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Disassembly of the *Drosophila* nuclear lamina in a homologous cell-free system

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SUMMARY

Stage 14 *Drosophila* oocytes are arrested in first meiotic metaphase. A cell-free extract of these oocytes catalyzes apparent disassembly of purified *Drosophila* nuclei as well as of nuclear lamin polymers formed in vitro from isolated interphase lamins. Biochemically, the oocyte extract catalyzes lamin solubilization and phosphorylation as well as characteristic changes in one- and two-dimensional gel mobility. A previously unidentified soluble lamin isoform is easily seen after in vitro disassembly. This isoform is detectable but present only in very small quantities in vivo and is apparently derived specifically from one of the two interphase lamin isoforms. Cell-free nuclear lamina disassembly is ATP-dependent and addition of calcium to extracts blocks disassembly as judged both morphologically and biochemically. This system will allow enzymological characterization of cell-free lamina disassembly as well as molecular analysis of specific *Drosophila* mutants.

Key words: nuclear lamina, nuclear lamins, meiosis, *Drosophila melanogaster*

INTRODUCTION

Nuclear disassembly, the process in higher eukaryotes whereby the nucleus breaks down during both meiosis and mitosis, is characterized by three key events. These are: (1) breakdown and vesicularization of the nuclear membrane; (2) chromosome condensation; and (3) nuclear lamina depolymerization with solubilization and redistribution of its major polypeptide components, the nuclear lamins. To date, several vertebrate systems have been established to study nuclear disassembly in vitro (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Suprynowicz and Gerace, 1986; Newport and Spann, 1987; Nakagawa et al., 1989; Pfaller et al., 1991). A cell-free nuclear disassembly system from the invertebrate, *Spisula*, was also reported (Dessev et al., 1989).

In vertebrates, hyperphosphorylation was correlated with lamin solubilization during mitosis (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Nakagawa et al., 1989). Recently, this has been investigated extensively (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990; Heitlinger et al., 1991; Lüscher et al., 1991; Peter et al., 1991). Similar studies were performed with *Spisula* (Dessev et al., 1991). In many systems, cdc2 kinase is clearly the major and perhaps sole mitotic kinase (see e.g. Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990; Peter et al., 1992; Molloy and Little, 1992; Eggert et al., 1993; for a review, see Nigg, 1992). However, the potential role of other kinases remains unclear (Ward and Kirschner, 1990; Hocevar and Fields, 1991; Lüscher et al., 1991; Peter et al., 1992; Hocevar et al., 1993).

In *Drosophila*, at least one major gene, coding for lamin Dm_0†, is expressed during oogenesis, early embryogenesis and in tissue culture cells (Smith et al., 1987; Gruenbaum et al., 1988; Smith and Fisher, 1989). The protein encoded by this gene is assembled into the nuclear envelope where differential phosphorylation leads to the generation of two interphase isoforms, lamins Dm_1 and Dm_2 (Smith et al., 1987). During both meiosis and mitosis, conversion of lamins Dm_1 and Dm_2 to a third isoform, lamin Dm_{mit}, correlates with nuclear disassembly (Smith and Fisher, 1989). In *Drosophila*, lamin hyperphosphorylation does not occur coincident with M-phase-specific solubilization. Rather, the overall amount of phosphate per lamin molecule remains relatively constant among isoforms; however, the lamin amino acid residues which are phosphorylated differ during the cell cycle (Smith and Fisher, 1989).

Here we report that an extract of wild-type stage 14 *Drosophila* oocytes catalyzes the disassembly of exogenous *Drosophila* nuclei, purified from either embryos or tissue culture cells. This extract will also catalyze disassembly of polymers formed in vitro from purified interphase lamins. In both contexts, in vitro disassembly occurs in conjunction with apparent conversion of lamins Dm_1 and Dm_2 to lamin Dm_{mit}. This system promises to be useful both for enzymological char-

†Unless indicated otherwise, for *Drosophila* the term lamin refers solely to protein products encoded by the *Drosophila* lamin Dm_0 gene.

acterization of in vitro lamina disassembly and in the analysis of specific *Drosophila* mutants.

MATERIALS AND METHODS

Antibodies

Affinity-purified, phosphatase-labeled goat anti-rabbit IgG was from Kirkegaard and Perry (Gaithersburg, MD). Horseradish peroxidase-conjugated sheep anti-mouse and anti-rabbit IgG were from Amersham International (Amersham, Great Britain). Affinity-purified rhodamine-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). Anti-*Drosophila* lamin antibodies were affinity purified (Fisher and Smith, 1988) from rabbit antiserum using a lamin β-galactosidase fusion protein (Gruenbaum et al., 1988) or a bacterially expressed portion of *Drosophila* lamin Dm_0 as the immobilized affinity ligand. Monoclonal anti-*Drosophila* lamin antibody 84 (ADL84) was generated from mice immunized with affinity-purified interphase *Drosophila* lamins Dm_1 and Dm_2. Details of monoclonal antibody characterization will be reported separately (Stuurman et al., unpublished data).

Methods

Much of the methodology has been detailed previously (Smith et al., 1987; Smith and Fisher, 1989; Lin and Fisher, 1990). *Drosophila melanogaster* (Oregon R, P2 strain) were maintained in mass culture and embryos collected according to Allis et al. (1977). Immunoprecipitation with polyclonal antibodies was according to Smith et al. (1987). SDS-PAGE was according to Laemmli (1970) as modified (Fisher et al., 1982) or on minigels as indicated in figure legends. Proteins were transferred electrophoretically from gels to sheets of nitrocellulose (Harlow and Lane, 1988) and the resulting immunoblots were processed and probed with primary antibodies. Bands of reactivity were visualized either by chemiluminescence (using the ECL detection kit, Amersham International, Amersham, Great Britain) or colorimetrically (McGadey, 1970) with calf alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Blake et al., 1984; Smith and Fisher, 1984).

Preparation of *Drosophila* oocyte extracts

Between 100 and 500 virgin *Drosophila* females, 7-10 days old, were anesthetized by chilling and placed in a plastic Petri dish, 14 cm in diameter, on ice, in 10 ml of 140 mM NaCl, 10 mM $KHPO_4$ pH 7.5 (25°C) containing 10 mg/ml polyvinylpyrrolidone (PBS/PVP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK). To release egg chambers, flies were crushed with the bottom of a glass Petri dish, 9 cm in diameter. The homogenate was filtered through a 250 μm nylon mesh to remove large fly body parts. The filtrate was passed through a 125 μm nylon mesh on which only stage 14 egg chambers were retained (Jacobs-Lorena and Crippa, 1977). In this paper, stage 14 egg chambers will be referred to as stage 14 oocytes (see Mahowald and Kambysellis, 1980; Ashburner, 1989). Stage 14 oocytes were washed extensively on the mesh with PBS/PVP, transferred to a 1.5 ml centrifuge tube and washed by repeated mixing and settling in cold disassembly wash buffer (Newport and Spann, 1987) containing 240 mM β-glycerophosphate, pH 7.2 (25°C), 60 mM EGTA, 45 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). Oocytes were then washed in cold mitotic dilution buffer (MDB; Newport and Spann, 1987), containing 40 mM β-glycerophosphate, pH 7.2, 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM DTT, after which they were homogenized in a minimal volume of MDB using a 0.2 ml glass micro tissue grinder (#357848, Wheaton, Millville, NJ) in an ice-water bath. Homogenates were centrifuged at 12,000 ***g*** for 10 minutes at 4°C. Supernatants (stage 14 *Drosophila* oocyte extracts) were withdrawn and either used fresh or aliquoted, quick-frozen and stored in liquid N_2 for later use. Extracts typically contained 20-25 mg protein/ml.

Preparation of nuclei from *Drosophila* embryos and tissue culture cells

Nuclei were prepared from frozen, dechorionated 0 to 15-hour-old *Drosophila* embryos essentially as previously described for in vitro nuclear run-on transcription assays (Fisher et al., 1989) except that nonionic detergent (0.5% Triton X-100) was omitted from buffers. Embryos were Dounce-homogenized on ice in 10 mM Tris-HCl, pH 8.0 (25°C), 5 mM $MgCl_2$, 1.3 M sucrose, 0.5 mM DTT and 1 mM PMSF (Buffer A). Homogenates were filtered through 125 μm nylon mesh, and filtrates were centrifuged at 10,000 ***g*** for 10 minutes at 4°C over a cushion of Buffer A. Purified nuclei were washed in MDB containing 40% glycerol (MDBG), centrifuged at 10,000 ***g*** for 10 minutes and resuspended at 2,000 units/ml (Fisher et al., 1982) in MDBG. Nuclei were then aliquoted, frozen by immersion in liquid N_2 and stored at −70°C.

Nuclei were prepared from *Drosophila* Schneider S2 and K_c tissue culture cells, essentially as described for embryo nuclei but with the following modifications. Cells were broken either by Dounce homogenization or by vortexing in Buffer A, and nuclei were isolated by centrifuging homogenates at 2,000 ***g***. After purification, nuclei were suspended at a final concentration of about 2×10^8/ml in MDBG, aliquoted, frozen and stored as described for embryo nuclei. For radiolabeling, cells were suspended in methionine-free Schneider cell medium at a density of 1×10^7 cells/ml. [^{35}S]methionine (#NEG-009L, New England Nuclear, Boston, MA) was added to a final concentration of 50 μCi/ml and cells were incubated in this medium for approximately 40 hours (Smith et al., 1987).

Immunoaffinity purification and in vitro polymerization of interphase *Drosophila* lamins

Immunoaffinity purification of interphase *Drosophila* lamins Dm_1 and Dm_2 was as described (Lin and Fisher, 1990). All procedures were performed at 4°C unless otherwise indicated. Embryo or tissue culture cell nuclei, purified as described above, were digested at 23°C (McConnell et al., 1987) with 10 μg/ml of DNase I and 8 μg/ml of RNase A, after which they were extracted sequentially with 2% Triton X-100 followed by 0.5 M NaCl. The 0.5 M NaCl extract was batch adsorbed by overnight incubation to a Protein A-Sepharose column containing covalently coupled, affinity-purified rabbit anti-*Drosophila* lamin antibodies. The column was washed, and purified *Drosophila* lamins Dm_1 and Dm_2 were eluted (Lin and Fisher, 1990). The eluate was equilibrated with MDB by open dialysis for at least 2 hours on 0.05 μm filters (#VMWP 025 00, Millipore, Bedford MA). Typically, greater than 75% of the purified lamins formed polymers during dialysis, as judged by recovery in the pellet fraction after sedimentation at 12,000 ***g***. Aliquots of lamin polymers were quick-frozen in liquid N_2 and stored at −70°C.

Indirect immunofluorescence

Indirect immunofluorescence of isolated nuclei was performed essentially as previously described for larval cryosections (Smith and Fisher, 1984; McConnell et al., 1987). Samples were placed on gelatin-coated glass slides, allowed to air dry and then probed with affinity-purified rabbit anti-*Drosophila* lamin IgG at a final concentration of 1-2 μg/ml followed by affinity-purified rhodamine-conjugated donkey anti-rabbit IgG at a final concentration of 1.5 μg/ml. After probing and washing, specimens were mounted in a solution of 1 μg/ml 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). Samples were examined with a Leitz Ortholux II epifluorescence microscope equipped with a 40× objective and photographed using an Orthomat W camera.

Nuclear lamina disassembly reactions

Nuclei, lamin polymers formed in vitro and oocyte extracts were

thawed on ice immediately before use. Standard reaction mixtures were prepared on ice and included 2.7 mM ATP, 13.3 mM phosphocreatine and 50 μg/ml creatine phosphokinase. Reactions were incubated at 23°C and at various times, aliquots were removed and centrifuged at 4°C for 10 minutes at 12,000 ***g***. Supernatant and corresponding pellet fractions were analyzed separately. For morphological analyses, samples were not fractionated but were fixed in 3.7% formaldehyde and processed as described above.

Treatment of lamins with calf alkaline phosphatase

Calf alkaline phosphatase treatment was performed essentially as previously described (Smith et el., 1987). Immunoprecipitated lamins were extracted from Protein A-Sepharose (Pharmacia, Uppsala, Sweden) by boiling in SDS-PAGE loading buffer and protein was recovered by precipitation with 10% trichloroacetic acid. Precipitates were washed with acetone, dried and resolubilized. Calf alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to a final concentration of 500 units/μl and samples were incubated for 1 hour at 37°C. Reactions were stopped by addition of 1/20 volume of 20% SDS followed by boiling.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of immunoprecipitated *Drosophila* lamins was performed essentially as described by Hochstrasser et al. (1988). Isoelectric focusing was in 8.5 cm long tube gels containing 2% (w/v) pH 5-8 ampholines (Sigma Chemical Co., St Louis, MO) for 14,000 V-hours without prerunning. Tube gels were equilibrated for 5 minutes in 60 mM Tris-HCl, pH 6.8 (23°C), 2% SDS, 100 mM DTT, and were loaded onto a standard 8% polyacrylamide separating gel with a 4% polyacrylamide stacking gel (Laemmli, 1970). Second-dimension electrophoresis was performed in an apparatus capable of running multiple gels simultaneously (Idea Scientific, Minneapolis, MN). Blotting of proteins to nitrocellulose and colorimetric detection of lamins was as described above.

RESULTS

An extract of stage 14 *Drosophila* oocytes catalyzes the breakdown of exogenous *Drosophila* nuclei

At stage 14, the final stage of *Drosophila* oogenesis, the oocyte germinal vesicle (nucleus) breaks down and the oocyte within the egg chamber arrests in metaphase of meiosis I (see Mahowald and Kambysellis, 1980). Coincidently, germinal vesicle lamins are solubilized and redistributed throughout the oocyte; biochemically, this correlates with conversion of interphase lamin isoforms Dm_1 and Dm_2 to meiotic/mitotic lamin isoform Dm_{mit} (Smith and Fisher, 1989).

When purified *Drosophila* embryo nuclei were added to a concentrated extract of stage 14 *Drosophila* oocytes, the results shown in Figs 1 and 2 were obtained. Morphologically, exogenous nuclei (Fig. 1A) lost refractility within 15 minutes (Fig. 1D) and disappeared completely within 30 minutes (Fig. 1G). Progressive changes in DNA structure were also seen. Initially, nuclei stained intensely and discretely with the DNA-specific dye, DAPI (Fig. 1B). After 15 minutes, most DAPI-staining material was absent from the field and much of that which remained appeared distorted (Fig. 1E). After 30 minutes, most fields were devoid of DAPI-staining material (Fig. 1H) but, occasionally, large aggregates were seen after 60-120 minutes of incubation (not shown, but see Fig. 7E). In our estimation, the disappearance of DAPI-staining material did not reflect large-scale DNA degradation and high levels of DNase activity, as exogenous DNA remained relatively intact in stage 14 oocyte extracts (not shown). Clearly, further investigation of this phenomenon is warranted.

Indirect immunofluorescence was also performed on these

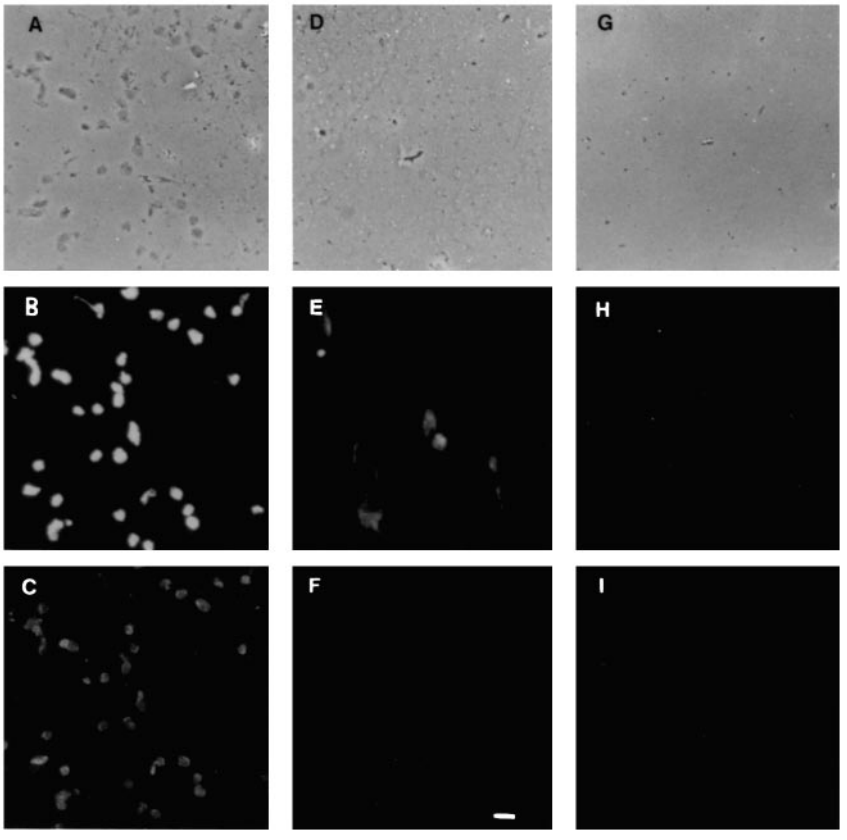

Fig. 1. Disassembly of the *Drosophila* nuclear lamina catalyzed by the stage 14 oocyte extract; morphological analysis. 20 μl of oocyte extract and 4 units of embryo nuclei (about 4 μg total protein/unit; see Fisher et al., 1982) were incubated at 23°C in a final volume of 80 μl. 10 μl samples were removed at each time point and processed as described (Materials and Methods) for morphological analysis. (A,B,C) Samples at 0 minutes of incubation; (D,E,F) at 15 minutes of incubation; (G,H,I) at 30 minutes of incubation. (A,D,G) Phase-contrast micrographs. (B,E,H) DAPI staining. (C,F,I) Immunofluorescence staining with affinity purified polyclonal anti-lamin antibodies. Bar in F represents 10 μm and applies to all panels.

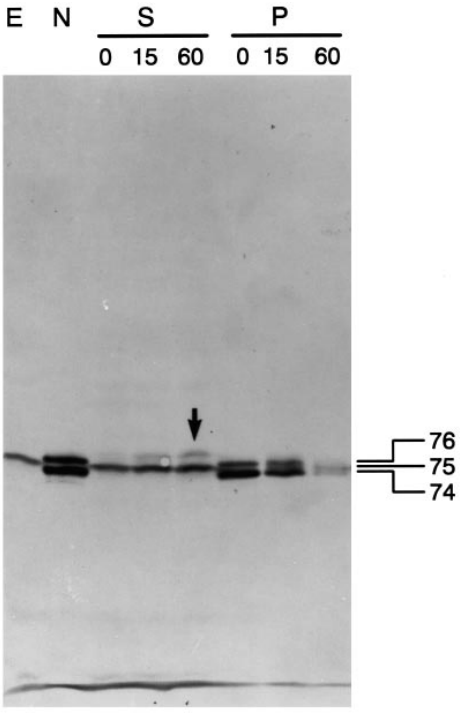

Fig. 2. Disassembly of the *Drosophila* nuclear lamina catalyzed by the stage 14 oocyte extract; immunoblot analysis of lamin isoform interconversion. 32 µl of stage 14 *Drosophila* oocyte extract and 9.6 units of embryo nuclei were incubated at 23°C in a final volume of 80 µl. 20-µl samples were fractionated at various times, subjected to SDS-7% PAGE and immunoblot analysis with affinity-purified polyclonal anti-lamin antibodies (Materials and Methods). Lane E, 8 µl of oocyte extract alone; lane N, 2.4 units of embryo nuclei alone. Supernatant (S) and corresponding pellet (P) fractions are shown after 0, 15 and 60 minutes of incubation. The migration positions of the different lamin isoforms are indicated to the right of the figure; 76 kDa, interphase lamin Dm_2; 75 kDa, meiotic/mitotic lamin Dm_{mit}; and 74 kDa, interphase lamin Dm_1. The downpointing arrow in lane S 60 designates lamin $Dm_{mit\text{-}s}$; lamin $Dm_{mit\text{-}s}$ is also visible in lane S 15 and barely visible in lane S 0.

same specimens with anti-*Drosophila* lamin IgG. Initial staining revealed a morphologically typical nuclear lamina (Fig. 1C). As the incubation progressed, this staining was lost (Fig. 1F and I). Large aggregates of DAPI-staining material seen late in the incubation did not stain with anti-lamin antibodies (not shown, but see Fig. 7F). Incubation of purified *Drosophila* nuclei for greater than 90 minutes in buffer minus oocyte extract did not result in any changes detectable by light microscopy (not shown).

Biochemical analyses were performed in parallel with morphological studies. At various time points during the course of in vitro disassembly, aliquots of the reaction mixture were separated into supernatant and pellet fractions by sedimentation at 12,000 ***g*** and proteins were subjected to SDS-PAGE and immunoblot analysis (Fig. 2). An extract of stage 14 *Drosophila* oocytes (Fig. 2, lane E) contained only lamin Dm_{mit} (Smith and Fisher, 1989; see also Lin and Fisher, 1990). Purified *Drosophila* nuclei (Fig. 2, lane N) contained lamins Dm_1 and Dm_2 (Smith et al., 1987; see also Lin and Fisher, 1990). Lamin Dm_{mit} (apparent mass of 75 kDa) migrates with a gel mobility intermediate to lamins Dm_1 (apparent mass of 74 kDa) and Dm_2 (apparent mass of 76 kDa).

Lamin Dm_{mit} derived from the oocyte extract was seen in the supernatant fraction of nuclear disassembly reactions before incubation (Fig. 2, lane S 0). With time, this soluble species increased as did a previously unrecognized soluble species that migrated more slowly than nuclear lamin Dm_2 (designated by the downpointing arrow, Fig. 2, lane S 60). This more slowly migrating soluble species was designated lamin $Dm_{mit\text{-}s}$. Although not visible initially in (Fig. 2, lane E) or after prolonged incubation of oocyte extract alone (see Fig. 6C), this species was found in limited quantities in vivo (see Fig. 5C, lane E). Examination of the corresponding pellet fractions showed that interphase nuclear lamins Dm_1 and Dm_2, readily identified initially (Fig. 2, lane P 0), were almost completely absent after 60 minutes of incubation (Fig. 2, lane P 60). As in morphological analyses, incubation of purified nuclei in buffer alone for more than 90 minutes was without effect on the gel mobility or sedimentation properties of the interphase nuclear lamins (not shown). Treatment with calf alkaline phosphatase of lamins immunoprecipitated from supernatant and pellet fractions both before and after in vitro disassembly resulted in the appearance of a single band at approximately 74 kDa after SDS-PAGE and immunoblot analysis (not shown, but see Smith et al., 1987).

The presence of endogenous lamin Dm_{mit} in stage 14 oocyte extracts led to ambiguity regarding the origins of the lamin Dm_{mit} seen after in vitro disassembly of *Drosophila* embryo nuclei. To distinguish exogenous from endogenous lamin, *Drosophila* Schneider S2 tissue culture cells were labeled in vivo with [^{35}S]methionine. Labeled nuclei were purified and added to unlabeled stage 14 oocyte extract. After incubation, samples were fractionated, denatured and subjected to immunoprecipitation with affinity-purified anti-*Drosophila* lamin antibodies followed by SDS-PAGE and fluorography. Although disassembly of radiolabeled Schneider cell nuclei was considerably less efficient than that seen with *Drosophila* embryo nuclei, significant changes in sedimentation properties and gel mobility were observed (Fig. 3). After 120 minutes of incubation, soluble (radiolabeled) species with the expected SDS-PAGE mobilities of both lamins Dm_{mit} and $Dm_{mit\text{-}s}$ were seen, whereas before incubation, only pelletable interphase lamins Dm_1 and Dm_2 were detected (Fig. 3). After incubation in stage 14 oocyte extract, radiolabeled nuclei purified from K_c cells, another *Drosophila* tissue culture cell line, gave results similar to those seen with Schneider cell nuclei (not shown). Addition of fresh extract at 120 minutes did not result in further breakdown of *Drosophila* tissue culture cell nuclei (not shown). In contrast, incubation of purified, radiolabeled *Drosophila* Schneider or K_c cell nuclei in buffer alone for more than 120 minutes was without effect on the sedimentation properties or gel mobility of the interphase lamins (not shown).

Disassembly catalyzed by stage 14 oocyte extracts is similar for polymers assembled from interphase *Drosophila* lamins purified from either embryos or tissue culture cells

To test the ability of the stage 14 oocyte extract to disassemble lamin polymers formed in vitro, solubilized interphase lamins were immunoaffinity purified from both embryos and tissue culture cells and polymerized as described (Materials

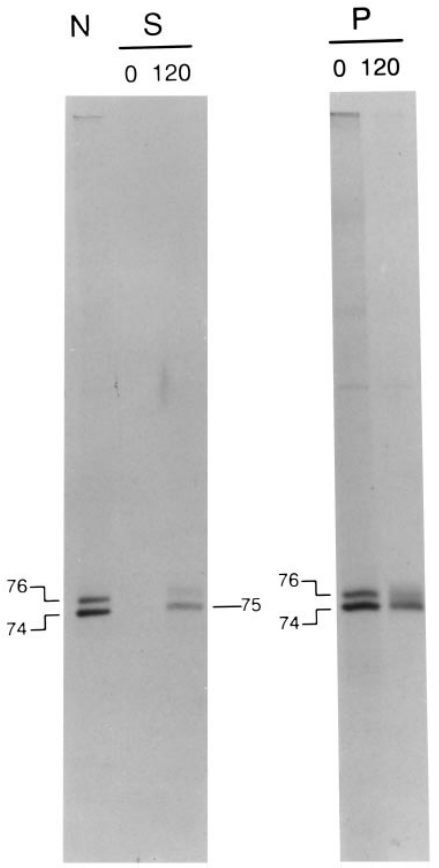

Fig. 3. Disassembly of the [^{35}S]methionine-labeled *Drosophila* tissue culture cell nuclear lamina; immunochemical analysis. Schneider S2 cells were labeled with [^{35}S]methionine as described (Materials and Methods). 3×10^7 purified nuclei were mixed with 26 µl of oocyte extract and incubated at 23°C in a final volume of 150 µl. 45 µl samples were fractionated at 0 and 120 minutes. Lamins were immunoprecipitated from the supernatant (S) and corresponding pellet (P) fractions with affinity-purified polyclonal anti-lamin antibodies. Samples were subjected to SDS-7% PAGE and fluorography. Lane N, lamins immunoprecipitated from 9×10^6 [^{35}S]methionine-labeled nuclei alone. The mobilities of lamins Dm_1 (74 kDa), Dm_2 (76 kDa), and Dm_{mit} (75 kDa) are indicated.

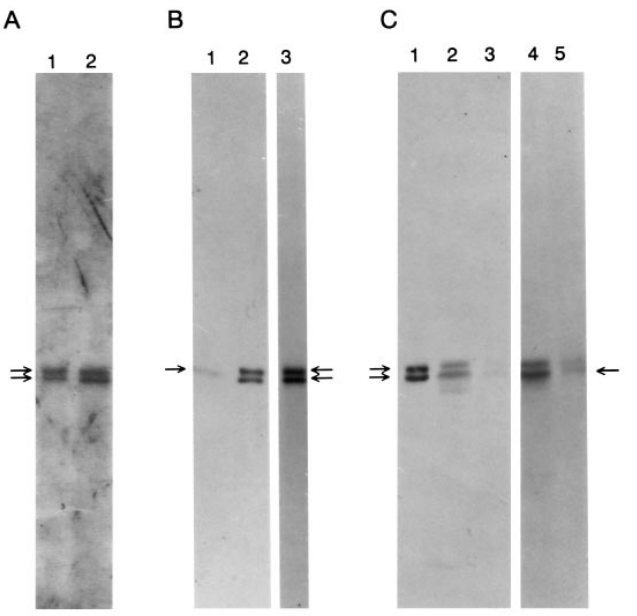

Fig. 4. Disassembly kinetics of lamin polymers assembled with lamins purified from embryo versus [^{35}S]methionine-labeled K_c tissue culture cell nuclei. Lamin polymers (about 420 ng) assembled in vitro from affinity-purified lamins isolated from either embryo or [^{35}S]methionine-labeled K_c nuclei were mixed with 6.3 µl of oocyte extract in a final volume of 158 µl. 75 µl samples were separated into supernatant and pellet fractions after 90 minutes of incubation at 23°C and analyzed by SDS-7% PAGE. (A) Coomassie Blue stained gel showing lamin polymers formed in vitro from lamins isolated from embryo (lane 1) and K_c (lane 2) nuclei. (B) lanes 1 and 2, immunoblot probed with affinity-purified polyclonal anti-lamin antibody of 3 µl of oocyte extract alone (lane 1) and 200 ng of lamin polymers from embryo nuclei (lane 2). Lane 3, fluorograph of 200 ng of ^{35}S-labeled K_c cell lamin polymers. (C) analysis of polymers after incubation in oocyte extract. Lane 1, polymers alone. Supernatant (lane 2) and corresponding pellet (lane 3) fractions of embryo lamin polymers visualized by immunoblot analysis; supernatant (lane 4) and corresponding pellet (lane 5) fractions of ^{35}S-labeled K_c cell lamin polymers visualized by fluorography. Single arrows indicate the mobility of lamin Dm_{mit} (75 kDa); double arrows indicate the mobilities of the interphase lamins Dm_1 (74 kDa) and Dm_2 (76 kDa). The minor immunoreactive band migrating faster than lamin Dm_1 is apparently a proteolytic breakdown product (see e.g. Smith and Fisher, 1989).

and Methods). Tissue culture cell lamins were ^{35}S-labeled. These polymers were then incubated in stage 14 oocyte extract and fractionated. Initially, interphase lamins were pelletable and had the expected SDS-PAGE mobilities of lamins Dm_1 and Dm_2 (Fig. 4A and B). After 90 minutes of incubation in the stage 14 oocyte extract, almost all of the pelletable interphase lamin isoforms from both sources appeared in the supernatant fraction, and the gel mobility of these isoforms was that of lamins Dm_{mit} and $Dm_{mit\text{-}s}$ (Fig. 4C).

Lamin $Dm_{mit\text{-}s}$ is related to both lamins Dm_{mit} and Dm_2

A monoclonal antibody, ADL84, was raised against authentic *Drosophila* lamins. The antibody was specific for lamin Dm_1 and did not recognize lamin Dm_2. A disassembly experiment was performed with nuclei and subjected to immunoblot analysis as above (Fig. 5). The resulting blot was probed with rabbit polyclonal anti-lamin antibodies that recognize all *Drosophila* nuclear lamin isoforms (see Lin and Fisher, 1990), and reactivity was visualized colorimetrically (Fig. 5A). The same blot was reprobed with mouse ADL84 and reactivity was detected by chemiluminescence (Fig. 5B). As will be demonstrated elsewhere (Stuurman et al., unpublished data), ADL84 reacted with lamin Dm_1 only and showed no reactivity with lamin Dm_2 (compare Fig. 5A and B, lanes N; see also Fig. 5C and D, lanes N). Apparently the epitope for ADL84 is post-translationally modified in lamin Dm_2, abolishing reactivity of this isoform. ADL84 also reacts with the soluble lamin isoform, Dm_{mit} (Fig. 5A and B, lanes E; see also Fig. 5C and D, lanes E), but does not react with lamin $Dm_{mit\text{-}s}$ present in oocyte extracts incubated for 90 minutes with nuclei (compare Fig. 5A and B, lanes S 90). Lamin $Dm_{mit\text{-}s}$ is soluble and presumably has the same posttranslational modification(s) which renders lamin Dm_{mit} soluble. Data shown in Fig. 5 suggest that

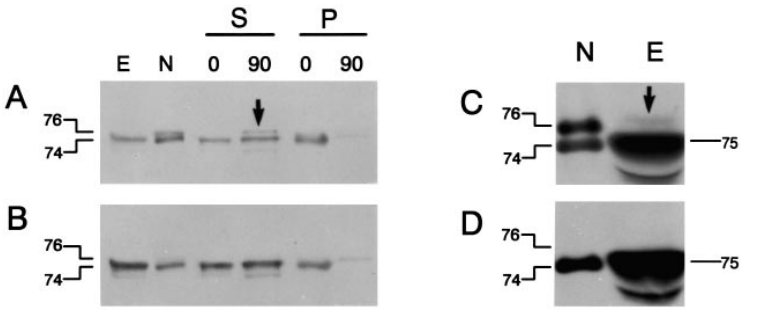

Fig. 5. Lamin Dm_{mit-s} is not recognized by mouse monoclonal antibody ADL84. A and B, 0.3 units of nuclei purified from 0-15 hour-old embryos were incubated with 5 μl of oocyte extract in a final volume of 25 μl. 10 μl samples were fractionated into supernatant (S) and pellet (P) fractions after 0 and 90 minutes of incubation at 23°C. Samples were subjected to SDS-7% PAGE followed by immunoblot analysis. (A) Immunoblot was probed first with affinity-purified rabbit polyclonal anti-lamin antibodies, visualized colorimetrically after incubation with calf alkaline phosphatase-conjugated goat anti-rabbit IgG; (B) The same blot reprobed with mouse monoclonal antibody ADL84 and visualized by chemiluminescence after incubation with horseradish peroxidase conjugated sheep anti-mouse IgG. Lanes E, 2 μl of extract alone; lanes N, 0.12 unit of nuclei alone. (C and D) 1 unit of embryo nuclei (lanes N) and 8 μl of oocyte extract (lanes E) were subjected to SDS-7% PAGE followed by immunoblot analysis. (C) Immunoblot was probed first with affinity-purified rabbit polyclonal anti-lamin antibodies, visualized colorimetrically after incubation with calf alkaline phosphatase-conjugated goat anti-rabbit IgG; D, the same blot reprobed with mouse monoclonal antibody ADL84 and visualized by chemiluminescence after incubation with horseradish peroxidase conjugated sheep anti-mouse IgG. The mobilities of lamins Dm_1 (74 kDa), Dm_2 (76 kDa), and Dm_{mit} (75 kDa) are indicated in each panel. The downpointing arrows in A, lane S 90, and C, lane E, indicate lamin Dm_{mit-s}.

it also contains the modification which distinguishes lamin Dm_2 from lamin Dm_1 and, thus, does not react with ADL84.

A soluble lamin isoform with the SDS-PAGE mobility and immunoreactivity of lamin Dm_{mit-s} was detected in minute quantities in unfractionated stage 14 *Drosophila* oocyte extracts (Fig. 5C and D). Fig. 5C shows an immunoblot of nuclei and oocyte extract probed with polyclonal anti-lamin antibodies. Fig. 5D shows the same blot reprobed with ADL84. Neither nuclear lamin Dm_2 (Fig. 5C, lane N) nor the small amount of lamin Dm_{mit-s} detected in the oocyte extract with polyclonal anti-lamin antibodies (Fig. 5C downpointing arrow) reacts with ADL84 (Fig. 5D).

Two-dimensional gel analysis of *Drosophila* nuclear lamin isoforms generated in vivo and in vitro

Further analysis of *Drosophila* lamin isoforms was performed using two-dimensional gel electrophoresis. After either silver staining (Fig. 6A) or immunoblot analysis (Smith et al., 1987; Havel et al., 1992), interphase lamins Dm_1 and Dm_2 were detected as multiple spots, indicating multiple posttranslationally modified forms. Meiotic lamin Dm_{mit} in the stage 14 oocyte extract (Fig. 6B) exhibited an entirely different pattern. Incubation of extract alone for 90 minutes at 23°C did not alter the pattern of oocyte-derived lamin Dm_{mit} significantly (Fig. 6C). When embryo nuclei were added to the extract and incubated for 90 minutes, a pattern (Fig. 6D) distinct from the interphase lamins and indistinguishable from that of the extract alone was found, indicating that embryo nuclear lamin was converted to forms highly similar, if not identical with meiotic lamin Dm_{mit}.

Nuclear lamina disassembly in extracts of stage 14 *Drosophila* oocytes is blocked by calcium

In other disassembly systems (see e.g. Lohka and Masui, 1984; Lohka and Maller, 1985), addition of calcium blocks events associated with nuclear disassembly and promotes nuclear formation. Accordingly, $CaCl_2$ was added to *Drosophila* oocyte cell-free lamina disassembly reactions. Morphologically, nuclei remained intact in Ca^{2+}-containing extract for 90 minutes (Fig. 7A-C) or longer (not shown). In contrast, parallel incubation of these same nuclei in extract without added Ca^{2+} resulted in complete nuclear lamina breakdown (Fig. 7D-F). Before incubation in stage 14 *Drosophila* oocyte extract, nuclei

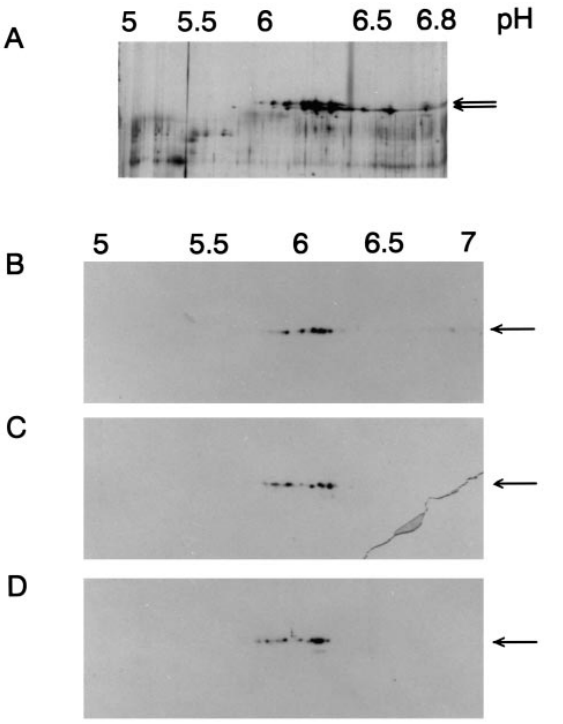

Fig. 6. Two-dimensional gel analysis of *Drosophila* lamin isoforms after cell-free lamina disassembly. Disassembly reactions were as described in the legend to Fig. 1. Lamins were immunoaffinity purified from unfractionated samples and subjected to two-dimensional gel electrophoresis (Materials and Methods). (A) Purified interphase lamins Dm_1 and Dm_2 detected by silver staining of resulting gels. (B-D) Detection was by immunoblot analysis with affinity-purified polyclonal anti-lamin antibodies; (B) lamins from oocyte extract alone, no incubation; (C) lamins from oocyte extract incubated for 90 minutes at 23°C; (D) lamins from oocyte extract with added embryo nuclei, incubated for 90 minutes at 23°C. The pH gradient in the first dimension is indicated at the top of the figure. Arrows indicate the migration position of lamin Dm_{mit} (75 kDa) in the second dimension. Immunoblots were developed to show lamin Dm_{mit}.

appeared similar (Fig. 7G-I) to those observed after 90 minutes in Ca^{2+}-containing extract. The aggregates of DAPI-staining material apparent after lamina disassembly (Fig. 7E) are of

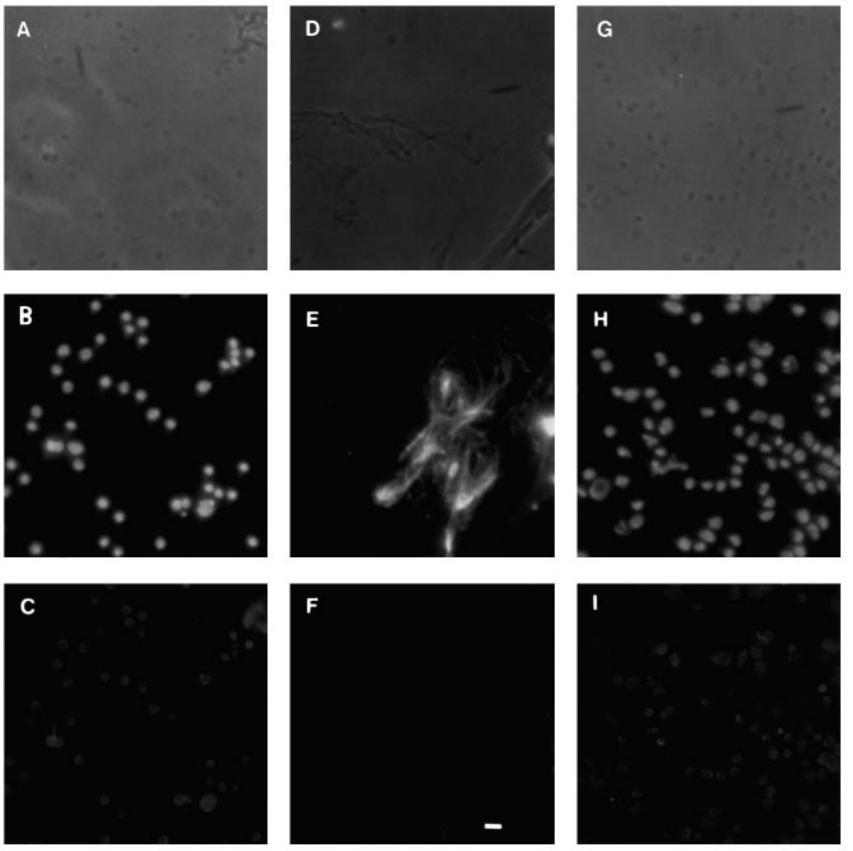

Fig. 7. Lamina disassembly is blocked by $CaCl_2$; morphological analysis. 3 μl of oocyte extract was preincubated for 10 minutes at 23°C in final volume of 20 μl in the presence or absence of 4 mM $CaCl_2$. One unit of embryo nuclei was added to each reaction and samples were incubated for an additional 90 minutes at 23°C. 5 μl samples were processed as described (Materials and Methods). (A,B,C) Reactions in the presence of 4 mM $CaCl_2$. (D,E,F) Reactions without added $CaCl_2$. (G,H,I) 0.5 units of nuclei alone, before incubation in oocyte extract. (A,D,G) Phase contrast microscopy. (B,E,H) DAPI staining. (C,F,I) Immunofluorescence after staining with affinity-purified polyclonal anti-lamin antibodies. Bar in F represents 10 μm and applies to all panels.

unknown significance but may represent decondensed chromatin (see Newport, 1987).

In conjunction with morphological studies, immunoblot analysis of lamina disassembly reactions executed in the absence or presence of Ca^{2+} was performed. In the absence of exogenous Ca^{2+}, interphase lamin isoforms largely disappeared from the pellet fraction (Fig. 8, 0, lane P) and isoforms with the gel mobility of meiotic/mitotic lamins appeared in the supernatant fraction (Fig. 8, 0, lane S). In the presence of 4 mM $CaCl_2$, no such changes were seen; lamins Dm_1 and Dm_2 remained in the pellet fraction (Fig. 8, 4, lane P) and neither lamin Dm_{mit} nor lamin $Dm_{mit\text{-}s}$ accumulated in the supernatant fraction (Fig. 8, 4, lane S).

In vitro disassembly of the *Drosophila* nuclear lamina requires an ATP-regenerating system

In vitro disassembly of the nuclear lamina was examined in the presence and absence of an ATP-regenerating system (Fig. 9). With an ATP-regenerating system, interphase lamins disappeared completely from the pellet fraction after 60 minutes of incubation (compare Fig. 9A, lanes P 0 and P 60). A corresponding increase was seen in the supernatant fractions over the same time course and the appearance of lamin $Dm_{mit\text{-}s}$ was noted (compare Fig. 9A, lanes S 0 and S 60). In the absence of an ATP-regenerating system, there was much less of a decrease in the amount of pelletable interphase lamin isoforms (compare Fig. 9B, lanes P 0 and P 60), nor was there any appreciable increase in the soluble lamin isoforms Dm_{mit} and $Dm_{mit\text{-}s}$

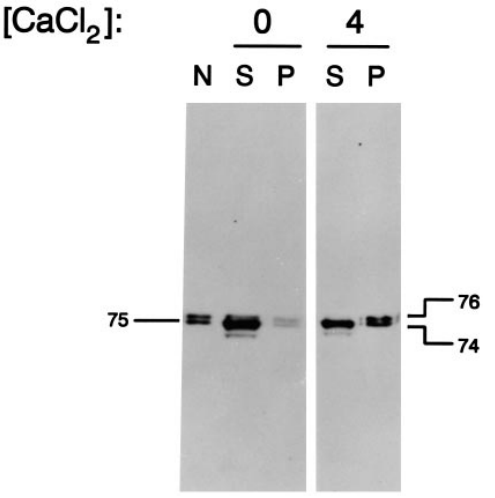

Fig. 8. Lamina disassembly is blocked by $CaCl_2$; immunoblot analysis after minigel SDS-PAGE of lamin isoform interconversion. 1.5 μl of oocyte extract and 0.5 unit of embryo nuclei were prepared in a final volume of 10 μl. $CaCl_2$ was added to one sample to a final concentration of 4 mM. Samples were incubated for 90 minutes at 23°C, separated into supernatant (S) or pellet (P) fractions and subjected to SDS-7% PAGE on minigels and immunoblot analysis with affinity-purified polyclonal anti-lamin antibodies. Lane N, 0.5 unit of embryo nuclei alone. The mobilities of lamins Dm_1 (74 kDa), Dm_2 (76 kDa), and Dm_{mit} (75 kDa) are indicated. The minor immunoreactive band migrating faster than lamin Dm_1 is apparently a proteolytic breakdown product (see e.g. Smith and Fisher, 1989).

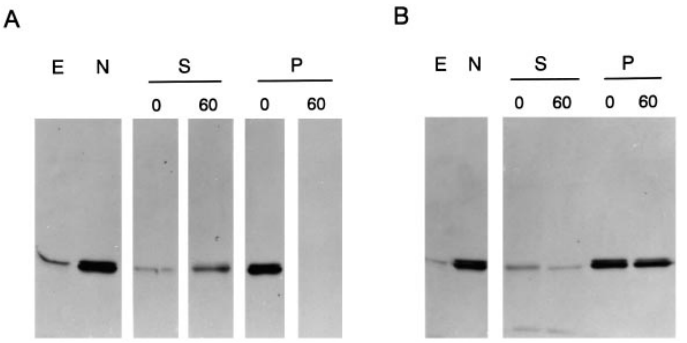

Fig. 9. Lamina disassembly requires an ATP regenerating system. 2 µl of oocyte extract was added to 2 units of embryo nuclei and incubated at 23°C in a final volume of 20 µl. 5 µl samples were removed at 0 and 60 minutes of incubation,. fractionated into supernatants (S) and pellets (P), and subjected to SDS-7% PAGE minigels followed by immunoblot analysis with affinity-purified polyclonal anti-lamin antibodies. (A) Reaction in the presence of an ATP regenerating system (2.7 mM ATP, 13.3 mM phosphocreatine, 50 µg/ml creatine phosphokinase); (B) reaction in the absence of an ATP regenerating system. E, extract alone; N, nuclei alone.

(compare Fig. 9B, lanes S 0 and S 60). In conjunction, no apparent change in nuclear morphology could be detected by light microscopy after incubation in oocyte extract without an ATP-regenerating system (not shown).

DISCUSSION

A cell-free extract of stage 14 *Drosophila* oocytes induced breakdown of both the nuclear lamina assembled in vivo within exogenous nuclei and polymers formed in vitro from immunoaffinity-purified *Drosophila* lamins Dm_1 and Dm_2. This was demonstrated morphologically as well as biochemically. Although the disassembly activity of extracts varied considerably from preparation to preparation, in general, much less stage 14 oocyte extract was required for polymer disassembly. After either in vitro disassembly reaction, interphase lamins Dm_1 and Dm_2 were no longer seen; instead the amount of meiotic/mitotic lamin isoform Dm_{mit} was increased and a novel lamin isoform, $Dm_{mit\text{-}s}$, was readily detected. Lamin $Dm_{mit\text{-}s}$ was detectable in very small quantities in unfractionated stage 14 *Drosophila* oocyte extracts and is presumably one of the possible intermediates in the conversion of lamin Dm_2 to Dm_{mit} in vivo. In vivo, lamin Dm_2 is generated by phosphorylation of lamin Dm_1 (Smith et al., 1987). Thus, there are at least two possible explanations to account for its increased abundance after disassembly in vitro: either the phosphatase required to remove the phosphate group responsible for conversion of lamin Dm_1 to Dm_2 in vivo is relatively inactive (e.g. has a limited capacity) in vitro or, alternatively, the interphase kinase that phosphorylates Dm_1 to convert it to Dm_2 is overly active (thus specifically phosphorylating lamin Dm_{mit} after it is generated to produce lamin $Dm_{mit\text{-}s}$). At present, it is impossible to distinguish between these mechanisms.

It was also noted repeatedly that after in vitro disassembly monitored by immunoblot analysis (e.g. see Fig. 2), less soluble lamin isoforms were recovered than pelletable lamin isoforms were present at the outset. This apparent loss of material was not seen after fluorographic analysis of ^{35}S-labeled tissue culture cell lamins (e.g. see Figs 3 and 4). As a result, it seems likely that apparent losses detected by immunoblot analysis are a technical artifact due perhaps to relatively poor transfer efficiency, relatively poor binding to nitrocellulose or relatively poor immunoreactivity with anti-lamin antibodies of M-phase *Drosophila* lamin isoforms.

In performing these studies, it was noted that the nuclear lamina of *Drosophila* tissue culture cell nuclei disassembled considerably more slowly (less efficiently?) than the lamina of embryo nuclei. This was not a property of the lamins themselves in that polymers formed from embryo lamins Dm_1 and Dm_2 disassembled with similar kinetics to polymers formed from tissue culture cell lamins Dm_1 and Dm_2 (Fig. 4). Presumably, some property of the tissue culture cell nucleus relative to the embryo nucleus renders the former less susceptible to lamina disassembly mediated by the stage 14 oocyte extract. Although similar differences were noted in vertebrates (Newport and Spann, 1987), the basis for such observations remains obscure. By taking advantage of the stage 14 *Drosophila* oocyte disassembly system, it may be possible to elucidate this observation further.

Cell-free disassembly of the *Drosophila* nuclear lamina could be blocked by addition of $CaCl_2$ to stage 14 oocyte extracts. In vivo, it is thought that release of oocytes from meiotic arrest is triggered by an increase in intracellular calcium (Meyerhof and Masui, 1977). Indeed, addition of calcium to vertebrate egg extracts can block disassembly of exogenous nuclei therein and promote nuclear formation in vitro (Lohka and Masui, 1984; Lohka and Maller, 1985). Thus *Drosophila* closely resembles vertebrates with respect to lamina breakdown. It remains to be determined if calcium-containing *Drosophila* extracts can support cell-free nuclear formation.

Cell-free disassembly of the *Drosophila* nuclear lamina was ATP-dependent as well as coincident with lamin phosphorylation (N. Maus, unpublished) and solubilization. Moreover, treatment of lamins with calf alkaline phosphatase obliterated the characteristic changes in lamin SDS-PAGE mobility that accompanied disassembly. These observations in conjunction with previous in vivo results (Smith and Fisher, 1989) suggest that lamina disassembly is catalyzed by protein kinase-dependent phosphorylation. Indeed, on the basis of studies in vertebrates, this notion is widely accepted (see e.g. Gerace and Blobel, 1980; Ottaviano and Gerace, 1985). Unfortunately, attempts to demonstrate a direct role for cdc2 kinase, the mitotic kinase of vertebrates, have thus far proven unsuccessful (N. Maus, unpublished). Nevertheless, the system we developed will facilitate precise dissection of the enzymology of *Drosophila* nuclear lamina disassembly in vitro. In addition, a conspicuous strength of *Drosophila* as a higher eukaryotic experimental system is the ease with which systematic genetic analyses may be performed. The stage 14 oocyte extract described here can be prepared from *Drosophila* lines mutated in specific genes coding for factors believed to be relevant to nuclear disassembly in vivo. Similarly, unknown lines may be screened biochemically to delineate gene products which influence lamina breakdown. In these ways, previously suspected nuclear disassembly factors may be further characterized and new factors may be discovered.

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