Heat Shock-induced Changes in the Structural Stability of Proteinaceous Karyoskeletal Elements In Vitro and Morphological Effects In Situ

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Abstract. Karyoskeletal protein fractions prepared from *Drosophila melanogaster* embryos contain morphologically identifiable remnants of nuclear pore complexes and peripheral lamina as well as what appears to be an internal nuclear "matrix" (Fisher, P. A., M. Berrios, and G. Blobel, 1982, *J. Cell Biol.*, 92: 674–686). Structural stability of these proteinaceous assemblies is dependent on thermal incubation in vitro (37°C, 15 min) before subfractionation of nuclei. In the absence of such incubation, >90% of the total karyoskeletal protein including major polypeptide components of internal "matrix," pore complexes, and the peripheral lamina, is solubilized by 1 M NaCl. In vivo heat shock induces karyoskeletal stabilization resembling that resulting from thermal incubation in vitro. Immunocytochemical studies have been used to establish the effects of heat shock on the organization and distribution of major karyoskeletal marker proteins in situ. Taken together, these results are consistent with the notion that in vivo, regulation of karyoskeletal plasticity (and perhaps form) may be a functionally significant component of the *Drosophila* heat shock response. They also have broad practical implications for studies pertaining to the structure and function of karyoskeletal protein (nuclear "matrix") fractions isolated from higher eukaryotic cells.

INSOLUBLE proteinaceous fractions may be obtained from cell nuclei by low-speed centrifugation after nuclease digestion and sequential extraction with nonionic detergents and NaCl. When prepared from higher eukaryotic cells, all such fractions contain morphologically identifiable remnants of the peripheral nuclear lamina to which nuclear pore complexes are apparently attached (Aaronson and Blobel, 1974, 1975; see Franke, 1974 for a review of the earlier literature; see Fisher, 1987*a* for a more recent review). A residual intranuclear meshwork of proteinaceous fibrils, here termed the internal nuclear "matrix," is also observed in a number of preparations from organisms as diverse as mammals and yeast (see for examples, Berezney and Coffey, 1977; Capco et al., 1982; Fisher et al., 1982; Potashkin et al., 1984; Wu et al., 1987; see Berezney, 1984; Fisher, 1987*a* for reviews). Franke and colleagues have adopted the term karyoskeleton and suggested that these isolated proteinaceous structures are derived, at least in the case of the residual nuclear lamina and pore complexes, from bona fide karyoskeletal elements of the intact cell (Benavente et al., 1984; see also Franke, 1987). The recently discovered homologies between the nuclear lamins A and C and intermediate filament proteins such as vimentin (McKeon et al., 1986; D. Fisher et al., 1986; see Franke, 1987 for a recent review) suggests that some karyoskeletal elements may in fact be directly related, in an evolutionary sense, to well-characterized elements of the cytoskeleton. These results have recently been elegantly complemented morphologically by electron microscopic analyses of lamin proteins and lamina fibrils in vitro (Aebi et al., 1986).

Work in our laboratory has focused on the identification and characterization of proteins associated with karyoskeletal fractions prepared from *Drosophila melanogaster*. Major polypeptide components of the nuclear lamina (lamins) (Smith and Fisher, 1984; Smith et al., 1987), nuclear pore complexes (gp-188, a 188-kD glycoprotein) (Filson et al., 1985), and putative internal matrix (DNA topoisomerase II) (Berrios et al., 1985) have been localized in situ and characterized in vitro. The nuclear lamins are ubiquitous among higher eukaryotes as is gp-188. (The rat liver homologue is apparently slightly larger and has been designated gp-190 [Gerace et al., 1982].) Similarly, evidence indicates that DNA topoisomerase II is a significant component of nuclear matrix fractions from calf thymus (Halligan et al., 1985) as well as mitotic chromosome scaffolds from chickens and humans (Earnshaw et al., 1985; Earnshaw and Heck, 1985; Gasser et al., 1986).[1]

1. While the inclusion of DNA topoisomerase II in this group of karyoskeletal proteins might be considered premature, there is little doubt regarding the others. The *Drosophila* lamins are among the most abundant polypeptides in the nuclear matrix–pore complex–lamina (NMPCL) fraction, are immunochemically homologous to all three rat liver lamins as well as to the

Recently, several reports have noted the effects of heat on the structure and composition of karyoskeletal protein fractions. Mirkovitch et al. (1984) first reported that the use of divalent cations (Ca^{2+}) to stabilize type I chromosome scaffolds (Lebkowski and Laemmli, 1982) from *Drosophila* tissue culture cells could be obviated by brief incubation in vitro at 37°C. Laemmli and colleagues have subsequently utilized mild thermal incubation in vitro as a technique to stabilize karyoskeletal preparations in order to characterize specific DNA attachment sites (Gasser and Laemmli, 1986*a*, *b*). In a more systematic study of the proteins involved, Evan and Hancock (1985) reported that the apparent association of oncoprotein $p62c^{myc}$ with human karyoskeletal preparations was similarly dependent on heat treatment in vitro and could also be promoted by heat shock in vivo.

The controversy surrounding the biological significance of the internal nuclear matrix is due at least in part to the differences encountered by different investigators attempting to prepare nuclear matrix fractions under slightly varied conditions and from different organisms. It seemed likely that a detailed and systematic analysis of karyoskeletal stability as a function of heat treatment in vitro might provide useful insights into a potentially critical operational parameter. The apparent response of karyoskeletal elements to heat shock in vivo suggested that insights into the molecular cell biology of heat shock might also be obtained. Our current findings demonstrate that karyoskeletal resistance to solubilization by nonionic detergents and NaCl can be induced as an artifact of heat treatment in vitro, and that similar effects occur upon heat shock in vivo. A detailed immunocytologic analysis of morphologic effects in situ has also been performed.

Materials and Methods

Materials

Triton X-100 was from New England Nuclear, Boston, MA. DNAse I, phenylmethylsulfonyl fluoride (PMSF), *N*-ethyl maleimide (NEM), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), DL-dithiothreitol (DTT), and polyoxyethylene sorbitan monolaureate (Tween 20) 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 Western blots was also from Sigma Chemical Co. (Type VII S). 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. Nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH. Sodium dodecyl sulfate (SDS) was from British Drug House, Poole, United Kingdom. Acrylamide was from Eastman Kodak Co., Rochester, NY.

Xenopus lamins (P. Fisher, unpublished observations), are recognized by a human autoimmune antiserum with demonstrable specificity for the mammalian lamins (McKeon et al., 1983), share sequence homology with mammalian vimentin (Slaughter and Fisher, unpublished observation) similar to that recently reported for human lamins A and C (McKeon et al., 1986; D. Fisher et al., 1986), disassemble and reassemble with the nuclear envelope during mitosis (Berrios et al., 1985), and have been specifically localized to the nuclear lamina by immunoelectron microscopy (Smith et al., 1987). Similarly, the 188-kD *Drosophila* NMPCL glycoprotein (gp-188) is homologous, based on biochemical, immunochemical, and immunocytologic data (Filson et al., 1985), to the rat liver glycoprotein (gp-190) localized to the nuclear pore complex by Gerace et al. (1982). At this point, both the nuclear lamina and pore complexes are generally thought to be genuine elements of the karyoskeleton in vivo and the nuclear lamins and gp-188 fulfill essentially all the criteria by which karyoskeletal proteins are defined.

Antibodies

Specific IgG fractions were from Cappel Laboratories, Cochranville, PA. Monoclonal antibodies AGP-26 and AGP-78, directed against the *Drosophila* nuclear pore complex glycoprotein (gp-188), were ammonium sulfate purified from hybridoma culture supernatants (Filson et al., 1985). Monoclonal antibodies T40, T50, and U25 directed against the *Drosophila* lamins were the generous gift of Dr. Peter Symmons, University of Tübingen, Federal Republic of Germany (Risau et al., 1981). Polyclonal antibodies against the *Drosophila* lamins were prepared (Fisher et al., 1982) and affinity purified either as previously (Smith and Fisher, 1984) or as more recently described (Smith et al., 1987). Polyclonal anti-DNA topoisomerase II antisera was either as specified previously (Berrios et al., 1985) and was the generous gift of Dr. Neil Osheroff, Vanderbilt University, Nashville, TN, or was raised in our laboratory against the SDS-hydroxylapatite-purified topoisomerase II subunit identified by immunoblot analyses using the original anti-topoisomerase II antiserum. The new anti-topoisomerase II antiserum was prepared by injecting SDS-PAGE-purified antigen exactly as previously described (Fisher et al., 1982). Anti-topoisomerase II antisera were demonstrated to be highly specific by affinity purification experiments as previously (Berrios et al., 1985). Both anti-topoisomerase II antisera gave identical results on Western blots, as well as by indirect immunofluorescence and both were effective at neutralizing the enzymatic activity of DNA topoisomerase II in vitro. They were therefore used interchangeably. Monoclonal antibodies directed against *Drosophila* hsp-70 were the generous gift of Dr. Susan Lindquist, University of Chicago.

Methods

Most of the methods have been described previously. *Drosophila melanogaster* (Oregon R, P2 strain) were grown in mass culture according to Allis et al. (1977). *Drosophila* Schneider 2 tissue culture cells (Schneider, 1972) were maintained in monolayer culture as previously (Smith et al., 1987). SDS-PAGE was according to Laemmli (1970) and proteins were transferred to nitrocellulose passively (Fisher et al., 1982). Blots were probed with antisera or specific IgG fractions and bands of antibody reactivity were visualized 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 of alkaline phosphatase activity on blots was according to McGadey (1970). Additional experimental details are provided in the figure legends.

Indirect Immunofluorescence Microscopy

Indirect immunofluorescence analyses were performed using permeabilized whole cells and cryosections as previously described (Fisher et al., 1982; Smith and Fisher, 1984). All data presented were obtained with larval cryosections. This material was considered optimal for a number of reasons. First, favorable cryosections of the larger nuclei present at this stage of development allowed for an unambiguous assignment of antigens either to the nuclear periphery or nuclear interior as well as maximal resolution of intranuclear detail in the localization of DNA topoisomerase II. Such resolution was not possible with whole mount preparations derived either from embryos or tissue culture cells (e.g., compare Fisher et al., 1982 with Smith and Fisher, 1984; Berrios et al., 1985). Secondly, cryosections of whole larvae allowed us to examine essentially all of the cell and tissue types present in the organism in a single preparation and thus exclude any tissue-specific effects. (In this respect, we feel that third instar larvae are similar to embryos. Embryos are nothing more than premature first instar larvae and except for absolute size, the later stage embryos with which we performed the bulk of our biochemical analyses resemble the third instar stage both anatomically and histologically.) Thirdly, and most importantly, cryosections were generated in a way that we feel minimizes artifactual redistribution of antigens. Living larvae are quick-frozen in embedding medium by immersion directly into liquid nitrogen and blocks are sectioned immediately or stored for brief periods of time at −70°C. Sections are then stored at −70°C until use. In our experience, samples have been stored for up to 2 yr in this way without obvious deterioration. All of the cryosections used in the current analysis were used within 2–4 wk of their initial preparation. Upon removal from the −70°C freezer, cryosections were thawed directly into 3.7% formaldehyde in 140 mM NaCl, 10 mM $KHPO_4$, pH 7.5 (PBS). The sections were fixed for 2 min, washed briefly in PBS alone, and then probed immediately with specific antibodies. There were therefore no lengthy permeabilization steps nor were there any treatments with nonphysiologic solvents such as acetic acid or ethanol. These solvents were a necessary part of the fixation and permeabilization procedure when whole-mount specimens were used. We therefore feel that cryosections offer the minimal poten-

tial for generation of morphologic artifacts in vitro. Nevertheless, all results obtained with cryosections for both the lamins and DNA topoisomerase II were confirmed using Schneider 2 tissue culture cells and various whole-cell preparations obtained both from third instar larval tissues and from embryos. These materials were fixed and permeabilized by sequential treatments with PBS plus 3.7% formaldehyde, 45% acetic acid plus 3.7% formaldehyde, 95% ethanol, and PBS. Results in all cases were independent of the source material or of the specific immunofluorescence technique employed.

Preparation of the Drosophila Nuclear Matrix–Pore Complex–Lamina (NMPCL)[2] Fraction

All of the various extraction conditions reported in this paper were explicitly derived from our standard protocol for preparation of the NMPCL fraction from 6–18-h-old embryos as previously described (Fisher et al., 1982). All procedures were performed at 4°C unless otherwise indicated. 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 $MgCl_2$, and protease inhibitors NEM (2.5 mM), PMSF (1 mM), and TPCK (1 mM). 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. The purified nuclear pellet was resuspended in 1 vol of nuclease digestion buffer (buffer B) containing 20 mM Tris HCl and 5 mM $MgCl_2$. Nuclei were digested for 15 min at 37°C or at 23°C as indicated with 10 μg/ml DNAse I and 8 μg/ml RNAse A. Nuclease digestion at the different temperatures was monitored and judged effective by the loss of nuclear viscosity after SDS denaturation. Residual proteinaceous structures were recovered by centrifugation at 2,000 *g* for 10 min. The pellet was resuspended in 0.9 vol of buffer C containing 10 mM Tris HCl, pH 7.5, 0.1 mM $MgCl_2$, 290 mM sucrose; 0.1 vol of 20% (vol/vol) Triton X-100 was added and the suspension was incubated for 10 min on ice. Residual proteinaceous structures were recovered by centrifugation at 2,000 *g* for 10 min. The pellet was resuspended in 0.5 vol of buffer C supplemented with Tris HCl, pH 7.5, to a final concentration of 100 mM; 0.5 vol of 2 M NaCl was then added (final salt concentration of 1 M), and the suspension was incubated for 10 min on ice. Residual proteinaceous structures were recovered by centrifugation at 10,000 *g* for 10 min and the salt extraction step was repeated once. The final NMPCL pellet fraction was resuspended in 1 volume of buffer C and denatured immediately by addition of boiling SDS.

All key experiments were performed using both fresh and frozen embryos, as well as *Drosophila* Schneider 2 tissue culture cells. In light of reports that the stability of nuclear matrix preparations from some mammalian sources may involve oxidative artifacts (Kaufmann et al., 1981), experiments were also repeated both plus and minus the reducing agent, DTT, as well as with and without 2% Triton X-100 included in the original extraction buffer (buffer A). It was found that Triton X-100 was necessary to insure complete cell breakage during Dounce homogenization when fresh embryos or tissue culture cells were used. Cell breakage was essentially 100% in the absence of Triton X-100 when frozen embryo starting material was employed. No significant differences were observed in comparing fresh vs. frozen embryos, either plus or minus DTT or plus or minus Triton X-100, or in comparing embryos with tissue culture cells. All results shown were obtained with quick-frozen embryos, Dounce homogenized in buffer A in the absence of Triton X-100.

Results

The Polypeptide Composition of Drosophila Embryo Karyoskeletal Fractions Depends upon Incubation at 37°C during Preparation As Well As on the Stage of Fractionation at Which Thermal Incubation Is Performed

The *Drosophila* embryo NMPCL fraction is typically prepared by digestion of purified nuclei with DNAse I and RNAse A for 15 min at 37°C (Fisher et al., 1982). Subsequent extraction with Triton X-100 removes the lipidic component of the nuclear membranes as well as a minor amount of protein, presumably associated with those membranes. Salt extraction (1 M NaCl) results in quantitative solubilization of histones and partial solubilization of a number of putative karyoskeletal proteins (see below). Nevertheless, ~40% of the total nuclear protein (2% of the embryo protein) remains associated with the NMPCL fraction (Fisher et al., 1982). These results are shown as a control in Fig. 1 *A*. In the experiment shown, typical NMPCL morphology (Fisher et al., 1982) was observed by phase-contrast microscopy (not shown). Clearly defined pore complexes and a peripheral lamina can be routinely observed in these preparations upon appropriate electron microscopic analysis (Fisher et al., 1982). When nuclei were digested with nucleases for 15 min at 23°C instead of 37°C, different results were obtained. >90% of the total nuclear protein was solubilized by sequential extraction with 2% Triton X-100 and 1 M NaCl (Fig. 1 *A*). No residual structures were identifiable by phase-contrast microscopy (not shown). Comparable electron microscopic analyses have not been performed.

The extractability of specifc NMPCL marker proteins was monitored by Western blot analyses on these same subcellular fractions (Fig. 1 *B*). When nuclei were digested with nucleases at 37°C, ~60% of the *Drosophila* lamins was salt soluble as was 5–10% of the gp-188 (Filson et al., 1985) and 20–40% of the DNA topoisomerase II (Berrios et al., 1985). These results are shown as controls in Fig. 1 *B*. When comparable fractions from nuclei digested at 23°C were analyzed, 80–100% of each of these major NMPCL marker proteins was found to be soluble in 1 M NaCl (Fig. 1 *B*). Further analysis by velocity gradient centrifugation after salt extraction from the NMPCL fraction indicated that the lamins behaved homogeneously with a sedimentation coefficient of ~6 S (data not shown). Sedimentation analyses of gp-188 and DNA topoisomerase II have not been performed.

Thermal incubation experiments initially performed with purified nuclei were repeated using crude embryo homogenates. Results from these experiments are shown in Fig. 2. Subcellular fractionation of total protein was monitored by SDS-PAGE (Fig. 2 *A*). The subcellular fractionation of specific karyoskeletal polypeptides was followed using Western blot analyses (Fig. 2 *B*). SDS-PAGE comparison of the NMPCL fractions derived from nuclei that had been rigorously purified before thermal incubation with those obtained from crude extracts subjected to similar heat treatments revealed striking differences (Fig. 2 *A*). NMPCL fractions prepared after thermal incubation in the crude extract (Fig. 2 *A*, lanes *c*) at either 23 or 37°C, contained ~10–20 times as much total protein as NMPCL fractions prepared after incubation of purified nuclei (Fig. 2 *A*, lanes *n*). As was observed with the purified nuclei, 37°C incubation of the crude homogenate led to a substantial increase in the amount of protein ultimately associated with the NMPCL fraction, relative to that obtained after 23°C incubation of the crude homogenate.

Despite the dramatic differences in the fractionation of total embryo proteins, marker-protein fractionation results obtained after thermal incubation of crude extracts (Fig. 2 *B*) were essentially identical with those obtained when purified nuclei were employed (compare Fig. 2 *B* with Fig. 1 *B*). In-

2. *Abbreviation used in this paper*: NMPCL, nuclear matrix-pore complex-lamina.

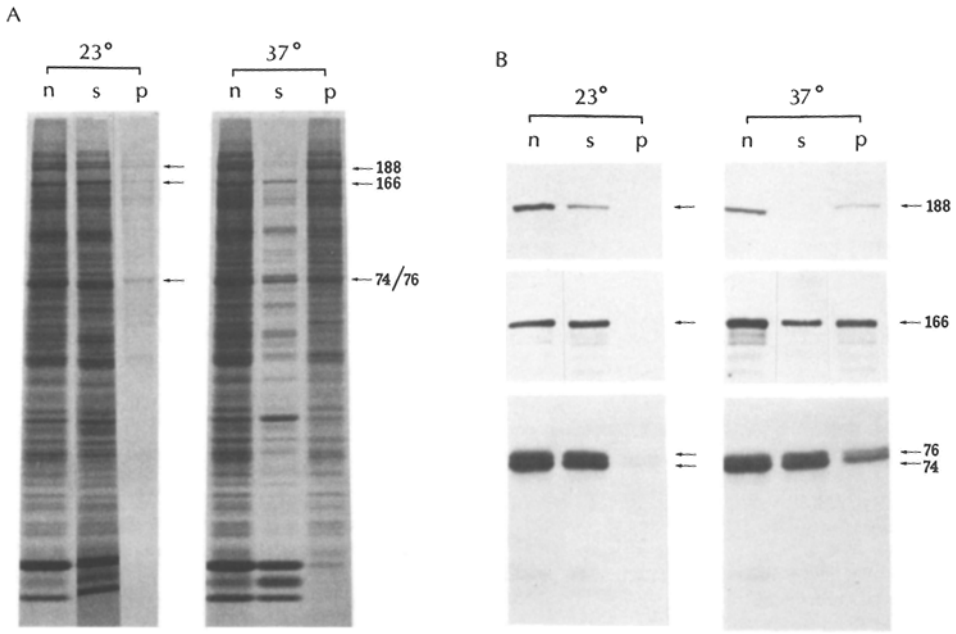


Figure 1. SDS-PAGE and Western blot comparison of nuclear protein fractionation after nuclease digestion at 23 vs. 37°C. (*A*) Coomassie Blue-stained gels. NMPCL fractions were prepared as described (see Materials and Methods) with nuclease digestion performed at either 23 or 37°C as indicated. Fractions loaded in each lane: *n*, purified nuclei; *s*; pooled supernatant fractions from nuclease digestion, Triton X-100 extraction, and two sequential NaCl extractions; *p*, residual NMPCL pellet fraction following complete extraction. Equivalent amounts (20 U, see Materials and Methods) of each fraction were loaded in each lane. Electrophoresis was on an SDS-7–15% polyacrylamide gradient gel. Gels were stained with Coomassie Blue, destained, and photographed. Arrows to the right of each panel indicate the migration position of gp-188 (*188*), DNA topoisomerase II (*166*), and the *Drosophila* lamins (*74/76*). (*B*) Western blot comparison of karyoskeletal marker-protein fractionation from nuclei after nuclease digestion at 23 vs. 37°C. Aliquots of those fractions shown in *A* were electrophoresed on an SDS-7% continuous concentration polyacrylamide gel. Equivalent amounts (8 U) of each fraction were loaded in the individual lanes. After electrophoresis, proteins were blot transferred to nitrocellulose. Two identical sets of blots were prepared. One was divided into two portions, the top third (indicated by the marker arrow, *188*) was probed with pooled monoclonal anti-gp-188 antibodies AGP-26 and AGP-78, each at a dilution of 1:50 relative to the tissue culture supernatant from which they were derived; the bottom third (indicated by marker arrows, *74* and *76*) was probed with affinity-purified anti-lamin antibodies at a final dilution of 1:2,000 relative to the antiserum from which they were purified; identical results were obtained with monoclonal anti-lamin antibodies (not shown). The second set of blots was probed with anti-DNA topoisomerase II antiserum (indicated by the marker arrow, *166*) at a final dilution of 1:1,000. Only the regions of interest on all three blots are shown. Blots were developed in the phosphatase stain reagents for 10–20 min.

cubation of crude homogenates at 37°C before nuclear isolation and subfractionation resulted in the stable association of the majority of cellular DNA topoisomerase II and gp-188, and ~50% of the nuclear lamins with a detergent-resistant, high salt-resistant NMPCL fraction. In the absence of 37°C incubation, all of these NMPCL proteins were nearly completely solubilized by 1 M NaCl.

In order to determine whether or not the effects of varying the temperature of nuclease incubation were related to a direct effect of temperature on the activity of the nucleases, nuclei were prepared as usual, and incubated before the addition of nucleases for various times, either at 37°C, or on ice. The various aliquots were then all incubated with nucleases for 15 min at 23°C and extracted with Triton X-100 and NaCl as above. Two control fractions were digested immediately with nucleases at either 23 or 37°C, without preincubation (as in Fig. 1) and then processed similarly. SDS-PAGE analysis and Coomassie Blue staining were performed as in Figs. 1 and 2 (not shown). As presented in Fig. 1, without specific preincubation, and after 23°C nuclease digestion, nuclear protein was solubilized nearly quantitatively by detergent and salt extraction; the NMPCL fraction was largely devoid of protein. Preincubation of nuclei for 45 min on ice resulted in a significant increase in the amount of NMPCL protein observed after nuclease digestion, and detergent and salt extraction (not shown). As the proportion of the preincubation time spent at 37°C was increased to 45 min, there was a further increase in the amounts of NMPCL protein obtained (not shown). Results obtained after 45 min of preincubation at 37°C, followed by 23°C incubation with nucleases were identical to those obtained without preincubation, but after 37°C nuclease incubation (see Fig. 1 *A*, 37°C, lane *p*). Examination of supernatant fractions revealed a compensatory decrease in extractable protein as a result of 37°C preincubation; the fractionation of the histones seemed unaffected by the varying conditions of nuclear preincubation (not shown).

It also appeared that preincubation on ice alone had a demonstrable effect similar to that observed with 37°C preincubation. This is in apparent contrast to results obtained by Evan and Hancock (1985) who reported that incubation of nuclear fractions at 5°C for as long as 16 h did not promote insolubilization of p62c^{myc} and that temperatures of at least 35°C were necessary for an effect in vitro.

Western blot analyses of the NMPCL fractions generated in the nuclear preincubation experiment described above are shown in Fig. 3. NMPCL pellet fractions were probed with antibodies directed against *Drosophila* gp-188 (Fig. 3 *A*, *top*), DNA topoisomerase II (Fig. 3 *B*) and the *Drosophila* lamins (Fig. 3 *A*, *bottom*). Results for each polypeptide were similar to each other and reflected the overall pattern of protein fractionation seen by Coomassie Blue staining as described above (not shown). Some loss of the two larger polypeptides, perhaps due to proteolysis, was observed during the nuclear preincubation at 37°C.

Karyoskeletal Stabilization In Vitro Can Be Induced by Heat Shock In Vivo

We have previously reported the apparent conversion of lamin Dm_2 (apparent mass of 76 kD) into lamin Dm_1 (apparent mass of 74 kD) during heat shock; in these experi-

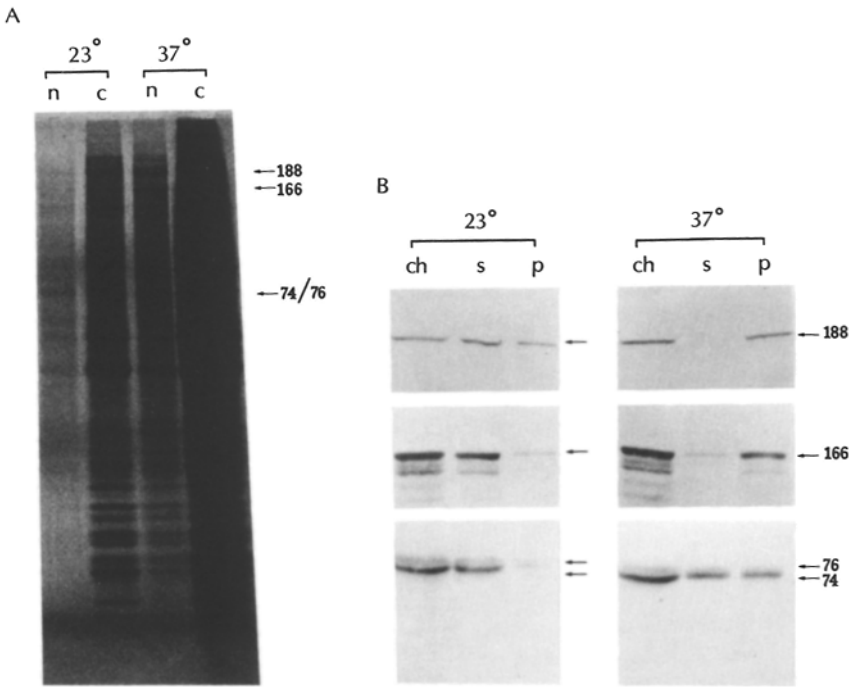


Figure 2. SDS-PAGE and Western blot comparison of NMPCL fraction polypeptide composition after thermal incubation of the crude homogenate vs. incubation of purified nuclei. (*A*) SDS-7–15% polyacrylamide gradient PAGE; equivalent amounts (20 U) were loaded in each lane. NMPCL fractions were prepared after nuclease digestion of purified nuclei (lanes *n*) or crude homogenates (lanes *c*) at either 23 or 37°C as indicated. Gels were stained with Coomassie Blue, destained, and photographed. Marker positions are as previously. *23°C*, lane *n* and *37°C*, lane *n* were loaded with aliquots of the identical NMPCL pellet fractions shown in Fig. 1 *A*, lanes *p* generated at 23 and 37°C, respectively. (*B*) Western blot analysis of karyoskeletal marker–protein fractionation after thermal incubation of crude embryo extracts. Embryos were Dounce homogenized in the standard manner and the filtered crude homogenate was incubated with nucleases at concentrations similar to those used to digest purified nuclei. Digestion was either at 23 or 37°C as indicated. After digestion, a NMPCL fraction was generated by centrifugation and extraction with Triton X-100 and NaCl, exactly as described when purified nuclei were used as the starting material (see Materials and Methods). Equivalent amounts (12 U) of crude homogenate (lanes *ch*), pooled supernatants (lanes *s*), and NMPCL pellet (lanes *p*) were electrophoresed as in Fig. 1 *B* and transferred to nitrocellulose. Two identical sets of blots were prepared. Blots were probed with AGP-26 and AGP-78 (*top*); anti–topoisomerase II (*middle*), and affinity-purified anti–lamin antibodies (*bottom*) exactly as described in the legend of Fig. 1 *B*. Marker positions are indicated as previously.

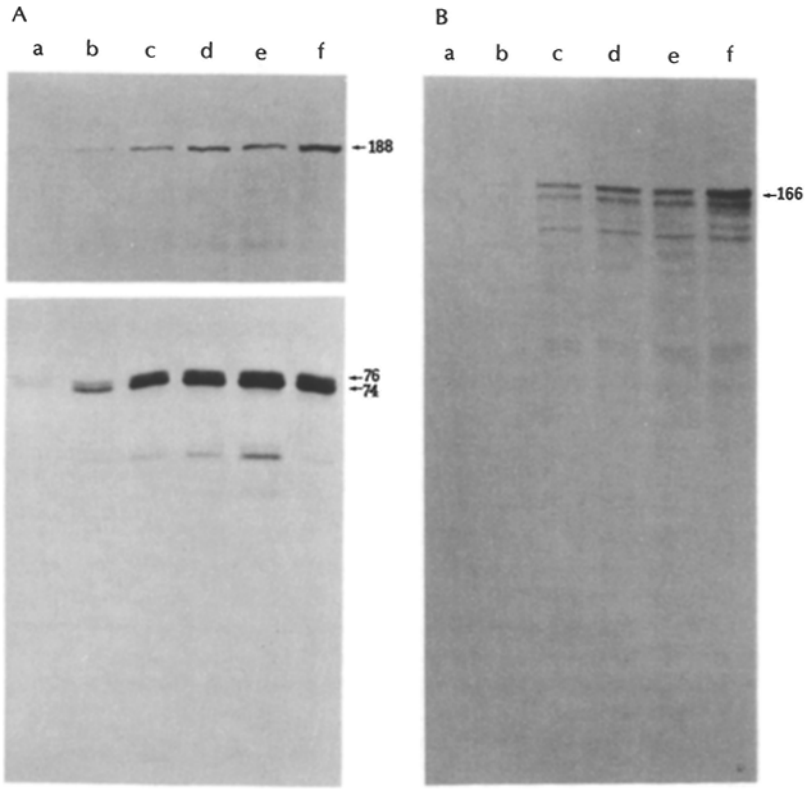


Figure 3. Western blot analysis of karyoskeletal marker–protein fractionation from nuclei after 37°C preincubation and digestion with nucleases at 23°C. Aliquots of NMPCL fractions prepared after thermal preincubation of purified nuclei at 37°C followed by nuclease digestion at 23°C except as indicated (lane *f*), were electrophoresed on an SDS-7% continuous concentration polyacrylamide gel. Equivalent amounts (12 U) were loaded in each lane. After electrophoresis, proteins were blot transferred to nitrocellulose. Two identical sets of blots were prepared. Lane headings are as follows: lane *a*, no preincubation; lane *b*, 45 min on ice; lane *c*, 30 min on ice followed by 15 min at 37°C; lane *d*, 15 min on ice followed by 30 min at 37°C; lane *e*, 45 min at 37°C; lane *f*, NMPCL pellet fraction prepared from nuclei after nuclease digestion at 37°C, exactly as indicated in Fig. 1 and without any preincubation. (*A*) Upper third was probed with monoclonal anti–gp-188 antibodies, AGP-26 and AGP-78, pooled, and diluted exactly as in Fig. 1 *B*. Bottom two-thirds was probed with pooled monoclonal anti–lamin antibodies T40, T50, and U25 (Risau et al., 1981) each diluted 1:1,000 relative to the ascites fluid from which they were originally derived. Identical results were obtained with affinity-purified polyclonal antibodies (not shown). (*B*) Entire blot was probed with anti–topoisomerase II antiserum exactly as in Fig. 1 *B*.

ments, there was no change in the total amount of lamin protein detected in crude embryo extracts, even after prolonged heat shock (Smith and Fisher, 1984). We have recently been able to demonstrate that this interconversion of lamin isoforms during heat shock is the result of an apparently specific dephosphorylation event (Smith et al., 1987). When similar analyses of gp-188 and DNA topoisomerase II were performed after varying periods of heat shock (36.5°C) in vivo,

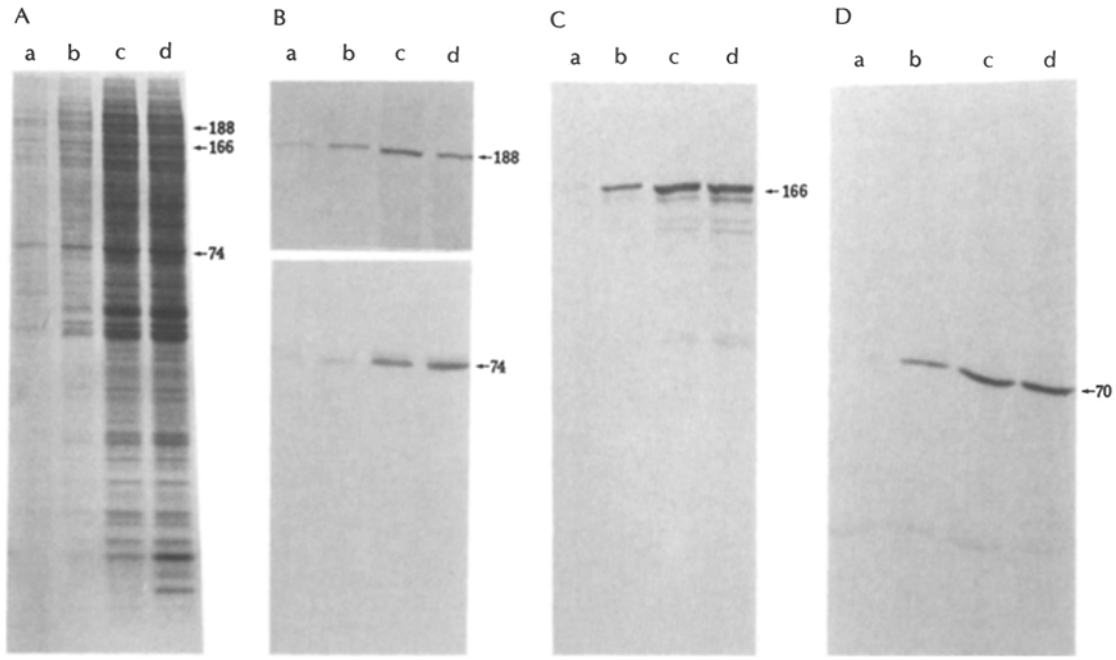


Figure 4. SDS-PAGE and Western blot analysis of the effects of heat shock in vivo on the polypeptide composition of the NMPCL fraction. Embryos were subjected to heat shock (36.5°C) in vivo for 0 (lanes *a*), 30 (lanes *b*), 60 (lanes *c*), and 120 (lanes *d*) min. NMPCL fractions were prepared from embryos at each of these time points exactly as described in Materials and Methods and Fig. 1 with nuclease digestion at 23°C. (*A*) Equivalent amounts of each NMPCL fraction (20 U) were electrophoresed on an SDS-7–15% polyacrylamide gradient gel. (*B* and *C*) Identical NMPCL aliquots were electrophoresed on an SDS-7% continuous concentration polyacrylamide gel. Two identical segments were loaded and run in parallel and each blot transferred to nitrocellulose. (*B*) The top portion was probed with monoclonal anti-gp-188 antibodies exactly as described in the legend for Fig 3; the bottom portion was probed with monoclonal anti-lamin antibodies, also as described in Fig. 3. (*C*) An entire duplicate blot was probed with anti-DNA topoisomerase II antiserum, also exactly as described in the legend of Fig. 3. (*D*) Equivalent aliquots (8 U) of the filtered crude homogenate were electrophoresed in parallel on an SDS-7% polyacrylamide gel and the proteins were blot transferred to nitrocellulose. The blot was probed with monoclonal anti-*Drosophila* hsp-70 antibodies at a dilution of tissue culture supernatant of 1:100. ~90% of the detectable hsp-70 remained in the postnuclear supernatant after purification of nuclei and none was detectable in the final NMPCL fraction (not shown).

no changes in the SDS-PAGE mobilities or amounts of either of these two proteins were detected (data not shown). We have not yet investigated changes in levels of in vivo phosphorylation for gp-188 or DNA topoisomerase II.

NMPCL fractions were prepared from heat shocked *Drosophila* embryos by the standard fractionation procedure, using a 23°C nuclease incubation exactly as described in Fig. 1. With increasing time of heat shock in vivo, increasing amounts of NMPCL protein were recovered after fractionation in vitro (Fig. 4 *A*). Western blot analyses of these same fractions are shown in Fig. 4, *B* and *C*. Examination of the blot probed with anti-lamin antibodies showed the anticipated conversion from a mixture of lamins Dm_1 and Dm_2 before heat shock (Fig. 4 *B*, *bottom*, lane *a*; see also anti-lamin blots shown in Figs. 1 and 3) to predominantly lamin Dm_1 after 1 and 2 h of heat shock (Fig. 4 *B*, *bottom*, lanes *c* and *d*). Effects of in vivo heat shock on the fractionation of all of the major NMPCL marker proteins were similar to those observed for total NMPCL protein (Fig. 4 *A*) and resemble results obtained after thermal incubation in vitro (Figs. 1–3). The results shown in Fig. 4 are also similar to observations reported by Evan and Hancock (1985) regarding the effects of in vivo heat shock on the association of $p62c^{myc}$ with the nuclear matrix fraction. Identical cell fractionation results to those shown for *Drosophila* embryos in Fig. 4 were obtained when in vivo heat shock experiments were performed using Schneider 2 tissue culture cells (not shown).

Also shown as a control in Fig. 4 (*D*) is a Western blot of the total embryo homogenates obtained at the various time points during the heat shock response that was probed with monoclonal antibodies directed against the major *Drosophila* heat shock protein, hsp-70, thus documenting the extent of the heat shock response in these embryos. The majority of hsp-70 was not associated with the purified nuclear fraction and that which remained was extracted nearly quantitatively from the NMPCL fraction with 1 M NaCl (not shown). This result is in apparent contrast to previous reports that hsp-70 was associated with nuclear matrix fractions (see e.g., Levinger and Varshavsky, 1981; see Pelham, 1986 for a review). This discrepancy is of uncertain significance.

Immunofluorescence Analysis of the Effects of Heat Shock on the Distribution and Immunoreactivity of Major Karyoskeletal Marker Proteins In Situ

Indirect immunofluorescence analysis has previously been used effectively to study the reorganization of intranuclear actin as well as changes in the morphology of cytoplasmic intermediate filaments in response to heat shock in mammalian cells (Welch and Suhan, 1985). The availability of highly specific antibodies directed against the lamins, gp-188 and DNA topoisomerase II, afforded us the opportunity to examine the distribution of these karyoskeletal marker pro-

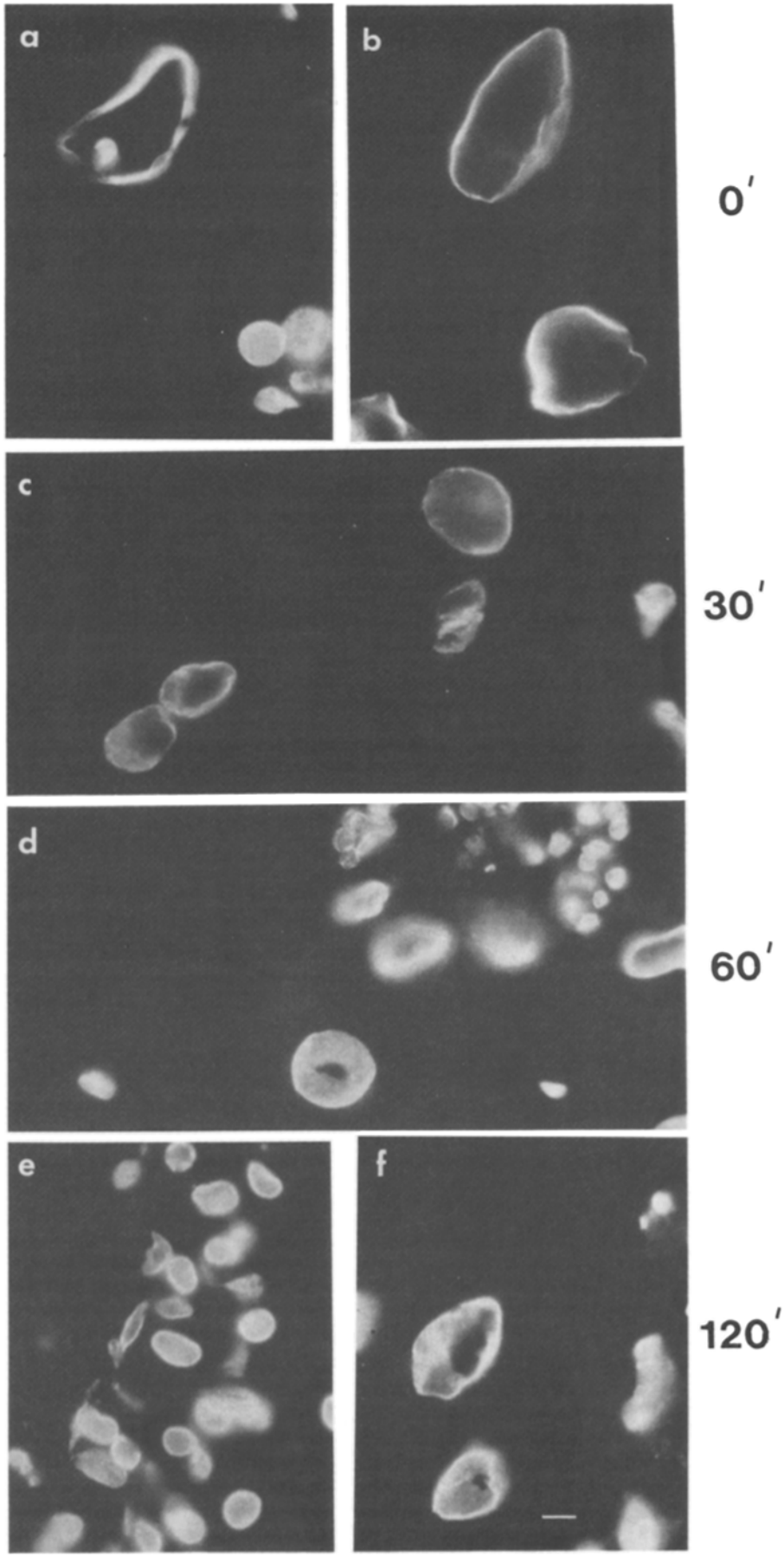
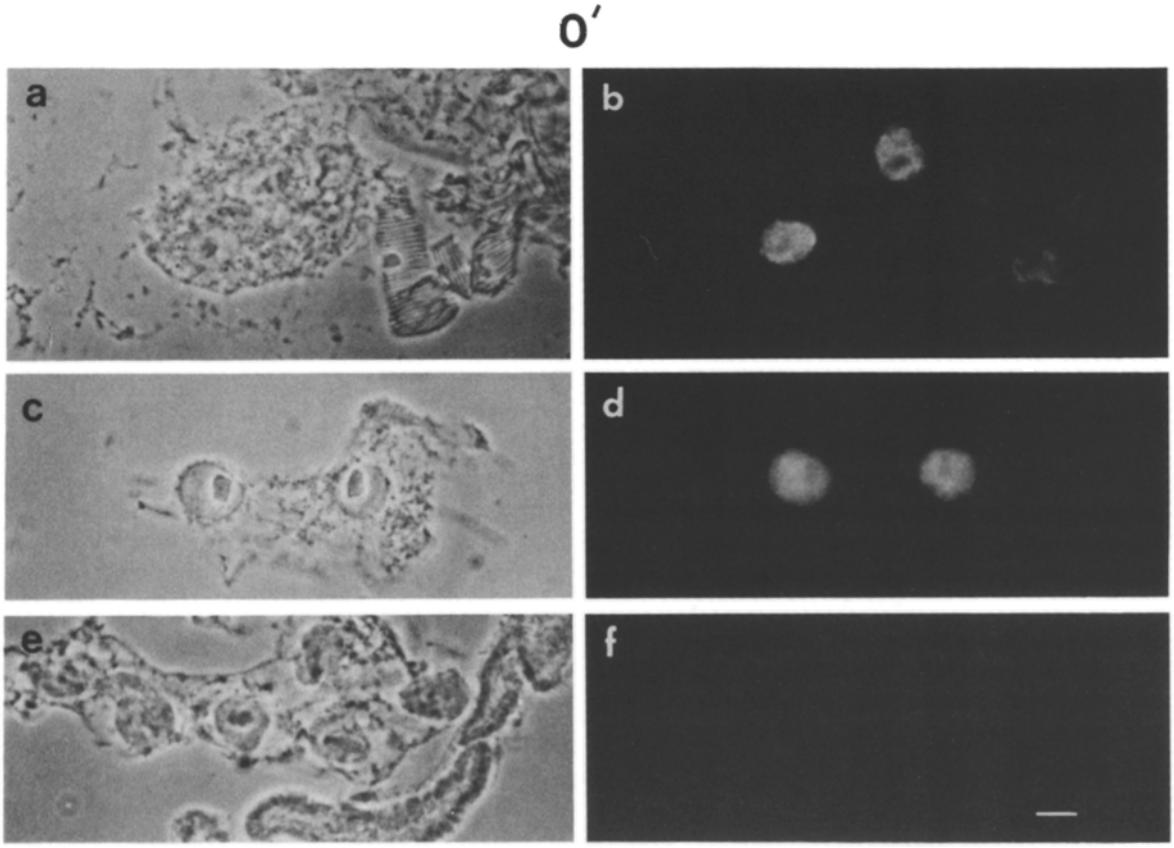


Figure 5. Indirect immunofluorescence localization of the lamins before and after heat shock in *Drosophila* larval cryosections. Cryosections from *Drosophila* third instar larvae that had been subjected to varying periods of heat shock (36.5°C) were prepared and probed as described (see Materials and Methods) with affinity-purified anti-lamin antibodies. Times of heat shock were 0 (*a* and *b*), 30 (*c*), 60 (*d*), and 120 min (*e* and *f*). Briefly, larvae were frozen alive directly in embedding matrix by immersion in liquid nitrogen; sections of ~6–8 μm were cut and were stored at −70°C until use. Before probing with specific antibodies, sections were thawed directly into 3.7% formaldehyde in PBS (2 min), and then washed briefly in PBS alone (5 min). No additional fixation or permeabilization steps were used. Affinity-purified polyclonal antibodies were diluted 1:100 relative to the specific antibody concentration of the unfractionated anti-lamin antiserum from which they were derived. Immunofluorescence detection was with rhodamine-conjugated affinity-purified goat anti-rabbit IgG antibodies diluted to a specific antibody concentration of 20 μg/ml. Identical results to those shown here were obtained with whole Schneider 2 tissue culture cells as well as with embryonic cells that had been fixed and permeabilized by sequential treatments with 3.7% formaldehyde, 45% acetic acid plus 3.7% formaldehyde, and 95% ethanol (not shown). Bar, 10 μm (applies to all panels).


0′
a
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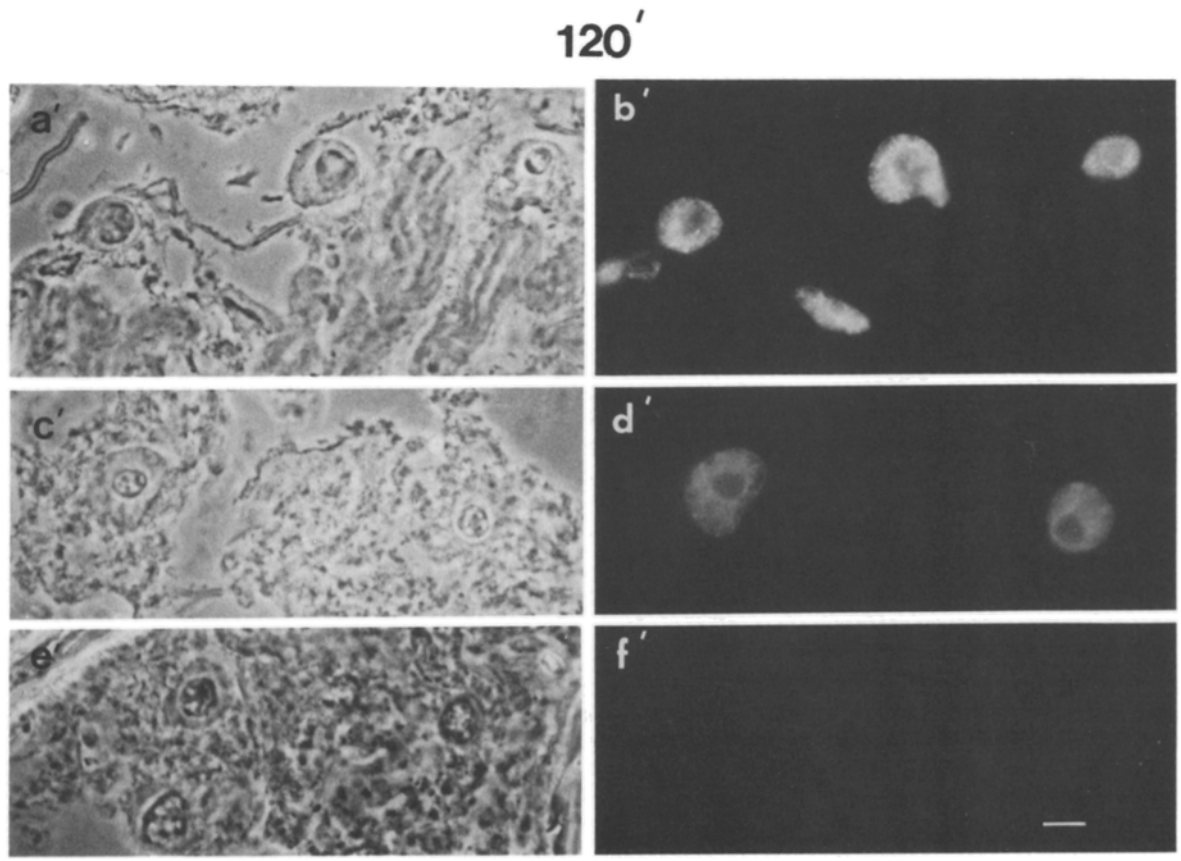
120′
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teins in *Drosophila* cell nuclei during the heat shock response. Preliminary analyses were performed with permeabilized whole cells derived from embryos, a variety of larval tissues, and Schneider 2 tissue culture cells and larval cryosections. In all cases, the extent of the heat shock response was monitored by indirect immunofluorescence analyses with monoclonal anti-hsp-70 antibody. Results with all of the different cell types were similar. However, for a number of reasons, detailed in Materials and Methods, we chose to focus our attention on larval cryosections for detailed and systematic analyses. Results of some of these analyses are shown in Figs. 5 and 6.

The data shown in Fig. 5 document the observation that there were no demonstrable changes in the distribution of the *Drosophila* lamins over a 120-min time course of heat shock. Only immunofluorescence results are shown. (See Fig. 6 for representative examples of phase-contrast morphology in these preparations.) Results shown were obtained with an affinity-purified polyclonal IgG fraction. Two representative panels are shown at each of the extreme time points; only a single panel is shown at each of the intermediate time points. In favorable specimens, the plane of the section passed through the centers of one or more nuclei such that the peripheral localization of the lamins could be unequivocally established. In other cases, a prominent nuclear "rim," characteristic of antigens concentrated in the nuclear envelope was apparent. Identical results were obtained with permeabilized whole Schneider 2 tissue culture cells as well as with embryonic cells prepared for immunofluorescence analyses under entirely different conditions (not shown).

It was clear from the data in Fig. 5 that the nuclear lamina itself did not appear to be undergoing any sort of gross structural rearrangement. Nevertheless, we have been able to demonstrate that at the biochemical level, heat shock results in the quantitative interconversion of lamin isoforms as the result of an apparently specific dephosphorylation event (Smith et al., 1987). We therefore reasoned that subtle changes in lamina morphology might in fact be taking place and if so, these changes might be reflected in perturbations of immunoreactivity with individual monoclonal anti-lamin antibodies. (It has been suggested for example that subtle structural rearrangements might account for changing reactivity of monoclonal anti-lamin antibodies with mammalian nuclei during development [Schatten et al., 1985].) We therefore decided to screen each of the available monoclonal antibodies (Risau et al., 1981) using similar cryosections to those shown in Fig. 5. As with the affinity-purified polyclonal reagents, no changes in antibody staining were observed with any of the three monoclonal anti-lamin antibodies during the 120-min time course of heat shock (not shown).

We performed similar experiments to those shown in Fig. 5 with each of two monoclonal antibodies directed against gp-188. As with the lamins, there were no detectable changes in immunoreactivity either qualitative, or quantitative, during a 120-min time course of heat shock (not shown).

To complete the morphologic analysis, we probed cryosections with antibodies directed against *Drosophila* DNA topoisomerase II. Results are shown in Fig. 6 and were at 0 (*a–f*) and 120 min of heat shock (*a′–f′*) as indicated. Phase-contrast and fluorescence micrographs are shown for each field. Two representative fields are shown at each time point. The specimens in *e*, *f*, *e′*, and *f′* were probed with preimmune serum taken from the same rabbit which was used to produce the anti-topoisomerase II antibodies.

A change in nucleolar appearance was the most notable consequence of heat shock to be appreciated from the phase-contrast micrographs. Nucleoli were expanded and increasingly refractile after 120 min of heat shock. These results are consistent with previous observations of Welch and Suhan (1985). Examination of the earlier time points (not shown) suggested that this effect was progressive. Diamidinophenylindole staining indicated that DNA was largely excluded from these well-delineated nucleoli but that DNA was otherwise distributed apparently normally throughout the interior of the nucleus (not shown). This observation is consistent with a previous report (Foe and Alberts, 1985) that in *Drosophila* embryos that have reached the stage of development where they are able to synthesize heat shock proteins (cycle 14), gross changes in chromatin morphology were not observed in response to heat shock.

When similar cryosections were stained for DNA topoisomerase II by indirect immunofluorescence, different patterns were observed in heat-shocked vs. control materials. Only the extreme time points are shown; intermediate effects were seen at the intermediate time points. In the control samples, anti-topoisomerase antibodies decorated a diffuse and relatively amorphous reticular network that apparently spanned the nuclear interior (Fig. 6, *b* and *d*). Relative nucleolar exclusion was seen as previously reported (Berrios et al., 1985). After 120 min of heat shock, nucleolar exclusion was more extreme and a clear ring of topoisomerase staining was observed around the prominent nucleoli; in some instances, spokes of material appeared to radiate out from this ring (Fig. 6, *b′* and *d′*). Counterstaining of these same sections with diamidinophenylindole showed that in both the control and the heat-shocked nuclei, the concentration of topoisomerase II did not correlate with localized concentration of DNA; if anything the opposite was true (not shown). Regions rich in DNA showed relatively less DNA topoisomerase II staining. This may either reflect the true distribution of antigen, or differential accessibility of the topoisomerase as an inverse function of the local DNA con-

Figure 6. Indirect immunofluorescence localization of DNA topoisomerase II before and after heat shock in *Drosophila* larval cryosections. Cryosections were prepared as described in the legend to Fig. 5. The time of heat shock is as indicated above the panels. Both phase-contrast (*a*, *a′*; *c*, *c′*; *e*, *e′*) and fluorescence micrographs (*b*, *b′*; *d*, *d′*; *f*, *f′*) are shown. Panels *a–d* and *a′–d′* were probed with anti-topoisomerase II antiserum diluted 1:100. Panels *e*, *f*, *e′*, and *f′* were probed with preimmune serum from the identical rabbit, also diluted 1:100. Detection was with rhodamine-conjugated affinity-purified goat anti-rabbit IgG antibodies as in Fig. 5. The antiserum used for the experiments shown was prepared against the denatured topoisomerase II polypeptide purified to apparent homogeneity under harshly denaturing conditions (see Materials and Methods). Identical results were obtained with antiserum prepared against native *Drosophila* DNA topoisomerase II conventionally purified by Shelton et al. (1983) and with affinity-purified anti-topoisomerase II IgG fractions (not shown). Identical results were also obtained when Schneider 2 tissue culture cells as well as embryonic cells, fixed and permeabilized as described in the legend to Fig. 5, were probed with these various antibody fractions (not shown). Bars, 10 μm (apply to all panels).

centration. The inverse correlation between the apparent distribution of DNA and DNA topoisomerase II was not observed for the nucleolus which contained neither DNA nor DNA topoisomerase II.

Discussion

It seems apparent that mild heat treatment, either in vitro or in vivo, has profound effects on the structural stability of proteinaceous karyoskeletal assemblies purified from *Drosophila melanogaster* embryos. Such stability is manifested by the resistance of karyoskeletal proteins to extraction in 1 M NaCl. Similar behavior of a number of mammalian nuclear "matrix" proteins has been reported by Evan and Hancock (1985).

The identification of heat as an important variable in the isolation of stable karyoskeletal structures in vitro has a number of practical implications. Several of these have been noted by Evan and Hancock (1985) but we feel that they should be reemphasized in the present context. Much of the controversy surrounding the significance of the internal nuclear matrix revolves around the difficulties encountered in developing preparative procedures that are reproducible among organisms and in different laboratories. Our current observations and those of Evan and Hancock suggest a new perspective from which to appreciate these difficulties. Results indicate that there is a substantial and heretofore, largely unrecognized (or at least undocumented) potential for the generation of systematic thermal artifacts during cell fractionation studies in vitro. Moreover, the fact that we observed significant karyoskeletal stabilization after a 45-min incubation on ice, in apparent contrast with the behavior of $p62c^{myc}$ (Evan and Hancock, 1985), suggests that not only the absolute temperature may be critical, but in addition, the total time required to proceed through a given preparation may influence the outcome substantially. The longer a preparative procedure takes, depending for example on the scale of the procedure or the difficulty of cell breakage, the more prone it is to artifact.

Our observation that the composition of the *Drosophila* NMPCL fraction depends significantly on the stage of purification at which thermal incubation is performed (Fig. 2) is particularly disturbing in terms of interpreting the biological significance of the identification of any given polypeptide as a component of a karyoskeletal protein fraction. This would imply that the purity of nuclei obtained prior to nuclease incubation determines to a large degree, the ultimate NMPCL composition. Also of some concern is the intentional inclusion of in vitro thermal stabilization steps as part of the preparative procedure in studies of putative karyoskeletal attachment sites of DNA (Gasser and Laemmli, 1986*a*, *b*). It seems possible that this practice might introduce as many artifacts as it resolves. It would be interesting to compare results in these sorts of experiments between nuclei stabilized by thermal incubation in vitro and those subjected only to heat shock in vivo.

Also from a practical perspective, it is generally accepted that the nuclear lamina and pore complexes are by definition, resistant to extraction in vitro with nonionic detergents and solutions of high salt concentration (for an explicit statement of this dogma, see Franke, 1987). Recently, Davis and Blobel (1986) reported the identification of a novel pore complex component, but felt it necessary to equivocate because a significant proportion of this polypeptide was extracted from their nuclear pore complex–lamina fractions by high salt. In light of our current observations, we feel that such equivocation was probably unnecessary. That is, salt extractability of karyoskeletal proteins both in mammalian systems (Evan and Hancock, 1985) and in *Drosophila* can vary substantially over a fairly narrow range of conditions and does not seem to really reflect absolutely upon the "karyoskeletalness" of the protein in question. We would therefore suggest that the dogmatic criterion of resistance to salt extraction to define pore complex and lamina components is perhaps too rigid and should be modified to take into account current observations regarding thermal effects both in vitro and in vivo.

From a biological perspective, our current observations may be of some interest in further elucidation of karyoskeletal structure and function and perhaps in understanding the heat shock response as well. Several well-characterized karyoskeletal marker proteins, among them the nuclear lamins, gp-188 and DNA topoisomerase II,[3] all behave similarly in response to heat shock in vivo and thermal incubation in vitro. Our data on the effects of heat shock in vivo indicate that the biologic changes that take place in the cell during heat shock are reflected by a consistent set of changes in karyoskeletal stability in vitro.

Do heat shock-induced changes in the structural stability of isolated karyoskeletal elements correlate with any alterations in nuclear morphology in vivo? In their analysis of the morphological effects of heat shock in mammalian cells, Welch and Suhan (1985) touched upon a number of relevant issues. By transmission electron microscopic analysis, they were able to detect specific morphologic changes both intranuclearly and in the cytoplasm. The most notable change in cytoskeletal morphology reported by Welch and Suhan (1985) was their observation that intermediate filaments appeared to collapse around the nucleus. Within the nucleus, they reported nucleolar swelling which they were able to relate to the appearance of the 72-kD mammalian heat shock protein within nucleoli as well as the appearance of well-defined intranuclear actin bundles. Profound effects of heat shock on nucleolar morphology have been reported previously (e.g., see Pelham, 1984). No specific mention was made by Welch and Suhan (1985) of changes in nuclear envelope structures such as lamina and pore complexes and it is difficult to come to any certain conclusions from the micrographs shown. Changes if any were subtle.

Welch and Suhan (1985) were able to obtain perhaps their most compelling evidence for morphologic effects, both cytoplasmic and nuclear, from indirect immunofluorescence analyses using antibodies directed against the intermediate filament protein, vimentin, as well as against actin and the mammalian 72-kD heat shock protein. We therefore decided to follow this approach in analyzing heat shock effects on karyoskeletal proteins. Effects of heat shock on the appearance and distribution of the lamins as well as gp-188 were not detectable. In the case of the lamins, reactivity was qualitatively and quantitatively unaltered with respect both to an

3. As noted above, final designation of DNA topoisomerase II as a "karyoskeletal" protein in the same class as the lamins and gp-188 is premature. However, the fact that DNA topoisomerase II behaves similarly to the lamins and gp-188 in response to heat shock may be taken as further evidence toward such designation.

affinity-purified polyclonal IgG fraction and with each of three distinct monoclonal antibodies. Identical immunofluorescence staining patterns were obtained with both cryosections and permeabilized whole Schneider 2 tissue culture cells as well as with embryonic cells prepared for immunofluorescence analyses under entirely different conditions. For gp-188, no heat shock-dependent changes in immunofluorescence staining were seen with either of two different monoclonal antibodies.

These results with anti-lamin and anti-gp-188 antibodies are not surprising. Pore complex and lamina morphology have been documented as invariant over a wide range of conditions. These include examination of isolated karyoskeletal protein fractions that we now know would have been inadvertently heat stabilized during preparation (e.g., Fisher et al., 1982). If morphology itself was responsive to thermal effects, such morphologic constancy would surely not have been the case. Rather, it is our contention that heat shock affects the stability or resiliency of these structures without actually altering their morphology. It should be recalled that the higher eukaryotic cell normally regulates the stability of these structures during the cell cycle—at the onset of mitosis, they are destabilized to the extent that they depolymerize and disassemble; at the end of mitosis, reassembly and restabilization is brought about (see Fisher, 1987*b* for a review).

In contrast to results with anti-lamin and anti-gp-188 antibodies, results with anti-topoisomerase II antibodies apparently showed some subtle morphologic effects of heat shock. A rather discrete "ring" of staining was seen to materialize around the nucleolus and in the best preparations, spokes of material seemed to radiate out from this ring. When these preparations were counterstained with the DNA-specific dye DAPI, it was noted that the staining pattern was different. Areas of increased anti-topoisomerase II immunofluorescence corresponded to regions where there appeared to be relatively less DNA. At this point, we cannot be certain what these observations signify. However, as with the lamins, similar results were obtained with both cryosections and Schneider 2 tissue culture cells as well as with embryos, each prepared differently for immunofluorescence analyses. Further, these results were obtained with each of two polyclonal antisera raised against topoisomerase II antigen purified in two distinct ways. We are therefore confident that the results shown in Fig. 6 accurately reflect the distribution of DNA topoisomerase II in the specimens that are shown.

In conclusion, we must caution that thermal stabilization in vitro cannot be fully equated with events that occur during heat shock in vivo. This is demonstrated most explicitly by our observations regarding the lamins. The specific conversion of lamin Dm_2 to Dm_1 observed in response to heat shock in vivo is not evident after thermal stabilization in vitro. This would imply that the interconversion of lamin isoforms is of itself, not a requirement for lamina stabilization, at least in vitro. For the moment, we would like to suggest that there is an intrinsic biochemical tendency of karyoskeletal assemblies to stabilize or rigidify (denature) upon thermal stress. In vitro, this process goes unchecked and is essentially complete within 15–30 min (Fig. 3). We think it likely that in vitro "rigidification" is somewhat random, prone to artifact and may be irreversible under nondenaturing conditions. Nevertheless, based on solubilization of karyoskeletal proteins in harsh denaturants (SDS or urea) in the absence of reducing agents (see, e.g., Filson et al., 1985), in vitro rigidification does not appear to necessarily involve covalent crosslinking, either oxidative or otherwise, at least in *Drosophila*.

In vivo, rigidification is much slower (complete in 1–2 h) (Fig. 4) and is reversible, at least to a degree (McConnell and Fisher, unpublished). Hence, it is tempting to speculate that rigidification in vivo may be precisely regulated. Evan and Hancock (1985) noted a similar discrepancy between effects in vitro and in vivo. They pointed out that thermal stabilization in vitro could be achieved by exposure to temperatures of 37°C, the normal growth temperature of the organism. In vivo, it was necessary to go to temperatures of 42°C to demonstrate heat shock effects. They speculated that in vivo, constitutive levels of the heat shock proteins might serve to mitigate thermal stress.

What is remarkable is that both in vitro and in vivo, the process seems to be a concerted one. That is, the kinetics of rigidification are more or less identical for each of the three karyoskeletal marker proteins that was monitored.

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