; Luderus et al., 1992; Luderus et al., 1994; Shoeman and Traub, 1990; Zhao et al., 1996) and to chromosomal proteins (Burke, 1990; Glass et al., 1993; Glass and Gerace, 1990; Hoger et al., 1991; Taniura et al., 1995; Yuan et al., 1991). Binding of lamins to chromatin is specific and depends on the integrity of the chromosomes. Lamin A binds in vitro to poly-nucleosomes with a dissociation constant of about $1x10^{-9}$ M (Yuan et al., 1991). A binding site for mammalian lamins A and B was localized at the tail domain (Taniura et al., 1995). In the latter study, the dissociation constant of the tail domain binding to interphase chromatin was estimated to be in the range of $3x10^{-7}$ M and the binding was mediated by histones. Since lamins form large polymers in vivo, the actual association between the lamin filament and chromatin may be stronger. A specific binding site to mitotic chromosomes was also found in the rod domain. However, the in vivo relevance of this binding is not yet clear since the rod domain binding occurred only under acidic, non-physiological, conditions (Glass et al., 1993). Chicken lamin B and *Drosophila* lamin Dm_0 polymers also bind specifically to M/SARs fragments (Luderus et al., 1992; Luderus et al., 1994). These DNA sequences are several hundred base pairs long with several stretches of AT rich sequences and are likely to form an "open" form of chromatin. Indeed, the binding to these sequences could be competed to some extent with single strand DNA (Luderus et al., 1994). The binding of *Drosophila* lamin Dm_0 to M/SARs is mediated by the rod domain and requires its polymerization (Zhao et al., 1996). Lamin-DNA interactions can occur, for example, in the centromeric regions since the l20p1.4 *Drosophila* centromeric sequence has DNA composition similar to M/SAR and it binds specifically to polymers of *Drosophila* lamin Dm_0 (Baricheva et al., 1996). Lamin polymers can also bind strongly to telomeric sequences (Shoeman and Traub, 1990).

B. Biological roles of the nuclear lamina

Several functions have been ascribed to the nuclear lamina concerning nuclear organization and activity. These functions include: (i) regulating the size, shape and assembly of the nuclear envelope, (ii) a role in higher order chromatin organization by providing docking sites for chromatin , (iii) a role in DNA replication, (iv) a possible role in differentiation, as indicated by the change in lamina composition during development. In addition, the nuclear lamina is a major substrate for signals that control the cell cycle and lamins are specifically degraded in apoptosis (Nigg, 1992; Oberhammer et al., 1994).

(i) Nuclear envelope disassembly.

During mitosis, the nuclear envelope breaks down in prophase and starts to reassemble at late anaphase. Nuclear lamins and lamina associated proteins are likely to play a role both in the assembly and disassembly of the nuclear envelope. Disassembly of the nuclear lamina is controlled by phosphorylation of sites outside the rod domain of lamins that prevents the head-to tail association of the lamin molecules. For example, mutations in Ser-22 and Ser-392 of human lamin A in transfected COS cells prevented phosphorylation at these sites and blocked the disassembly of the nuclear lamina during mitosis (Heald and McKeon, 1990).

(ii) Nuclear envelope assembly depends on lamins and on lamin-associated proteins. Microinjection of lamin antibodies into cultured PtK2 cells resulted in daughter nuclei that remained arrested in a telophase-like configuration, and telophase-like chromatin that remained inactive (Benavente and Krohne, 1986). In mammalian cell-free extracts, antibodies directed against type A or B lamins blocked vesicles binding to chromatin, which is the first step of nuclear envelope assembly (Burke and Gerace, 1986). Similarly, anti-lamin Dm_0 antibodies blocked the interaction between vesicles and chromatin in a *Drosophila* cell-free system that assembles nuclei from sperm chromatin (Ulitzur et al., 1992; Ulitzur et al., 1997). The role of lamin proteins in the association between nuclear vesicles and chromatin in *Xenopus* extracts has been the subject of debate; Depletion of lamin B3 from the assembly extract did not prevent the formation of nuclear envelopes consisting of membranes and nuclear pores. These lamin B3-depleted nuclei were small, fragile and failed to replicate their DNA (Jenkins et al., 1995; Meier et al., 1991; Newport et al., 1990). In contrast, Dabauvalle et al. (Dabauvalle et al., 1990) were able to block the formation of nuclear envelopes by using an antibody directed against both lamins B2 and B3. A major reason for the discrepancy between the above studies could be that *Xenopus* extracts contain lamins B2 and B1, in addition to lamin B3 (Lourim et al., 1996; Lourim and Krohne, 1993). In cell-free extracts of *Xenopus* eggs and *Drosophila melanogaster* it was shown that trypsinization of the membrane fraction abolished its ability to bind demembranated sperm chromatin and hence to support assembly of the nuclear envelope (Ulitzur et al., 1997; Wilson and Newport, 1988). Possible target proteins for the Trypsin treatment are IMPs. Indeed, several studies suggest a role for LBR, LAP1 and LAP2 in nuclear assembly. LAP2 associates with chromosomes at the same time as lamins, which suggests a role for LAP2 in initial events of nuclear envelope reassembly (Foisner and Gerace, 1993). A recent study (Yang et al., 1997) shows that LAP1 and LAP2 become completely dispersed throughout ER membranes during mitosis and proposes that the reassembly of the nuclear envelope at the end of mitosis involves sorting of IMPs to chromosome surfaces by binding interactions with lamins and chromatin. Pyrpasopoulou et al. (Pyrpasopoulou et al., 1996) analyzed the role of LBR in providing chromatin docking sites for nuclear vesicles by binding in vitro reconstituted vesicles of nuclear envelopes to chromatin. The results of this study suggest that LBR is involved in providing chromatin anchorage site at the nuclear envelope. It was also suggested that the homologue of LBR in sea urchin targets membranes to chromatin and later anchors the membrane to the lamina (Collas et al., 1996). The essential role of otefin in the assembly of the nuclear envelope was recently demonstrated in a *Drosophila* cell-free system (Ashery-Padan et al., 1997b). The similar phenotype obtained when otefin or lamin Dm_0 activities are inhibited (Ashery-Padan et al., 1997b) is probably due to the fact that otefin and lamin are part of the same protein complex in the vesicle fraction (Goldberg et al., 1997). In summary, the above data implies that the assembly of nuclear membranes following mitosis requires the function of protein complexes containing both peripheral and integral membrane proteins including: lamin, otefin, LAP2 and LBR.

Lamin genes are not present in significant homology in the yeast *Saccharomyces cerevisiae* (Gruenbaum, Y., unpublished observations) and in the protozoon *Amoeba proteus* (Schmidt et al., 1995). In addition, the lamina-associated proteins LAP1, LAP2 and otefin are not present in significant homology in *Saccharomyces cerevisiae* (Gruenbaum, Y., unpublished observations), while LBR is the enzyme sterol C14 reductase (reviewed in Gerace and Foisner, 1994). One possible explanation for the appearance of lamins only in organisms with an open mitosis concerns their roles in nuclear envelope breakdown at the begining of mitosis and nuclear reassembly at the end of mitosis. These activities are not required in organisms with a closed mitosis. The involvement of the nuclear lamina in nuclear organization, development and DNA replication may have appeared later in evolution.

(iii). Nuclear and chromatin organization.

The nuclear lamina is a major component of the nuclear matrix. It was, therefore, suggested that a lamin filamentous meshwork is involved in nuclear and chromatin organization. An example for a direct involvement of a lamin protein in nuclear organization comes from an ectopic expression of the mouse sperm-specific lamin B3 in cultured somatic cells. This ectopic expression resulted in transformation of the nuclear morphology from spherical to hook-shaped (Furukawa and Hotta, 1993). Also, depletion of soluble lamin B3 from *Xenopus* nuclear assembly extracts gave in vitro assembled nuclei that were small and fragile (Meier et al., 1991; Newport et al., 1990). Another evidence for the role of lamin in nuclear organization comes from the analysis of flies mutated in the *Drosophila* lamin Dm_0 gene. Flies homozygous for a strong mutation in the lamin Dm_0 gene had an aberrant nuclear structure and died following 9-16 hours of development. The dissociation of chromatin from the nuclear membrane was one of the first phenotypes observed in these flies (Osman, 1992). A weak mutation in the lamin Dm_0 gene (<20% of lamin expression) resulted in a retarded development, reduced viability,

sterility, and impaired locomotion. The nuclei in these mutant flies are enriched in nuclear pore complexes, in cytoplasmic annulate lamellae and contain defective nuclear envelopes (Lenz-Bohme et al., 1997). In vitro studies support the role of nuclear lamin in chromatin organization. As discussed above, the nuclear lamina interacts in vivo with chromatin, and lamin proteins can bind histones and specific DNA sequences.

The *Drosophila* YA protein is needed to initiate embryonic cleavage divisions (Lopez et al., 1994). Ya is likely to be involved in mediating the association of chromosomes with the lamina (Goldberg et al., 1997), thus contributing to the organization of the nucleus in a developmental stage-specific manner (Lopez and Wolfner, 1997). Nuclei in YA-deficient eggs and embryos have abnormal chromosome condensation states (Liu et al., 1995), ectopically expressed YA associates with polytene chromosomes in vivo, and YA can associate with chromosomes in vitro (Lopez et al., 1994; Lopez and Wolfner, 1997).

(iv). DNA replication requires nuclear lamins.

Several reports demonstrated that, during interphase, lamin B molecules are present in foci in the nucleoplasm, in addition to their presence in the nuclear envelope. These foci coincide with sites of DNA replication (Goldman et al., 1992; Moir et al., 1994; Spann et al., 1997). In addition, nuclei assembled in *Xenopus* egg extracts that were depleted of lamin B3 were unable to initiate DNA replication. These lamin B3-depleted nuclei had continuous nuclear envelopes and nuclear pores and were able to import proteins required for DNA synthesis such as PCNA, MCM3, ORC2 and DNA polymerase α (Goldberg et al., 1995; Meier et al., 1991; Newport et al., 1990; Spann et al., 1997). Addition of purified lamin B3 to the depleted extracts could rescue lamina assembly and DNA replication. Microinjection of a truncated human lamin, that was utilized as a dominant negative mutant to perturb lamin organization in mammalian cells, caused a dramatic reduction in DNA replication (Spann et al., 1997). Nuclear lamins are likely to be required for the elongation phase of DNA replication since the distribution of MCM3, ORC2, and DNA polymerase α that are required for the initiation stage of DNA replication was not affected by the depletion of lamin B3 activity (Spann et al., 1997).

II. Results and discussion

A. Mutations in the *Drosophila* lamin Dm_0 gene reveal that it is an essential gene that is required for nuclear organization.

During egg chamber development, large amounts of lamin Dm_0 are secreted by the nurse cells into the developing *Drosophila* oocyte (Ashery-Padan et al., 1997b; Smith and Fisher, 1989; Ulitzur et al., 1992). The amounts of lamin Dm_0 RNA and protein that are maternally stored in the oocyte are sufficient for the assembly of many thousands of nuclei. In addition, lamin Dm_0 is a very stable protein with an estimated half life of about 24 hr (Dr. Paul A. Fisher, personal communication). Therefore, flies mutated in their lamin Dm_0 gene are expected to show a phenotype only following the consumption of the large maternal pool of lamin Dm_0.

Drosophila melanogaster (canton S) males were mutagenized with ethyl methane sulphanate (ems) and offspring flies mutated in their second chromosome were crossed with flies containing the deletion Df(2L) gdh-A (Knipple et al., 1991). This deletion is between 25D7-26A7 bands and contains the 25F1 locus of lamin Dm_0 (Gruenbaum et al., 1988). One of the complementation groups was specific for a mutation in lamin Dm_0 since it could be specifically rescued by a P-element mediated transformation with a CaspeR vector (Pirrotta, 1988) containing 1.2 kb upstream sequences of lamin Dm_0 and either the complete genomic lamin Dm_0 gene (EcoRI-EcoRI fragment; Osman et al., 1990) or the two first exons and part of the third exon of the genomic lamin gene (EcoRI-HindIII fragment; Osman et al., 1990) ligated to the HindIII-EcoRI fragment of lamin Dm_0 cDNA (Gruenbaum et al., 1988). A second mutagenesis screen utilized a P-element targeted gene mutation, using the Birm-2/Birm-2; ry/ry line (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990). Candidate lines for a mutation in lamin Dm_0 were crossed with an ems-mutated line in lamin Dm_0 (Osman, 1992). One of the isolated mutations, termed PM-15, was analyzed in more details. PM-15/PM-15 or PM-15/Df(2L)gdh-A flies showed an abnormal chromatin organization following 9-16 hr of development. The variability in the time of phenotype appearance is likely to be due to differences in the amounts of the maternal pool of lamin Dm_0. Flies homozygous or trans-heterozygous for these mutations eventually die. Therefore, lamin Dm_0 is an essential gene of the fruit fly (Osman, 1992). One of the first phenotypes in embryos homozygous for a mutation in lamin Dm_0 gene is the detachment of the peripheral chromatin from the nuclear envelope. This detachment occurs in many regions of affected nuclei and is followed by condensation of chromatin (**Fig. 2**. compare panels **C,D** to panels **A,B**). The later phenotypes of these embryos include nuclei aggregation and formation of cytoplasmic annulate lamellae (**Fig. 2E**). A *Drosophila* line mutated in its lamin Dm_0 gene (Lenz-Bohme et al., 1997), in which the amounts of lamin Dm_0 protein are reduced to less than 20% of their normal levels, also revealed enrichment in annulate lamellae and in nuclear envelope clusters. These

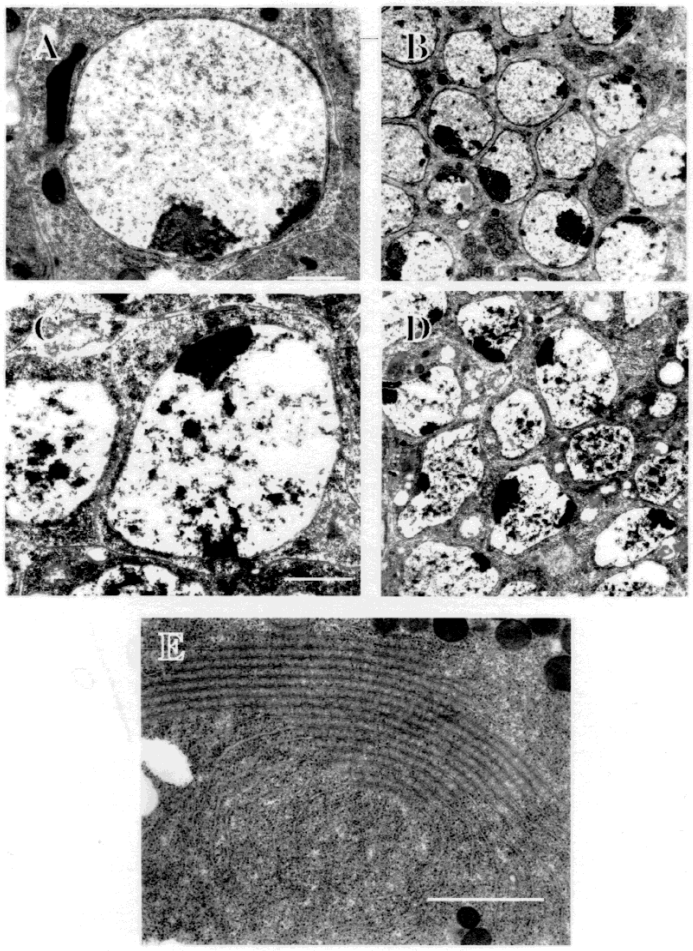

Figure 2. A mutation in lamin Dm_0 gene results in dissociation of chromatin from the nuclear envelope and accumulation of annulate lamellae (Osman et al., 1990). Embryos mutated in lamin Dm_0 showed a visible phenotype following 9-16 hr of development. Electron microscope analysis of PM-15/PM-15 cells (**C,D**) revealed chromatin dissociation from the nuclear envelope as compared to normal cells (**A,B**). (**E**) Annulate lamellae in PM-15/Df(2L)gdh-A embryos following degradation of nuclei. The bars in panels A,C,E represent 1 µm. The bars in panels B,D represent 5 µm.

flies showed reduced viability, retardation in their development, sterility, and impaired locomotion. In some cells, defective nuclear envelopes were also observed (Lenz-Bohme et al., 1997). In summary, these studies demonstrate the essential role of lamins in nuclear and chromatin organization.

B. Filament assembly properties of lamin Dm_0 and derivative proteins.

The assembly properties of lamin Dm_0 were investigated in vitro using bacterially expressed and purifies lamin Dm_0 and derivatives (Ulitzur et al., 1992). To test for the ability of filamentous protein to polymerize we used the sedimentation test (Heitlinger et al, 1991), which is based on the separation of pelletable polymers from soluble protein, following incubation under various chemicals and pH conditions. Reduction of salt concentration from 0.5 M NaCl to 50-150 mM NaCl in pH range of 5-9 was sufficient to induce 35-95% polymerization of lamin Dm_0 protein. Electron microscope analysis of negative stained pellets confirmed the formation of filamentous structures (**Fig. 3**). The observed paracrystals were characterized by a distinct stain-excluding pattern with 25 nm axial repeat unit, which is half the size of the lamin rod domain (**Fig. 3 A,B**). Figure 3C shows a relatively rare case which reveals that these paracrystals are composed of separate lamin filaments. These filaments are 8-10 nm wide, which is the normal size of cytoplasmic intermediate filaments. Although there is no evidence for the existence of paracrystals in vivo, it is noteworthy that the width of these paracrystals fits is in the size range of lamin fibers that were visualized in *Drosophila* cells in vivo (Paddy et al., 1990).

The ability of the isolated rod domain of lamin Dm_0 (amino acids 55-413) to polymerize was analyzed utilizing bacterially expressed protein that was purified to near ho-

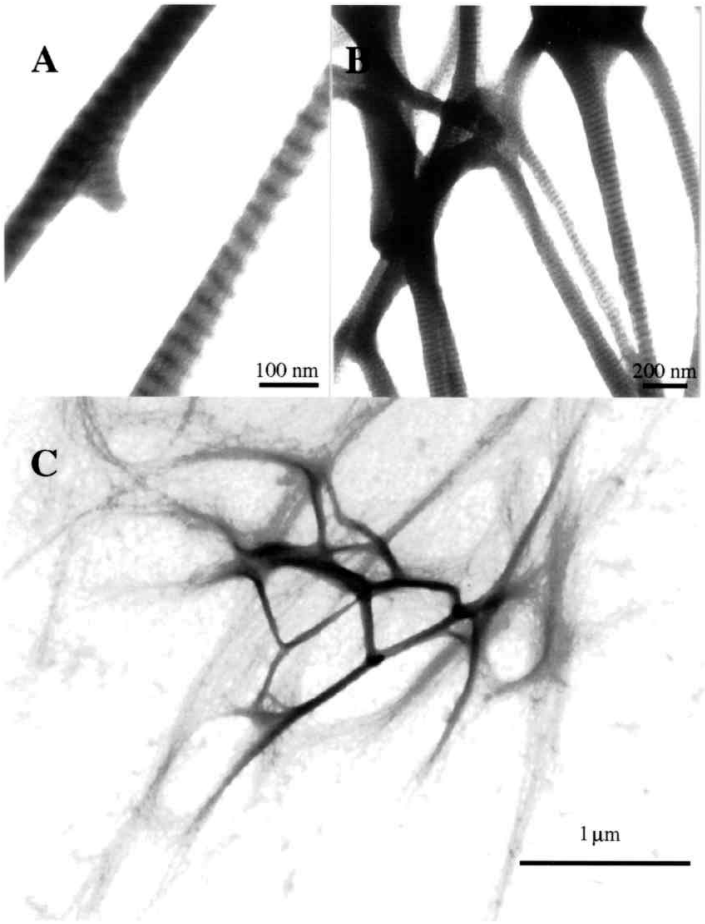

Figure 3. Supramolecular structures formed by lamin Dm_0 at low ionic strength. Lamin Dm_0 protein at 2 mg/ml in buffer H (30 mM Tris-HCl pH 7.5, 1 mM DTT) containing 0.5 M NaCl was diluted 5 times in buffer H and incubated for 80 min on ice. Samples were placed on electron microscope grid and negatively stained with 1% uranyl acetate. Tightly packed paracrystals exhibit ~25 nm axial repeat unit and their thickness ranged between 40-200 nm (**A,B**). The thick paracrystalline arrays are composed of a large number of thin filaments (**C**).

mogeneity. Unlike the complete lamin Dm_0 molecule, polymerization of the isolated rod domain was salt-independent. However, under acidic conditions (pH 5.5) and in the presence of 25 mM $CaCl_2$, the isolated rod domain was organized in higher order structures, as judged by the sedimentation test (**Fig. 4A**) and by electron microscope analysis (**Fig. 4B**). The filamentous structure of the polymerized rod domain resembled that of the complete lamin protein, but lacked the 25 nm repeat unit. Under neutral and basic pH conditions the rod domain was organized into dimers which were 52 nm long and about 0.5 nm in diameter (not shown). In summary, these results demonstrate that the rod domain contains enough information to form the lamin filaments and that sequences outside the rod domain are required for the proper organization of the lamin filaments and for their assembly under physiological conditions.

C. Interaction between lamin Dm_0 and chromatin.

Our previous analysis demonstrated that lamin Dm_0 can interact specifically with sperm chromatin (Ulitzur et al., 1992). These experiments also showed that the addition of bacterially expressed lamin Dm_0 to *Drosophila* embryonic extracts that can assemble nuclei from sperm chromatin resulted in increased amounts of lamin Dm_0 around the peripheral chromatin (Ulitzur et al., 1992). The mitotic chromosome assay that measures the association between lamin and mitotic CHO chromosomes (Glass et al., 1993; Glass and Gerace, 1990) was used to analyze domains in lamin Dm_0 protein that are capable of interaction with chromatin. When lamin Dm_0 protein was incubated for 30-60 min at 22^{o}C with isolated mitotic chromosomes, in the presence of excess amounts of either 5% BSA or 10% FCS, a strong lamin staining was observed following immunofluorescence analysis with anti-lamin antibodies. The staining was mostly peripheral to the chromosomes and included aggregates of lamin (not shown). These aggregations are probably due to the organization of lamin Dm_0 into polymers since the

aggregates were absent when mitotic chromosomes were incubated with lamin Dm_0 containing the mutation R64>H (Zhao et al., 1996), which impairs the ability of lamin Dm_0 to form filaments (**Fig. 5B**). The tail domain of lamin Dm_0 (amino acids 425-622) contains specific binding site(s) to chromatin since it bound specifically to the mitotic chromosomes (**Fig. 5A**). The R64>H mutant protein was incubated with mitotic chromosomes in the presence of hundred fold molar excess of the isolated tail domain in order to find other possible domains in lamin that bind chromatin. As shown in **Fig. 5C**, staining with affinity purified polyclonal antibodies against the rod domain of lamin Dm_0 gave intensity levels that were close to background levels. In conclusion, lamin Dm_0 binds specifically to chromatin and its binding site(s) are localized to its tail domain. The specific lamin sequence that binds to chromatin, the affinity of its binding and the target chromosomal proteins are currently under investigation.

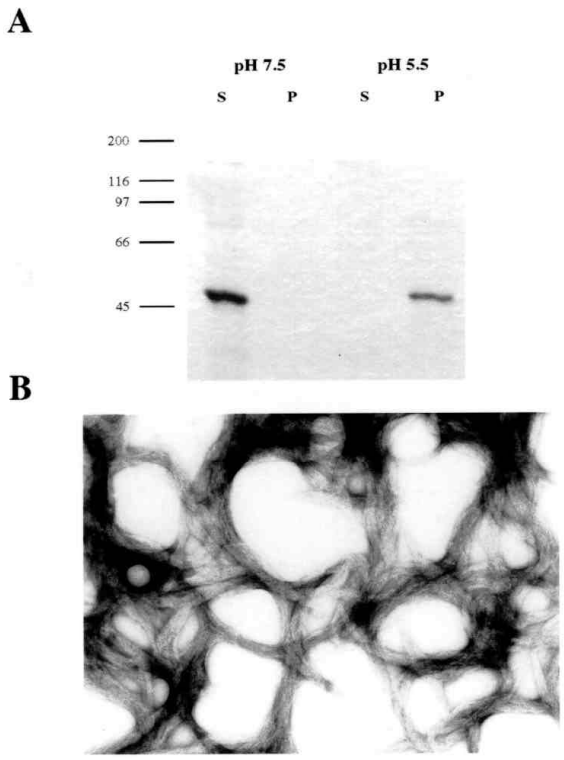

Figure 4. Polymerization properties of the isolated rod domain of lamin Dm_0. (A) Isolated rod domain protein (2 mg/ml) in buffer H was diluted 5 folds in buffer H or in 50 mM sodium citrate pH 5.5, 25 mM $CaCl_2$. Pellet (p) and supernatant (s) were separated by 30 min centrifugation at 15,000xg, boiled in sample loading buffer and subjected to SDS-10% PAGE stained with Comassie Brillant blue. The position of the size markers are shown on the left of the panel. The rod domain polymerized at pH 5.5 but not at pH 7.5. (B) The pellet fraction was placed on electron microscope grid and negatively stained with 0.75% uranyl acetate.

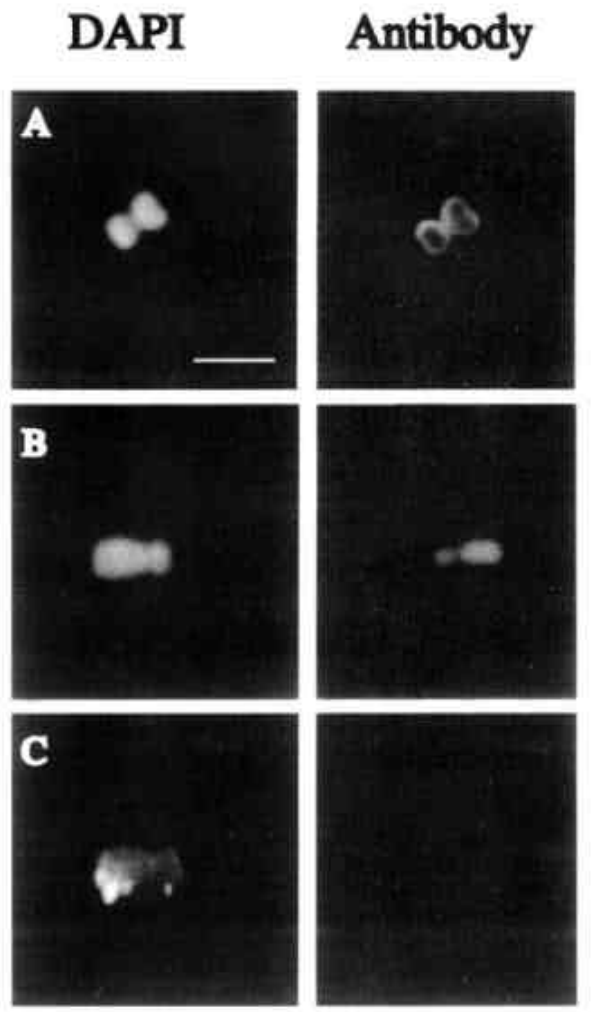

Figure 5. Binding of lamin Dm_0 to chromosomes. Lamin Dm_0 protein mutated in Arginine 64 (R64>H) (Zhao et al., 1996), which is impaired in its ability to form head-to-tail polymers (Stuurman et al., 1996; Zhao et al., 1996), bound specifically to mitotic chromosomes (B). The tail domain of lamin Dm_0 (amino acids 425-622) also bound specifically to mitotic chromosomes (A). The tail domain of lamin Dm_0 could compete for the binding of the complete lamin Dm_0 molecule to mitotic chromosomes since addition of a hundred fold molar excess of the tail domain could efficiently compete for the binding of R64>H (C). DAPI staining of DNA, left panels; antibody staining, right panels. Affinity purified polyclonal antibodies against the rod domain of lamin Dm_0 B,C; monoclonal antibody 611A3A6 anti-lamin Dm_0, A. This monoclonal antibody recognize an epitope in the tail domain. The bar represents 6μm and applies to all panels.

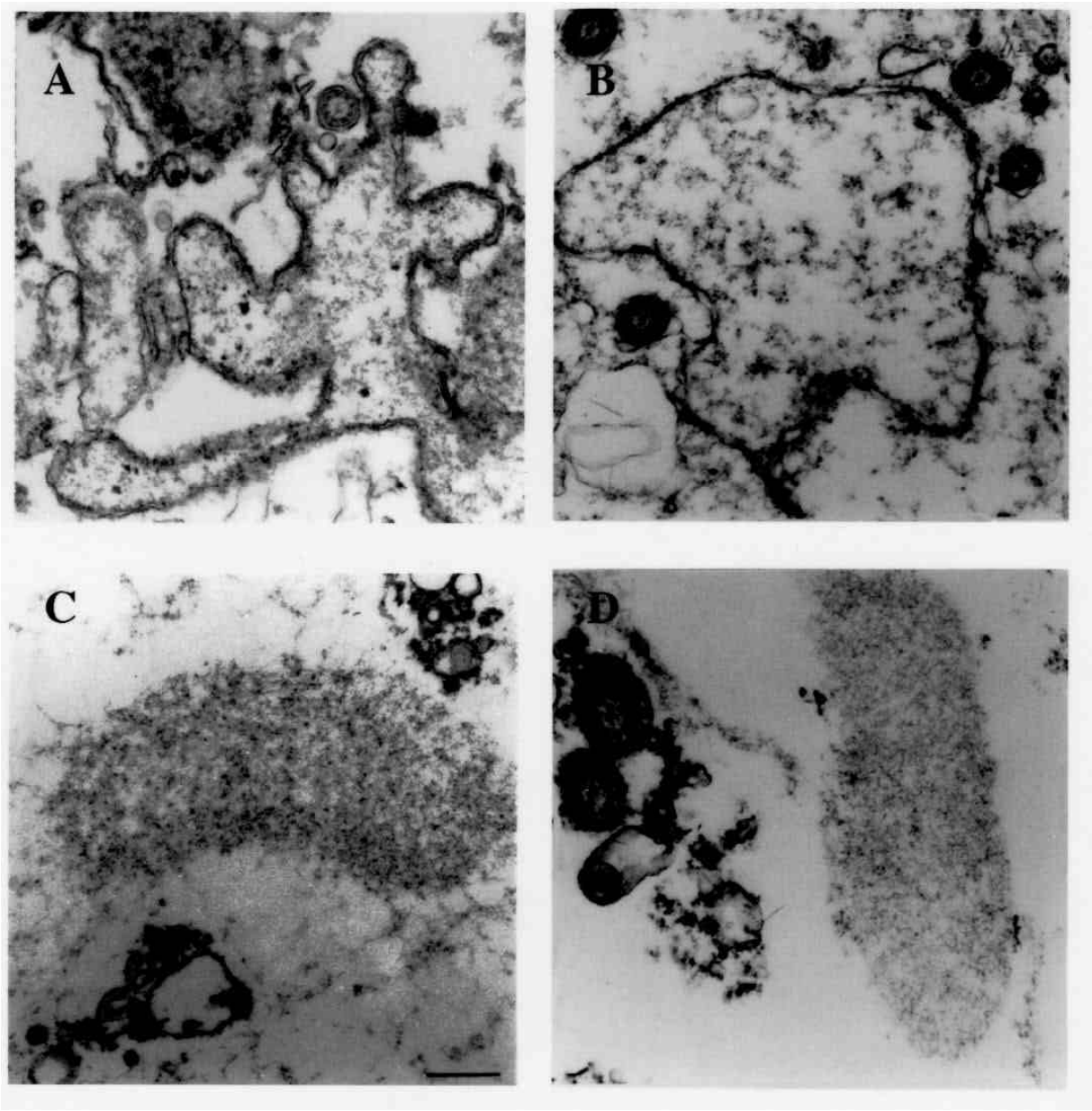

Figure 6. Inhibition of lamin Dm_0 and otefin activity prevents the *in vitro* nuclear envelope assembly in *Drosophila* embryonic extracts. Twenty microliters of embryonic extracts were preincubated for 90 min with either 100 µg polyclonal anti-lamin Dm_0 antibodies (**C**), 100 µg polyclonal anti-otefin antibodies (**D**), or 100 µg of preimmune serum antibodies (IgG fraction), (**A,B**). Sperm chromatin was added and the incubation proceeded for additional 90 min. Samples from the two experimental systems were viewed by standard transmission electron microscope. Decondensed chromatin was enveloped with nuclear membranes in preimmune antibodies-treated extracts (A,B) but not in anti-lamin Dm_0 (C) or anti-otefin (D) antibodies-treated extracts. The bar represents 1 µm.

D. Lamin and otefin are essential for nuclear envelope formation

To analyze lamin Dm_0 and otefin function in nuclear envelope formation, 0-6 hr old *Drosophila* embryo extracts, in which interphase-like nuclei can be assembled from sperm chromatin (Berrios and Avilion, 1990; Crevel and Cotterill, 1991; Ulitzur and Gruenbaum, 1989), were incubated with either 100-300 µg polyclonal anti-*Drosophila* lamin Dm_0 or anti-*Drosophila* otefin antibodies (IgG fractions). Incubation of the extract under the same conditions with 100-300 µg of preimmune rabbit sera (IgG fraction) or with normal rabbit IgG served as controls. No membrane assembly was observed when lamin Dm_0 or otefin activities were inhibited

(Ashery Padan et al., 1997; Ulitzur et al., 1992; Ulitzur et al., 1997). Electron microscope (**Fig. 6**), light, and fluorescent microscope analyses (not shown) 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. 6C,D**). Incubation of the extract with preimmune sera, (IgG fraction), or with commercially available normal rabbit IgG fraction had no effect on nuclear assembly, the presence of membranes around the chromatin was observed (**Fig. 6A,B**). Addition of 2 µg of interphase lamin isolated from *Drosophila* embryos to extracts that were preincubated with the anti-lamin Dm_0 antibodies restored binding of vesicles to chromatin (Ulitzur et al., 1997).

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