

# Biosynthesis and Interconversion of *Drosophila* Nuclear Lamin Isoforms during Normal Growth and in Response to Heat Shock

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**Abstract.** Two major immunocross-reactive polypeptides of the *Drosophila* nuclear envelope, distinguishable in interphase cells on the basis of one-dimensional SDS-PAGE mobility, have been localized to the nuclear lamina by immunoelectron microscopy. These have been designated lamins Dm<sub>1</sub> and Dm<sub>2</sub>. Both lamins are apparently derived posttranslationally from a single, primary translation product, lamin Dm<sub>0</sub>. A pathway has been established whereby lamin Dm<sub>0</sub> is processed almost immediately upon synthesis in the cytoplasm to lamin Dm<sub>1</sub>. Processing occurs posttranslationally, is apparently proteolytic, and has been

reconstituted from cell-free extracts in vitro. Processing in vitro is ATP dependent. Once assembled into the nuclear envelope, a portion of lamin Dm<sub>1</sub> is converted into lamin Dm<sub>2</sub> by differential phosphorylation. Throughout most stages of development and in Schneider 2 tissue culture cells, both lamin isoforms are present in approximately equal abundance. However, during heat shock, lamin Dm<sub>2</sub> is converted nearly quantitatively into lamin Dm<sub>1</sub>. Implications for understanding the regulation of nuclear lamina plasticity through normal growth and in response to heat shock are discussed.

**T**HE nuclear lamina is a proteinaceous network of intermediate filament-like fibrils that lies immediately adjacent to the inner nuclear membrane and surrounds the nuclear contents (Aebi et al., 1986; also, see reviews by Franke et al., 1981; Gerace and Blobel, 1982; Gerace et al., 1984; Gerace, 1986; Franke, 1987). It has been suggested that the lamina serves to anchor nuclear pore complexes (Aaronson and Blobel, 1974, 1975), presumed passageways for nucleocytoplasmic exchange of macromolecules (see, for example, Feldherr et al., 1984), and to provide structural support to maintain the integrity of the interphase nucleus (Gerace et al., 1978; Gerace and Blobel, 1980; Gerace and Blobel, 1982). It has also been proposed that the lamina provides attachment sites for the organization of interphase chromosomes (Gerace et al., 1978; Lebkowski and Laemmli, 1982; Agard and Sedat, 1983; Burke and Gerace, 1986).

In mammalian cells, the nuclear lamina is composed primarily of three proteins, designated lamins A, B, and C (Gerace et al., 1978). Lamins A (70 kD) and C (60 kD) share considerable primary amino acid sequence and secondary structural homology, both with each other and with intermediate filament proteins such as vimentin; in human cells, lamins A and C are apparently encoded by distinct messages transcribed from a common gene (McKeon et al., 1986; D. Fisher et al., 1986). Lamin B (67 kD) is a distinct protein based on peptide map analyses (Gerace and Blobel, 1982; Kaufmann et al., 1983). However, the identification of a monoclonal antibody that recognizes all three mammalian

lamins (Burke et al., 1983) indicates that all may have evolved from a common precursor. In *Xenopus*, four lamins, designated lamins I-IV, have been identified and found to be differentially expressed during development (Benavente et al., 1985; Stick and Hausen, 1985; Benavente and Krohne, 1985). Differential expression of mammalian lamins during embryogenesis in the mouse has also been reported (Schatten et al., 1985).

In vitro translation studies performed in conjunction with in vivo pulse-chase analyses have shown that mammalian lamin A is apparently synthesized as a precursor, lamin A<sub>0</sub>, ~2 kD larger than mature lamin A (Laliberte et al., 1984; Gerace et al., 1984; Ottaviano and Gerace, 1985; Lehner et al., 1986). Lamin A<sub>0</sub> is processed into lamin A after assembly into the nuclear envelope (Ottaviano and Gerace, 1985; Lehner et al., 1986). Lamin A<sub>0</sub> is not present at significant steady-state levels in the nucleus but is only detectable in the context of in vivo pulse-chase or in vitro translation experiments. Most recently, a short-lived ( $t_{1/2} = 3$  min) precursor of lamin B has also been identified in avian cells (Lehner et al., 1986).

During interphase, the mammalian lamins are localized exclusively to the nuclear envelope but are redistributed throughout the cell during mitosis. Mitotic dissolution of the lamina correlates with hyperphosphorylation of all three lamins (Gerace and Blobel, 1980). All three lamins also exhibit a basal level of interphase phosphorylation (Ottaviano and Gerace, 1985).

Initial SDS-polyacrylamide gradient gel analyses of nuclear lamina-enriched fractions prepared from *Drosophila melanogaster* embryos demonstrated the presence of a single major protein band of nearly identical SDS-PAGE mobility to lamin A from rat liver (Fisher et al., 1982). Subsequent immunoblot analyses on high-resolution continuous concentration gels indicated that there were in fact two immunologically homologous species of apparently 74 and 76 kD, respectively, that had not been well resolved on the gradient gels. Indirect immunofluorescence analyses of *Drosophila* larval cryosections indicated that these two polypeptides were confined to the nuclear envelope during interphase (Smith and Fisher, 1984). During mitosis, these polypeptides were apparently redistributed throughout the cell (Berrios et al., 1985). McKeon et al. (1983) have reported the identification of two *Drosophila* polypeptides of similar molecular mass that are recognized by a human autoimmune serum with demonstrable specificity for the mammalian lamins. On the basis of considerable circumstantial evidence therefore, we suggested that these two *Drosophila* polypeptides be tentatively designated lamins (Smith and Fisher, 1984). We have recently noted that these putative *Drosophila* lamins cross-react weakly with all three mammalian lamins on immunoblots and that anti-*Drosophila* lamin antiserum stains the nuclear envelopes of *Xenopus* liver cells as well as oocytes (Gara, L., and P. Fisher, unpublished observations). Others have also reported the identification of *Drosophila* nuclear envelope polypeptides of similar molecular mass based on immunofluorescent staining with monoclonal antibodies (Risau et al., 1981; Fuchs et al., 1983). These monoclonal antibodies have been shown to recognize the putative *Drosophila* lamins (Smith and Fisher, 1984).

In identifying these two major nuclear envelope polypeptides in *Drosophila* embryos, it was originally noted that the relative abundance of these two species varied during embryogenesis and in response to heat shock (Smith and Fisher, 1984). Early in embryogenesis, only the 74-kD polypeptide was identified. In later embryos, both forms were present in approximately equal amounts. During heat shock of these older embryos however, the 76-kD form was apparently processed quantitatively into the 74-kD form.

In the present article, we report the further characterization of these nuclear envelope polypeptides. We have used immunoelectron microscopy to confirm the localization of these two species specifically to the nuclear lamina. In vitro translation studies as well as in vivo pulse-chase experiments were initiated in order to elucidate further the biosynthesis and posttranslational processing of the *Drosophila* lamins. A detailed pathway for the biosynthesis and posttranslational modification of the *Drosophila* lamins during cellular interphase is proposed. Implications for regulation of nuclear lamina structure during normal growth and in response to heat shock are discussed.

## Materials and Methods

### Materials

Triton X-100, rabbit reticulocyte lysate, [<sup>35</sup>S]methionine, and [<sup>32</sup>P]orthophosphate were from New England Nuclear (Boston, MA). DNase I, phenylmethylsulfonyl fluoride (PMSF), *N*-ethyl maleimide, *L*-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), DL-dithiothreitol (DTT), 2-mercaptoethanol, polyoxyethylene sorbitan monolaureate (Tween 20),

octylphenoxy polyethoxyethanol (Nonidet P-40), *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (Hepes), 2-(*N*-cyclohexylamino)ethane sulfonic acid (Ches), 1,4-piperazinediethane-sulfonic acid (Pipes), cyanogen bromide (CNBr),<sup>1</sup> hexokinase, trypsin, and chymotrypsin were from Sigma Chemical Co. (St. Louis, MO). Calf alkaline phosphatase that was used for coupling to IgG fractions for colorimetric immunodetection of antibodies on immunoblots was also from Sigma Chemical Co. (type VII S). Calf alkaline phosphatase ("nuclease-free" grade) used for dephosphorylation experiments with highly purified lamins was from Boehringer Mannheim Biochemicals (Indianapolis, IN), as were gamma-thiol-adenosine triphosphate ( $\gamma$ -S-ATP) and puromycin. Protein A-Sepharose CL-4B was from Pharmacia Fine Chemicals (Piscataway, NJ). Phage T7 RNA polymerase, 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, and *p*-nitro blue tetrazolium chloride were from United States Biochemical Corp. (Cleveland, OH). RNase A (RAF grade) was from Worthington Biochemical Corp. (Freehold, NJ). Hydroxylapatite Bio-Gel HTP was from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). Fetal bovine serum was from KC Biologicals (Lenexa, KS). Ampholines were from LKB Produkter (Uppsala, Sweden). SDS was from British Drug House (Poole, England). Reagents used for electron microscopy were from Polysciences, Inc. (Warrington, PA). Acrylamide, methylene bisacrylamide, and X-Omat XAR x-ray film were from Eastman Kodak Co. (Rochester, NY). All other chemicals were obtained commercially, were of reagent grade, and were used without further purification.

### Antibodies

Specific affinity-purified IgG fractions were from Cappel Laboratories (Cochranville, PA) except as otherwise indicated. Gold-conjugated (10 nm) goat anti-rabbit IgG was from Janssen Pharmaceutica (Piscataway, NJ). Monoclonal antibodies T40, T50, and U25 directed against the *Drosophila* lamins were the generous gift of Dr. Peter Symmons (Risau et al., 1981). Polyclonal antibodies against the *Drosophila* lamins were prepared (Fisher et al., 1982) and affinity-purified essentially as previously (Smith and Fisher, 1984) but in larger scale, using antigens immobilized by coupling to CNBr-activated Sepharose (March et al., 1974). Specific IgG fractions were eluted from affinity columns using a pH 2.3 elution step only.

### Methods

Much of the methodology has been described previously. *Drosophila melanogaster* (Oregon R, P2 strain) were grown in mass culture according to Allis et al. (1977). SDS-PAGE was according to Laemmli (1970) and proteins were transferred to nitrocellulose passively (Fisher et al., 1982). Blots were probed with antiserum or specific IgG fractions and bands of antibody reactivity were visualized colorimetrically according to Blake et al. (1984) as modified by Smith and Fisher (1984). Calf alkaline phosphatase was glutaraldehyde conjugated to affinity-purified goat anti-IgG antibodies according to Avrameas (1969); colorimetric detection was according to McGadey (1970). Additional experimental details are provided in the figure legends.

### Isolation of *Drosophila* RNA

Washed and dechorionated embryos were suspended in 5 embryo vol of RNA extraction buffer (0.15 M sodium acetate, 50 mM Tris-HCl, pH 9.0, 5 mM EDTA, 1% [wt/vol] SDS) according to Schlieff and Wensink (1981), and Dounce homogenized with five strokes of the tight-fitting pestle. To this embryo homogenate was added 0.5 vol of buffer-saturated phenol preheated to 60°C. The mixture was incubated at 60°C for 5 min with occasional mixing and then cooled to room temperature and 0.5 vol of chloroform was added. This mixture was shaken at room temperature for 5 min, and spun at 10,000 *g* for 15 min at 4°C. The aqueous phase was withdrawn and reextracted as above until no visible material remained at the phase interface. To the aqueous phase was added 0.1 vol 3 M sodium acetate, pH 5.5, and 2.5 vol ethanol and the RNA was precipitated at -20°C overnight. For use in translations in vitro, the ethanol-precipitated RNA was collected by centrifugation at 10,000 *g*, washed with 70% ethanol, dried, resuspended in H<sub>2</sub>O, and stored at -70°C.

1. *Abbreviations used in this paper:* CNBr, cyanogen bromide; IPA, immunoprecipitation buffer A; <sup>7</sup>mGp, 7-methyl-guanosine 5'-monophosphate; MSM, modified Shields' medium;  $\gamma$ -S-ATP, gamma-thiol-adenosine triphosphate.

## ***In Vitro Translation***

Preparation of wheat-germ lysate and *in vitro* translation in this lysate were according to Erikson and Blobel (1983). Translation in rabbit reticulocyte lysate was performed as suggested by the vendor, essentially as originally described by Pelham and Jackson (1976). Routinely, the translation systems were programmed with 800–1,000 µg/ml total *Drosophila* RNA.

Translation in *Drosophila* embryo lysate was done according to Scott et al. (1979) as follows. 6–18-h-old embryos were dechorionated with 50% Cloxox in 0.7% NaCl, 0.1% (vol/vol) Triton X-100. Embryos were then washed extensively with 140 mM NaCl, 10 mM KHPO<sub>4</sub>, pH 7.5, and Dounce homogenized with five strokes of the tight-fitting pestle in an equal volume of 10 mM Hepes, pH 7.5, 5 mM 2-mercaptoethanol. The lysate was then centrifuged at 40,000 g for 20 min at 4°C. The supernatant was collected and aliquots were quick-frozen in liquid nitrogen and stored at –70°C until use. The translation mix was essentially the same as that used in the wheat-germ lysate translation except the final concentration of potassium acetate was 80 mM, magnesium acetate was 0.3 mM, and spermidine (0.6 mM) was substituted for spermine. Inasmuch as we were interested in translation of homologous mRNA in the *Drosophila* lysate, we did not attempt to deplete this lysate of endogenous mRNA by micrococcal nuclease digestion. Translation of the lamins in the *Drosophila* embryo lysate was therefore independent of the addition of exogenous embryo RNA. The reactions were carried out at 25°C for 60 min.

## ***7-Methyl-Guanosine 5'-Monophosphate (<sup>7</sup>mGp)*** ***"Synchronized" Translation in Rabbit Reticulocyte Lysate***

Translation was performed in the usual manner using 800 µg/ml total RNA isolated from 6–18-h-old embryos. After 12 min of incubation, <sup>7</sup>mGp was added to 2 mM to block further protein chain initiation. Total incorporation of [<sup>35</sup>S]methionine plateaued after ~30 min in the presence of <sup>7</sup>mGp; under similar conditions incorporation was linear for at least 60 min in the absence of <sup>7</sup>mGp. Aliquots taken at the various time points as indicated above each lane were SDS denatured and processed for immunoprecipitation, SDS-PAGE, and fluorography as described below.

## ***Posttranslational Processing of Wheat Germ In Vitro Translation Products with Drosophila Embryo Lysate***

*Drosophila* embryo lysate was prepared as described above for *in vitro* translation reactions. Translations in wheat-germ lysate were for 60 min after which, the translation was quenched by the addition of RNase A to a final concentration of 20 µg/ml and incubation continued for 15 min at 37°C. After the RNase treatment, an equal volume of *Drosophila* lysate was added and the reaction mixture was incubated at 25°C.

Hexokinase inhibition of lamin-processing activity was assayed as follows. After translation in the wheat-germ lysate, 10 U of hexokinase in 10 µl of 50 mM NaHPO<sub>4</sub>, pH 7.0, 100 mM glucose was added to 100 µl of translation mixture to deplete the mix of endogenous ATP (Schlossman et al., 1984). Incubation with hexokinase was for 5 min at 25°C, after which 100 µl of the *Drosophila* embryo lysate was added as above. For γ-S-ATP inhibition studies, γ-S-ATP was added to reaction mixtures after completion of translation to a final concentration of 12 mM, and before addition of the *Drosophila* embryo lysate.

## ***Purification of Drosophila Embryo Nuclei***

Procedures for the preparation of nuclear fractions from *Drosophila* embryos were exactly as previously described (Fisher et al., 1982). Volumes refer to the original volume of embryo starting material. There are ~40,000–50,000 individual organisms/ml of packed embryos. One unit is defined as the amount of material derived from 1 µl of embryos, i.e., 40–50 organisms. Washed and dechorionated embryos that had been quick-frozen in liquid nitrogen were thawed directly into 9 vol of extraction buffer (buffer A) containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl<sub>2</sub>, and protease inhibitors *N*-ethyl maleimide, PMSF, and TPCK. Embryos were broken in a Dounce homogenizer and nuclei were purified by low-speed centrifugation (2,000 g for 10 min). The nuclear pellet was resuspended and washed twice in 5 vol of buffer A by similar centrifugation.

## ***SDS-PAGE Purification of Drosophila Embryo Lamins***

Lamins Dm<sub>1</sub> and Dm<sub>2</sub> (see below) were separated from each other by preparative SDS-PAGE and individually recovered by shake-elution from the gel. Lamins derived from ~2,500 U of nuclei were electrophoresed on a preparative (no wells) SDS-7% (wt/vol) polyacrylamide gel. The gel was stained with 0.5% (wt/vol) Coomassie Blue in H<sub>2</sub>O for 30 min, then destained with water until the protein bands were clearly visible. The lamins were excised from the gel and the gel fragments were homogenized with a motor-driven teflon pestle in 5 vol of H<sub>2</sub>O. To this was then added 50 vol of 100 mM NaHPO<sub>4</sub>, pH 6.8, and 0.1% (wt/vol) SDS, and the crushed gel was gently agitated for 24 h. Elution was performed a total of three times in succession and the eluates were pooled and concentrated by adsorption to individual hydroxylapatite columns (~2 ml each) essentially according to Moss and Rosenblum (1972). The lamins were eluted from the column with 0.5 M NaHPO<sub>4</sub>, pH 6.8, 0.1% (wt/vol) SDS.

## ***In Vivo Labeling of Schneider 2 Tissue Culture Cells***

Schneider cells (line 2) were maintained in monolayer culture (Schneider, 1972) and exponentially growing cells were harvested by low-speed centrifugation and resuspended at a final concentration of ~10<sup>7</sup> cells/ml in methionine-free medium. Cells were incubated in methionine-free medium for 15 min after which [<sup>35</sup>S]methionine (1,000 Ci/mmol) was added to 500 µCi/ml, and cells were labeled for the times indicated in the figure legends. Chase conditions were as described in the individual figure legends.

Pulse labeling with [<sup>32</sup>P]orthophosphate was performed similarly. Growing cells were concentrated as above and then preincubated in phosphate-free medium for 15 min. [<sup>32</sup>P]orthophosphate was then added to 600 µCi/ml, and cells were incubated for 20 min. Chase conditions were as described in the figure legend. Labeling to steady state was done for 24 h in low-phosphate medium (10<sup>-4</sup> M phosphate) containing 50 µCi/ml [<sup>32</sup>P]orthophosphate.

## ***Subcellular Fractionation of Radiolabeled Schneider 2 Cells***

Labeled Schneider cells were harvested by low-speed centrifugation, washed with 140 mM NaCl, 10 mM KHPO<sub>4</sub>, pH 7.5 (PBS), and lysed in a Dounce homogenizer (five strokes, tight-fitting pestle) in the standard extraction buffer used for embryo homogenization (buffer A) containing in addition, 2% (vol/vol) Triton X-100. Homogenates were then fractionated into a crude nuclear pellet and postnuclear supernatant by centrifugation at 10,000 g for 5 min. The nuclear pellet was resuspended with an equal volume of extraction buffer as used in the initial homogenization. To both supernatant and nuclear pellet fractions was then added 0.5 vol of 20% (wt/vol) SDS, giving a final concentration of 7% (wt/vol) SDS. Samples were boiled and immunoprecipitated as described below. For generation of total Schneider cell lysates, cells were washed as above in PBS then lysed by Dounce homogenization directly into buffer containing 0.4% (wt/vol) SDS and boiled.

## ***Immunoprecipitation of Lamins***

Immunoprecipitation was performed essentially according to Chang et al. (1979). Protein A-Sepharose was suspended in buffer containing 20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) SDS (immunoprecipitation buffer A, buffer IPA). Either unfractionated anti-lamin antiserum or affinity-purified anti-lamin IgG was then added in order to saturate the protein A-Sepharose. Antibody fractions were allowed to incubate with the protein A-Sepharose for 90 min at 37°C. After incubation, the protein A-Sepharose beads containing bound affinity-purified antibody were washed three times in buffer IPA. SDS-denatured samples to be immunoprecipitated were first reduced by the addition of 10 mM DTT, then alkylated by incubation with 100 mM iodoacetamide for 30 min at room temperature. After reduction and alkylation, samples were made 5:1, Triton X-100 to SDS, by the addition of an appropriate amount of 20% (vol/vol) Triton X-100. Samples were then diluted with buffer IPA to a final concentration of 2% (vol/vol) Triton X-100, 0.4% (wt/vol) SDS. The protein A-Sepharose-antibody complex was added to each sample, and incubations were for 90 min to 2 h at 37°C on a rotator. After this incubation, the Sepharose beads were recovered by centrifugation and washed three times in buffer IPA and once in buffer IPA without detergents. Immunoprecipitates were either resolubilized in SDS-PAGE loading buffer for

immediate gel analysis, or as indicated in the individual figure legends for further biochemical manipulations.

### **CNBr Cleavage of SDS-denatured Proteins**

CNBr digestion of SDS-denatured polypeptides was essentially according to Nikodem and Fresco (1979) and exactly as recently reported (Berrios and Fisher, 1986). Protein samples were denatured by boiling for 2–4 min in 2% (wt/vol) SDS, 20 mM DTT. They were then chilled briefly on ice and protein was precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 10% (wt/vol). The TCA precipitates were resolubilized in 80  $\mu$ l of 88% (wt/wt) formic acid, and 100  $\mu$ l of 20 mg/ml CNBr dissolved in 70% formic acid was added to each sample (final CNBr concentration of 11 mg/ml). Digestions were performed at 23°C for 2 h. CNBr digestions were stopped by the addition of 10 vol of cold water, followed by the addition of TCA to a final concentration of 10% (wt/vol). Protein precipitates were collected by centrifugation, resolubilized, and processed for SDS-PAGE and immunoblot analysis in the standard manner. Proteins that had been purified by SDS-PAGE were found to be extremely resistant to cleavage by CNBr, perhaps owing to oxidation of methionine residues during electrophoresis. Accordingly, when SDS-PAGE-purified proteins were analyzed by CNBr cleavage, we used a final CNBr concentration of 110 mg/ml. This was 10 times the concentration typically used for proteins that had not been subjected to SDS-PAGE. Nevertheless, CNBr cleavage of these gel purified proteins was relatively limited (e.g., compare Fig. 2, A with C).

### **Chymotryptic Digestion of SDS-denatured Proteins**

Chymotryptic mapping of SDS-denatured proteins was performed similarly to CNBr cleavage, also as recently reported (Berrios and Fisher, 1986) and was essentially according to Cleveland et al. (1977). After TCA precipitation, samples were resolubilized at a final protein concentration of  $\sim$ 0.5 mg/ml in 100 mM Tris-HCl, pH 6.8, 0.5% (wt/vol) SDS; boiled, cooled to 37°C, and digested with chymotrypsin at a final concentration of 40  $\mu$ g/ml for 32 min. Incubations were terminated by the addition of an equal volume of 2% (wt/vol) SDS, followed immediately by immersion in a boiling water bath. SDS-PAGE and immunoblot analyses were performed as above.

### **pT7 Cloning and In Vitro Transcription**

Full-length lamin cDNA clones cDNL2800 and cDNL3000, isolated by Gruenbaum, Sedat, and colleagues (detailed characterization to be reported at a later time) were cloned into the pT7 transcription vector, pT7-1 (Tabor and Richardson, 1985). The EcoRI-ended clones were ligated to the EcoRI-digested pT7 vector. Orientation of the clones was determined by restriction analysis. In vitro transcription with purified phage T7 RNA polymerase was according to Chamberlain and Ring (1973). Runoff transcription was performed by linearizing the pT7 clones (pT7cDNL2800, and pT7cDNL3000) with XbaI which cuts just 3' of the insert. A standard reaction (50  $\mu$ l) contained 40 mM Tris-HCl, pH 8.0, 15 mM MgCl<sub>2</sub>, 1  $\mu$ g of linearized template DNA, 1 mM of each nucleoside triphosphate, 5 mM DTT, 0.5 mg/ml BSA, and 12–25 U of T7 RNA polymerase. Reactions were for 30 min at 37°C. The reaction was stopped by phenol/chloroform extraction, and the RNA synthesized was ethanol precipitated at  $-20^{\circ}\text{C}$  overnight. The resulting ethanol pellet was resuspended in H<sub>2</sub>O and stored at  $-70^{\circ}\text{C}$  until use. This RNA was routinely used at 200  $\mu$ g/ml in the wheat-germ in vitro translation system.

### **Transcription and Translation of a Truncated Lamin Polypeptide**

Full-length lamin cDNA clone pT7cDNL2800 was used to generate a truncated lamin polypeptide as follows. There is a unique Bam-HI site present 1,200 bp from the 5' end of both lamin cDNA clones. pT7cDNL2800 was cut with Bam HI and transcribed in vitro as described above to generate a truncated lamin mRNA. The truncated message was then used to program translation in wheat-germ lysate in the usual manner. After 60 min, the translation reaction was divided into two fractions. One fraction was kept on ice for 20 min, while the other fraction was treated with 2 mM puromycin for 20 min at 25°C to liberate the nascent polypeptide chains from the ribosomes (Blobel and Sabatini, 1971). Posttranslational processing of the in vitro translation products by *Drosophila* embryo lysate was as detailed above for both the puromycin-treated and untreated samples. Immunoprecipitation, SDS-PAGE, and fluorography were also as above. Efficient pro-

cessing was only seen in the puromycin-treated sample and only those results are shown.

### **Calf Alkaline Phosphatase Digestion of the *Drosophila* Lamins**

Calf alkaline phosphatase digestion experiments were performed either individually with lamins Dm<sub>1</sub> and Dm<sub>2</sub> SDS-PAGE purified from *Drosophila* embryos, or with a mixture of lamins Dm<sub>1</sub> and Dm<sub>2</sub> immunoprecipitated from either a <sup>32</sup>P-labeled Schneider cell lysate or from an unlabeled embryo lysate. Embryo lamins, gel purified and concentrated on an SDS-hydroxylapatite column as described above, were precipitated by the addition of 0.1 vol of 100% (wt/vol) TCA. The TCA precipitate was resolubilized in 200  $\mu$ l of 50 mM glycine, pH 9.6, 0.2% (wt/vol) SDS. Immunoprecipitated samples were recovered by solubilizing the antigen-antibody complex from the protein A-Sepharose beads directly into this same buffer. To each sample was then added an equal volume of 50 mM glycine, pH 9.6, 1% (vol/vol) Triton X-100, 10 mM MgCl<sub>2</sub>, and calf alkaline phosphatase to a final concentration of 500 U/ml. Samples were incubated at 37°C for the times indicated in the figure legends and the reaction was stopped by the addition of 0.1 vol of 20% (wt/vol) SDS. Samples were boiled and processed for SDS-PAGE in the standard manner.

### **Two-dimensional Gel Electrophoresis**

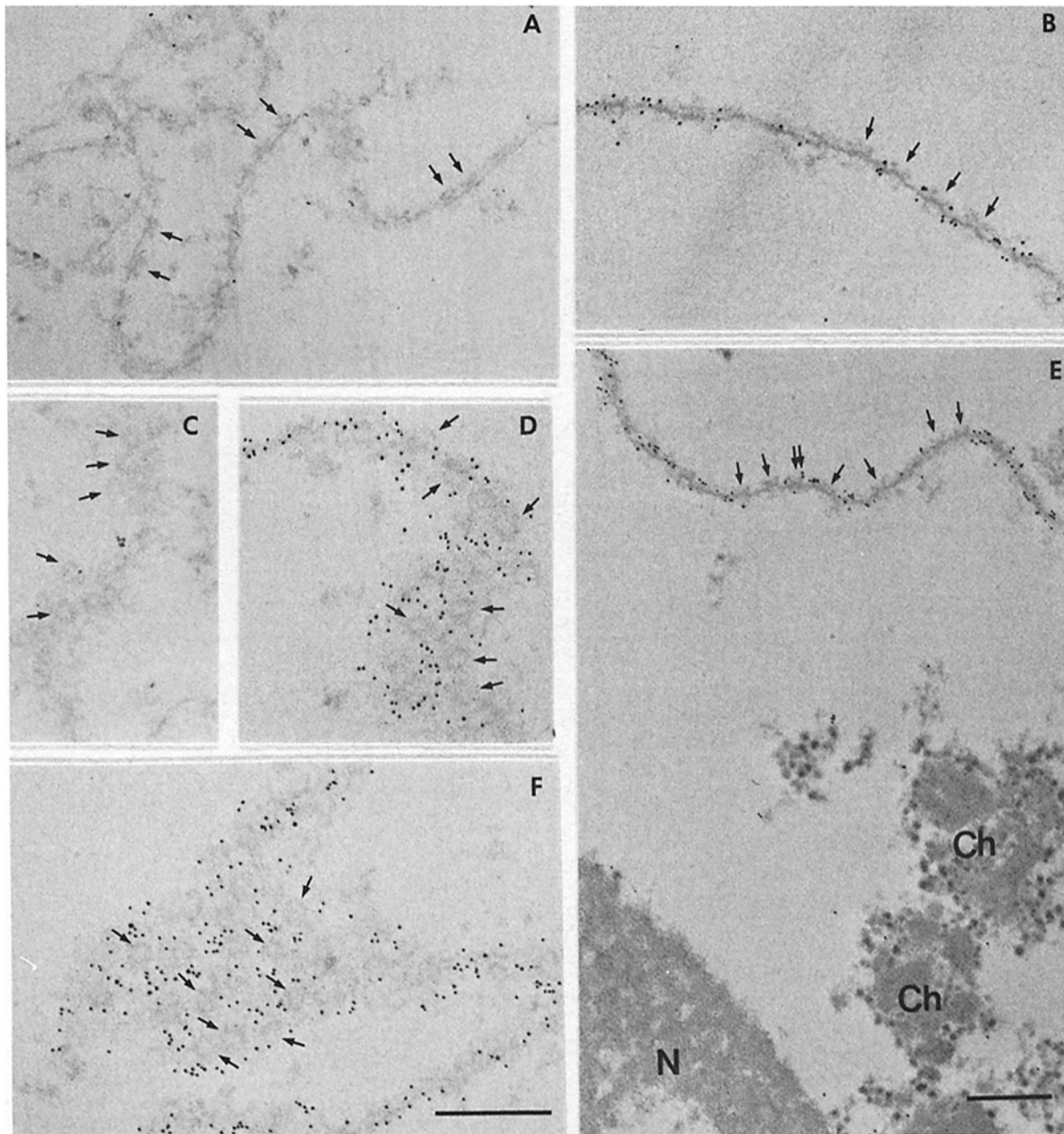
Isoelectric focusing gel electrophoresis was done essentially according to O'Farrell (1975) as follows. Immunoprecipitates were solubilized by boiling in isoelectric focusing sample buffer (50 mM Na-Ches, pH 9.5, 2% (wt/vol) SDS, 65 mM DTT, 10% (vol/vol) glycerol). The sample was then loaded at the basic end of a 5% (wt/vol) polyacrylamide tube gel containing 3% (vol/vol) each of pH 5–7 and pH 6–8 ampholytes. After focusing, the tube gels were equilibrated in 10% (vol/vol) glycerol, 62.5 mM Tris-HCl, pH 6.8, 5% (vol/vol) 2-mercaptoethanol, and 2.3% (wt/vol) SDS for 30 min and loaded onto a standard SDS-polyacrylamide slab gel. After electrophoresis gels were processed routinely for subsequent analyses.

### **Phosphoamino Acid Analysis**

Phosphoamino acid analyses were performed essentially as described (Brugge and Darrow, 1982; Golden et al., 1986). Schneider cells were metabolically labeled with 50  $\mu$ Ci/ml [<sup>32</sup>P]orthophosphate in low phosphate medium (10<sup>-4</sup> M) for 24 h. Cells were lysed directly into buffer containing SDS and immunoprecipitated with anti-lamin antibodies. Immunoprecipitates were electrophoresed on an SDS-7% (wt/vol) polyacrylamide gel. Lamin bands were located by autoradiography of the dried gel. The bands were excised from the gel and rehydrated, and phosphoamino acids were eluted from the gel fragments after digestion with 50  $\mu$ g of trypsin in 1 ml of 50 mM ammonium carbonate, pH 8.5. Samples were lyophilized and then hydrolyzed with 6 N HCl for 2 h at 110°C. After hydrolysis, samples were again lyophilized and electrophoresed on Whatman grade 3 MM chromatographic paper (Whatman, Inc., Clifton, NJ) for 1.75 h at 4,000 V. Electrophoresis buffer was pyridine/acetic acid/H<sub>2</sub>O (1:10:89), pH 3.5. After electrophoresis, chromatograms were air-dried, stained with ninhydrin to visualize the phosphoamino acid standards, and autoradiographed to detect <sup>32</sup>P label.

### **Immunoelectron Microscopy**

Immunoelectron microscopic analyses were performed with nuclei extruded from *Drosophila melanogaster* third-instar larval salivary glands as recently described (Berrios and Fisher, 1986). Third-instar larval salivary glands were dissected under a stereo microscope and nuclei were extruded into modified Shields' medium (Shields et al., 1975) buffered with Pipes (MSM-Pipes) and containing 18 mM MgSO<sub>4</sub>, 5.0 mM CaCl<sub>2</sub>, 40 mM KCl, 24 mM NaCl, 5 mM Pipes, pH 6.8, 0.5% (vol/vol) Triton X-100, and 0.5% (vol/vol) Nonidet P-40. To obtain fully extruded nuclei, the glands were squashed between a clean microscope slide and a siliconized coverslip, and gentle pressure was applied. After squashing, the coverslip was removed. Extruded nuclei were initially fixed for 30 min in a freshly prepared solution containing 2.7% (vol/vol) paraformaldehyde, 0.1% (vol/vol) glutaraldehyde in MSM-Pipes. Samples were washed free of fixative and incubated at room temperature for 6–12 h with the primary antibody diluted in MSM-Pipes. Samples were washed with four changes of MSM-Pipes for a total time of  $\sim$ 1 h and then incubated 6–12 h with 10-nm gold-conjugated affinity-purified



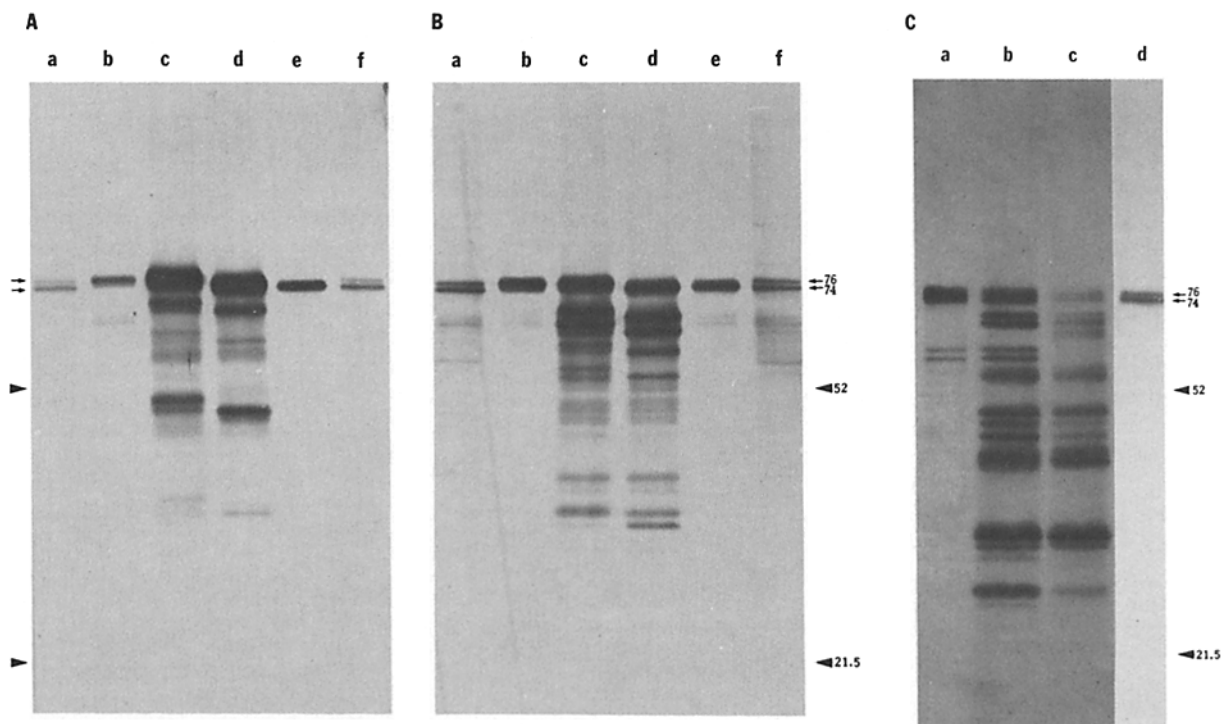
**Figure 1.** Immunoelectron microscopic localization of the *Drosophila* lamins. (A and C) Samples were probed with preimmune serum diluted 1:100 in MSM-Pipes (Materials and Methods). (B, D, E, and F) Samples were probed with anti-lamin antiserum diluted 1:100. Identical results to those shown in B, D, E, and F were obtained with affinity-purified anti-lamin IgG fractions (not shown). (A, B, and E) Sections cut perpendicularly to the plane of the nuclear envelope; (C, D, and F) tangential views. Downpointing arrows in the various panels designate selected pore complexes. Apparent labeling of pore complexes is observed only rarely; an example of such is indicated by the double arrow in E. We do not feel that this labeling is significant. Also indicated in E are chromosomes (Ch) and the nucleolus (N). (A, B, C, and E) Magnification is 25,000; bar in E represents 500 nm and applies to all of these panels. (D and F) Magnification is 36,400; bar in F represents 500 nm and applies to both.

goat anti-rabbit IgG diluted 1:2 in MSM-Pipes. This was followed by four brief washes in MSM-Pipes. Samples were then fixed for electron microscopy in 2.5% (vol/vol) paraformaldehyde, 3.1% (vol/vol) glutaraldehyde in MSM-Pipes, postfixed for 15 min in 1% (wt/vol) OsO<sub>4</sub>, washed in MSM-Pipes, dehydrated in ethanol and propylene oxide, and embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969). Sections ~60 nm thick were stained with uranyl acetate and Reynolds' lead citrate and examined with a JEOL 100B transmission electron microscope at 80 kV (JEOL USA, Peabody, MA).

## Results

### **Antibodies Directed against the Major 74- and 76-kD Nuclear Envelope Polypeptides Bind Specifically to the *Drosophila* Nuclear Lamina**

The preparation of highly specific antisera and affinity-purified IgG fractions directed against the major 74- and 76-



**Figure 2.** One-dimensional SDS-PAGE peptide map comparisons of lamins Dm<sub>1</sub> and Dm<sub>2</sub>. SDS-7–15% polyacrylamide gradient gel electrophoresis and immunoblot analysis were performed as described (Materials and Methods). Blots were probed with affinity-purified anti-lamin IgG at a dilution of 1:1,000 relative to the specific antibody concentration of the unfractionated antiserum. (A) CNBr digestion of gel-purified lamins; (lanes *a* and *f*) undigested nuclei (5 U); (lane *b*) 100 ng of undigested lamin Dm<sub>2</sub>; (lane *c*) 5 μg of lamin Dm<sub>2</sub> digested with 110 mg/ml CNBr for 2 h; (lane *d*) 5 μg of lamin Dm<sub>1</sub> digested similarly; (lane *e*) 100 ng of undigested lamin Dm<sub>1</sub>. (B) Chymotryptic digestion of gel-purified lamins; conditions were exactly as described in Materials and Methods; lanes were loaded as in A. (Lanes *c* and *d*) Digestion of lamins Dm<sub>2</sub> and Dm<sub>1</sub>, respectively. (C) CNBr peptide map comparison of *Drosophila* lamins identified in embryos with those obtained from Schneider 2 tissue culture cells. CNBr digestion and immunoblot analysis of lamins from 6–18-h-old embryos (lanes *a* and *b*) and Schneider cells (lanes *c* and *d*) were performed essentially as detailed above in A; 200 U of nuclei were digested with 11 mg/ml CNBr for 2 h. (Lane *a*) 5 U of embryo nuclei before CNBr treatment; (lane *b*) 200 U of embryo nuclei after digestion with CNBr; (lane *c*) 200 U of Schneider cell nuclei after digestion with CNBr; (lane *d*) 5 U of Schneider cell nuclei before CNBr treatment. Two major endogenous proteolytic breakdown products of the embryo lamins, commonly encountered in previous analyses, are seen in the sample before CNBr treatment (lane *a*). These fragments are also seen after CNBr digestion (lane *b*). These fragments were not observed in the sample of Schneider cell lamins used for this experiment, either before (lane *d*) or after CNBr treatment (lane *c*); the CNBr map of the Schneider cell lamins is otherwise identical to that obtained for the embryo lamins. SDS-PAGE mobility markers shown are rabbit IgG heavy chain (52 kD) and soybean trypsin inhibitor (21.5 kD).

kD polypeptides identified in nuclear envelope-enriched fractions obtained from *Drosophila melanogaster* embryos has been reported (Fisher et al., 1982; Smith and Fisher, 1984). Monoclonal antibodies with similar specificities have also been identified (Risau et al., 1981) and characterized (Smith and Fisher, 1984). Immunofluorescent localization of these two polypeptide species in *Drosophila* larval cryosections has shown that both are apparently localized exclusively to the nuclear periphery (nuclear envelope) in interphase cells (Smith and Fisher, 1984; Filson et al., 1985); during mitosis, these polypeptides are apparently redistributed throughout the mitotic cell (Berrios et al., 1985). In situ analyses were performed at the electron microscopic level using polyclonal antibodies to probe salivary gland nuclei isolated from *Drosophila* third-instar larvae. Nuclei were prepared, detergent-permeabilized, and probed with specific antibody fractions. They were then fixed, embedded, and sectioned for electron microscopy. When probed with antibody fractions directed against the 74- and 76-kD *Drosophila* nuclear envelope polypeptides, followed by col-

loidal gold-conjugated goat anti-rabbit IgG antibodies, only the nuclear lamina was labeled; nuclear pore complexes were essentially unreactive (Fig. 1). Immunoelectron microscopic analyses were also performed after chemical etching of fixed and embedded sections. This technique preserves the membranous architecture of the nuclear envelope. Results obtained with both affinity purified polyclonal antibodies (Matz, E., and M. Berrios, unpublished data) and with monoclonal antibody T40 (Paddy, M., and J. Sedat, personal communication) demonstrated that labeling was restricted to the nucleoplasmic face of the nuclear envelope as would be expected for the nuclear lamina.

From this point forward, it seems reasonable to refer to these two *Drosophila* nuclear envelope polypeptides as lamins (also see Discussion). Because it is not yet certain whether these polypeptides resemble mammalian lamins A and C or lamin B, we have given them a provisional designation of lamins Dm<sub>1</sub> (74 kD) and Dm<sub>2</sub> (76 kD). The Dm in this nomenclature refers to the organism, *Drosophila melanogaster*.



### One-dimensional SDS-PAGE Peptide Map Comparisons between Lamins Dm<sub>1</sub> and Dm<sub>2</sub>

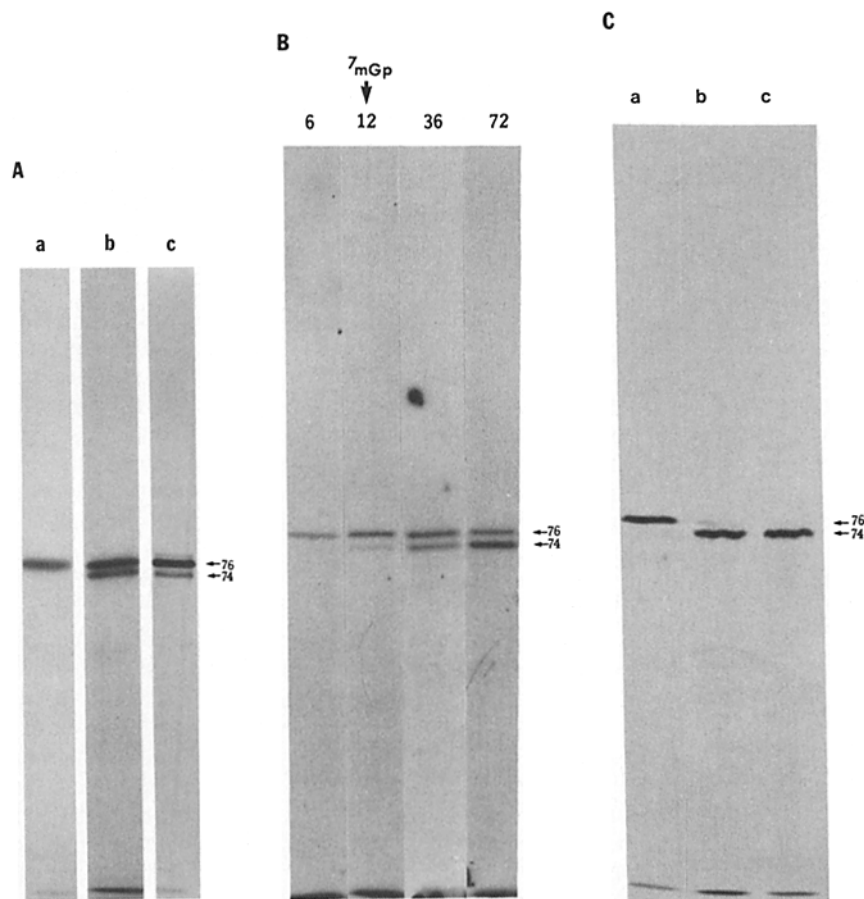
*Drosophila* lamins Dm<sub>1</sub> and Dm<sub>2</sub> are immunochemically indistinguishable (Smith and Fisher, 1984). Both polypeptides cofractionate during preparation of nuclear envelope enriched fractions from *Drosophila* embryos and subsequently copurify through selective urea solubilization, urea-DEAE cellulose chromatography, and SDS-hydroxylapatite chromatography (Filson et al., 1985). We sought to further determine their degree of homology by peptide mapping as shown in Fig. 2. Lamins Dm<sub>1</sub> and Dm<sub>2</sub> were purified by preparative SDS-PAGE and subjected to either CNBr (Fig. 2 A) or chymotryptic digestion (Fig. 2 B). The peptide fragments were visualized by immunoblot analysis. With either digestion procedure, both lamins appeared to have nearly identical maps, except that for a number of the fragments, the 2-kD difference between lamins Dm<sub>1</sub> and Dm<sub>2</sub> seems to have been preserved. It should be noted that these maps are generated by partial digestion such that a single structural difference in two otherwise identical proteins can be represented in a large number of different fragments. Apparently, these peptide fragments contain the moiety which results in the 2-kD dif-

ference in the mature, uncut species. The CNBr maps for the lamins from Schneider cells were identical to the maps of the embryo lamins (Fig. 2 C).

### Biosynthesis of *Drosophila* Nuclear Lamins In Vitro

Preliminary results of in vivo pulse-chase analyses performed in *Drosophila* Schneider 2 tissue culture cells demonstrated that lamin Dm<sub>1</sub> (apparent mass of 74 kD) was synthesized in the cytoplasm and assembled into the nuclear envelope as such (not shown, but see Figs. 5 and 10 below). Once in the nucleus, lamin Dm<sub>1</sub> was processed to lamin Dm<sub>2</sub> (apparent mass of 76 kD) posttranslationally. We attempted to corroborate these observations by performing in vitro translation studies.

Contrary to our expectations based on preliminary in vivo analyses, in vitro translation of *Drosophila* embryo mRNA in a wheat germ cell-free translation system followed by immunoprecipitation with affinity-purified anti-lamin IgG resulted in the identification of a single polypeptide species with the expected SDS-PAGE mobility of lamin Dm<sub>2</sub>, i.e., 76 kD (Fig. 3 A, lane a). When in vitro translations were performed in either rabbit reticulocyte lysate (Fig. 3 A, lane b)



**Figure 3.** In vitro translation of the *Drosophila* lamins. (A) Unfractionated *Drosophila* RNA (800  $\mu$ g/ml) was used to program translation of the lamin polypeptides in three in vitro systems; (lane a) wheat-germ lysate; (lane b) rabbit reticulocyte lysate; and (lane c) *Drosophila* embryo lysate. Each translation reaction contained [<sup>35</sup>S]methionine (1 mCi/ml, 1,000 Ci/mmol). After translation, lamin polypeptides were immunoprecipitated with anti-lamin IgG and electrophoresed on an SDS-7% polyacrylamide gel. The gel was fluorographed (Materials and Methods) and exposed to Kodak XAR film for 24 h at  $-70^{\circ}$ C. Identical results were obtained with poly(A)<sup>+</sup> mRNA (not shown). (B) Kinetic analysis of in vitro synthesis of *Drosophila* lamins in rabbit reticulocyte lysate. In vitro translation of *Drosophila* total RNA was performed in the reticulocyte lysate system. At 12 min (arrow) after the start of translation, <sup>7</sup>mGp was added to a final concentration of 2 mM and the reaction was continued. Inhibition of initiation by <sup>7</sup>mGp was monitored by measuring total methionine incorporation throughout the course of the reaction. Incorporation was essentially complete after 30 min; in the absence of <sup>7</sup>mGp, incorporation of methionine was linear for at least 60 min (not shown). At the times indicated above each lane, aliquots were taken and lamins were immunoprecipitated with affinity-purified anti-lamin antibodies. Resulting immunoprecipitates were then electro-

phoresed. Gels were fluorographed for 48 h at  $-70^{\circ}$ C. (C) Posttranslational processing of the lamin primary translation product by a cell-free *Drosophila* lysate. Wheat-germ in vitro translation of *Drosophila* RNA was performed under standard conditions. After translation for 60 min, the reaction was quenched with the addition of nonradioactive methionine to a final concentration of 10 mM and digestion with RNase A (10  $\mu$ g/ml) for 15 min at  $37^{\circ}$ C. After RNase digestion, an equal volume of *Drosophila* embryo lysate was added, and the incubation was continued at  $37^{\circ}$ C. (Lane a) 0 time before addition of the *Drosophila* lysate; (lane b) 15 min after addition of *Drosophila* lysate; (lane c) 30 min after addition of *Drosophila* lysate. Lamins were immunoprecipitated from each sample and analyzed by SDS-PAGE and fluorography. Exposure was for 24 h at  $-70^{\circ}$ C.

or *Drosophila* embryo lysate (Fig. 3 A, lane c) polypeptides with the expected gel mobilities of both lamins Dm<sub>1</sub> and Dm<sub>2</sub> were identified.

Two different mechanisms were considered to account for the differences observed among the three different in vitro translation systems. On the one hand, it seemed plausible that both lamin polypeptides were synthesized as primary translation products from separate messages, but that only the message coding for the 76-kD polypeptide was efficiently translated in the wheat-germ lysate. However, both putative mRNA species might be efficiently translated in vitro in either the rabbit reticulocyte or the *Drosophila* embryo lysate.

Alternatively, it seemed possible that the 76-kD polypeptide was the only primary translation product in vitro and that the 74-kD polypeptide was derived by posttranslational modification catalyzed by an activity present in both the rabbit reticulocyte and *Drosophila* embryo lysate, but not in the wheat germ extract. In either mechanism, the exact relationship among lamins Dm<sub>1</sub> and Dm<sub>2</sub> synthesized in vivo, and the 74- and 76-kD polypeptides synthesized in vitro was not immediately obvious.

We first approached the elucidation of this problem by examining the kinetics of biosynthesis of 74- and 76-kD lamins in the rabbit reticulocyte lysate. The in vitro translation was programmed with embryo mRNA in the usual manner but after 12 min, <sup>7</sup>mGp, a specific inhibitor of translation initiation in vitro (Rothman and Lodish, 1976) was added to the mix to "synchronize" the translation reaction. Aliquots of the in vitro translation mix, taken at the time of addition of <sup>7</sup>mGp and at various time points thereafter, were analyzed by immunoprecipitation with specific anti-lamin IgG and SDS-PAGE in the standard manner. Results of these experiments are shown in Fig. 3 B. Early in the course of the reaction, only a 76-kD lamin was identified. However, as the reaction was allowed to proceed, a majority of the 76-kD species was apparently processed posttranslationally into the 74-kD form. Similar results were obtained in a synchronized *Drosophila* embryo cell-free translation system (data not shown), suggesting that in both systems, the 76-kD species was the only primary translation product.

It was still a formal possibility that in the synchronized translation reaction, the 76-kD lamin was synthesized rapidly but then degraded, whereas the 74-kD lamin was also synthesized as a primary translation product, but much less efficiently, i.e., at a much slower rate. We would have to further postulate that once synthesized, the 74-kD lamin was nevertheless stable relative to the 76-kD form, and was therefore the majority species identified after prolonged incubation. In order to exclude this last possibility, we "uncoupled" in vitro translation from the putative posttranslational processing event. Results of this experiment are shown in Fig. 3 C. *Drosophila* embryo mRNA was translated in the wheat-germ lysate such that only the 76-kD lamin was synthesized (Fig. 3 C, lane a). The in vitro translation reaction was first quenched by the addition of excess unlabeled methionine followed by termination with RNase A. An equal volume of cell-free extract prepared from *Drosophila* embryos was then added to the wheat-germ lysate. Incubation at 25°C was continued for 15 (Fig. 3 C, lane b) and 30 min (Fig. 3 C, lane c), at the end of which time, the 76-kD primary translation product was completely processed, posttranslationally, into the 74-kD form. These results suggest therefore that the only

primary in vitro translation product of *Drosophila* nuclear lamin mRNA has an apparent mass of 76 kD.

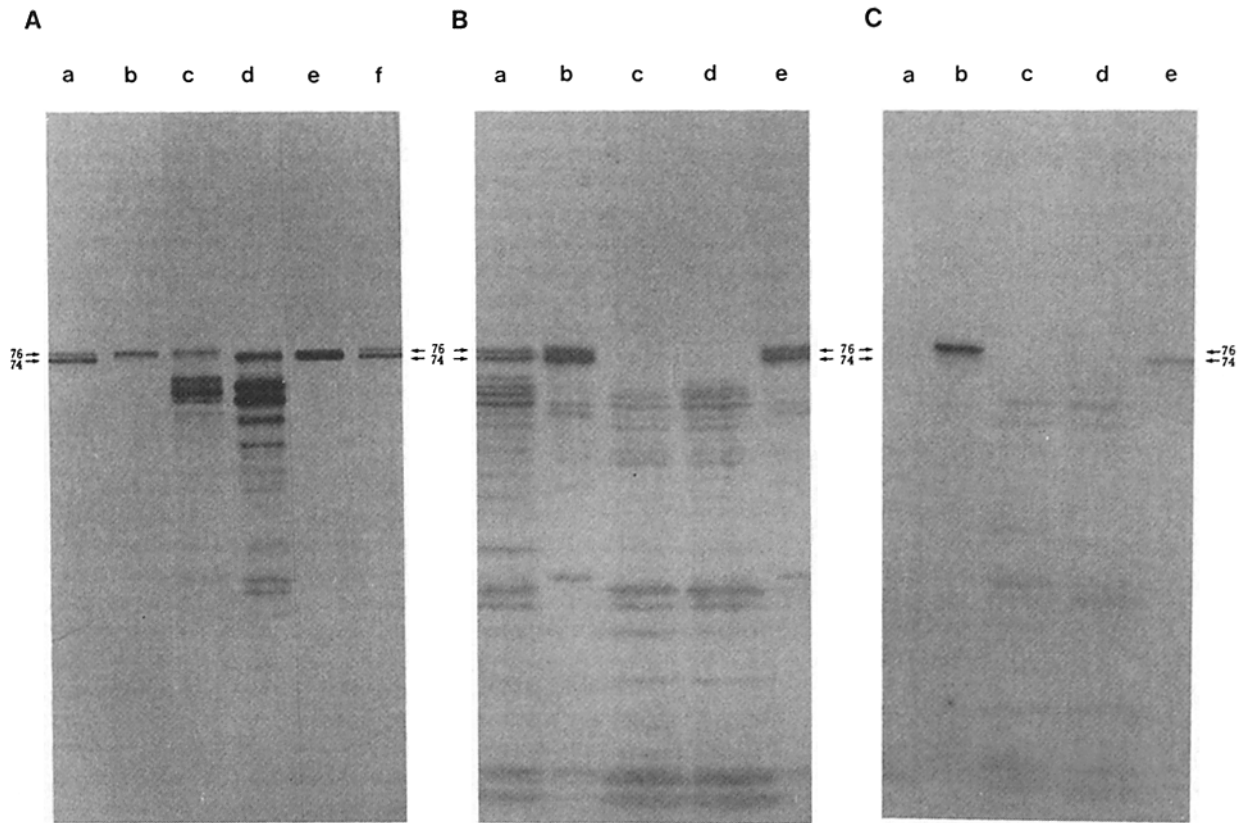
#### **Peptide Map Comparisons of Lamins Dm<sub>1</sub> and Dm<sub>2</sub> Synthesized In Vivo with 76- and 74-kD Lamins Synthesized In Vitro**

It seemed imperative at this point to elucidate the exact relationships among the 74- and 76-kD lamins synthesized in vitro and lamins Dm<sub>1</sub> (74 kD) and Dm<sub>2</sub> (76 kD) identified in vivo. Authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> have highly similar one-dimensional peptide maps after either chymotryptic or CNBr digestion (Fig. 2), thus supporting the notion that both lamin forms were derived posttranslationally from a single polypeptide precursor. However, the two are readily distinguishable upon peptide map analyses due to the fact that the putative posttranslational modification that results in the mobility difference between lamins Dm<sub>1</sub> and Dm<sub>2</sub> is apparently represented in a number of the partial digestion products derived from the uncut lamins. As a result, there is a 2-kD shift in several of the otherwise identical fragments common to both maps (Fig. 2). It was therefore possible to obtain further insights into the relationships between lamin forms synthesized in vitro and authentic polypeptides purified from the organism by peptide map analyses, as follows.

Results of chymotryptic mapping are shown in Fig. 4. Fig. 4 A shows the results obtained when lamins Dm<sub>1</sub> and Dm<sub>2</sub>, SDS-PAGE purified from *Drosophila* embryo nuclei were compared. Detection was by immunoblot analysis. Lanes a and f are control lanes and were loaded with unfractionated nuclei. Lanes b and e were loaded with gel-purified lamins Dm<sub>2</sub> and Dm<sub>1</sub>, respectively, before digestion with chymotrypsin. Lanes c and d show the comparison of lamins Dm<sub>2</sub> (lane c) and Dm<sub>1</sub> (lane d) after incubation with chymotrypsin. It is clear that the shift in SDS-PAGE mobility seen with the uncut lamins is preserved in several of the fragments generated by chymotrypsin. Most notable among them are the two major fragments in each of lanes c and d migrating immediately ahead of the intact lamins.

Comparison of authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> with lamins synthesized and processed posttranslationally in vitro is shown in Fig. 4, B and C. The radiolabeled 76-kD lamin was synthesized in the wheat-germ lysate and an aliquot was processed into the 74-kD form by addition of *Drosophila* embryo extract exactly as described in Fig. 3. Both lamin forms were immunoprecipitated separately and added to a nuclear fraction prepared from *Drosophila* embryos that contained both authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> as standards. An immunoblot of the experiment is shown in Fig. 4 B. An autoradiogram of this blot is shown in Fig. 4 C. Lane a in each panel shows a control fraction to which no radiolabeled in vitro synthesized lamins were added. Lanes b and c show a nuclear fraction to which the 76-kD form synthesized in vitro was added. Lanes d and e show a similar fraction to which the 74-kD polypeptide synthesized in vitro was added. Both lanes b and e represent aliquots taken before digestion with chymotrypsin. Lanes c and d show the results after incubation with chymotrypsin. By immunoblot analysis, the distinctive large fragments differentially derived from the authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> can be distinguished. The digestion has proceeded to a slightly greater extent in lanes c and d than in lane a. There is thus a relative depletion in the



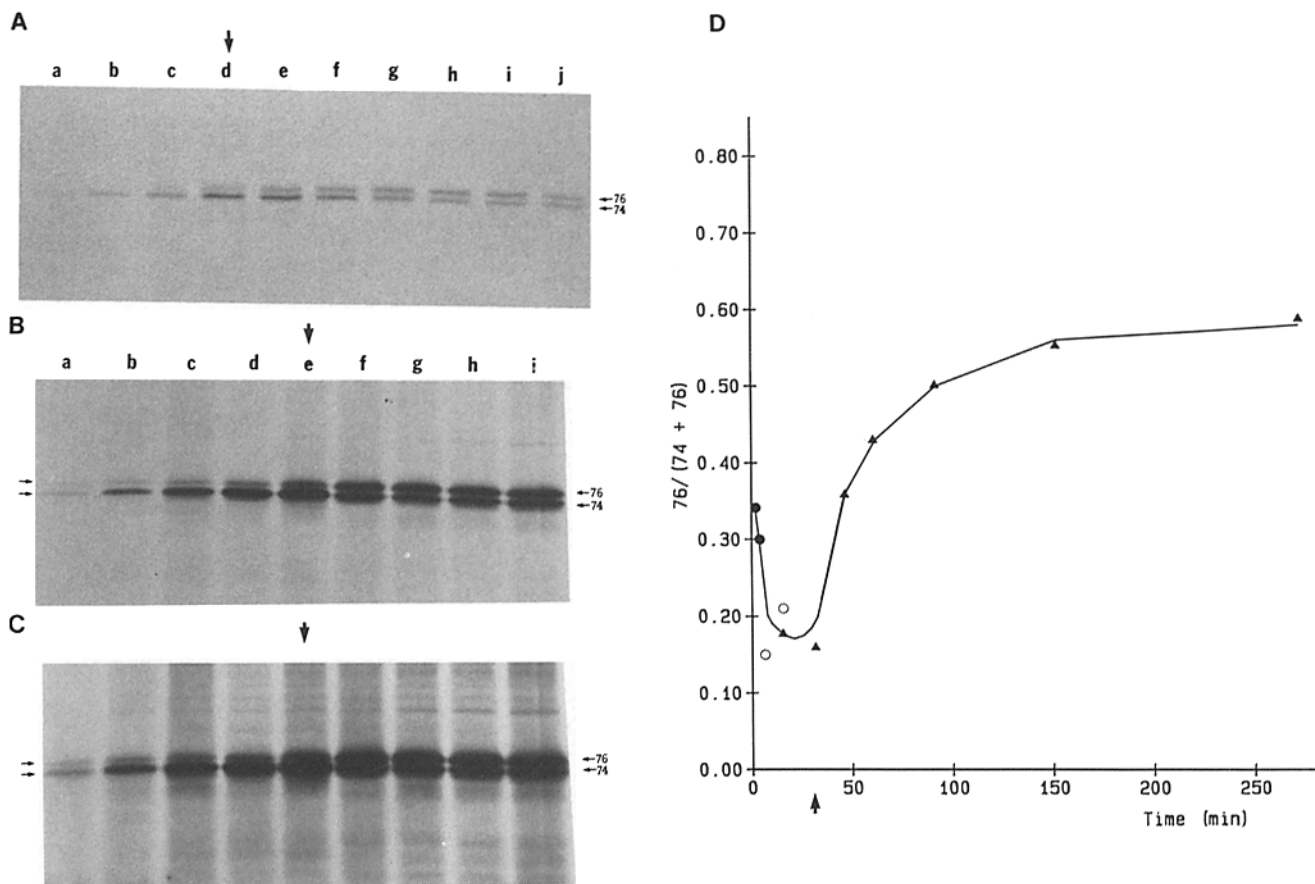


**Figure 4.** Chymotryptic peptide map comparisons of authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> with 76- and 74-kD forms synthesized in vitro. SDS-polyacrylamide gradient gel electrophoresis and immunoblot analysis were performed as described (Materials and Methods). (A) Immunoblot analysis, chymotryptic mapping of authentic lamins purified from *Drosophila* embryo nuclei by SDS-PAGE as above (Fig. 2 B). (Lanes a and f) 5 U of unfractionated nuclei were loaded in each lane; (lane b) ~50 ng of undigested lamin Dm<sub>2</sub> was loaded; (lane c) ~2.5 µg of lamin Dm<sub>2</sub>, digested with chymotrypsin was loaded; (lane d) ~3.5 µg of lamin Dm<sub>1</sub>, digested with chymotrypsin was loaded; (lane e) ~70 ng of undigested lamin Dm<sub>1</sub> was loaded. (B and C) Chymotryptic mapping of 76-kD lamin synthesized in wheat-germ lysate in vitro and 74-kD lamin derived from 76-kD lamin as a result of posttranslational processing by *Drosophila* embryo lysate. (B) Immunoblot analysis; (lane a) 200 U of unfractionated nuclei, digested with chymotrypsin exactly as previously (Berrios and Fisher, 1986), were loaded; (lane b) <sup>35</sup>S-labeled 76-kD lamin was immunoprecipitated from 50 µl of wheat-germ in vitro translation mixture, mixed with 10 U of unfractionated nuclei and loaded directly on the gel; (lane c) <sup>35</sup>S-labeled 76-kD lamin was immunoprecipitated from 950 µl of wheat-germ in vitro translation mixture, mixed with 190 U of unfractionated nuclei and digested with chymotrypsin as in lane a; (lane d) <sup>35</sup>S-labeled 76-kD lamin from 950 µl of wheat-germ in vitro translation mixture was processed to 74-kD lamin by incubation for 30 min with an equal volume of *Drosophila* embryo lysate exactly as detailed in the legend to Fig. 3, immunoprecipitated, mixed with 190 U of unfractionated nuclei, and digested with chymotrypsin as in lane c; (lane e) <sup>35</sup>S-labeled 76-kD lamin from 50 µl of wheat-germ in vitro translation mixture was processed to lamin Dm<sub>1</sub> by incubation for 30 min with an equal volume of *Drosophila* embryo lysate, immunoprecipitated, mixed with 10 U of unfractionated nuclei, and loaded directly on the gel. (C) Fluorogram of the blot shown in B; fluorography was for 35 d at -70°C.

largest fragments and an apparently compensatory increase in some of the smaller ones. This is even more apparent in comparing the digestion patterns shown in Fig. 4 B with those for the gel-purified lamins shown in A. (In general, we have encountered some difficulty in digesting proteins with chymotrypsin after SDS-PAGE and elution from the gel. This problem is even more pronounced with CNBr [compare Fig. 2, A vs. C].) It was immediately apparent that the autoradiogram shown in Fig. 4 C was different from the blot shown in B, particularly in the critical region immediately below the uncut lamins. In contrast with authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> (Fig. 4 A), the 76-kD form synthesized in vitro gave rise to a nearly identical pattern of fragments as the 74-kD form. Moreover, by directly superimposing the

autoradiogram shown in Fig. 4 C on the stained immunoblot (B), we were able to discern that both radiolabeled polypeptides synthesized in vitro (i.e., the 76-kD primary translation product and the 74-kD form derived from it by in vitro processing) generated maps that were nearly coincident with authentic lamin Dm<sub>1</sub>.

The results of chymotryptic mapping shown in Fig. 4 were corroborated by CNBr analyses (not shown). As was seen with chymotrypsin, CNBr maps for both in vitro forms (i.e., the 76-kD form and the 74-kD form) were nearly identical with each other; in the region of the map which we feel is diagnostic (immediately below the uncut lamins), both showed a pattern which was identical with authentic lamin Dm<sub>1</sub>.



**Figure 5.** In vivo kinetic analysis of *Drosophila* lamin biosynthesis. (A) Schneider cells were pulse-labeled with [<sup>35</sup>S]methionine for 32 min as described (Materials and Methods). Arrow above lane *d* indicates the time point at which labeling was terminated. Cells were then washed and resuspended in normal medium and incubated for times as indicated below; (lanes *a*, *b*, *c*, and *d*) 4, 8, 16, and 32 min during the labeling period, respectively; (lanes *e*, *f*, *g*, *h*, *i*, and *j*) 15, 30, 60, 120, 240, and 1,020 min of chase, respectively. At each time point, aliquots of  $\sim 10^6$  cells were lysed in SDS, and lamins were recovered by immunoprecipitation and analyzed by SDS-7% PAGE. Fluorography was for 2 wk at  $-70^\circ\text{C}$ . (B) Schneider cells were pulse-labeled with [<sup>35</sup>S]methionine for 32 min as in A. Arrow above lane *e* indicates the end of the labeling period. Cells were then washed, resuspended in standard medium with nonradioactive methionine, and incubated for the times indicated below; (lanes *a*, *b*, *c*, *d*, and *e*) 2, 4, 8, 16, and 32 min during the labeling period, respectively; (lanes *e*, *f*, *g*, *h*, and *i*) 120, 240, 480, and 1,260 min of chase, respectively. At each time point, aliquots of  $\sim 10^6$  cells were lysed in SDS, and lamins were recovered by immunoprecipitation and analyzed by SDS-7% PAGE. Fluorography was for 3 wk at  $-70^\circ\text{C}$ . (C) Fluorography of the identical gel was for 3 mo at  $-70^\circ\text{C}$ . Only the regions of interest are shown in A, B, and C. (D) Quantitative densitometry of fluorograms shown in A, B, and C. Arrow on the abscissa indicates the end of the labeling period. The amount of 76-kD lamin calculated as a fraction of the total of 76-kD lamin plus 74-kD lamin is plotted on the ordinate as a function of time during pulse-chase analysis of lamin labeling in Schneider cells. (●) Data plotted were obtained from lanes *a* and *b* of C; (○), data plotted were obtained from lanes *c* and *d* of B; (▲), data plotted were obtained from lanes *c*-*i* of A.

### Lamin Dm<sub>2</sub> Is Derived from Lamin Dm<sub>1</sub> Posttranslationally

The similarity in peptide maps between lamins Dm<sub>1</sub> and Dm<sub>2</sub> (Figs. 2 and 4 A) suggested that both might be derived from a single primary translation product as a result of some posttranslational modification(s). Results of in vitro translation experiments suggested that this primary translation product was a 76-kD polypeptide distinguishable from both lamins Dm<sub>1</sub> and Dm<sub>2</sub> by one-dimensional SDS-PAGE and peptide map analysis, respectively. This hypothesis was supported by the results of in vivo pulse-chase experiments performed in Schneider 2 tissue culture cells (Fig. 5). Schneider cells were labeled with [<sup>35</sup>S]methionine for 32 min and then incubated in medium with unlabeled methionine for the times indicated in Fig. 5 A. At each time point the cells were

lysed into boiling SDS and the lamins were specifically immunoprecipitated with affinity purified anti-lamin antibodies. Early in the labeling period, only lamin Dm<sub>1</sub> was detectable (Fig. 5 A, lanes *a* and *b*). With increasing periods of chase, lamin Dm<sub>2</sub> appeared until approximately equal steady-state levels were achieved (Fig. 5 A, lanes *c*-*g*). Lamins Dm<sub>1</sub> and Dm<sub>2</sub> remained in apparent equilibrium from this point forward. There was no evidence of further net conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub>, even after extended chase periods (data not shown).

On the basis of results presented in Figs. 2-5 A, the following mechanism could be proposed to account for essentially all of the observations both in vitro and in vivo. The *Drosophila* nuclear lamin was synthesized as a single primary translation product, henceforth designated lamin Dm<sub>0</sub>, with

an apparent mass of 76 kD. An activity present both in the rabbit reticulocyte lysate and in *Drosophila* embryo extracts, but not in the wheat-germ lysate, was able to process lamin Dm<sub>0</sub> (76 kD) posttranslationally to lamin Dm<sub>1</sub> (74 kD). In vivo, this reaction would have to be postulated to occur so rapidly such that in our initial in vivo pulse-chase analyses (Fig. 5 A), lamin Dm<sub>0</sub> had not been identified because of an extremely short half-life. Lamin Dm<sub>1</sub> therefore appeared as the first form after pulse-labeling with [<sup>35</sup>S]methionine. Once lamin Dm<sub>1</sub> was generated, a portion of it was modified to produce lamin Dm<sub>2</sub>. The similarities in the peptide maps between lamins Dm<sub>0</sub> and Dm<sub>1</sub> reflected the fact that these two forms were more closely related in the biosynthetic pathway than lamins Dm<sub>0</sub> and Dm<sub>2</sub>, despite the coincidence of apparent mass between the latter two species.

#### ***Processing of Lamin Dm<sub>0</sub> to Dm<sub>1</sub> Appears to Take Place Immediately after Synthesis In Vivo***

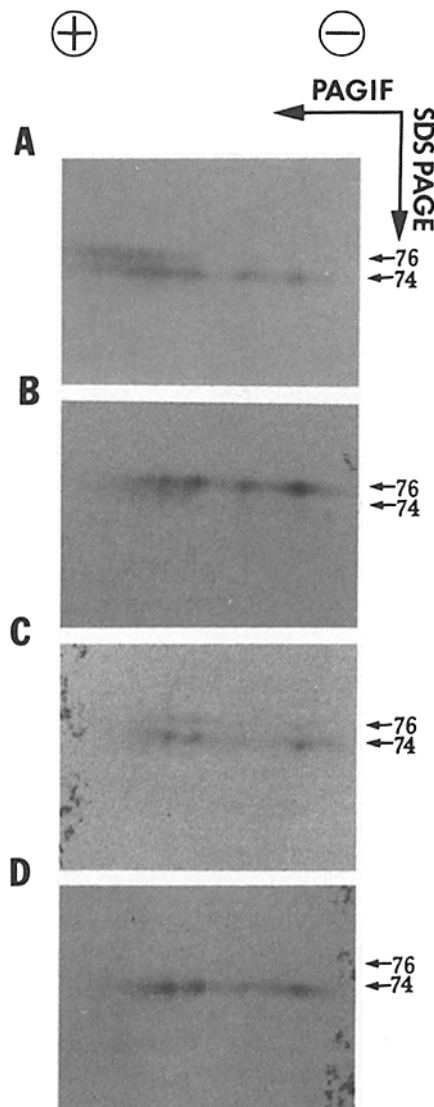
Further evidence for the pathway delineated above was obtained from detailed in vivo pulse-chase analyses similar to that shown in Fig. 5 A, but performed with very short sampling times and prolonged fluorography. These results are shown in Fig. 5, B and C; two autoradiographic exposures of the same gel are shown. At the earliest time point, 2 min (lane a), there is clearly some 76-kD lamin and despite the weakness of the signal, the amount of 76-kD lamin (putatively, lamin Dm<sub>0</sub>) relative to the 74-kD form (Dm<sub>1</sub>) is greater at the earliest time point than at time points immediately subsequent. This is particularly apparent on the longer exposure shown in panel C.

Quantitative densitometric analysis of the data shown in Fig. 5 is presented in Fig. 5 D. We feel that this analysis confirms our impression that there is an extremely short-lived 76-kD form of the lamin present shortly after synthesis in vivo that is kinetically distinguishable from the more readily identifiable 76-kD form, lamin Dm<sub>2</sub> that is formed after ~30–60 min.

On the basis of the pulse-chase kinetics shown in Fig. 5, we are forced to conclude that processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub>, if it occurs as we propose, occurs nearly immediately after synthesis in the cytoplasm. This is in contrast to observations of Ottaviano and Gerace (1985) and Lehner et al. (1986) who reported that processing of a superficially similar mammalian lamin A precursor, lamin A<sub>0</sub>, occurred from 1–2 h posttranslationally, and only after lamin incorporation into the nuclear envelope. It is similar however, to the processing of a putative lamin B precursor described in avian fibroblasts (Lehner et al., 1986). It should be stressed that the processing of lamin Dm<sub>0</sub>, although rapid, is probably posttranslational in a strict mechanistic sense, i.e., as regards potential coupling with protein synthetic events. Processing can be readily accomplished posttranslationally in vitro (Fig. 3) and is catalyzed by a soluble cell-free *Drosophila* extract that is completely free of microsomal membranes (see below).

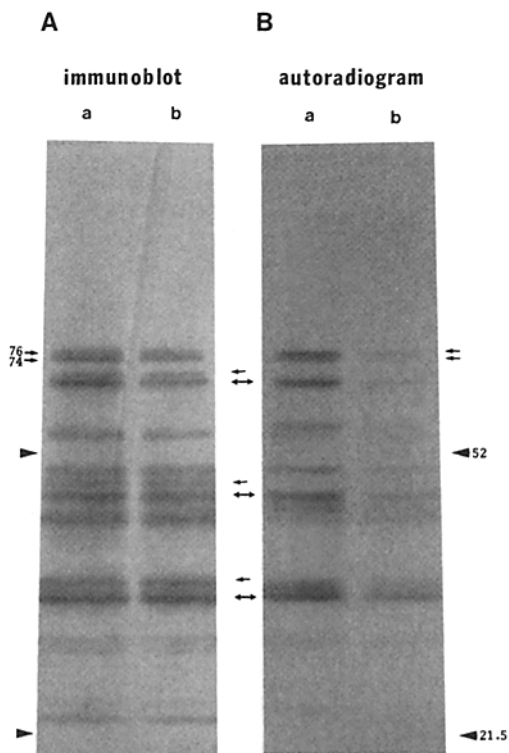
#### ***Processing of Lamin Dm<sub>0</sub> to Lamin Dm<sub>1</sub> Does Not Involve Detectable Charge Modification***

Initial attempts to characterize the nature of the posttranslational modification resulting in the conversion of lamin Dm<sub>0</sub> to Dm<sub>1</sub> focused on direct analyses of NH<sub>2</sub>-terminal se-



**Figure 6.** Two-dimensional gel analysis of lamin forms synthesized in vitro. <sup>35</sup>S-labeled lamins were synthesized in vitro in wheat-germ lysate and processed posttranslationally by *Drosophila* embryo extract exactly as described in the legend to Fig. 3. Authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub>, unlabeled, were immunoprecipitated from an SDS-lysate of *Drosophila* embryo nuclei. Authentic lamins from 10 U of embryo nuclei were mixed with radiolabeled lamins synthesized in vitro. Two-dimensional gel analyses were performed under standard conditions. Gels were blotted to nitrocellulose and resultant immunoblots probed with affinity purified anti-lamin antibodies. Visualization of immunoreactive spots was colorimetric. One such blot is shown in A. For visualization of radiolabeled lamins synthesized in vitro, identical blots were impregnated with 2,5-diphenyloxazole (Fisher et al., 1982) and fluorographed. Fluorography was for 2 wk at -70°C. (B) Wheat-germ in vitro translation product before incubation with *Drosophila* embryo lysate; (C) 10-min processing with embryo lysate, (D) 20-min processing with embryo lysate.

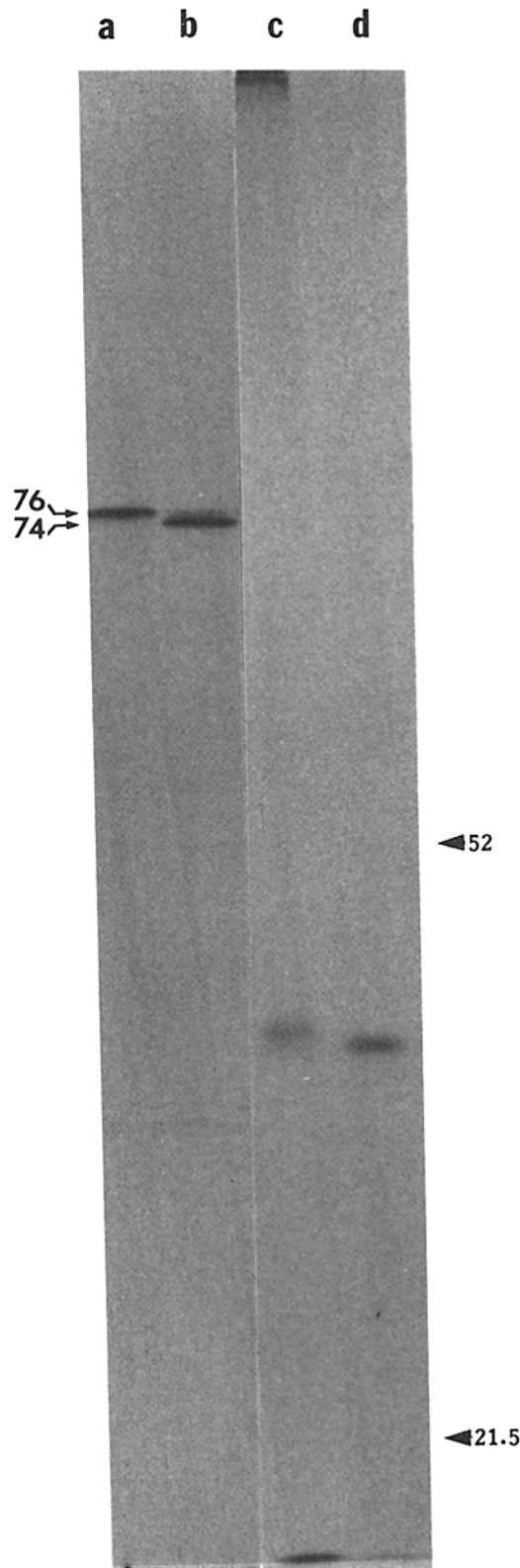
quence in the expectation that the possibility of NH<sub>2</sub>-terminal proteolysis would be most easily evaluated in this way. Unfortunately, repeated attempts to sequence the NH<sub>2</sub>-termini of the intact lamins, either purified unlabeled from *Drosophila* embryos, or by trying to take advantage of radiochemical sequencing techniques after in vitro translation



**Figure 7.** One-dimensional CNBr peptide map comparison of authentic lamins with cDNA clone-encoded polypeptides. RNA transcribed from pT7cDNL2800 and pT7cDNL3000 was used to program a wheat-germ lysate. Labeled lamins were immunoprecipitated to remove wheat-germ proteins and then mixed with 200 U of *Drosophila* embryo nuclei. CNBr digestion was for 2 h at room temperature as described; after digestion samples were recovered by TCA precipitation and electrophoresed on an SDS-7-15% polyacrylamide gradient gel. The gel was blotted to nitrocellulose and the blot probed with affinity-purified anti-lamin IgG to visualize the digestion pattern of the authentic, nonradiolabeled embryo lamins. (A) Immunoblot; detection of immunoreactivity was colorimetric. (B) Autoradiogram of blot shown in A; lane a was loaded with in vitro translation product encoded by pT7cDNL2800; lane b was loaded with in vitro translation product encoded by pT7cDNL3000. Double arrows between the panels indicate the migration positions of fragments derived from authentic lamin Dm<sub>1</sub>; single left-pointing arrows indicate the migration positions of fragments derived from authentic lamin Dm<sub>2</sub>. Molecular mass markers (arrowheads) to the right of B are rabbit IgG heavy chain (52 kD) and soybean trypsin inhibitor (21.5 kD). Fluorography was for 2 wk at -70°C.

were unsuccessful. Sequencing of several internal fragments derived from the authentic lamins by CNBr digestion and purified by HPLC was readily accomplished (Slaughter, C., and P. Fisher, unpublished observation) suggesting that problems with NH<sub>2</sub>-terminal sequencing might likely be due to chemically blocked NH<sub>2</sub>-termini for lamins synthesized in vivo. (Elaborate precautions were taken to prevent artifactual blocking of NH<sub>2</sub>-termini during handling of purified proteins in vitro before attempts at sequencing.) Two different amino acids, methionine and leucine, were used to attempt radiochemical sequencing. Except, for the NH<sub>2</sub>-terminal methionine, neither residue was identified in the first 20 cycles of the sequenator.

In the absence of direct sequence information, we sought



**Figure 8.** In vitro processing of full-length and specifically truncated cDNA clone-encoded lamins. T7 RNA polymerase-directed transcripts of full-length lamin cDNA clone pT7cDNL2800 (lanes a and b) and Bam HI-truncated clone pT7cDNL1200 (lanes c and d) were synthesized in vitro (lanes a and c) and processed by the addition of the embryo lysate (lanes b and d). The truncated transcript from pT7cDNL1200 does not possess a translation stop sequence. To liberate the truncated lamin polypeptide from the ribo-

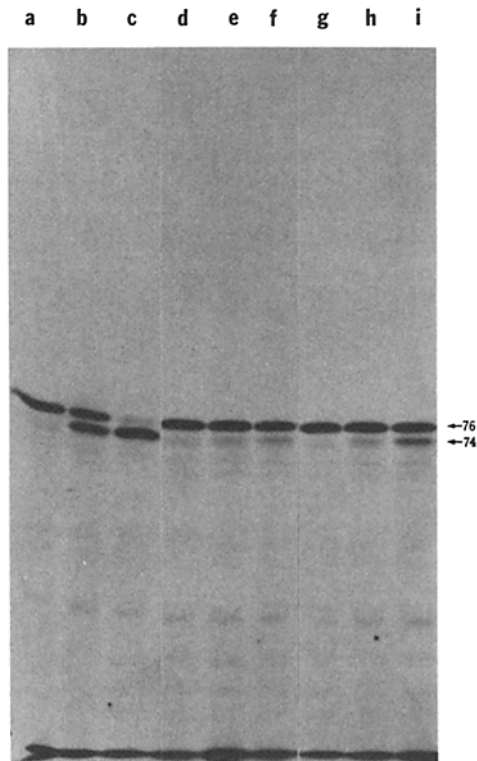
to obtain insights into the nature of the conversion of lamin Dm<sub>0</sub> to lamin Dm<sub>1</sub> by two-dimensional gel analyses. These results are shown in Fig. 6. Authentic lamins Dm<sub>1</sub> and Dm<sub>2</sub> exist as many charge isoforms, due in part to posttranslational phosphorylation (see below). These results are shown as a control in Fig. 6 A. When lamin Dm<sub>0</sub> synthesized in the wheat-germ lysate was analyzed similarly, multiple spots were also identified (Fig. 6 B), but in contrast with lamin Dm<sub>2</sub>, all were in the more basic region of the map. (It is not known whether the apparent heterogeneity of lamin Dm<sub>0</sub> synthesized in vitro reflects posttranslational modification or aggregation during the isoelectric focusing step of two-dimensional gel analysis.) Two-dimensional gel analysis after posttranslational processing of lamin Dm<sub>0</sub> to lamin Dm<sub>1</sub> in vitro showed the expected shift in SDS-PAGE mobility but without any apparent effect on the number or absolute mobility of any of the lamin isoforms; two time points in the digestion are shown (Fig. 6, C and D). At the intermediate time point, ~30% of lamin Dm<sub>0</sub> was not yet processed and provides an internal control for the mobility of lamin Dm<sub>0</sub> forms in this analysis (Fig. 6 C). Complete processing is evident in Fig. 6 D.

### Processing of Lamin Dm<sub>0</sub> to Lamin Dm<sub>1</sub> Occurs on the NH<sub>2</sub>-terminal "Half" of the Protein

We in collaboration with Dr. John Sedat and colleagues have recently succeeded in identification and partial characterization of full-length cDNA clones coding for *Drosophila* lamin Dm<sub>0</sub> (details to be published at a later time). In the present study, we took advantage of the availability of these clones to partially localize the site of lamin Dm<sub>0</sub> processing with respect to the primary structure of the protein.

cDNL2800 and cDNL3000, two different full-length cDNA clones of lamin Dm<sub>0</sub>, were cloned into the pT7 in vitro transcription vector (Tabor and Richardson, 1985) and lamin mRNA was transcribed from the resultant clones (designated pT7cDNL2800 and pT7cDNL3000, respectively) with T7 RNA polymerase. These mRNAs were used to program in vitro translation in wheat-germ lysate and the product was analyzed in several ways. Peptide map comparisons with lamin Dm<sub>0</sub> synthesized from authentic embryo mRNA (not shown) and lamins Dm<sub>1</sub> and Dm<sub>2</sub> purified from the embryo confirmed the identity of cDNL2800 and cDNL3000 as coding for the *Drosophila* lamin (Fig. 7). Addition of *Drosophila* embryo extract, as performed in Fig. 3, demonstrated the in vitro conversion of pT7-encoded lamin Dm<sub>0</sub> to lamin Dm<sub>1</sub> (Fig. 8, lanes a and b). By truncating the pT7cDNL2800 clone at the 3'-end, we were able to synthesize a truncated lamin polypeptide of ~30–35 kD in vitro, containing the

some after translation in the wheat-germ lysate, puromycin was added to 1 mM and incubation was continued at 25°C for 20 min before the addition of the *Drosophila* embryo lysate. Processing by the activity in the embryo lysate was for 15 min. If puromycin pretreatment of the truncated polypeptides was not done before the addition of the embryo lysate, processing was ~50% as efficient as that shown in lane d. Lamin polypeptides were immunoprecipitated with anti-lamin antibodies and run on an SDS-10% polyacrylamide gel. Fluorography was for 2 d at -70°C. Markers to the right of lane d indicate the migration position of IgG heavy chain (52 kD) and soybean trypsin inhibitor (21.5 kD) on comparable gels.

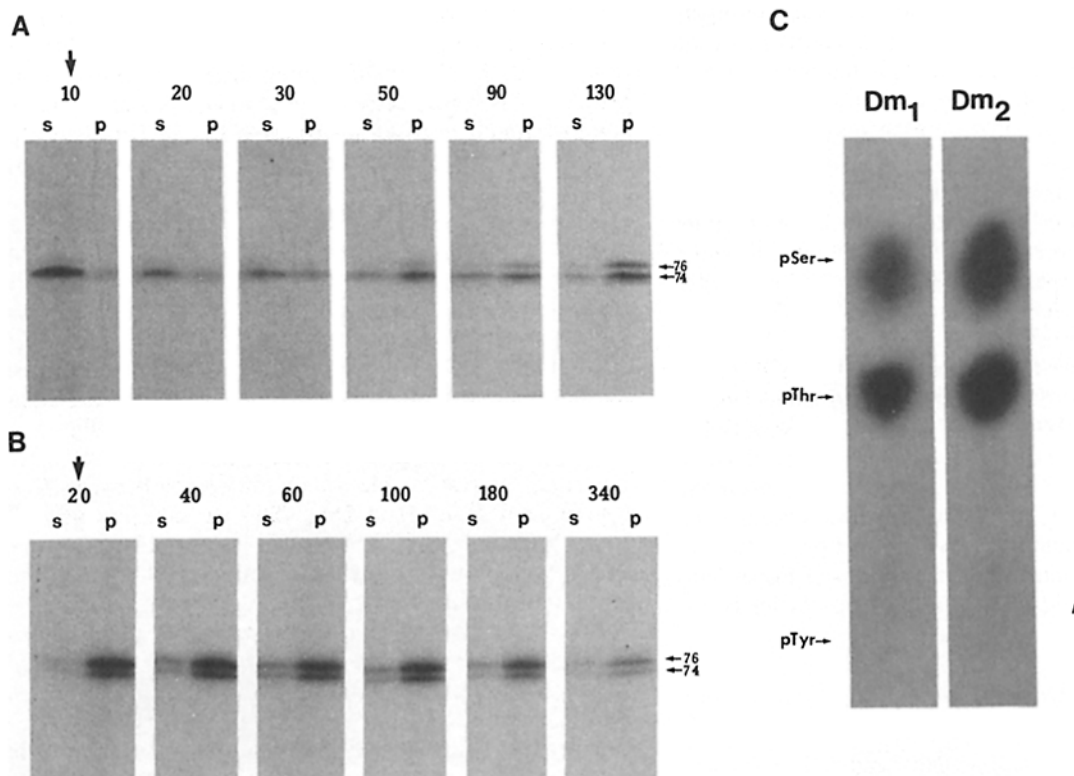


**Figure 9.** Inhibition of in vitro lamin processing reaction by  $\gamma$ -S-ATP and hexokinase-mediated ATP depletion. In vitro translation of *Drosophila* mRNA was performed in wheat-germ lysate. An incubation mixture of 300  $\mu$ l was divided into three aliquots for the posttranslational processing assay with *Drosophila* embryo lysate. An equal volume of *Drosophila* lysate was added to each aliquot. Before addition of the *Drosophila* lysate, however,  $\gamma$ -S-ATP was added to one aliquot (lanes d-f); a second fraction was treated with 10 U of hexokinase plus 10 mM glucose for 10 min at 25°C to deplete the free ATP before the addition of the embryo lysate (lanes g-i). Each assay was incubated with the embryo lysate for 0 min (lanes a, d, and g); 15 min (lanes b, e, and h); and 30 min (lanes c, f, and i). Lamin polypeptides were immunoprecipitated with anti-lamin antibodies and processed as described previously. Fluorography was for 48 h.

proper NH<sub>2</sub>-terminus but lacking the COOH-terminal half of the protein. This NH<sub>2</sub>-terminal fragment was an acceptable substrate for the lamin-processing activity (Fig. 8, lanes c and d). An NH<sub>2</sub>-terminal fragment of ~18 kD, synthesized similarly, was not efficiently processed; however, this analysis was compromised by technical difficulties in the analysis of this small lamin fragment and the negative result should be regarded as preliminary. On the basis of these results, it seems reasonable to conclude that processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub> occurs on the NH<sub>2</sub>-terminal half of the protein.

### Identification and Partial Characterization of an Enzymatic Activity that Catalyzes the Processing of Lamin Dm<sub>0</sub> to Dm<sub>1</sub>

Our ability to apparently reconstitute the processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub> in vitro allowed us to characterize the processing activity enzymologically. Results of some of these analyses are shown in Fig. 9. The positive control for this pro-



**Figure 10.** In vivo kinetic analysis of *Drosophila* lamin assembly into nuclei and subsequent phosphorylation. (A) Schneider cells were pulse-labeled for 10 min with [<sup>35</sup>S]methionine as described (Materials and Methods). Arrow above the pair of lanes designated 10 indicates the time point at which labeling was terminated. Cells were then washed and incubated in normal medium with nonradioactive methionine. At the time points indicated in minutes above each pair of lanes, cells were lysed in buffer containing 2% (vol/vol) Triton X-100 and separated into supernatant (s) and nuclear pellet (p) fractions (Materials and Methods). Lamin polypeptides were immunoprecipitated from each fraction and analyzed by SDS-7% PAGE as described in the legend of Fig. 5. Fluorography was for 3 wk at -70°C. (B) Schneider cells were incubated for 20 min in phosphate-free medium containing 600 μCi/ml [<sup>32</sup>P]orthophosphate. Arrow above the pair of lanes designated 20 indicates the time point at which labeling was terminated. Cells were then washed, resuspended in normal medium, and incubated for the times indicated in minutes above each pair of lanes. Aliquots of cells were lysed and fractionated into supernatant (s) and nuclear pellet (p) fractions exactly as described in A. Immunoprecipitation of lamins and SDS-PAGE were also as above. Autoradiography was for 60 h at room temperature without an intensifier screen. (C) Phosphoamino acid analysis of *Drosophila* lamins. ~10<sup>8</sup> Schneider cells were labeled to steady state (24 h) with 50 μCi/ml [<sup>32</sup>P]orthophosphate in low-phosphate medium. Lamins were immunoprecipitated with anti-lamin IgG. Phosphoamino acid analysis was performed as described (Materials and Methods). Migration positions of phosphoamino acid standards are indicated to the left of the figure: phosphoserine (p-Ser), phosphothreonine (p-Thr), phosphotyrosine (p-Tyr). Arrow indicates direction of paper electrophoresis. Fluorography was for 90 h at -70°C with an intensifier screen.

cessing experiment is shown in lanes a-c. Processing was effectively inhibited by γ-S-ATP (lanes d-f) and was sensitive to ATP depletion by hexokinase (lanes g-i). Processing was also strongly inhibited by N-ethyl maleimide and EDTA and at least partially by chymostatin (data not shown). A number of other agents, among them TPCK, PMSF, antipain, and leupeptin had little or no effect.

Analysis of the lamin-processing activity by velocity sedimentation through a linear glycerol gradient was also performed (not shown). Processing activity was detectable in the top two fractions of the gradient. This corresponded to a sedimentation coefficient of 2-2.5 S based on the position of a cytochrome c standard. Preliminary attempts to purify the lamin-processing activity using ion exchange chromatography indicated that the activity could be chromatographed on DEAE-cellulose with >50% recovery (data not shown). Activity eluted from the column at ~300 mM potassium acetate.

#### **Lamin Dm<sub>1</sub> Is Converted into Lamin Dm<sub>2</sub> by Differential Phosphorylation after Assembly into the Nuclear Envelope**

Having reconstituted and partially characterized the biosynthesis and posttranslational processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub>, we next turned our attention to the apparent conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub>. Cell fractionation experiments were performed in conjunction with pulse-chase analyses to determine first the subcellular localization for this event. Results are shown in Fig. 10. The pulse-labeling and chase conditions were as described for the experiments shown in Fig. 5. At each time point the cells were lysed in buffer containing 2% (vol/vol) Triton X-100 and centrifuged at 10,000 g for 10 min to generate a nuclear pellet and a postnuclear supernatant fraction. Each fraction was then SDS-denatured as described for the whole cell lysates (Fig. 5) and lamins were immunoprecipitated and analyzed by SDS-PAGE. At the end of the 10-min [<sup>35</sup>S]methionine pulse, newly synthe-



sized lamin Dm<sub>1</sub> was found almost exclusively in the supernatant fraction (Fig. 10 A, lane 10 s). With increasing time after addition of unlabeled methionine, lamin Dm<sub>1</sub> was first seen to shift from the postnuclear supernatant into the nuclear pellet fraction; it was only subsequent to this shift that the conversion from lamin Dm<sub>1</sub> to lamin Dm<sub>2</sub> appeared to take place (Fig. 10 A, lanes 20, 30, 50, 90, and 130).

Insight into the biochemical nature of the posttranslational modification leading to conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub>, was obtained from results of the two-dimensional gel analyses shown in Fig. 6 A. It appeared that there were multiple isoelectric forms of both lamins. Although there was considerable overlap in the isoelectric-focusing dimension, lamin Dm<sub>2</sub> was, in general, somewhat more acidic than lamin Dm<sub>1</sub>. Results shown were obtained with lamins purified

from both embryos (Fig. 6 A) and Schneider 2 tissue culture cells (not shown). Consistent with the impression obtained from Fig. 6, the *Drosophila* lamins distributed over a broad pH range when isoelectric focused to equilibrium in liquid glycerol gradients (data not shown). Lamin Dm<sub>1</sub> had a pI between 6.0 and 6.4 while lamin Dm<sub>2</sub> focused between 5.8 and 6.1.

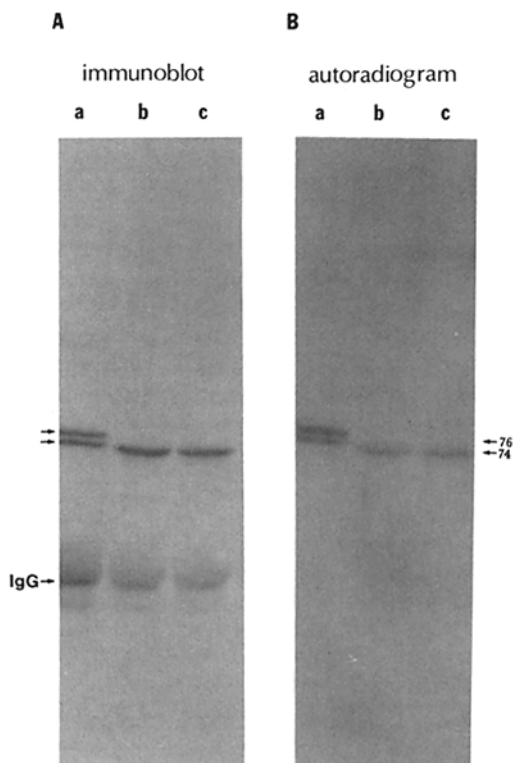
Ottaviano and Gerace (1985) reported that phosphorylation of the mammalian lamins occurred after assembly of newly synthesized protein into the nuclear envelope. The kinetics and subcellular localization of the conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub> that we observed (Figs. 5 and 10 A) resembled that reported by Ottaviano and Gerace for the interphase phosphorylation of newly synthesized lamins in mammalian cells. The results of two-dimensional gel analysis (Fig. 6 A) also supported the hypothesis that differential phosphorylation might be responsible for the difference between *Drosophila* lamins Dm<sub>1</sub> and Dm<sub>2</sub>. This hypothesis was confirmed both by in vivo <sup>32</sup>P-labeling analyses (Fig. 10, B and C) and by in vitro experiments performed with purified calf alkaline phosphatase (Fig. 11).

Schneider cells were labeled in vivo with [<sup>32</sup>P]orthophosphate for 20 min and then incubated in medium with non-radioactive phosphate for the times shown in Fig. 10 B. Cell fractionation followed by immunoprecipitation and SDS-PAGE analysis were exactly as in the experiment shown in Fig. 10 A. At the earliest time point taken (at the end of the labeling period), the <sup>32</sup>P-labeled lamins were found almost exclusively in the nuclear pellet fraction. No significant phosphorylation of lamins in the postnuclear supernatant was observed. At later time points, there appeared to be detectable amounts of <sup>32</sup>P-labeled lamins in the postnuclear supernatant fractions. Similar observations were noted by Ottaviano and Gerace (1985) who suggested that this material might represent a small amount of hyperphosphorylated lamin solubilized in mitotic cells. We think it is also possible that a small pool of "soluble" (i.e., Triton X-100 extractable) lamins may exist during interphase and might be reflective of lamina remodeling thought to occur during normal cell growth.

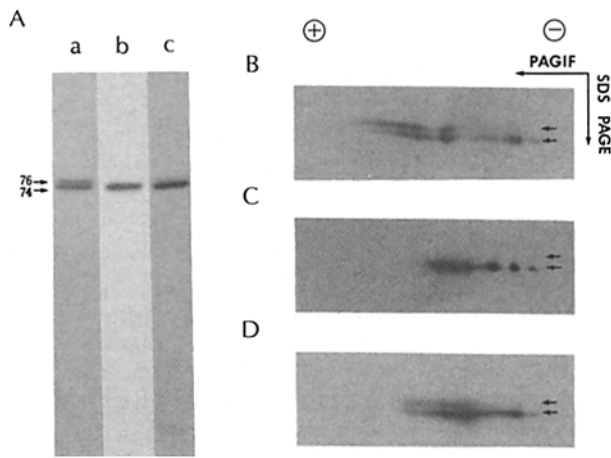
Both lamins Dm<sub>1</sub> and Dm<sub>2</sub> were labeled with [<sup>32</sup>P]orthophosphate. However, based on a comparison between <sup>32</sup>P and <sup>35</sup>S labeling (Fig. 10, B vs. A, respectively), it was apparent that lamin Dm<sub>2</sub> was hyperphosphorylated relative to lamin Dm<sub>1</sub> during the short time course of this experiment. This was also seen when the cells were grown in [<sup>32</sup>P]orthophosphate for extended periods of time to label the lamins to steady state (data not shown). In contrast with the kinetics of <sup>35</sup>S incorporation, <sup>32</sup>P labeling showed a relatively rapid turnover of phosphate from both lamin polypeptides. Label half-lives of ~3–4 h were estimated for each.

Phosphoamino acid analysis of the lamins labeled to steady state showed that both were phosphorylated at threonine and serine residues; phosphotyrosine was not detected. Lamin Dm<sub>2</sub> had ~40% more associated phosphate than lamin Dm<sub>1</sub>. The additional phosphate on lamin Dm<sub>2</sub> appeared to be predominantly at serine residues (Fig. 10 C). (Quantitative analysis of the data shown in Fig. 10 C was performed by direct scintillation counting of spots after excision from the paper chromatogram.)

In vitro experiments with purified calf alkaline phosphatase were used to corroborate results of in vivo pulse-chase



**Figure 11.** Effect of calf alkaline phosphatase on the SDS-PAGE mobilities of the *Drosophila* lamins. SDS-PAGE and immunoblot analysis was performed as described (Materials and Methods). Schneider cells were labeled to steady state with [<sup>32</sup>P]orthophosphate as described in the legend to Fig. 10. Cells were lysed into boiling SDS and <sup>32</sup>P-labeled lamins were immunoprecipitated with anti-lamin antibodies. Immunopurified lamins were treated with calf alkaline phosphatase as described (Materials and Methods). Samples were then electrophoresed on an SDS-7% polyacrylamide gel and blotted to nitrocellulose. The blot was probed with anti-lamin IgG as described in the legend to Fig. 2. (A) Immunoblot, as indicated, lamins were digested for 0 (lane a), 30 (lane b), and 270 min (lane c) with alkaline phosphatase. (The band of immunoreactivity indicated IgG in A results from detection of the heavy chain of the rabbit anti-lamin IgG used for immunoprecipitation of lamins prior to alkaline phosphatase treatment that is recognized by the goat anti-rabbit IgG used for colorimetric detection of rabbit primary antibodies on the immunoblot.) (B) Autoradiogram, as indicated, of the blot shown in A. Autoradiography was for 2 wk at room temperature without an intensifier screen.



**Figure 12.** One- and two-dimensional gel analysis of the conversion of embryo lamin Dm<sub>2</sub> to lamin Dm<sub>1</sub> after alkaline phosphatase treatment in vitro and during heat shock in vivo. (A) SDS-7% PAGE; lamins were immunoprecipitated with affinity-purified polyclonal antibodies, electrophoresed, and blot transferred to nitrocellulose. Blot was probed with affinity purified polyclonal antibodies as in Fig. 2. Lamin protein from ~8 U of embryos was loaded in each lane. (Lane a) Lamins purified from control embryos; (lane b) aliquot of fraction run in lane a was treated with purified calf alkaline phosphatase before electrophoresis; (lane c) lamins purified from embryos that had been subjected to heat shock (60 min at 36.5°C) before homogenization. (B–D) Two-dimensional gel analysis of lamin fractions shown in A; lamin protein derived from ~20 U of embryos was loaded on the first-dimension gel. After the second-dimension electrophoresis, proteins were blot transferred to nitrocellulose and blots probed with affinity-purified polyclonal anti-lamin antibodies as in A. (B) Control; (C) control after treatment with purified alkaline phosphatase; (D) lamin protein purified from heat-shocked embryos. (A small amount of lamin Dm<sub>2</sub> was observed after 60 min of heat shock in the particular experiment shown in D. This serves as an internal control for the relative SDS-PAGE mobilities of the two lamin forms. In other comparable experiments, lamin Dm<sub>2</sub> was completely undetectable after 60 min of heat shock [not shown].)

studies shown in Fig. 10. When <sup>32</sup>P-labeled Schneider cell lamins were treated with calf alkaline phosphatase, lamin Dm<sub>2</sub> was converted quantitatively to lamin Dm<sub>1</sub> as determined by immunoblot analysis (Fig. 11 A). When this blot was autoradiographed, it was observed that ~80% of the total <sup>32</sup>P label initially associated with the lamins was removed by the phosphatase treatment (Fig. 11 B). The phosphate that remained associated with lamin Dm<sub>1</sub> after conversion from Dm<sub>2</sub> was apparently resistant to removal by calf alkaline phosphatase, even following extended periods of enzyme incubation (Fig. 11 B, compare lane b with lane c). The significance of this observation is uncertain.

Lamins Dm<sub>1</sub> and Dm<sub>2</sub> that had been SDS-gel purified from *Drosophila* embryo nuclei were also treated with calf alkaline phosphatase (data not shown, but see Fig. 12 A for alkaline phosphatase treatment experiments with embryo lamins). Consistent with results shown in Fig. 11, treatment with alkaline phosphatase had no effect on the SDS-PAGE mobility of lamin Dm<sub>1</sub>. However, with increasing time of incubation with purified alkaline phosphatase, the majority of embryo lamin Dm<sub>2</sub> was processed to lamin Dm<sub>1</sub>. EDTA,

a specific inhibitor of calf alkaline phosphatase, was found to completely block this conversion of lamin Dm<sub>2</sub> to lamin Dm<sub>1</sub>.

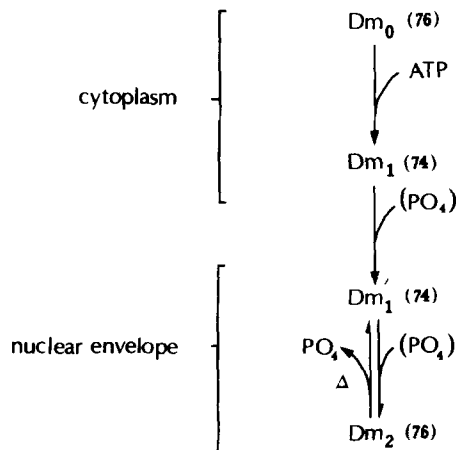
We think it also noteworthy that unlike authentic lamin Dm<sub>2</sub>, the 76-kD in vitro translation product, lamin Dm<sub>0</sub> was completely resistant to the action of purified calf alkaline phosphatase (not shown).

### Lamin Dm<sub>2</sub> Is Apparently Converted to Lamin Dm<sub>1</sub> In Vivo by Dephosphorylation during Heat Shock

During heat shock in *Drosophila* embryos, lamin Dm<sub>2</sub> appears to be converted nearly quantitatively into lamin Dm<sub>1</sub> (Smith and Fisher, 1984). Treatment of a mixture of lamins Dm<sub>1</sub> and Dm<sub>2</sub> derived from embryos (Fig. 12 A, lane a) with highly purified calf alkaline phosphatase resulted in the apparently quantitative conversion of lamin Dm<sub>2</sub> into lamin Dm<sub>1</sub> (Fig. 12 A, lane b). This was accompanied by a demonstrable shift toward the basic end of the isoelectric focusing gel (Fig. 12, compare B vs. C). A similar shift in both the one- (Fig. 12 A, lane c) and the two-dimensional gel mobility of the lamins was recorded after heat shock in vivo (Fig. 12 D).

### Discussion

Based on the results presented in this article, a detailed pathway may be proposed for the biosynthesis and posttranslational modification of the *Drosophila* lamins during cellular interphase. This pathway is shown in Fig. 13. A single primary translation product, lamin Dm<sub>0</sub> (apparent mass of 76 kD) is synthesized in the cytoplasm and processed almost immediately to lamin Dm<sub>1</sub> (apparent mass of 74 kD). This processing event has been reconstituted from cell-free extracts in vitro. Processing is apparently ATP-dependent and occurs on the NH<sub>2</sub>-terminal half of the lamin polypeptide. Once processed, the newly synthesized lamin Dm<sub>1</sub> is assembled into the nuclear envelope where at least two distinguishable phosphorylation events take place. Lamin Dm<sub>1</sub>



**Figure 13.** Biosynthesis and posttranslational modification of the *Drosophila* lamins: a proposed pathway. In the phosphorylation reactions, the PO<sub>4</sub> is written in parentheses so as not to imply that inorganic phosphate is the phosphate source in vivo but rather, to simply present the net reaction. We assume that ATP is actually the PO<sub>4</sub> donor through a protein kinase-mediated reaction but have no direct evidence to support this assumption.

appears to first be phosphorylated without change in the one-dimensional SDS-PAGE mobility. However, further phosphorylation results in an altered mobility to a form designated lamin Dm<sub>2</sub> (apparent mass of 76 kD). This form is readily distinguished from lamin Dm<sub>0</sub> by a number of criteria. Lamins Dm<sub>1</sub> and Dm<sub>2</sub> appear to be in equilibrium during normal cell growth. During heat shock, this equilibrium appears to shift quantitatively, or nearly so, toward lamin Dm<sub>1</sub>.

A biosynthetic precursor form of mammalian lamin A, designated lamin A<sub>0</sub>, has been described by Laliberte et al. (1984), by Gerace and colleagues (Ottaviano and Gerace, 1985; Gerace et al., 1984) and by Lehner et al. (1986). Like lamin Dm<sub>0</sub>, lamin A<sub>0</sub> was initially identified after in vitro translation and based upon the observation that it migrated ~2 kD slower than lamin A upon one-dimensional SDS-PAGE. However, in contrast with lamin Dm<sub>0</sub>, it appears that lamin A<sub>0</sub> is processed to lamin A, only subsequent to assembly into the mammalian nucleus (Ottaviano and Gerace, 1985; Lehner et al., 1986). Further characterization of lamin A<sub>0</sub> and the processing of lamin A<sub>0</sub> to lamin A has not been reported, and it is difficult to conclude with certainty whether or not the observations we have made with *Drosophila* lamin Dm<sub>0</sub> are homologous.

In this regard, we think it noteworthy that it has been suggested (D. Fisher et al., 1986) that the precursor sequence of lamin A<sub>0</sub> may be localized to the carboxyl-terminal domain of the protein, based on the observation that mammalian lamins A and C share identical NH<sub>2</sub>-terminal sequences at the mRNA level (McKeon et al., 1986; D. Fisher et al., 1986), yet it appears that only lamin A is synthesized as a polypeptide precursor. This would be in obvious contrast to our results regarding the NH<sub>2</sub>-terminal localization of lamin Dm<sub>0</sub> processing. It might be informative to determine whether or not mammalian lamin A<sub>0</sub> could serve as a substrate for the lamin-processing activity partially purified from *Drosophila* embryos.

In terms of the kinetics and subcellular localization of in vivo processing, it would appear that the conversion of lamin Dm<sub>0</sub> to Dm<sub>1</sub> in *Drosophila* tissue culture cells more nearly resembles the reported processing of a putative lamin B precursor in avian fibroblasts (Lehner et al., 1986). It is particularly intriguing to note that when these authors attempted to synthesize avian lamin B in a rabbit reticulocyte lysate in vitro, they were able to identify both the precursor form of avian lamin B as well as the mature form. Lehner and colleagues went so far as to speculate that this result might be due to processing of the lamin B precursor in the reticulocyte lysate. Given our current observations on the in vitro biosynthesis and processing of *Drosophila* lamin Dm<sub>0</sub> in the reticulocyte lysate (Fig. 3 B), it seems likely that this was indeed the case for the avian lamin. As above, it might be informative to challenge the *Drosophila* lamin-processing activity with avian lamin B precursor as a substrate.

Attempts to elucidate the biochemical nature of the processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub> have been only partially successful. Processing is sensitive to ATP depletion, appears to be restricted to the NH<sub>2</sub>-terminal portion of the polypeptide, does not involve detectable charge modification and as originally defined, results in a 2-kD decrease in the apparent mass of the protein. The strong inhibition of the in vitro processing reaction by  $\gamma$ -S-ATP in conjunction with the lack of

charge shift suggests that the processing activity is not a protein kinase (see, e.g., Burke and Gerace, 1986). Albeit largely on the basis of exclusion, our current results seem most consistent with the notion of proteolysis. ATP-dependent mechanisms of proteolysis (both with and without prior ubiquitination) have been well documented (for a review, see, e.g., Ciechanover et al., 1984). Proteolysis has also been assumed to account for the processing of mammalian lamin A<sub>0</sub> to lamin A (Laliberte et al., 1984; Lehner et al., 1986; D. Fisher et al., 1986).

Despite the circumstantial evidence for NH<sub>2</sub>-terminal proteolytic conversion of lamin Dm<sub>0</sub> to Dm<sub>1</sub>, several attempts to confirm this hypothesis with respect to the *Drosophila* lamins by direct NH<sub>2</sub>-terminal radiolabel sequence analysis of lamins Dm<sub>0</sub> and Dm<sub>1</sub> synthesized in vitro have been unsuccessful. Two different labeled amino acids, leucine and methionine, have been used and, with the exception of the NH<sub>2</sub>-terminal methionine, in neither case were we able to obtain positive results within the first 20 residues. Attempts at NH<sub>2</sub>-terminal sequence analysis of the authentic *Drosophila* lamins were frustrated in that the NH<sub>2</sub>-termini of both lamins Dm<sub>1</sub> and Dm<sub>2</sub> were apparently blocked. Similar observations have been reported for the human lamins (D. Fisher et al., 1986). Further sequence analysis will be essential, but will only be considered after completion of the nucleic acid sequence for the 5'-end of the *Drosophila* lamin cDNA clones. We must point out that in the absence of direct protein sequence information, we cannot be certain that the processing of lamin Dm<sub>0</sub> to Dm<sub>1</sub> in vitro is identical with that which apparently occurs in vivo. Although our peptide map and two-dimensional gel comparisons between authentic lamin Dm<sub>1</sub> and putative lamin Dm<sub>1</sub> synthesized from lamin Dm<sub>0</sub> in vitro would seem to rule out gross discrepancies, subtle differences between the two might not be apparent from our analyses to date.

It is impossible to provide any certain insights into the biological significance of our current observations and those of others regarding the synthesis of vertebrate lamins A and B in precursor form. If they are in fact homologous, i.e., results obtained in *Drosophila* and those obtained with vertebrate cells, it seems likely that the synthesis of at least some lamins as biosynthetic precursors is of general importance. Nevertheless, it is clear both from our results and those of others (Gerace et al., 1984; Ottaviano and Gerace, 1985; Lehner et al., 1986) that the processing of these lamin precursors does not represent a typical secretory or membrane insertional event. In the case of the *Drosophila* lamin, although the processing occurs rapidly in the cytoplasm, it is independent of added membranes, occurs with good efficiency posttranslationally, and can be catalyzed by 2-2.5-S activity isolated from a soluble extract (100,000-g supernatant) prepared from *Drosophila* embryos entirely in the absence of detergents. Our greatest hope in ultimately understanding the biological significance of the *Drosophila* lamin precursor stems from the fact that we have apparently been able to reconstitute the lamin-processing reaction in vitro, and that it has been possible to generate substrates for this reaction from lamin cDNA clones expressed in the pI7 in vitro transcription vector. It should therefore be possible to further localize the site of lamin processing on the protein by deletion analysis and then to perform site-directed mutagenesis in the region of interest. Once we have been able to

identify mutants that cannot be efficiently processed by the lamin-processing activity in vitro, it should then be possible to transform such mutant lamins back into the organism in order to elucidate effects in vivo.

It appears that only a single type of lamin can be identified in *Drosophila melanogaster* embryos. Two discrete forms of this lamin that had previously been identified on one-dimensional SDS-polyacrylamide gels are apparently generated by differential phosphorylation of a single polypeptide precursor. Phosphorylation has been shown to result in similar mobility shifts of other proteins (see, e.g., Shih et al., 1982). However, phosphorylation-dependent changes in the SDS-PAGE mobilities of the mammalian lamins have not been noted despite detailed analyses of differential lamin phosphorylation through the mammalian cell cycle (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985).

We have however noted that there appear to be several operationally distinguishable types of phosphorylation that take place with the *Drosophila* lamins. Both lamins Dm<sub>1</sub> and Dm<sub>2</sub> are phosphorylated in vivo. Therefore, it would seem that not all phosphorylation events lead to a demonstrable change in one-dimensional SDS-PAGE mobility. The phosphate group or groups that do result in the conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub> are completely sensitive to the action of calf alkaline phosphatase. In contrast, a fraction of the phosphate associated with lamin Dm<sub>1</sub> appears resistant to phosphatase treatment, even after extended incubation. Phosphoamino acid analyses have indicated that *Drosophila* lamin phosphorylation takes place with approximately equal frequency at both serine and threonine residues. This last observation is in contrast with results of Ottaviano and Gerace (1985). They reported a marked predominance of phosphoserine in phosphoamino acid analyses of the mammalian lamins. This difference is of uncertain significance.

Ottaviano and Gerace (1985) also reported that the mammalian lamins contained ~0.27–0.46 mol of phosphate/mol of protein during cellular interphase. We have attempted to quantitate lamin phosphorylation stoichiometry in *Drosophila* based on <sup>32</sup>P incorporation relative to lamin protein as estimated by Coomassie Blue staining of SDS-polyacrylamide gels. Although data from these experiments were at the limits of sensitivity with respect to the quantitation of protein by Coomassie Blue staining, it is our impression that the *Drosophila* lamins contain between 2 and 4 mol of phosphate/mol of protein. That such is indeed the case is also corroborated by the number of discrete spots identified on two-dimensional gels and by the fact that most of these spots are conspicuously shifted toward the basic region of the map after treatment with calf alkaline phosphatase (Fig. 12). This impression is further supported by the following deductive argument. Each molecule of lamin Dm<sub>2</sub> must contain at least 1 mol of phosphate/mol of protein to effect the characteristic SDS-PAGE mobility shift. What is more, because lamin Dm<sub>1</sub> is phosphorylated to approximately two-thirds the level of lamin Dm<sub>2</sub> based on the ratio of <sup>32</sup>P label incorporated to immunoreactive lamin antigen, one can estimate the minimum amount of phosphate per mole of protein by solving simultaneous equations. If we assume that the phosphorylation of lamin Dm<sub>1</sub> = 0.67 × the phosphorylation of lamin Dm<sub>2</sub>, and that the conversion of lamin Dm<sub>1</sub> to Dm<sub>2</sub> requires the addition of a minimum of 1 mol phosphate/mol of protein, then the amount of phosphate per mole of Dm<sub>1</sub>

= 0.67 (Dm<sub>2</sub>), and the amount of phosphate per mole of Dm<sub>2</sub> = Dm<sub>1</sub> + 1. If these equations are solved algebraically, we can estimate that lamin Dm<sub>1</sub> has 2 mol of phosphate/mol of protein while lamin Dm<sub>2</sub> would be calculated to have 3 mol of phosphate/mol of protein. During most stages of development and in tissue culture cells, we have observed approximately equal amounts of lamins Dm<sub>1</sub> and Dm<sub>2</sub>. Thus we would estimate a minimum of 2.5 mol of phosphate/mol of total *Drosophila* lamin, ~5–10-fold higher than that reported for the interphase lamins in mammalian cells (Ottaviano and Gerace, 1985).<sup>2</sup>

Comparison of interphase lamin phosphorylation in *Drosophila* with that reported for mammalian cells has also revealed a number of similarities. In both organisms, phosphorylation appears to occur only after lamin assembly into the nuclear envelope, implicating the activity of a nuclear envelope protein kinase. The kinetics of in vivo assembly and incorporation of phosphate are also similar between the two systems. In neither organism was there any evidence of lamin phosphorylation at tyrosine residues, and the difference in the ratios of phosphoserine to phosphothreonine is simply that. Both phosphoamino acids are in fact detectable in both organisms.

At this point in time, we can offer no simple explanation for the differences in interphase phosphorylation between the *Drosophila* lamins and their mammalian counterparts. Obvious suggestions pertaining to developmental regulation of lamin phosphorylation during embryogenesis as compared with mammalian cells in culture can be excluded *a priori* when it is noted that our results have been obtained with *Drosophila* tissue culture cells as well as with embryos and have been corroborated using lamins obtained from essentially all developmental stages of the organism. However, the difference in lamin phosphorylation between *Drosophila* and mammalian cells may be more apparent than real. During heat shock, lamin phosphorylation decreases substantially and lamin Dm<sub>2</sub> is converted nearly quantitatively into lamin

2. During mitosis in mammalian cells, the lamins are hyperphosphorylated to levels 5–10-fold greater than those during interphase, but as noted above, without demonstrable alteration in one-dimensional SDS-PAGE mobility (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985). Attempts to study lamin phosphorylation during the mitotic cycle in *Drosophila* tissue culture cells have been largely frustrated by our inability to develop suitable protocols for cell synchrony. However, preliminary results using vinblastine in an attempt to block cells in mitosis have been informative to a degree. When cells are treated with low concentrations of vinblastine and postnuclear supernatant fractions are assayed for the appearance of soluble lamin, a single polypeptide form, with a mobility slightly slower than lamin Dm<sub>1</sub>, can be identified (Smith, D., unpublished observation). This soluble lamin comigrates exactly with the soluble lamin previously identified in early embryos (Smith and Fisher, 1984). Evidence indicates that this soluble lamin is present in the oocyte and may in fact be derived from "mitotic" breakdown of the nurse cell nuclei at between stages 10 and 11 of oocyte maturation (Smith, D., manuscript in preparation). (It has been postulated that soluble lamin pools identified during early embryogenesis in *Xenopus* have been similarly derived, in this case, from germinal vesicle breakdown during oocyte maturation [Benavente et al., 1985; Stick and Hausen, 1985].) Two-dimensional gel analysis of this latter form has shown it to be relatively acidic, consistent with phosphorylation to the same or greater degree as lamin Dm<sub>1</sub> (Smith, D., unpublished observation). It seems possible therefore, that a third form of the *Drosophila* lamin is generated by differential phosphorylation during mitosis. It is this form that may be comparable to the hyperphosphorylated forms of the mammalian lamins that show little or no change in their one-dimensional SDS-PAGE mobilities. Detailed results of these experiments will be reported at a later time.

Dm<sub>1</sub>. It is conceivable that this conversion is not a component of the "heat shock response" per se, but rather, represents a general response of cells to the temperature at which they are grown. Inasmuch as mammalian cells are normally grown at 37°C, a temperature typically used to induce heat shock in *Drosophila*, it may actually be appropriate to compare the levels of phosphorylation of the *Drosophila* lamins obtained from heat-shocked embryos with those prepared from normal mammalian cells. It appears from our current data (Fig. 12) that these values may in fact be comparable.

Further insights into the biological significance of changes in nuclear lamin phosphorylation during cellular interphase may be obtained upon consideration of recent observations both in our laboratory and elsewhere. In *Drosophila*, we have noted that in vivo heat shock results in increased structural stability (or rigidity) of isolated karyoskeletal fractions that include residual components of the nuclear lamina (McConnell et al., 1987). Karyoskeletal fractions from mammalian cells have been reported to behave similarly (Evan and Hancock, 1985).<sup>3</sup> In mammals, it is believed that during the cell cycle, lamina breakdown at the onset of mitosis is triggered at least in part by hyperphosphorylation and consequent solubilization of the lamins (for a review, see Fisher, 1987). During interphase, maintenance of the lamins in a partially phosphorylated state has been proposed to serve as a means of maintaining lamina plasticity and allowing for addition of monomer (or oligomer) units and lamina growth (Ottaviano and Gerace, 1985). It is tempting to speculate that temperature-induced hypophosphorylation of the *Drosophila* lamins is reflective of the opposite effect in vivo, that is, a reduction in lamina plasticity or increased structural rigidity. We would further postulate that increased structural rigidity in vivo is reflected by increased lamina stability upon tissue homogenization and cell fractionation in vitro (McConnell et al., 1987).

If one considers that a major function of the nuclear lamina may be to provide structural support to the nuclear envelope in maintaining the integrity of the intact nucleus, the following hypothesis may be suggested. In general terms, one might propose that the two elements that provide structural integrity to the nuclear envelope are the nuclear membranes and the nuclear lamina. It is apparent that nuclear envelope stability or rigidity must be maintained, but at the same time, considerable plasticity, allowing for nuclear growth, shape changes, and perhaps internal rearrangements, is necessary. As the temperature of a cell is increased, membrane fluidity might be expected to increase, thereby decreasing the contribution of the nuclear membranes to the structural stability of the envelope. To compensate, the nuclear lamins would dephosphorylate, thereby increasing the structural rigidity of the nuclear lamina and maintaining the overall resiliency of the envelope. At lower temperatures, the opposite events would occur. Decreased membrane fluidity at lower temperatures would be compensated for by increased lamin phos-

phorylation and hence, increased plasticity of the lamina. Overall stability of the nuclear envelope would remain relatively constant. Explicit experiments to test this hypothesis are currently being considered.

It is a pleasure to acknowledge Dr. M. Eisenberg for computer-assisted densitometry of autoradiograms, Ms. E. Matz for her expert assistance in immunoelectron microscopy, and Mr. A. Golden for assistance with the phosphoamino acid analysis.

These studies were supported by research grant GM-33132 from the National Institutes of Health. D. E. Smith was supported in part by National Institutes of Health training grant GM-07518. Y. Gruenbaum was supported in part by a Senior Postdoctoral Fellowship from the California Division of the American Cancer Society.

Received for publication 19 January 1987, and in revised form 8 April 1987.

## References

- Aaronson, R. P., and G. Blobel. 1974. On the attachment of the nuclear pore complex. *J. Cell Biol.* 62:746-754.
- Aaronson, R. P., and G. Blobel. 1975. Isolation of nuclear pore complexes in association with a lamina. *Proc. Natl. Acad. Sci. USA.* 72:1007-1011.
- Aebi, U., J. Cohn, L. Buhle, and L. Gerace. 1986. The nuclear lamina is a meshwork of intermediate-type filaments. *Nature (Lond.)* 323:560-564.
- Agard, D. A., and J. W. Sedat. 1983. Three-dimensional architecture of the polytene nucleus. *Nature (Lond.)* 302:676-681.
- Allis, C. D., G. L. Waring, and A. P. Mahowald. 1977. Mass isolation of pole cells from *Drosophila melanogaster*. *Dev. Biol.* 56:372-381.
- Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde: use of the conjugates for the detection of antigens and antibodies. *Immunochemistry.* 6:43-52.
- Benavente, R., and G. Krohne. 1985. Changes of karyoskeleton during spermatogenesis of *Xenopus*: expression of lamin L<sub>1</sub>, a nuclear lamina protein specific for the male germ line. *Proc. Natl. Acad. Sci. USA.* 82:6176-6180.
- Benavente, R., G. Krohne, and W. W. Franke. 1985. Cell type-specific expression of nuclear lamina proteins during development in *Xenopus laevis*. *Cell.* 41:177-190.
- Berrios, M., and P. A. Fisher. 1986. A myosin heavy chain-like polypeptide is associated with the nuclear envelope of higher eukaryotic cells. *J. Cell Biol.* 103:711-724.
- Berrios, M., N. Osheroff, and P. A. Fisher. 1985. *In situ* localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction. *Proc. Natl. Acad. Sci. USA.* 82:4142-4146.
- Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid sensitive method for detection of alkaline phosphatase conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.
- Blobel, G., and D. Sabatini. 1971. Dissociation of mammalian polyribosomes into subunits by puromycin. *Proc. Natl. Acad. Sci. USA.* 68:390-394.
- Brugge, J. S., and D. Darrow. 1982. Rous sarcoma virus-induced phosphorylation of a 50,000-molecular weight cellular protein. *Nature (Lond.)* 295:250-253.
- Burke, B., and L. Gerace. 1986. A cell-free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell.* 44:639-652.
- Burke, B., J. Tooze, and G. Warren. 1983. A monoclonal antibody which recognizes each of the nuclear lamin polypeptides in mammalian cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:361-367.
- Chamberlain, M., and J. Ring. 1973. Characterization of T7-specific ribonucleic acid polymerase: general properties of the enzymatic reaction and the template specificity of the enzyme. *J. Biol. Chem.* 248:2235-2244.
- Chang, C. N., P. Model, and G. Blobel. 1979. Membrane biogenesis: cotranslational integration of the bacteriophage f1 coat protein into an *Escherichia coli* membrane fraction. *Proc. Natl. Acad. Sci. USA.* 76:1251-1255.
- Ciechanover, A., D. Finley, and A. Varshavsky. 1984. The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. *J. Cell. Biochem.* 24:27-53.
- Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
- Erikson, A. H., and G. Blobel. 1983. Cell-free translation of messenger RNA in a wheat germ system. *Methods Enzymol.* 96:38-50.
- Evan, G. I., and D. C. Hancock. 1985. Studies on the interaction of the human *c-myc* protein with cell nuclei: p62<sup>myc</sup> as a member of a discrete subset of nuclear proteins. *Cell.* 43:253-261.
- Feldherr, C. M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* 99:2216-2222.
- Filson, A. J., A. Lewis, G. Blobel, and P. A. Fisher. 1985. Monoclonal antibodies prepared against the major *Drosophila* nuclear matrix-pore complex-lamina glycoprotein bind specifically to the nuclear envelope in situ. *J. Biol. Chem.* 260:3164-3172.

3. We think it noteworthy that Evan and Hancock (1985) observed that the nuclear lamina isolated from mammalian cells under normal growth conditions (37°C) was quite stable relative to other components of the mammalian nuclear matrix fraction; such was not the case with the *Drosophila* lamina (McConnell et al., 1987). The increased stability of the mammalian lamina relative to that isolated from *Drosophila* is consistent with our current interpretation of observations regarding the relative levels of lamin phosphorylation in the two different systems.

- Fisher, P. A. 1987. Disassembly and reassembly of nuclei in cell-free systems. *Cell*. 48:175-176.
- Fisher, P. A., M. Berrios, and G. Blobel. 1982. Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of *Drosophila melanogaster*. *J. Cell Biol.* 92:674-686.
- Fisher, D., N. Chaudhary, and G. Blobel. 1986. cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc. Natl. Acad. Sci. USA*. 83:6450-6454.
- Franke, W. W. 1987. Nuclear lamins and cytoplasmic intermediate filament proteins: a growing multigene family. *Cell*. 48:3-4.
- Franke, W. W., U. Scheer, G. Krohne, and E.-D. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91:(2, Pt. 2):39s-50s.
- Fuchs, J. P., H. Giloh, C.-H. Kuo, H. Saumweber, and J. W. Sedat. 1983. Nuclear structure: determination of the fate of the nuclear envelope in *Drosophila* during mitosis using monoclonal antibodies. *J. Cell Sci.* 64:331-349.
- Gerace, L. 1986. Nuclear lamina and organization of the nuclear architecture. *Trends Biochem. Sci.* 11:443-446.
- Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell*. 19:277-287.
- Gerace, L., and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. *Cold Spring Harbor Symp. Quant. Biol.* 46:967-978.
- Gerace, L., A. Blum, and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction: interphase and mitotic distribution. *J. Cell Biol.* 79:546-566.
- Gerace, L., C. Comeau, and M. Benson. 1984. Organization and modulation of nuclear lamina structure. *J. Cell Sci. Suppl.* 1:137-160.
- Golden, A., S. P. Nemeth, and J. S. Brugge. 1986. Blood platelets express high levels of the pp60<sup>src</sup>-specific tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA*. 83:852-856.
- Kaufmann, S. H., W. Gibson, and J. H. Shaper. 1983. Characterization of the major polypeptides of the rat liver nuclear envelope. *J. Biol. Chem.* 258:2710-2719.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Laliberte, J.-F., A. Dagenais, M. Fillion, V. Bibor-Hardy, R. Simard, and A. Royal. 1984. Identification of distinct messenger RNAs for nuclear lamin C and a putative precursor of nuclear lamin A. *J. Cell Biol.* 98:980-985.
- Lebkowski, J., and U. K. Laemmli. 1982. Non-histone proteins and long-range organization of HeLa interphase DNA. *J. Mol. Biol.* 156:325-344.
- Lehner, C. F., G. Furstenberger, H. M. Eppenberger, and E. A. Nigg. 1986. Biogenesis of the nuclear lamina: in vivo synthesis and processing of nuclear protein precursors. *Proc. Natl. Acad. Sci. USA*. 83:2096-2099.
- March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149-152.
- McConnell, M., A. M. Whalen, D. E. Smith, and P. A. Fisher. 1987. Heat shock induced changes in the structural stability of proteinaceous karyoskeletal elements in vitro and morphologic effects in situ. *J. Cell Biol.* In press.
- McGadey, J. 1970. A tetrazolium method for non-specific alkaline phosphatase. *Histochemie*. 23:180-184.
- McKeon, F. D., M. W. Kirschner, and D. Caput. 1986. Primary and secondary structural homologies between the major nuclear envelope and cytoplasmic intermediate filament proteins. *Nature (Lond.)*. 319:463-468.
- McKeon, F. D., D. L. Tuffanelli, K. Fukuyama, and M. W. Kirschner. 1983. Autoimmune response directed against conserved determinants of nuclear envelope proteins in a patient with linear scleroderma. *Proc. Natl. Acad. Sci. USA*. 80:4374-4378.
- Moss, B., and E. N. Rosenblum. 1972. Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes. A new method for the separation of polypeptide subunits. *J. Biol. Chem.* 247:5194-5198.
- Nikodem, V., and J. R. Fresco. 1979. Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. *Anal. Biochem.* 97:382-386.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Ottaviano, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 260:624-632.
- Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
- Risau, W., H. Saumweber, and P. Symmons. 1981. Monoclonal antibodies against a nuclear membrane protein of *Drosophila*: localization by indirect immunofluorescence and detection of antigen using a new protein blotting procedure. *Exp. Cell Res.* 133:47-54.
- Rothman, J. E., and H. F. Lodish. 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (Lond.)*. 269:775-780.
- Schatten, G., G. G. Maul, H. Schatten, N. Chaly, C. Simerly, R. Balczon, and D. L. Brown. 1985. Nuclear lamins and peripheral nuclear antigens during fertilization and embryogenesis in mice and sea urchins. *Proc. Natl. Acad. Sci. USA*. 82:4727-4731.
- Schlieff, R. F., and P. C. Wensink. 1981. Extracting RNA from *Drosophila* adults. In *Practical Methods in Molecular Biology*. P. Manor, editor. Springer-Verlag, New York. 166-167.
- Schlossman, D. M., S. L. Schmid, W. A. Braell, and J. E. Rothman. 1984. An enzyme that removes clathrin coats: purification of uncoating ATPase. *J. Cell Biol.* 99:723-733.
- Schneider, I. 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* 27:353-365.
- Scott, M. P., R. V. Storti, M. L. Pardue, and A. Rich. 1979. Cell-free protein synthesis in lysates of *Drosophila melanogaster* cells. *Biochemistry*. 18:1588-1594.
- Shields, G., A. Dubendorfer, and J. H. Sang. 1975. Differentiation in vitro of larval cell types from early embryonic cells of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* 33:159-175.
- Shih, T. Y., M. O. Weeks, P. Gruss, R. Dhar, S. Oroszlan, and E. M. Scolnick. 1982. Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. *J. Virol.* 42:253-261.
- Smith, D. E., and P. A. Fisher. 1984. Identification, developmental regulation and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* 99:20-28.
- Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31-43.
- Stick, R., and P. Hausen. 1985. Changes in nuclear lamina composition during early development of *Xenopus laevis*. *Cell*. 41:191-200.
- Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlling exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA*. 82:1074-1078.