

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

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ABSTRACT An affinity-purification method has been developed for the rapid, efficient, and precise elution of antibodies specifically bound to antigens immobilized on nitrocellulose after blot transfer from SDS polyacrylamide gels. The applicability of this technology has been demonstrated using antisera raised against the nuclear matrix-pore complex-lamina fraction prepared from *Drosophila melanogaster* embryos. In so doing, we have established the existence in whole embryo lysates, of two nearly identical forms of the predominant 74-kilodalton polypeptide previously identified in lower resolution studies of the nuclear matrix-pore complex-lamina fraction. These species, distinguishable on the basis of a slight difference in SDS PAGE mobilities on low concentration polyacrylamide gels, are immunochemically cross-reactive and have been localized exclusively to the nuclear periphery (nuclear envelope) by indirect immunofluorescence analyses of cryosections. The steady-state levels of these two polypeptides have been examined in total embryo lysates both as a function of embryogenesis and in response to heat shock. The larger species is not detectable in early embryos but approaches levels approximately equal to that of the smaller form by about the temporal midpoint of embryonic development. In response to heat shock, this larger form appears to be converted nearly quantitatively into the lower molecular weight polypeptide. These results, as well as the general reliability of the nitrocellulose blot immunoaffinity-purification methodology, have been substantiated through the use of monoclonal antibodies.

A method for the affinity purification of antibodies from diazotized paper blots of SDS polyacrylamide gels has recently been published by Olmsted (1). Although a detailed quantitative assessment of the efficiency of this technique was not presented in her report, Olmsted did note that in order to achieve signals with the affinity-purified IgG that were of comparable intensity to those seen with the unfractionated serum, she eluted antibodies from approximately 10 times the amount of paper used to characterize those eluates in the

reprobe. This, in conjunction with data showing significant residual signal remaining on the original blot after elution, suggested a relatively poor recovery of affinity-purified IgG. Further limitations of the procedure reported by Olmsted include the selection of diazotized paper for the original blot—nitrocellulose is easier to use, gives higher resolution, and is hence preferred for routine blotting applications—as well as the use of a radiolabeled protein A probe to detect the first antibody bound to the blot. This detection method necessi-

tates the delay (as well as expense) inherent in any autoradiographic procedure. Furthermore, it was specified that those portions of blots that were destined for subsequent elution of active antibody were not themselves probed with iodinated protein A or subjected to the rigors of autoradiography. Rather, regions of blots to be eluted were located by probing parallel strips excised from the same blots before incubation with protein A and aligning the resultant autoradiograms with the unprobed portion (1). It would seem difficult, using such an approach, to distinguish with confidence two closely migrating polypeptides.

In spite of the technical limitations outlined above, the potential applicability of blot-affinity purification of antibody probes to the characterization of biochemically complex subcellular fractions was readily apparent. We therefore set about to refine the technology of Olmsted (1) within the context of the nuclear matrix-pore complex-lamina (NMPCL)¹ fraction prepared from *Drosophila melanogaster* embryos.

SDS-polyacrylamide-gradient-gel analysis of the *Drosophila* NMPCL fraction has resulted in the observation of a quantitatively major band at the 74-kilodalton (kD) position (2). Antisera specifically directed against the polypeptide(s) contained in this band have been obtained by injecting SDS PAGE-purified antigen into rabbits; using these antisera for indirect immunofluorescence analyses of permeabilized whole cells, it has been possible to demonstrate an exclusively nuclear localization for the 74-kD antigen during cellular interphase. As a result of this in situ localization, and in conjunction with data regarding cell fractionation, apparent molecular weight and relative quantitative abundance, it has been suggested (2) that this species may be functionally analogous to one or more of the three nuclear lamina polypeptides commonly seen in mammalian as well as other vertebrate tissues (designated lamins A, B, and C in order of decreasing molecular weight) (3). This suggestion was made despite the lack of any demonstrable immunochemical crossreactivity between the *Drosophila* 74-kD polypeptide and the vertebrate lamins (2).

In recent experiments in our laboratory, the observation has been made that what had appeared upon SDS polyacrylamide gradient gel electrophoresis of the *Drosophila* NMPCL fraction to be a single predominant 74-kD polypeptide could in fact be resolved into two closely migrating species on higher resolution continuous concentration gels. Further, both species were equally immunoreactive by Western blot analysis with each of several antisera prepared (as cited above) against the SDS PAGE-purified 74-kD polypeptide. In light of the multiplicity of vertebrate nuclear lamina polypeptides and of the observation that two of the three, lamins A and C, shared considerable sequence homology (4-7), the resolution of the *Drosophila* 74-kD band into two, apparently immunocross-reactive species raised several questions with regard to *Drosophila* nuclear structure and in a more general context as well.

In the present paper, we report results detailing several modifications of the blot-affinity antibody purification technique of Olmsted (1) introduced in order to quantitate as well as broaden the scope and power of the method. We also describe studies using this approach that demonstrate the presence in *Drosophila* embryo lysates of two antigenically

related polypeptides that migrate in the 74-kD region upon SDS PAGE and can be localized exclusively to the nuclear periphery in situ. These results have been corroborated using monoclonal antibodies and extended to include an examination of the steady-state levels of these two species through embryogenesis and in response to heat shock.

MATERIALS AND METHODS

Specific IgG fractions were from Cappel Laboratories Inc. (Cochranville, PA). [¹⁴C]Formaldehyde was from New England Nuclear (Boston, MA). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). Acrylamide, methylene bisacrylamide, and X-Omat XAR x-ray film were from Eastman Kodak Co. (Rochester, NY). Polyoxyethylene sorbitan monolaureate (TWEEN 20), Fraction V-bovine serum albumin (BSA) and calf alkaline phosphatase (CAP) were from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies prepared against nuclear extracts of *Drosophila* tissue culture cells (8) were the generous gift of Dr. Peter Symmons (University of Tübingen, Federal Republic of Germany). Monoclonal antibodies against the 70-kD *Drosophila* heat shock protein (hsp-70) were the generous gift of Dr. Susan Lindquist (University of Chicago). 5-Bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, and *p*-nitro blue tetrazolium chloride were purchased from the United States Biochemical Corp. (Cleveland, OH). Embedding medium for cryosectioning was from Lipshaw Mfg. Co. (Detroit, MI). All other chemicals were obtained commercially and were of reagent grade.

Most of the methods have been previously described in detail (2, 9, 10). *D. melanogaster* (Oregon R, P2 strain) were grown in mass culture and embryos were collected essentially according to Allis et al. (11). Total embryo extracts were prepared by Dounce homogenization of the freshly harvested dechorionated embryos directly into 4 vol of boiling SDS (12% wt/vol), 200 mM Tris-HCl, pH 8.4, 100 mM dithiothreitol; aliquots of the extract were either electrophoresed immediately or stored frozen at -20°C. SDS PAGE was essentially according to Laemmli (12) as previously described (2, 9) and as detailed in the individual figure legends. Blotting of SDS gels onto nitrocellulose was essentially according to Southern (13) as described (2). Probing of blots with either crude antisera or affinity-purified IgG was as detailed below and in the individual figure legends. Antisera against the entire *Drosophila* NMPCL fraction were raised in rabbits as previously described for the rat liver pore complex-lamina fraction (2); the *Drosophila* protein was injected either in native form as prepared from the embryos or after SDS denaturation. In vitro ¹⁴C-labeling of proteins with retention of biological activity was performed by the reductive methylation procedure of Jentoft and Dearborn (14) exactly as previously (2). Protein determinations and staining of nitrocellulose blots for protein were performed according to Schaffner and Weissman (15).

Indirect Immunofluorescence: Indirect immunofluorescence analysis of whole *Drosophila* salivary gland cells was performed exactly as previously described (2). Cryosections (6 μm thick) of *Drosophila* third instar larvae were cut from blocks of embedding medium in which the animals had been frozen by immersion into liquid N₂. After sectioning, specimens were placed onto slides and stored at -70°C for up to 6 mo before use. Upon removal from the freezer, sections were immersed immediately in 3.7% formaldehyde in 140 mM NaCl, 10 mM KPO₄, pH 7.5 (phosphate-buffered saline [PBS]). After 1-2 min in formaldehyde-PBS, slides were placed into phosphate-buffered saline for two 10-min washes, after which the samples were incubated with the first and second antibodies, washed, and mounted exactly as described for whole cells and tissues (2). Specimens were examined and photographed using a Leitz Ortholux II microscope with epifluorescence illumination and an Orthomat W camera (E. Leitz, Inc., Rockleigh, NJ).

Antibody Probing of Nitrocellulose Blots: After completion of the transfer procedure, the nitrocellulose blots were incubated for 1 h in a minimal volume of 100 mg/ml BSA in PBS. Blots were then washed thoroughly with distilled water, air-dried, and stored at room temperature until use. Before the addition of the first antibody, blots were rehydrated in 0.5% (vol/vol) TWEEN 20 in PBS (TWEEN-PBS). The rehydrated blots were incubated overnight at room temperature in the first antibody diluted appropriately into TWEEN-PBS, washed at room temperature three times for 15 min each with TWEEN-PBS, washed at room temperature three times for 15 min each with TWEEN-PBS, incubated for 2 h at 37°C with calf alkaline phosphatase-conjugated goat antirabbit or goat antimouse IgG antibody diluted in TWEEN-PBS, and then washed again as above with TWEEN-PBS. Volumes of solution used for each of the above steps were based on the size of the blot being probed as previously reported (2). Goat IgG fractions were glutaraldehyde conjugated with calf alkaline phosphatase according to the procedure of Avrameas (16) and were used at approximately 1:40,000 dilutions relative to the originally reconstituted Cappel IgG stocks.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; CAP, calf alkaline phosphatase; hsp, heat shock protein; NMPCL, nuclear matrix-pore complex-lamina; PBS, phosphate-buffered saline.

After the final wash in TWEEN-PBS, blots were stained for alkaline phosphatase activity essentially according to McGadey (17). They were first rinsed briefly (1–2 min) in 50 mM Na-glycinate, pH 9.6, and then incubated at room temperature in 50 mM Na-glycinate, pH 9.6, 0.1 mg/ml of *p*-nitro blue tetrazolium chloride, 0.05 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, 4 mM MgCl₂. As above, the volume of staining solution used was based on blot size and in general was identical with that used for the first antibody solution. Times of staining were varied depending on the intensity of the response and are as indicated in the individual figure legends. With blots that were destined for subsequent elution, all of the above staining solutions contained in addition, 0.5% (vol/vol) TWEEN 20.

Elution of Antibodies from Nitrocellulose Blots: After staining for alkaline phosphatase activity as described above, blots were rinsed briefly in TWEEN-PBS and regions containing bands of interest were excised from the blot and transferred to microfuge tubes. Each fragment was then eluted with three 30-s washes with 5 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.5% (vol/vol) TWEEN 20, 100 µg/ml of BSA; these eluates were immediately neutralized by the addition of Na₂PO₄ to a final concentration of 50 mM. After the pH 2.3 elution, fragments were further washed with three similar aliquots of TWEEN-PBS, 100 µg/ml of BSA, followed by three washes with 3 M NH₄SCN, 150 mM KCl, 10 mM NaPO₄, pH 6.0, 100 µg/ml of BSA. After pooling individual aliquots, the three different eluates were maintained separately; further processing was as indicated in the individual figure legends. Elution volumes varied between 50 and 300 µl depending on the size of the nitrocellulose fragment being eluted and the specific purpose for which the eluate was being prepared; details are as specified in the individual figure legends.

RESULTS

The NMPCL prepared from *D. melanogaster* embryos is a biochemically complex subnuclear fraction representing ~2% of the total embryo protein and as much as 40% of the total nuclear protein (2). Work to date has focused largely on the immunocytochemical localization of the numerous polypeptide constituents found to make up this fraction and to this end, we have prepared several high titer polyspecific antisera by injecting the entire NMPCL fraction into rabbits. As expected, these antisera react with a large number of individual polypeptides as determined by Western blot analysis. (The results for one such serum are shown in Fig. 2, *lane S*; results with a similar serum prepared against the rat liver pore complex-lamina fraction have been reported previously [2].) In order to screen these antisera, it was initially anticipated that individual polypeptides could be purified, either by gel electrophoresis or otherwise, covalently coupled to Sepharose and used to affinity-purify monospecific IgG fractions. However, the diazotized paper blot elution procedure of Olmsted (1) suggested a much more convenient alternative. Further, at least two recent reports (18, 19) indicated that elution from nitrocellulose blots was similarly possible.

Our initial attempts to elute antibodies from nitrocellulose blots using what were then standard buffer conditions for first antibody incubations (2) led to extremely variable results and only very poor yields. A preliminary study of protein-nitrocellulose interactions led to the suggestion that the poor recoveries observed under these conditions were due to incomplete blocking of the nonspecific protein binding sites on the nitrocellulose (R. Fisher and P. Fisher, unpublished results). Although this problem might have been alleviated by using more exhaustive blocking conditions (as we have in fact done in switching from a 10-mg/ml of BSA blocking solution [2] to one containing 100 mg/ml of BSA), it was reasoned that if Western blots could be probed and analyzed in the continuous presence of an agent that directly prevented all nonspecific binding interactions between protein and nitrocellulose, the problem of poor recoveries due to incomplete blocking could be effectively circumvented. A recent report by Blake et al. (20) indicated that the detergent TWEEN 20

might be such a reagent; in the presence of TWEEN 20, blocking agents such as BSA were found to be dispensable. Further, if otherwise compatible, the use of a phosphatase-conjugated second antibody detection system such as that employed by these same investigators offered the advantage of allowing rapid and direct visualization of the first antibody prior to excision and elution from the nitrocellulose.

Quantitative Recovery of Rabbit Antigoat IgG Antibody from Nitrocellulose-bound Antigen

The results of our initial attempts to apply the probing and detection methodology of Blake et al. (20) (with modifications as described in Materials and Methods) to antibody elution from nitrocellulose blots are shown in Fig. 1. In this experiment, different concentrations of purified goat IgG were electrophoresed on an SDS polyacrylamide gel. One segment of the gel, fixed and stained with Coomassie Blue to demonstrate the linearly increasing protein concentrations is shown in Fig. 1A (*gel*). Parallel segments were quantitatively blot-transferred to nitrocellulose as described in Materials and Methods and then probed first with ¹⁴C-labeled rabbit antigoat IgG antibody and then with CAP-conjugated goat antirabbit IgG antibody. A fluorograph directly demonstrating the presence of the radiolabeled first antibody on such a blot is shown in Fig. 1A (*fluorograph*); the results of staining for alkaline phosphatase activity is shown in Fig. 1A (*blot*). Both the fluorograph and the blot stained for phosphatase activity demonstrate the same linear relationship of intensity with respect to the amount of antigen loaded on the original gel (Fig. 1A [*gel*]). When the heavy-chain band regions from a phosphatase-stained blot identical to that shown in Fig. 1A (*blot*) were excised and eluted using the sequential washes described in Materials and Methods, the results shown in Fig. 1, B and C, were obtained. As seen in Fig. 1B, recovery of ¹⁴C-labeled rabbit antigoat IgG antibody was linear with respect to the amount of goat heavy chain loaded on the SDS gel; the vast majority of the eluted antibody was recovered in the pH 2.3 wash with lesser, but nevertheless reproducible, amounts found in both the reequilibration wash after pH 2.3 elution and in the NH₄SCN eluate. Additional experiments have shown that the pH 2.3 eluate has the highest specific activity in antibody titration experiments (P. Fisher, unpublished observation). Overall, ~36 ng of rabbit antigoat IgG antibody was recovered per microgram of goat IgG heavy-chain antigen loaded on the original SDS gel. As shown in Fig. 1C, this represents a recovery of between 80 and 90% of the ¹⁴C-labeled IgG that was originally bound to the blot after the first antibody incubation (determined by counting the nitrocellulose segments in a standard non-aqueous scintillation cocktail).

Immunochemical Specificity of IgG Recovered after Affinity Purification Using Nitrocellulose-bound Antigen

The effectiveness of our affinity selection procedure for the generation of specific IgG fractions was assessed using antisera prepared against the entire NMPCL fraction obtained from *D. melanogaster* embryos. The antigen used to select the antibodies of interest was a total embryo extract prepared by Dounce homogenization of dechorionated whole embryos directly into boiling SDS. After electrophoresis on a prepara-

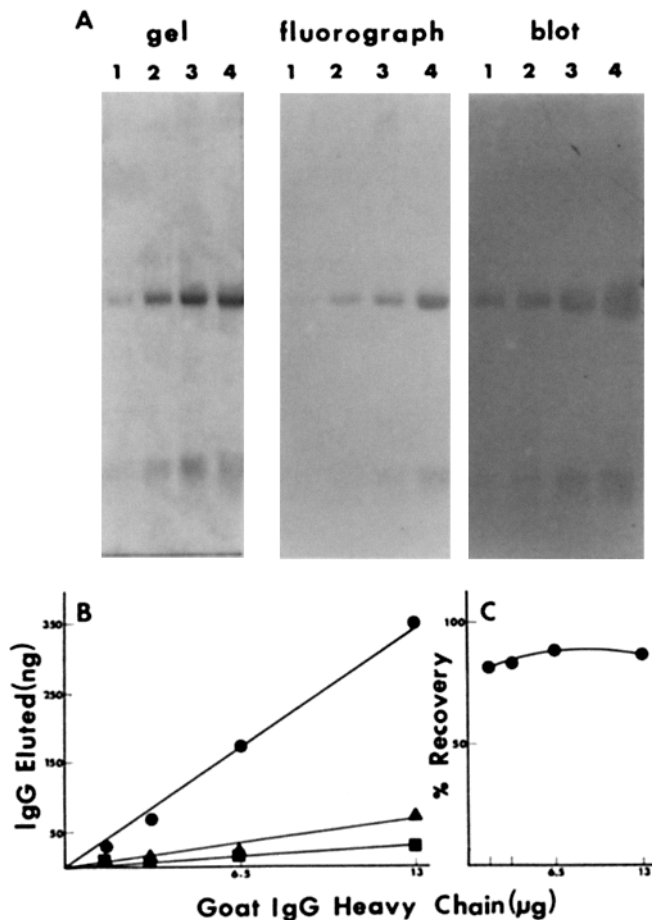


FIGURE 1 Recovery of rabbit antigoat IgG antibody from nitrocellulose-bound goat IgG heavy chain. (A) Purified goat IgG was electrophoresed on an SDS-10% polyacrylamide gel; 2.5, 5, 10, and 20 μg (i.e., 1.6, 3.3, 6.5, and 13 μg of heavy chain) were loaded in lanes 1-4, respectively, of each of three parallel segments. One segment was stained with Coomassie Blue (*gel*); the protein from the two parallel segments was blot transferred onto nitrocellulose. The two resultant blots were each probed with 30 μg of ^{14}C -labeled rabbit antigoat IgG antibody (42,500 cpm/ μg) followed after appropriate washing by standard incubation with calf alkaline phosphatase-conjugated goat antirabbit IgG antibody (see Materials and Methods). After staining for phosphatase activity (15 min, room temperature), heavy-chain band regions from one of the two blots were immediately excised and the bound IgG was eluted; the other segment was dried, impregnated with 2,5-diphenyloxazole (2), and fluorographed on Kodak XAR film for 18 h at -70°C (*fluorograph*). A photograph of the blot stained for phosphatase activity is also shown (*blot*). (B) Equal areas of nitrocellulose containing the heavy-chain band regions from each of lanes 1-4 were excised after probing and staining as described in A and were eluted with 100- μl aliquots of the appropriate elution solutions as described in Materials and Methods. Aliquots from each of the three different elution steps were pooled separately, diluted with an equal volume of water, and counted directly in a standard aqueous scintillation cocktail. (●), pH 2.3 eluate; (▲) TWEEN-PBS eluate; (■) NH₄SCN eluate. Amounts of IgG heavy-chain antigen corresponding to each eluate are as indicated on the abscissa. (C) Percent recovery as a function of the amount of antigen was calculated by counting the residual nitrocellulose segments after elution, and summing that with the total amount recovered in the eluates. The calculated recoveries thus represent the total amount eluted divided by the total of the eluates plus that which remained on the nitrocellulose.

tive (no individual wells) SDS polyacrylamide gel, the polypeptides were blot transferred to a sheet of nitrocellulose. Western blot analysis using pooled anti-native and anti-SDS

denatured NMPCL antisera to probe strips from this blot revealed several major immunoreactive species (Fig. 2, lane S). Six different reactive regions of this strip were excised and eluted; the eluates were then used to reprobe similar strips from the same blot. Numbers above each eluate probed strip correspond to the regions indicated to the left of the serum probed strip.

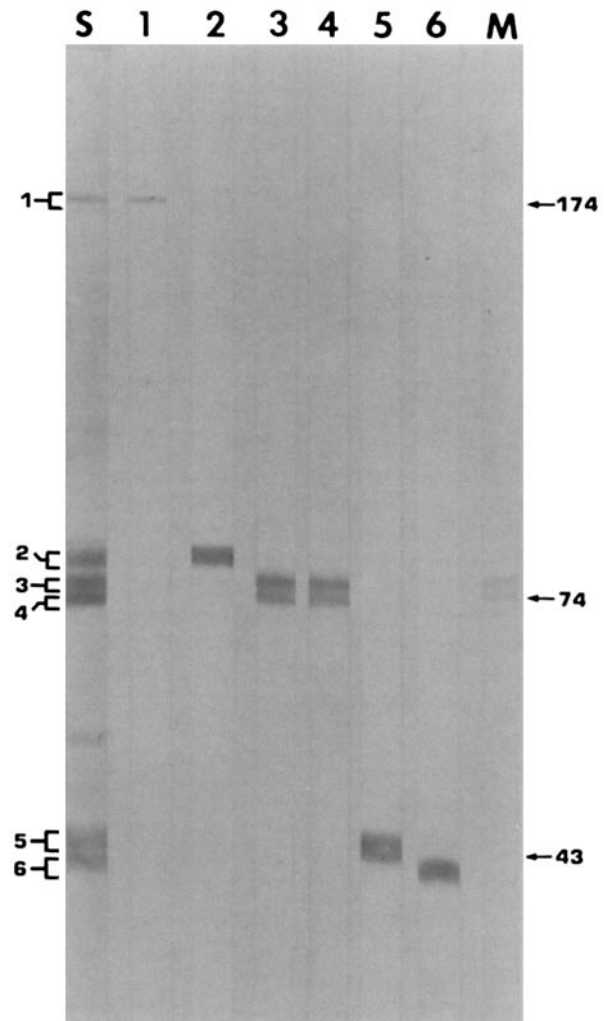


FIGURE 2 Affinity purification of specific antibodies against *Drosophila* embryo proteins. Equal volumes of two separate rabbit antisera raised against either the entire native or SDS-denatured *Drosophila* NMPCL fraction were pooled, and 125 μl of the pooled sera was used to probe each of two 0.75-cm nitrocellulose strips to which proteins of a *Drosophila* embryo lysate had been blot transferred after electrophoresis on an SDS-7% polyacrylamide gel. After standard second antibody probing and phosphatase staining (Materials and Methods, 10 min at room temperature), six regions of the nitrocellulose containing prominent bands were excised from one of the strips and separately eluted using three 100- μl aliquots of the pH-2.3 elution solution (Materials and Methods); the parallel strip was rinsed with water, dried, and photographed as shown in lane S. Labeling to the left of lane S designates those regions excised and eluted from the first strip. The six eluates were then used to probe six additional strips from the same blot, phosphatase staining was for 20-40 min at room temperature; the results are as indicated in lanes designated 1-6. Lane M shows the results of a strip also from the same blot probed with mouse monoclonal antibody T40 (8) ammonium sulfate purified from 10 μl of ascites fluid—phosphatase staining was for 40 min at room temperature; similar results were obtained with two other monoclonals, T50 and U25 (8) (not shown).

In addition to the questions of recovery and general specificity of the blot affinity-purified IgG as demonstrated in Figs. 1 and 2, one of our major concerns in using the blot-elution technique was the resolution with which two closely migrating but antigenically distinct polypeptides could be identified. This problem was elucidated by examination of the results obtained with the eluates from regions 2, 3, and 4 (corresponding to apparent molecular weights of ~79,000, 76,000, and 74,000, respectively) and of regions 5 and 6 of the blot shown in Fig. 2, lane *S*. When the eluate from region 2 was compared with that from region 3, it was apparent that the species migrating in these two closely spaced regions were in fact distinguishable. Eluate 2 was strongly reactive only with a very sharply defined band exactly coincident with the position on the original blot from which the eluate was derived. Neither the major species seen in Fig. 2, lane *S*, immediately below region 2 or the minor band seen immediately above it (apparent molecular weight of ~80,000) were observed to any significant degree in Fig. 2, lane 2. In contrast, eluate 3, although unable to recognize anything at the position of region 2, was apparently specific for two species. One, as expected, was coincident with region 3 whereas the second coincided with region 4. The eluate from region 4, in turn, showed a specificity identical with that from region 3, i.e., polypeptides at the positions of both regions 3 and 4 were recognized. Like the eluate from region 2 and in contrast to the cross-reactivity seen between eluates from regions 3 and 4, eluates from the two adjacent regions 5 and 6 showed little or no cross-reactivity.

As region 4 was coincident with the major 74-kD NMPCL polypeptide previously identified (2), we probed strips from the same embryo lysate blot shown in Fig. 2 with a highly specific polyclonal antiserum raised against the gel-purified 74-kD NMPCL polypeptide. An identical pattern of reactivity as is shown in Fig. 2, lanes 3 and 4, was observed (data not shown). Further, three monoclonal antibodies raised against a nuclear extract of *Drosophila* Kc tissue culture cells and shown to stain salivary gland nuclei in situ (8) all gave patterns of reactivity essentially identical with the eluates from both regions 3 and 4; one such result is shown (Fig. 2, lane *M*).

Comparison of Blot Affinity-purified IgG with Monoclonal Antibodies by Indirect Immunofluorescence

One of the goals in characterizing components of the *Drosophila* NMPCL fraction is to establish their in situ localization; indirect immunofluorescence analysis provides a convenient first-level approach to this problem. The results of indirect immunofluorescence comparing unfractionated antisera raised against the entire NMPCL fraction, blot affinity-purified anti-74- and 76-kD polypeptides IgG and monoclonal anti-74- and 76-kD polypeptides IgG are shown in Fig. 3. Fig. 3, *A* and *B*, shows phase-contrast and fluorescence micrographs, respectively, obtained after reaction of salivary gland tissue from third instar larvae with the crude antisera. Nuclei are barely discernible over a high background of diffuse cytoplasmic staining; both nuclear and cytoplasmic staining were specific with respect to nonimmune controls (data not shown). Reactivity of the anti-74- and 76-kD polypeptides IgG blot affinity-purified from these antisera is shown along with the companion phase-contrast micrographs in Fig. 3, *C–F*. The immunoeluate from the 74-kD region of a total

embryo lysate blot was used to probe either salivary gland tissue (Fig. 3, *C* and *D*) or cryosections (Fig. 3, *E* and *F*) from *Drosophila* third instar larvae. From the salivary gland squash shown in Fig. 3, *C* and *D*, it can be seen that the immunoeluate was highly specific for the salivary gland nuclei; this is similar to results obtained with antisera prepared against the gel-purified 74-kD NMPCL polypeptide (2). Nuclear specificity was confirmed by examination of cryosections which demonstrated that localization was further restricted to the nuclear periphery. This latter staining pattern was identical with that obtained with cryosections probed with any of the three available monoclonal anti-74- and 76-kD polypeptide antibodies; an example of such staining is shown in Fig. 3, *G* and *H*. (It is also noteworthy that, for these experiments, eluates from either the upper or the lower band of the 74- and 76-kD doublet as shown in Fig. 2, lanes 3 and 4, could be used interchangeably.)

Relative Steady-State Levels of the 74- and 76-kD Nuclear Envelope Polypeptides during Embryogenesis

In an attempt to attach some biological significance to the observation that there were apparently two forms of a major *Drosophila* nuclear envelope polypeptide present in embryo lysates, we examined the relative steady-state levels of these two polypeptides through the course of embryonic development. Embryos that were collected shortly after fertilization and oviposition (11) were allowed to progress through development to the point of hatching; at various time points, aliquots were frozen in liquid N₂ and then homogenized directly into boiling SDS. The accumulated samples were subjected to SDS PAGE followed by blot transfer to nitrocellulose. The resultant Western blots were probed either with monoclonal anti-74- and 76-kD polypeptides antibodies (Fig. 4*A*) or with a polyclonal antibody raised against the gel-purified 74-kD species (Fig. 4*B*). The results with either monoclonal or polyclonal reagents were similar and, as shown, demonstrated substantial differences in the relative steady-state levels of the two species of interest. Early in embryogenesis, the 74-kD form seemed to be present nearly exclusively while by the time of larval hatching, the 76-kD polypeptide was present in equivalent or slightly greater abundance. Also noteworthy in this experiment is the observation that, during the course of embryogenesis, there was at most, only a four- to fivefold increase (based on densitometric evaluation) in the total combined amounts of 74- and 76-kD proteins. This is despite the fact that there is an ~25–50-fold increase in the amount of total embryo DNA between hours 2 and 10 of embryonic development and as much as a 100,000-fold increase in the amount of embryo DNA and the number of cell nuclei between the time of fertilization (hour 0) and hatching of the first instar larva (hour 22) (21).

Effect of Heat Shock on the Steady-State Levels of the 74- and 76-kD Drosophila Nuclear Envelope Polypeptides

The well-documented effect of heat shock on nuclear function and composition (see reference 22 for a review; also 23–25) as well as the fact that one of the most abundant hsp's in *Drosophila*, hsp-70, is similar in molecular weight, led us to examine the effect of heat shock on the 74- and 76-kD

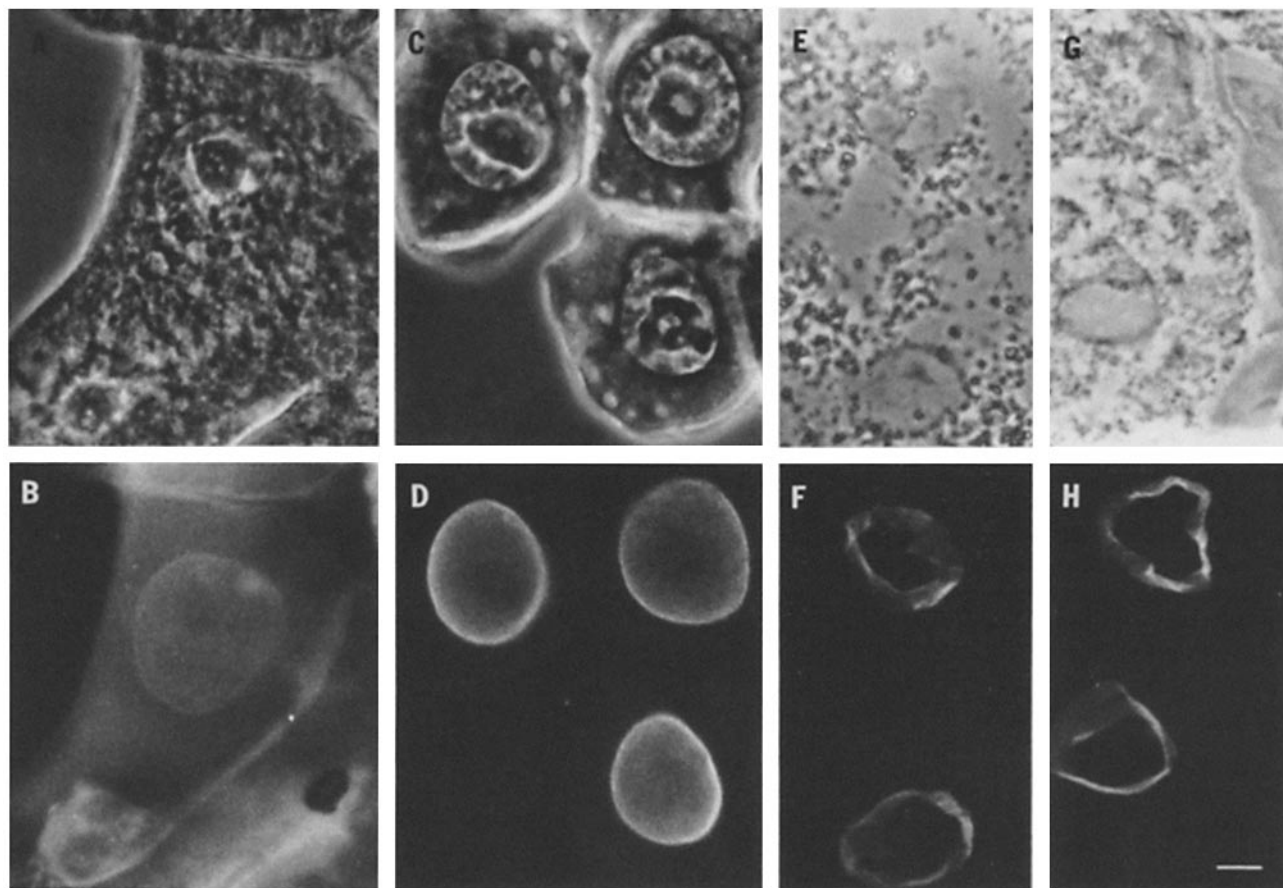


FIGURE 3 Characterization of immunopurified anti-74-kD polypeptide IgG by indirect immunofluorescence microscopy. Salivary gland squashes and 6- μ m-thick cryosections prepared from *Drosophila* third instar larvae were used for analyses. The bar shown in panel H denotes 10 μ m. (A and B) Phase-contrast and fluorescence micrographs, respectively, showing salivary gland tissue probed with a 1:200 dilution of the pooled antisera used to probe the strip shown in Fig. 2 lane S. (C and D) Phase-contrast and fluorescence micrographs of salivary gland tissue probed with a 1:4 dilution of the eluate from region 4 of the strip shown in Fig. 2 lane S; identical results were obtained with the eluate from region 3 (not shown). (E and F) Phase-contrast and fluorescence micrographs of cryosections probed with the region 4 eluate exactly as in C and D; identical results were obtained with the eluate from region 3 (not shown). (G and H) Phase-contrast and fluorescence micrographs of cryosections probed with a 1:200 dilution of monoclonal antibody T40 (8) ammonium sulfate purified from an equal volume of ascites fluid; identical results were obtained with monoclonal antibodies T50 and U25 (8) (not shown). All panels, \times 600.

Drosophila nuclear envelope polypeptides. A heterogeneous population of embryos, 5–20 h in age was exposed to heat shock (36.5°C), and at the times indicated (Fig. 5), aliquots were homogenized in boiling SDS. After SDS PAGE and blot transfer to nitrocellulose, the samples were probed with either monoclonal (Fig. 5A) or polyclonal (Fig. 5B) anti-74- and 76-kD polypeptide antibodies. In response to heat shock, the 76-kD form decreased dramatically with an apparently compensatory increase in the abundance of the 74-kD species. (This increase is more clearly seen in Fig. 5A; the intensity of the response with the polyclonal antiserum is such that the intensity of the lower band before heat shock is already near saturation for the assay system and thus any increases in this lower band response are difficult to appreciate. Experiments performed with lesser amounts of the polyclonal antiserum showed the same compensatory increase seen with the monoclonal antibodies (data not shown).) A blot made in parallel to those shown in Fig. 5 was probed with monoclonal antibodies directed against hsp-70 (26). The results of this experiment demonstrated that the expected heat-shock response had occurred in the embryos and that hsp-70 was distinct

from both the 74- and 76-kD nuclear envelope polypeptides (data not shown).

DISCUSSION

The previous report of Olmsted (1) describing the affinity purification of antibodies using antigens immobilized on diazotized paper blots presented a qualitative characterization of the affinity-purified IgG that was obtained. We have adapted this technology for antigens immobilized on nitrocellulose and have further presented a quantitative assessment of the technique. In using nitrocellulose as the inert support for immobilization of antigen, the problem of essentially irreversible binding of the specific antibody to the nitrocellulose was encountered. However, by using TWEEN 20 as originally introduced by Blake et al. (20), this problem may be avoided entirely. We have therefore employed this detergent in all the solutions used for our procedure and, in so doing, have achieved reproducibly high yields of affinity-purified IgG. Overall, we have been able to recover between 80 and 90% of as little as 60 ng of specific IgG initially bound to an

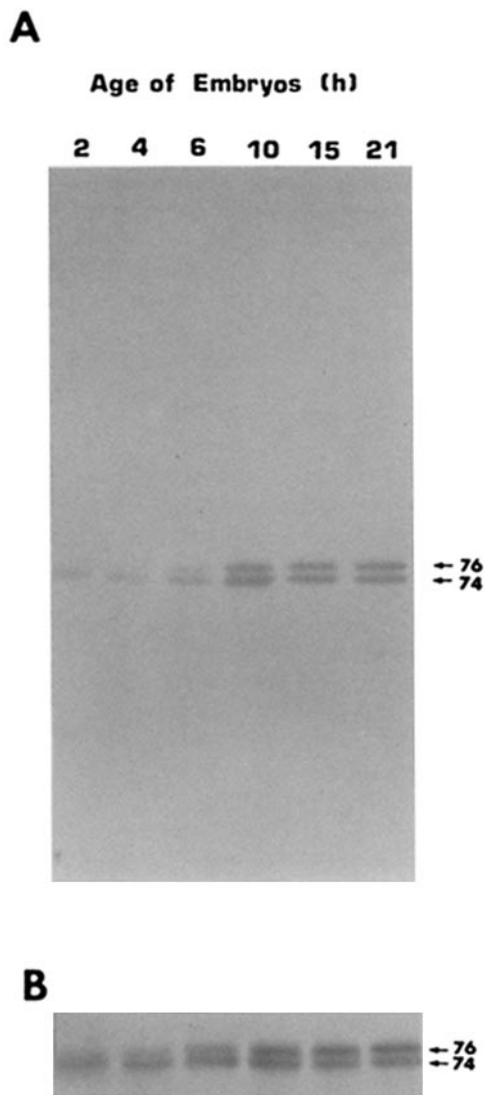


FIGURE 4 Steady-state levels of the 74- and 76-kD nuclear envelope antigen during *Drosophila* embryogenesis. *Drosophila* embryos were collected for 2 h from a well-fed population of adults, washed, and a dechorionated immediately in cold buffers and allowed to age in a well-humidified chamber maintained at 25°C. At various time points, aliquots of the developing embryos were removed and frozen in liquid N₂. The frozen samples were thawed by homogenization directly into boiling SDS, and aliquots from each sample were run on a standard SDS-7% polyacrylamide gel. Protein derived from the equivalent of 1.5 μl of settled dechorionated embryos was loaded in each lane. Polypeptides were blot transferred from the gels onto nitrocellulose. The numbers above each lane refer to the mean age in hours, of the embryos from which the respective protein extracts were derived. The range about each mean is ±1 h reflecting the 2-h collection time. The positions of the 74- and 76-kD polypeptides are indicated by arrows to the right of each panel. (A) The blot was probed with the pooled monoclonal antibodies T40, T50, and U25 in the standard manner; amounts of each antibody used were those ammonium sulfate purified from 10 μl of ascites fluid; phosphatase staining was for 20 min at room temperature. (B) A blot made in parallel to the one shown in (A) was probed with a rabbit antiserum raised against the gel purified 74-kD NMPCL polypeptide. The serum was used at a 1:1,000 dilution; phosphatase staining was for 5 min at room temperature. Only the portion of the blot containing the 74- and 76-kD region is shown.

immobilized antigen and have no reason to think that we are nearing a lower limit in this regard.

The steps used to elute antibody from the nitrocellulose

immobilized antigen include low pH wash, reequilibration to neutral pH, and NH₄SCN wash; when performed sequentially, >75% of the total IgG recovered is in the low pH eluate. Furthermore, in using a quantitative spot titration assay (2), we have been able to show that this IgG is of significantly higher specific antibody activity than that which is present in either of the other two washes (data not shown). However, the observation has also been made that for certain antigens and/or certain sera, the low pH elution and reequilibration have been ineffective and the NH₄SCN has been required in

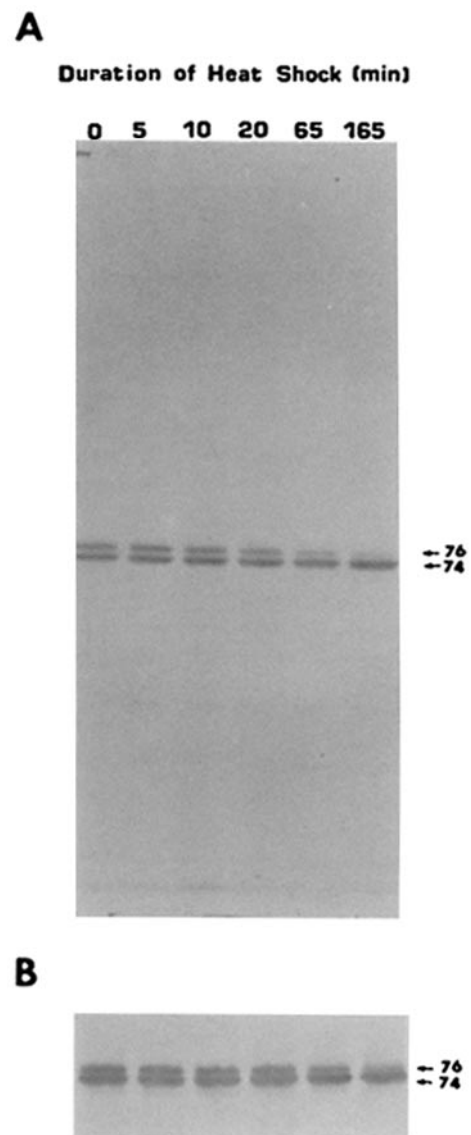


FIGURE 5 Effect of heat shock on the steady state levels of the 74- and 76-kD *Drosophila* nuclear envelope antigen. A heterogeneous population of embryos, 0-15 h in age, was washed and dechorionated in the standard manner, allowed to age for 5 h at 25°C, and then subjected to heat shock by incubation in a well-humidified chamber at 36.5°C. At various time points, aliquots were removed and homogenized directly into boiling SDS; samples were electrophoresed and blot transferred to nitrocellulose essentially as in Fig. 4. The numbers above each lane refer to the duration of heat shock in minutes. The positions of the 74- and 76-kilodalton polypeptides are indicated as in Fig. 4. (A) The blot shown was probed with monoclonal antibodies exactly as in Fig. 4A; phosphatase staining was for 40 min at room temperature. (B) The blot shown was probed with polyclonal rabbit antiserum exactly as in Fig. 4B; phosphatase staining was for 10 min at room temperature. Only a portion of the blot is shown.

order to recover reasonable quantities of active antibody (data not shown). In applying this technique, therefore, we would recommend that all three wash steps be tried and that the respective eluates be characterized in the context of the specific antigen of interest.

The use of an alkaline phosphatase-conjugated second antibody detection system in our procedures offers several advantages over radiolabeled protein A as described by Olmsted (1). Aside from the obvious handling problems of working with radioactive materials, we have also been able to avoid the delay and the expense inherent in autoradiographic detection of the first antibody. Furthermore, and perhaps most importantly, the ability to directly visualize the first antibody before excision from the nitrocellulose introduces a level of resolution that would be difficult to achieve with autoradiographic detection. It is noteworthy that as a result of this direct visualization of first antibody before elution, there is presumably a certain amount of CAP-conjugated second antibody present in the immunoaffinity-purified IgG. Although this would not be a problem in experiments in which the eluted antibody was used for reprobe of nitrocellulose blots, it was of some concern in fluorescence experiments where the CAP-conjugated goat antirabbit IgG antibody might be expected to compete with the fluorescein isothiocyanate-labeled goat antirabbit IgG antibody for binding to the first antibody. In practice however, this does not appear to be a problem, in all probability reflecting the extremely small amounts of CAP-conjugated second antibody that actually contaminate the immunoeluates. (In the event that any problems of this sort are encountered, the addition of nonimmune rabbit IgG to the blot elution buffers would be expected to provide an effective competitor for any CAP-goat antirabbit IgG antibody conjugate that was present.) Finally, it is of interest in this context that the antigen remains irreversibly bound to the nitrocellulose throughout the probing and elution procedure and is in fact stable to treatments as harsh as boiling in SDS (P. Fisher, unpublished observation). Thus, contamination of the affinity-purified IgG with antigen is not a problem and further, it is possible to reuse the eluted nitrocellulose for subsequent affinity purifications.

The high degree of resolution possible in our blot affinity-purification procedure has allowed us to distinguish two polypeptides migrating within 1–2 mm of each other on a one-dimensional SDS polyacrylamide gel. The obvious application of this approach after two-dimensional separations requires no further discussion. In addition, the fact that sufficient quantities of antibody are recovered for fluorescence analyses, as well as reprobing of nitrocellulose blots, makes this approach particularly useful in correlating immunocytochemical observations with polypeptide specificity for a given antibody. In our initial application of this technology to problems of NMPCL characterization, we have been able to obtain highly specific antibodies to a number of previously uncharacterized antigens. Further, it has been possible to demonstrate that two closely migrating species that react with a highly specific antiserum raised against the major 74-kD *Drosophila* NMPCL polypeptide purified by one-dimensional SDS PAGE are antigenically quite similar. This antigenic similarity has also been established using three monoclonal antibodies and, in fact, the immunoeluates obtained using the crudest available serum and totally unfractionated antigen (i.e., total embryo lysate) behave indistinguishably from the monoclonal antibodies based both on Western blot analysis and on indirect immunofluorescence.

It is of some interest that in their initial characterization of the monoclonal antibodies used in this study, Risau et al. (8) found that these reagents recognized a single 80-kD band in Kc cell extracts. It remains to be determined whether the apparent differences in size and in the number of species recognized are due to technical factors in the conditions of SDS PAGE or represent real distinctions between *Drosophila* embryos and tissue culture cells. Regardless of this however, the identification of these antibodies as being specific for the major 74-kD polypeptide present in the NMPCL fraction purified from *Drosophila* embryos suggests that they will be invaluable tools for the elucidation of nuclear structure in this organism.

The results of the indirect immunofluorescence studies presented in this paper demonstrate that the major 74-kD polypeptides found in the *Drosophila* NMPCL fraction are localized exclusively to the nuclear periphery in interphase cells. Analogous experiments using frozen sections of rat liver have previously been used to demonstrate a similar localization for the rat liver lamins (27) and recent results of immunoelectron microscopy have indicated that this localization is in fact restricted to the nuclear lamina (3, 6, 28). (These latter observations are in contrast to other results suggesting that the vertebrate lamin B may in fact be a component of the nuclear pore complex as well [29].) Similar immunoelectron microscopic studies remain to be performed with the *Drosophila* material, but based both on molecular weight and now upon in situ localization at the light microscopic level, it seems reasonable in the interim to regard these major 74- and 76-kD *Drosophila* polypeptides as analogs to vertebrate lamins. The availability of effectively monospecific polyclonal IgG fractions prepared by affinity purification using nitrocellulose blot immobilized polypeptides should greatly facilitate the further elucidation of this problem.

The exact biological significance of the observation that two forms of the major 74-kD *Drosophila* NMPCL antigen are present in whole embryo lysates remains to be determined. Both polypeptides are also found in the NMPCL fraction and subsequently co-purify through differential urea extraction from the NMPCL followed sequentially by chromatography on DEAE-cellulose in the presence of 8 M urea, and Sephacryl S-300 and hydroxylapatite, both in the presence of 0.1 % SDS (P. Fisher, manuscript in preparation). Although results similar to these have been obtained in comparative studies of vertebrate lamins A and C (3–7, 10), the relative biological significance of lamins A and C is only poorly understood and it is therefore difficult to confidently draw direct analogies among species.

Additional experiments with *Drosophila* have shown the steady-state levels of the two forms of the 74- and 76-kD antigen to be differentially regulated both through embryonic development and during heat shock. These differences constitute strong evidence of in vivo significance and are consistent with a precursor-product relationship between the 76- and 74-kD polypeptides, respectively. Immunoprecipitation analyses of in vitro translation products of *Drosophila* mRNA obtained at various stages of development will be necessary in order to rigorously assess this possibility. Nevertheless, this conclusion seems particularly attractive in light of the known patterns of transcription and translation during *Drosophila* embryogenesis (21), as well as the fact that during heat shock, there is little or no transcription or translation other than that directly involved in the synthesis of the heat shock proteins (22).

The changes in the combined steady-state levels of the 74- and 76-kD nuclear envelope polypeptides as well as the differential regulation of their relative abundance during embryogenesis raise several important biological questions. Based on the results shown in Fig. 4, it appears that the fertilized egg is laid by the female with as much as 25% of the 74- and 76-kD protein required to complete embryogenesis already present in some sort of storage form. Velocity sedimentation analyses in linear glycerol gradients have demonstrated that the majority of the 74-kD antigen found in early embryos is soluble (i.e., not associated with nuclei) and has a sedimentation coefficient of about 6 S (K. Doyle and P. Fisher, unpublished observations). (This is approximately the value that would be expected for a reasonably symmetrical homodimer.) Although an examination of changes in the levels of these species before fertilization, i.e., during oocyte maturation, will be required in order to further elucidate this observation, recent studies on the assembly of nuclei in *Xenopus* eggs have led to the suggestion of a similar phenomenon in that organism (30). With respect to *Drosophila*, there are at present, at least two differing hypotheses which may be advanced to guide further investigation. The first is that the 74-kD form represents the product of a maternal gene that is distinct from one coding for the 76-kD species that is ultimately expressed in the embryo. Alternatively, it may simply be that the 76-kD form of the protein is processed to the 74-kD form and that this processing event takes place shortly after synthesis and prior to the earliest time point in our analysis (1–3 h). Given the apparent conversion of the 76-kD species to the 74-kD form during heat shock, the latter mechanism seems plausible. However, the stability of the 76-kD species through the latter half of embryogenesis (Fig. 4) argues strongly for some sort of developmental regulation as well. Further experimentation and perhaps cloning of the gene or genes coding for these proteins will be required before any definitive conclusions can be drawn.

The apparent processing of the 76-kD nuclear envelope polypeptide into the 74-kD form during heat shock is likely of considerable significance. The heat shock response is generally thought to involve major changes in nuclear function and composition (22, 23, 25), and there are indications that the hsp's themselves may in fact be NMPCL components (24). At the present time, it seems premature to speculate as to the specific importance of our current observation; nevertheless, experimental elucidation of this phenomenon promises to be particularly relevant to our overall understanding of the heat shock response as well as to our appreciation of nuclear structure in general.

The preliminary observations that led to the experiments presented in this manuscript were made in collaboration with Dr. G. Blobel in his laboratory at The Rockefeller University; his numerous suggestions and generous encouragement have been invaluable. It is similarly a pleasure to acknowledge the advice and critical reading of this manuscript provided by Dr. M. Berrios and Dr. D. Bogenhagen. We would also like to express our appreciation to Dr. M. Blake for his suggestions concerning the use of TWEEN 20 and phosphatase-conjugated second antibodies and to Dr. P. Symmons and Dr. S. Lindquist for providing the monoclonal antibodies used in this work.

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Note Added in Proof: The recent work of Laliberté et al. (1984, *J. Cell Biol.* 98:980–985) indicates that mammalian lamin A is synthesized as a higher molecular weight precursor. This is consistent with similar results of Gerace et al. (*J. Cell Sci.*, in press) and supports the hypothesis that the 76-kD *Drosophila* nuclear envelope polypeptide is a precursor of the 74-kD species.

REFERENCES

- Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.
- 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.
- 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.
- Lam, K. S., and C. B. Kasper. 1979. Electrophoretic analysis of three major nuclear envelope polypeptides: topological relationship and sequence homology. *J. Biol. Chem.* 254:11713–11720.
- Shelton, K. R., L. L. Higgins, D. L. Cochran, J. J. Ruffolo, and P. M. Egle. 1980. Nuclear lamins of erythrocyte and liver. *J. Biol. Chem.* 255:10978–10983.
- 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.
- 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.
- 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.
- Berrios, M., G. Blobel, and P. A. Fisher. 1983. Characterization of an ATPase/dATPase activity associated with the *Drosophila* nuclear matrix-pore complex-lamina fraction: identification of the putative enzyme polypeptide by direct ultraviolet photoaffinity labeling. *J. Biol. Chem.* 258:4548–4555.
- Berrios, M., A. J. Filson, G. Blobel, and P. A. Fisher. 1983. A 174-kilodalton ATPase/dATPase polypeptide and a glycoprotein of apparently identical molecular weight are common but distinct components of higher eukaryotic nuclear structural protein subfractions. *J. Biol. Chem.* 258:13384–13390.
- Allis, C. D., G. L. Waring, and A. P. Mahowald. 1977. Mass isolation of pole cells from *Drosophila melanogaster*. *Dev. Biol.* 56:372–381.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.
- Jentoft, N., and D. G. Dearborn. 1979. Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J. Biol. Chem.* 254:4359–4365.
- Schaffner, W., and C. Weissman. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56:502–514.
- Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochimistry*, 6:43–52.
- McGadey, J. 1970. A tetrazolium method for non-specific alkaline phosphatase. *Histochemistry*, 23:180–184.
- Coudrier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:469–475.
- Talian, J. C., J. B. Olmsted, and R. D. Goldman. 1983. A rapid procedure for preparing fluorescein-labeled specific antibodies from whole antiserum: its use in analyzing cytoskeletal architecture. *J. Cell Biol.* 97:1277–1282.
- 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.
- Anderson, K. V., and J. A. Lengyel. 1979. Rates of synthesis of major classes of RNA in *Drosophila* embryos. *Dev. Biol.* 70:217–231.
- Ashburner, M., and J. J. Bonner. 1979. The induction of gene activity in *Drosophila* by heat shock. *Cell*, 17:241–254.
- Velazquez, J. M., B. J. DiDomenico, and S. Lindquist. 1980. Intracellular localization of heat shock proteins in *Drosophila*. *Cell*, 20:679–689.
- Levinger, L., and A. Varshavsky. 1981. Heat-shock proteins of *Drosophila* are associated with nuclease-resistant, high-salt-resistant nuclear structures. *J. Cell Biol.* 90:793–796.
- Velazquez, J. M., and S. Lindquist. 1984. HSP 70: Nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell*, 36:655–662.
- Velazquez, J. M., S. Sonoda, G. Bugaisky, and S. Lindquist. 1983. Is the major *Drosophila* heat shock protein present in cells that have not been heat shocked? *J. Cell Biol.* 96:286–290.
- Krohne, G., W. W. Franke, S. Ely, A. D'Arcy, and E. Jost. 1978. Localization of a nuclear envelope-associated protein by indirect immunofluorescence microscopy using antibodies against a major polypeptide from rat liver fractions enriched in nuclear envelope associated material. *Cytobiologie*, 18:22–38.
- Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826–837.
- Stick, R., and G. Krohne. 1982. Immunological localization of the major architectural protein associated with the nuclear envelope of the *Xenopus laevis* oocyte. *Exp. Cell Res.* 138:319–330.
- Forbes, D. J., M. W. Kirschner, and J. W. Newport. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into *Xenopus* eggs. *Cell*, 34:13–23.