MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 1187–1203
0270-7306/86/041187-17$02.00/0

Vol. 6, No. 4

Expression and Localization of *Drosophila melanogaster* hsp70 Cognate Proteins

KAREN B. PALTER,[1,2] MICHIKO WATANABE,[2] LESLIE STINSON,[1] ANTHONY P. MAHOWALD,[2] AND ELIZABETH A. CRAIG[1]*

Department of Physiological Chemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706,[1] and Department of Anatomy and Developmental Biology, Case Western Reserve University, Cleveland, Ohio 44106[2]

Received 15 October 1985/Accepted 30 December 1985

Monoclonal antibodies have been used to identify three proteins in *Drosophila melanogaster* that share antigenic determinants with the major heat shock proteins hsp70 and hsp68. While two of the proteins are major proteins at all developmental stages, one heat shock cognate protein, hsc70, is especially enriched in embryos. hsc70 is shown to be the product of a previously identified gene, Hsc4. We have examined the levels of hsp70-related proteins in adult flies and larvae during heat shock and recovery. At maximal induction in vivo, hsp70 and hsp68 never reach the basal levels of the major heat shock cognate proteins. Monoclonal antibodies to hsc70 have been used to localize it to a meshwork of cytoplasmic fibers that are heavily concentrated around the nucleus.

Drosophila melanogaster responds to growth at elevated temperature and a number of other stresses by suppressing normal protein synthesis and inducing or enhancing the synthesis of seven heat shock proteins (hsps), hsp70, -68, -83, -27, -26, -23, and -22 (4). The characteristic vigorous induction of a small number of evolutionarily conserved heat shock genes is now known to be a universal cellular response to stress. This response has been observed in a broad spectrum of eucaryotes, including plants, and also in the procaryote *Escherichia coli* (61). The mechanism of induction common to these inducers and the functions of the hsps are unknown. There is good evidence that the stress response is an adaptive mechanism that enables cells to survive a variety of environmental conditions that would otherwise be lethal (2, 17, 34, 36, 38).

It is clear that the *D. melanogaster* heat shock genes are not coordinately expressed during normal development. hsp83 is probably present in all normal cells, and its induction after heat shock results in only a few fold increase in protein (33, 40, 76). mRNAs for hsp27 and -26 are synthesized during oogenesis and can be detected until the blastoderm stage (76). All four small hsps are induced in response to ecdysone during puparium formation (8, 25, 26, 58).

In *D. melanogaster*, the Hsp70 gene family consists of both heat-inducible and constitutively expressed genes. hsp70 and hsp68 (which share 75% homology) are virtually absent under nonstress conditions (68). There are two copies of Hsp70 at 87A, three to four copies at 87C, and one copy of Hsp68 at 95D. Craig and colleagues (9, 23) have discovered a family of genes, 75 to 80% homologous to Hsp70, termed heat shock cognate genes, which are expressed under normal growth conditions. The three heat shock cognate genes identified, Hsc1, -2, and -4, map cytologically to 70C, 87D, and 88E, respectively. The levels of RNA from each of the cognate genes are different in adults, with Hsc4 approaching the level of actin and Hsc1 and Hsc2 being 30- and 60-fold less.

Proteins related to Hsp70 have now been found to be synthesized under normal growth conditions in a variety of organisms (20, 28, 35, 71, 72, 74). A family of genes encoding proteins homologous to the Hsp70 constitutive and inducible members has also been identified and isolated in yeasts (24). In *E. coli*, heat treatment stimulates the rates of synthesis of at least 17 proteins (39), of which DnaK and C62.5 have been shown to be homologous to hsp70 and hsp83, respectively, in *D. melanogaster* (5; J. C. A. Bardwell and E. A. Craig, unpublished results). The bacterial protein DnaK, which is 48% homologous to hsp70 (5), is an extremely abundant protein at normal growth temperatures.

We have identified three proteins present during normal *Drosophila melanogaster* development that share antigenic determinants with hsp70 and hsp68. We will refer to these proteins as heat shock cognate proteins (hscs). One of these proteins, hsc70, is shown to be the product of a previously identified gene, Hsc4. To understand the relationship among the various members of the Hsp70 gene family, we have examined the levels of these proteins in adult flies and larvae during heat shock and recovery. Monoclonal antibodies to the major hsc, hsc70, have been used to localize it to a meshwork of cytoplasmic fibers that are heavily concentrated around the nucleus. We discuss the implications of having constitutive hsp70-related proteins present during normal development and relate this to possible functions of the stress response.

MATERIALS AND METHODS

Fly culture and preparation of pole cell staged embryos. *D. melanogaster* (Oregon R, P_2 strain) was grown in mass culture according to procedures previously described (64). Embryos were collected by allowing flies to lay eggs on agar-molasses trays for 2-h periods, and then the embryos were aged at 25°C for 1.5 h. Aged embryos were collected on a 125-μm sieve (W. S. Tyler Co.) after being rinsed through 841- and 420-μm sieves to remove whole flies and body parts. Embryos were stored in water at 4°C to pool collections (not more than 4 h), dechorionated in 50% Chlorox at room temperature, and rinsed in 0.02% Triton X-100 (Calbiochem) at 4°C. The developmental stage of the embryos was monitored by removing a sample to view through a compound microscope.

* Corresponding author.

Isolation of pole cells and production of monoclonal antibodies. Enriched pole cell preparations were prepared according to Allis et al. (1) from staged *D. melanogaster* embryos. Cell preparations typically contained 60% pole cells, 35% blastoderm cells, and 5% postblastoderm cells, plus particulate material from embryos, such as yolk particles. BALB/c mice were injected intraperitoneally with 0.5 $\times 10^7$ to 1 $\times 10^7$ cells in complete Freund adjuvant (Difco Laboratories) for the first two injections and in incomplete Freund adjuvant (Difco) for the last three booster injections. Injections were administered on days 0, 30, 45, 60, and 85, and spleen cells were taken for the fusion 3 days after the last booster injection. Hybridomas were derived from a fusion of mouse myeloma cell line P3-AgX63.856 with mouse spleen cells as described by Oi and Herzenberg (42). Hybridoma cell lines were cloned by the method of limiting dilution.

Screening of hybridoma cell lines and antibody production. On day 16 of the fusion, hybridoma supernatants were withdrawn and tested for reaction with immunogen by solid-phase radioimmunoassay on polyvinylchloride plates, using goat anti-kappa light chain (Antibodies Inc.), iodinated by the chloramine-T method (65), as the second antibody. Over 95% of hybridoma wells (containing between one and three hybridoma cell lines) secreted antibody that reacted to the immunogen. Positive cell lines were then screened by indirect immunofluorescence of enriched pole cell preparations that had been fixed to albuminized glass slides, using 10% formaldehyde vapors. Hybridoma cell lines secreting antibody that reacted strongly to embryonic cells were cloned by limiting dilution and characterized by immunoblots of one- and two-dimensional gels. Antibody was obtained either from culture supernatants or as ascites fluid from mice that had been primed with 0.5 ml of pristane (Aldrich Chemical Co.) 10 days before injecting 5 $\times 10^6$ hybrid cells intraperitoneally. All monoclonal antibodies used in this study are mouse immunoglobulin G, unless otherwise indicated. Hybridoma cell lines were frozen in 90% fetal calf serum–10% dimethyl sulfoxide and stored in liquid nitrogen. Monoclonal antibodies 7.1N and 7.FB (68) and 7.10 are rat antibodies that were derived from hybridomas that had been directed against gel-purified hsp70 and were generous gifts from J. Velazquez and S. Lindquist. Monoclonal antibody α-int-fil is a mouse immunoglobulin G that recognizes a domain common to all classes of intermediate filament proteins in all organisms (47), including the 46,000-dalton protein in *D. melanogaster* (obtained from the American Type Culture Collection). Monoclonal antibody against α-tubulin was a generous gift of M. Fuller.

One- and two-dimensional gel electrophoresis and protein sample preparation. Acrylamide slab gels, 10% (0.75 mm thick), were prepared according to published procedures (30). Samples were solubilized either by sonication in Laemmli sample buffer and boiling for 5 min or by sonication in sonication buffer (2 mM Tris, 1 mM $MgCl_2$, pH 7.4) containing RNase (100 μg/ml; Worthington Diagnostics) and DNase I (20 μg/ml; Sigma Chemical Co.), incubation on ice for 15 min, addition of 2× Laemmli sample buffer, and boiling for 5 min. Samples were microfuged before being applied to the gel. We find that RNase and DNase treatment of samples increases the protein recovery (especially of non-heat-shocked samples) and makes protein recovery from different samples more uniform. Two-dimensional isoelectric focusing gels were prepared and run as described by O'Farrell (41). Protein samples from *D. melanogaster* ovaries (three ovaries per sample) were sonicated in sonication buffer containing RNase and DNase before addition of solid urea and lysis buffer A (41). Protein samples from larvae and adults were prepared by homogenizing animals in small Eppendorf tubes containing 50 μl of sonication buffer, using a glass pestle. Samples were then sonicated and processed as described above. Generally, three adults or six larvae were processed per sample, and one-third of each sample was loaded per gel. Gels were stained for 1 h in 0.1% Coomassie brilliant blue in 50% methanol–7.5% acetic acid and destained in a solution containing 5% methanol and 7.5% acetic acid.

RNA isolation. Adult flies were homogenized in equal amounts of homogenization buffer (7 M urea, 50 mM sodium acetate, pH 5.1, 10 mM EDTA, pH 6.0, 0.5% sodium dodecyl sulfate), and a mixture of phenol, chloroform, and isoamyl alcohol (24:24:1), using a Polytron homogenizer (Brinkmann Instruments). The suspension was stirred for 30 min prior to separation of phases by centrifugation. The aqueous phase was precipitated with ethanol. RNA was precipitated by addition of an equal volume of 4 M LiCl, maintaining the solution at 4°C for more than 10 h, and centrifugation. RNA concentration was measured by A_{260} and by ethidium bromide staining of 1% agarose–10 mM sodium phosphate (pH 7.0) gels.

Hybrid selection and cell-free translation. Hybrid selections were performed with two plasmids. Plasmid MG34P3 is a subclone in pBR322 of a 2.4-kilobase *Pst*I fragment of MG34 (9) that contains the entire 5′ noncoding region plus the protein-coding region through amino acid 350. Plasmid MG34VX is a subclone of an 800-base pair (bp) *Pvu*II-*Xba* fragment in pMT11 that contains the entire 160-bp 5′ noncoding region and terminates in the intron that precedes the ATG. Hybrid selection was carried out by the technique of Ricciardi et al. (48). A 5 μg portion of plasmid was linearized with *Hin*dIII, extracted with phenol and then chloroform, and precipitated with ethanol. DNA was denatured and fixed onto nitrocellulose as described before. Prehybridization was carried out for 2 h at 50°C in a 100-μl volume of 50% formamide–100 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8)–0.6 M NaCl. The prehybridization solution was removed and hybridization was performed for 2 h at 50°C in the same buffer, with the addition of 300 μg of RNA (isolated from 0- to 18-h embryos). Filter slices were washed 10 times in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate at 60°C, three times in 2 mM EDTA, pH 8, at room temperature, and once in 2 mM EDTA at 60°C for 1 min. The RNA was released by boiling in 200 μl of water for 90 s and ethanol precipitated with 20 μg of yeast tRNA. RNA was added to a rabbit reticulocyte translation system (56), and the protein products were subjected to two-dimensional gel electrophoresis and fluorography.

Northern blot analysis. Isolated fly RNA was separated by gel electrophoresis through 1% agarose gels containing formaldehyde, transferred to nitrocellulose (Schleicher & Schuell), and hybridized as described by Scott et al. (54), except hybridization was performed at 50°C and the final wash solution was 0.05× SET (5× = 0.75 M NaCl, 0.1 M Tris hydrochloride, 10 μM EDTA; pH 8.0). A 2-μg amount of total RNA was loaded per lane for all blots, except those probed for Hsp70, which contained only 1 μg of total RNA per lane. Cloned probes used were pB8, which contains one copy of the 87C Hsp70 gene; pMG34, which contains the Hsc4 cognate gene at 88E (9); pK60, which contains the 87E actin gene (62); pPW227, which contains the Hsp68 gene; pPW244, which contains the Hsp83 gene (21); J1S3, which contains the Hsp27 gene; and J1PR3, which contains the

Hsp23 gene (11). Autoradiographic exposures ranged from 3 h for the Hsp70 probe to 4 days for the actin probe, using an intensifying screen. Band intensities were quantified by densitometry (LKB Instruments, Inc.).

cDNA extension. A 76-bp *Hae*II fragment from the amino-terminal end of the protein-coding region of MG34 (amino acids 72 to 97) was used as a primer for cDNA extension. Conditions of hybridization and extension were as described by Craig et al. (9). The size of the extension product was determined by gel electrophoresis on 6% acrylamide-urea gels, using labeled denatured *Hae*III fragments of ϕX174 DNA as markers.

Immunoblot analysis. Immunoblot analysis was performed as described in Towbin et al. (63). Proteins were transferred to nitrocellulose, using a Hoeffer electrotransphor apparatus, and blocked with 3% gelatin (Difco) in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h at room temperature. Blots were incubated overnight at room temperature on a rotating shaker, with a 1:100 dilution of ascites fluid in 1% gelatin–TBS, rinsed three times in TBS-Tween (containing 0.05% Tween 20), 10 min per wash, incubated for 2 h with a 1:500 dilution of peroxidase-conjugated goat anti-mouse or -rat antibody (Cappell Laboratories), rinsed three times as before, and reacted with diaminobenzidine.

Labeling of *D. melanogaster* ovaries. Ovaries (three pairs) were dissected from adult flies in Robb's medium (49) minus methionine. They were transferred to a 10-μl drop of Robb's medium minus methionine containing 80 μCi of [^{35}S]methionine (Amersham Corp.) in a small petri dish, which was placed in a humid chamber in either a 25 or 37°C incubator. For heat-shocked samples, ovaries were preincubated in medium for 1 h prior to addition of label. After 1 or 2 h at the appropriate temperature, ovaries were rinsed in 500 μl of sonication buffer, briefly centrifuged to remove buffer, and suspended in 25 μl of sonication buffer. Samples were sonicated and processed for two-dimensional gel electrophoresis. An aliquot of each sample was trichloroacetic acid precipitated and total incorporation was between 0.3×10^7 and 1×10^7 cpm. Non-heat-shocked samples incorporate five times as much radioactivity as heat-shocked samples so the protein equivalent of one pair of ovaries was loaded per sample rather than equalizing the radioactivity loaded.

Heat shock of adult flies and larvae. For 1-h heat shocks, adult flies or larvae were transferred to preheated glass vials (containing moistened paper) and placed in a 37°C incubator. For longer heat shocks, flies or larvae were transferred to vials or bottles containing medium that had been preheated and placed in a 37°C incubator. For recovery experiments, flies or larvae were transferred to vials or bottles at room temperature. Flies seemed energetic during the first 4 h of heat shock at 37°C. Thereafter, they became progressively more sluggish, until by 8 h some flies started to die. Only flies that were still moving were selected for analysis. By 12 to 24 h, only a small percentage of flies survived. Flies were barely moving after prolonged heat shock. Nevertheless, when restored to room temperature, these flies were indistinguishable from untreated flies after <2 min. Flies that showed no movement did not recover, even after 24 h at room temperature. It is not known what kills flies at high temperature, but these experiments suggest that recovery is too rapid to involve new RNA or protein synthesis. No larvae died during the course of these experiments. However, some larvae pupated at long time points.

Embryonic cell cultures. Primary cultures of *D. melanogaster* embryonic cells were prepared and allowed to differentiate into a variety of cell types (55). Cultures were prepared from gastrula staged (4 to 6 h) embryos that had been collected on agar-molasses trays covered with autoclaved yeast. Embryos were rinsed with 0.02% Triton X-100, water, and 70% ethanol and dechorionated in Chlorox (2.5%) in 50% ethanol. They were rinsed with 70% ethanol and then with a 100-fold dilution of commercial Rocall II (National Laboratories) in 70% ethanol for 15 min to surface sterilize. Embryos were rinsed three times in cold balanced salt solution (7), and subsequent steps were performed in a tissue culture hood under sterile conditions. Embryos were homogenized in a Dounce homogenizer in 5 volumes of balanced salt solution on ice. The homogenate was filtered through 30-μm Nitex mesh, centrifuged for 10 min at 800 rpm in a clinical centrifuge, and washed three times in balanced salt solution. Cells were suspended at a concentration of 2×10^5 to 3×10^5 cells per ml in culture medium (59) containing 0.1 U of insulin (GIBCO Laboratories) per ml, and 2 ml of cells was plated in each 35-mm petri dish and allowed to differentiate overnight at 25°C.

Indirect immunofluorescence staining of *D. melanogaster* embryonic sections and primary embryonic cell cultures. Cell cultures were plated either directly onto petri dishes or onto cover glasses in petri dishes and allowed to differentiate. Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by either methanol at −10°C for 6 min and then PBS for 30 s (31) or 0.2% Triton X-100 in PBS for 2 min followed by a PBS wash (18). Cytoskeletal preparations were prepared as described by Osborn et al. (44). Specific antibodies were diluted into PBS and incubated with cells under cover glasses for 60 min at room temperature. The cells were rinsed with PBS and incubated for 30 min with a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappell Laboratories). After rinsing, dishes or cover glasses were mounted in phosphate-buffered glycerol and viewed in a Leitz microscope equipped with epifluorescence optics. Samples were photographed with Kodak 35-mm Tri-X film shot at either 800 or 1600 ASA and developed with Acufine. Staged *D. melanogaster* embryos were prepared for sectioning by the phase-partition fixation procedure (75). Dechorionated embryos were placed in the heptane phase of solution A for 5 min, fixed directly in solution C for 2 to 4 h at room temperature, and rinsed with water. Embryos were frozen in O.C.T. (Miles Scientific) on a sample holder for cryostat sectioning (Slee) by placing the metal holder on a block of dry ice. Sections, 6 μm, were melted onto subbed slides, fixed for 10 min in 10% formaldehyde vapors, and rinsed in PBS before antibody staining. Staining and visualization were as described above.

RESULTS

An hsc encoded by Hsc4 is abundant in unstressed *D. melanogaster* ovaries and embryos. We have produced monoclonal antibodies directed against preblastoderm staged embryonic antigens. In the course of screening such antibodies, it was noted that a high proportion (10 to 20%) was directed against an abundant 70,000-dalton protein. The 70,000-dalton protein, identified on two-dimensional gels by immunoblotting, is indicated in the Coomassie blue-stained gel of proteins from *D. melanogaster* ovaries (Fig. 1A) and the corresponding immunoblot (panel C). The 70,000-dalton protein is equally abundant in ovaries and embryos, but is approximately fivefold less abundant in larvae, pupae, and adults, although it remains a major protein (data not shown).

Heat shock of *D. melanogaster* ovaries at 37°C results in the detection of an additional 70,000-dalton protein by im-

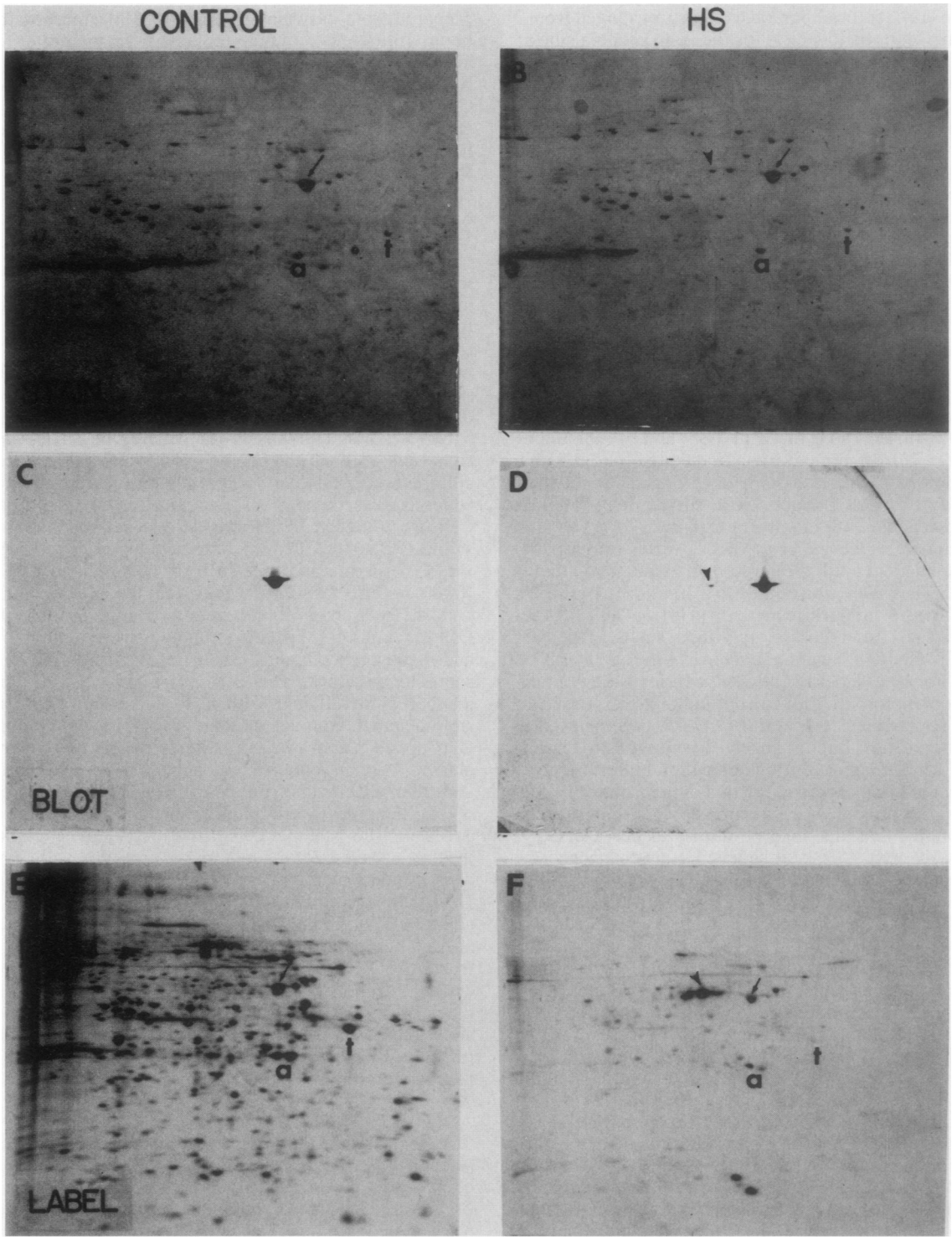


FIG. 1. Abundance of hsc70 in *D. melanogaster* ovaries. Ovaries were labeled with [^{35}S]methionine at 25°C for 2 h (A, C, E) or preheated to 37°C for 1 h before addition of label and then labeled for 2 h (B, D, F). Duplicate samples of ovary proteins were separated by two-dimensional gel electrophoresis and either stained with Coomassie blue (A, B) or transferred to nitrocellulose and reacted with monoclonal antibody IPC4 (C, D). The autoradiograms of (A) and (B) are shown in (E) and (F), respectively. Arrowheads indicate positions of hsp70 and arrows indicate hsc70; A, actin; T, tubulin. Acidic end of gel is at the right.

munoblot analysis (Fig. 1B and D), which comigrates with radioactively labeled hsp70 (panel F). Based on the abundance and developmental pattern of transcripts of the three known heat shock cognate genes (9), only Hsc4 (at cytological locus 88E) could encode the 70,000-dalton protein (henceforth called hsc70). The hsc70 protein is synthesized during oogenesis and Northern blot analysis shows that transcripts from Hsc4 are abundant in oocytes, indicating

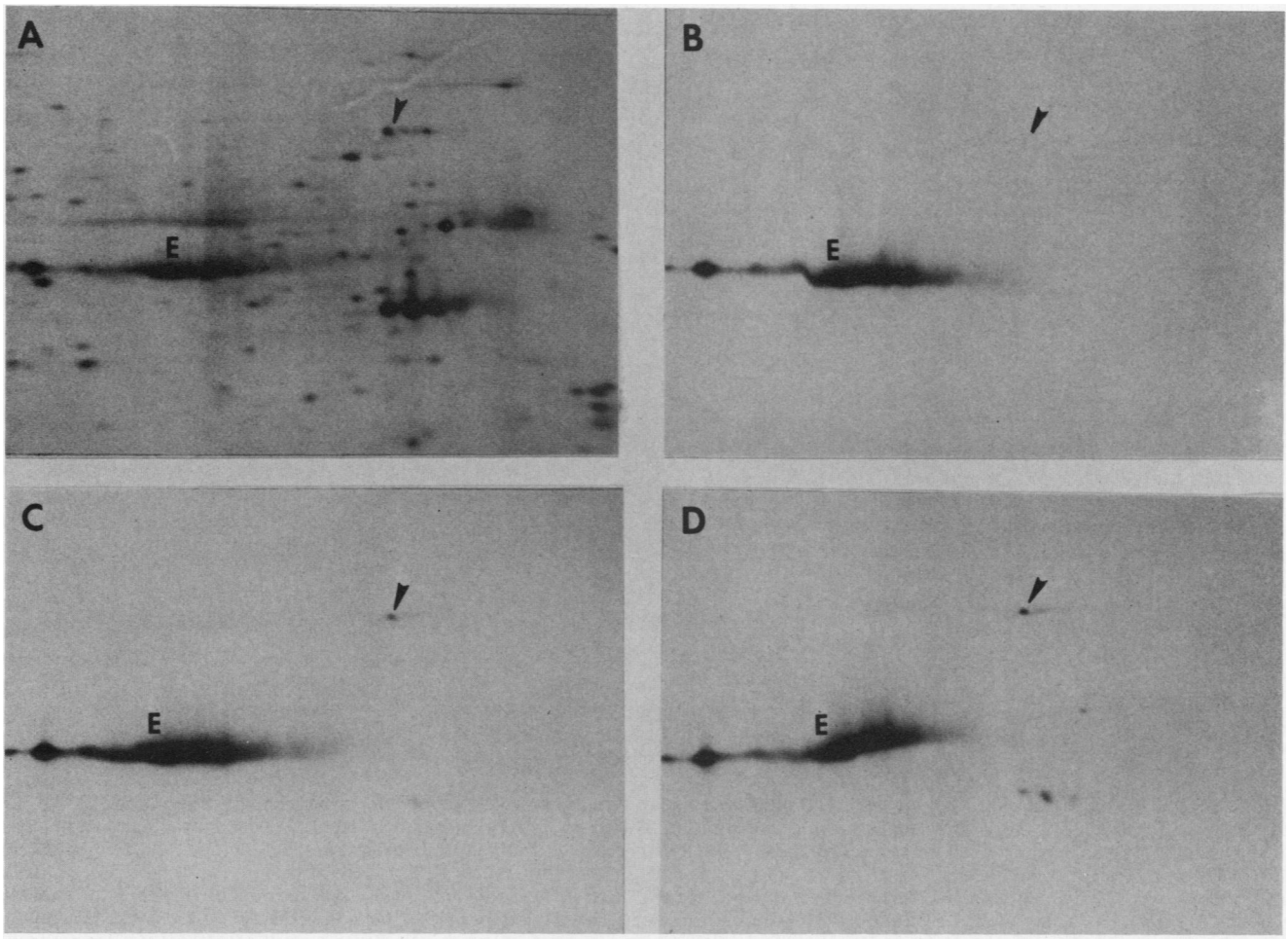


FIG. 2. hsc70 encoded by Hsc4. A 300-μg portion of RNA from 0- to 18-h embryos was hybridized to MG34P3 (Hsc4 protein-coding region) or MG34VX (Hsc4 5′ noncoding region), eluted, and translated by a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and subjected to two-dimensional gel electrophoresis. Translation of (A) input RNA; (B) 20 μg of yeast tRNA as control; (C) RNA hybridized to MG34P3; (D) RNA hybridized to MG34VX. Arrows indicate the positions of hsc70. Lower-molecular-weight protein triplet is due to slight contamination with actin message. Protein spots labeled E are endogenous proteins, which are present in the yeast tRNA control.

that they represent a large percentage of the maternal message stored in the egg (data not shown).

A direct demonstration that Hsc4 encodes hsc70 was obtained by hybrid selection of RNA, using cloned Hsc4 DNA, and in vitro translation of the selected RNA. Translation products were subjected to two-dimensional gel electrophoresis and fluorography (Fig. 2). A protein that comigrates with hsc70 on two-dimensional gels is the only 70,000-dalton protein selected by a plasmid containing a portion of the protein coding region of Hsc4 (panels A and C). Although the stringency of hybrid selection used is sufficient to prevent selection of Hsp70 RNA by the related Hsc4 gene (data not shown), we wanted to further minimize the chance that Hsc4 DNA would select RNA homologous to another cognate gene. We repeated the hybrid selection with a plasmid containing only 5′ noncoding sequences, which are usually highly divergent among members of multigene families. Results identical to those using the protein-coding region plasmid were obtained (panel D).

Identification of other cognate proteins by using monoclonal antibodies. As mentioned above, there are several genes homologous to Hsp70, whose products should be antigenically related. The monoclonal antibodies that we have produced against preblastoderm embryos or those produced by S. Lindquist and J. Velazquez against purified hsp70 include antibodies that recognize hsc70 and hsp70 with varying avidities, suggesting that they recognize different determinants (Fig. 3). For example, one recognizes only hsp70 (Fig. 3D), while another reacts strongly with hsc70 and is only weakly cross-reactive with hsp70 on immunoblots (Fig. 3G). One antibody (7.10) generated by Lindquist and Velazquez is of particular interest because it seems to recognize a determinant common to several proteins related to hsp70. In non-heat-shocked control samples, antibody 7.10 binds to hsc70, a 72,000-dalton protein (hsc72), and a minor 70,000-dalton protein (panel C). After heat shock, hsp68 and the four isoforms of hsp70 labeled with [^{35}S]methionine were also recognized (panel B).

It has not been possible to assign the new cognate proteins identified by these experiments to known cognate genes. hsc72 is a major protein at all developmental stages, a pattern that differs from the RNA expression patterns of the cognate genes located at cytological loci 70C and 87D. This suggests that there should be a fourth cognate gene. The

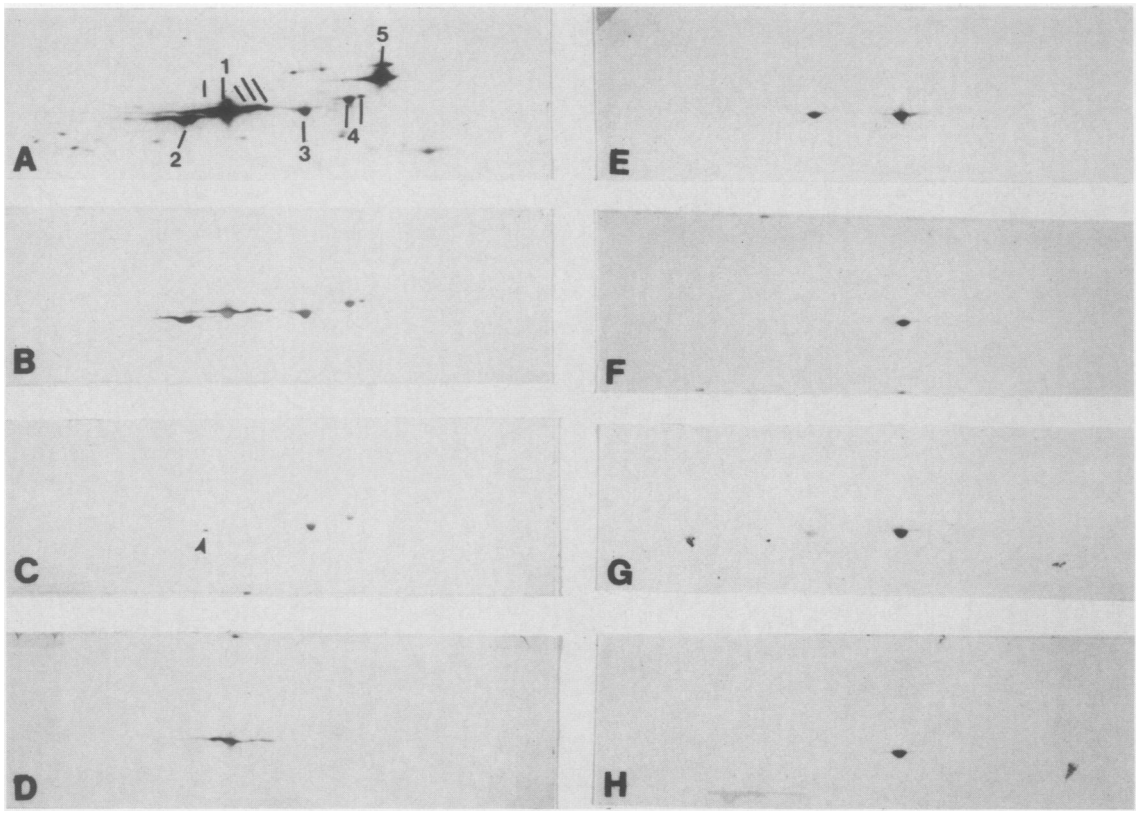


FIG. 3. Identification of hsp70-related proteins. Proteins from adult flies grown at 23°C (C, F, H) or heat shocked for 4 h at 37°C (B, D, E, G) were separated by two-dimensional gel electrophoresis, blotted to nitrocellulose, and reacted with monoclonal antibodies. [^{35}S]methionine-labeled heat-shocked salivary gland marker proteins were added to nonradioactive heat-shocked samples. (A) Autoradiogram of blot B showing [^{35}S]methionine salivary gland marker proteins: 1, hsp70 isoforms (pI 6.1); 2, hsp68 (pI 6.3); 3, hsc70 (pI 5.75); 4, hsc72 isoforms (pI 5.6); 5, hsp83 (pI 5.4). (B) Heat shock, antibody 7.10. (C) Control, antibody 7.10. Arrow indicates minor 70,000-dalton cognate protein. (D) Heat shock, antibody 7.FB (antibody 7.1N gives same pattern). Both antibodies 7.FB and 7.1N do not recognize proteins in control samples. (E) Heat shock, antibody IPC4 (antibody IPC34 gives the same pattern). (F) Control, antibody IPC4. (G) Heat shock, antibody IPC35. (H) Control, antibody IPC35. Blot G was allowed to develop for an extended period so that cross-reaction with hsp70 could be detected. Under such conditions, binding to hsc70 had already reached saturation so the relative staining of the two proteins overestimates the amount of cross-reaction to hsp70.

minor 70,000-dalton cognate protein (Fig. 3C) is present only at low levels (and is not visible after Coomassie blue staining) so it could be encoded by either Hsc1 or Hsc2.

Craig et al. (9) have shown that transcripts of the cognate genes show developmental regulation. The abundance of two of the cognate proteins also varies during development. The ratio of hsc70/hsc72 on two-dimensional gels is approximately 10:1 in unfertilized eggs and embryos (Fig. 1), 3:1 in adults (see Fig. 5), and 1:1 in late larvae (unpublished results). The enrichment of hsc70 in ovaries and embryonic tissue compared with somatic tissue can be visualized by comparing the amount of hsc70 in the head and thorax of female flies to the amount in their abdomens (Fig. 4). The enrichment of hsc70 in ovaries results in there being approximately two- to threefold more hsc70 in total adult female flies than in total adult male flies. Therefore, in experiments in which quantitation is important, either only female or only male flies were used.

Cognate proteins are synthesized during heat shock. During heat shock, synthesis of heat shock proteins is induced and most other protein synthesis is repressed. It was therefore of interest to see if cognate proteins behave like hsps or like most 25°C proteins. Figure 1E and F show the patterns of protein synthesis in ovaries labeled with [^{35}S]methionine at 25 and 37°C, respectively. At 37°C, almost all 25°C protein synthesis is arrested, but hsc70 and hsc72 continue to be synthesized, although at lower levels than at 25°C. The hsc72 synthesis rate is repressed to a lesser extent than that of hsc70, and hsc72 has probably been classified as an hsp (69). After heat shock, cognate proteins are the most abundantly synthesized non-heat shock proteins. Similar results are observed with salivary glands labeled after heat shock (Fig. 3A). There appears to be a mechanism that enables cognate proteins to escape repression to a greater extent than most 25°C proteins. This suggests that cognate proteins are not dispensable during heat shock.

In vivo regulation of heat shock and cognate proteins during prolonged heat shock. Previous work on the regulation of heat shock genes in *D. melanogaster* showed that rates of transcription of the genes were correlated with the severity of the stress (12) and that transcription was inhibited after appropriate accumulation of hsps had occurred, whether the temperature was maintained at 37°C or returned to 25°C. We examined the level of accumulation of hsps to compare their levels with those of the cognate proteins.

We have examined the time course of total accumulation of major hsp70-related proteins during prolonged heat shock at 37°C in adult flies. Protein levels in flies placed at 37°C for

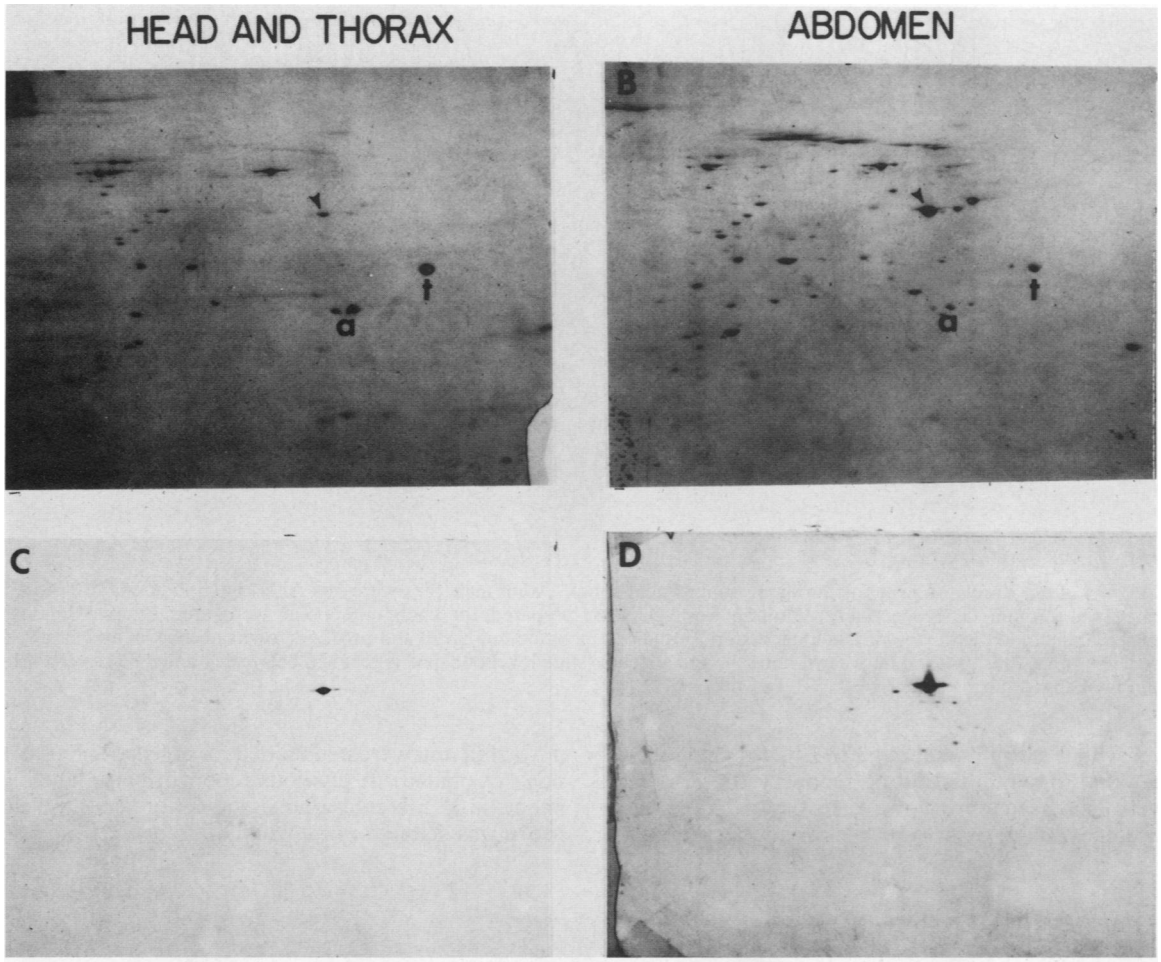


FIG. 4. Enrichment of hsc70 in female abdomens. Adult female flies were separated at the thorax, and protein samples containing head and thorax (A, C) or abdomen (B, D) were separated by two-dimensional gel electrophoresis. Six heads and thoraces or three abdomens were used for each sample to equalize the amount of total protein per sample. Gels were either stained with Coomassie blue (A, B) or in duplicate samples transferred to nitrocellulose and reacted with monoclonal antibody IPC4.

0 to 24 h were analyzed by two-dimensional gel electrophoresis of extracts and staining with Coomassie blue (Fig. 5). The amounts of most proteins, including hsc70 and hsc72, remained constant throughout the experiment. hsp70 increases in amount until it reaches a steady-state level, approximately one-third the amount of hsc70 (in adult male flies), after 1 to 2 h at 37°C. hsp68 accumulates at a slower rate but reaches a steady-state level equivalent to hsp70 after about 4 h. hsp70 probably accumulates at a faster rate than hsp68 because there are five times as many genes for hsp70 as for hsp68.

There is less than twofold more total hsp70-related protein at 37 than at 25°C. Results of prolonged heat shock in larvae are similar to those in adult flies (data not shown). The amounts of hsp70 and hsp68 that accumulate in ovaries (Fig. 1B and C) during prolonged heat shock are negligible compared with the amount of hsc70. This is partly because only the nurse cells and follicle cells, but not the oocyte proper, are transcriptionally active and hence capable of producing hsps and partly due to the enormous pool of maternal message for hsc70 that exists in ovaries. However, in vivo accumulation of hsp70 in ovaries is approximately threefold higher than in vitro (Fig. 1).

Work in other laboratories on prolonged heat shock of *D. melanogaster* tissue culture cells differs quantitatively from that just described. The total accumulation of hsp70 protein is reported to be massive after 8 h of heat shock (37). One observation pertinent to this discrepancy is the effect of heat shock on solubilization of protein samples prepared for gel electrophoresis. During the course of this work, samples solubilized by boiling in Laemmli sodium dodecyl sulfate sample buffer (30) showed five- to tenfold greater protein recovery (more extreme for molecular weights above 40,000) of heat-shocked samples than for control samples. Samples prepared after various times of recovery from heat shock gradually returned to the low preheat shock level of protein solubilization. This gel artifact, which is eliminated by RNase and DNase treatment of protein samples, is most likely responsible for the vast overestimation in the literature of hsp70 accumulation (see references 33, 37). The biological basis for the enhanced protein solubilization after heat shock is unknown.

Stability of hsps during recovery from heat shock. We have examined the stability of hsp70 and hsp68 during recovery from heat shock. After 1 h at 37°C, flies were allowed to recover at 23°C for various times. Protein levels of treated flies were analyzed by two-dimensional gel electrophoresis followed by staining with Coomassie blue (Fig. 6). hsp70

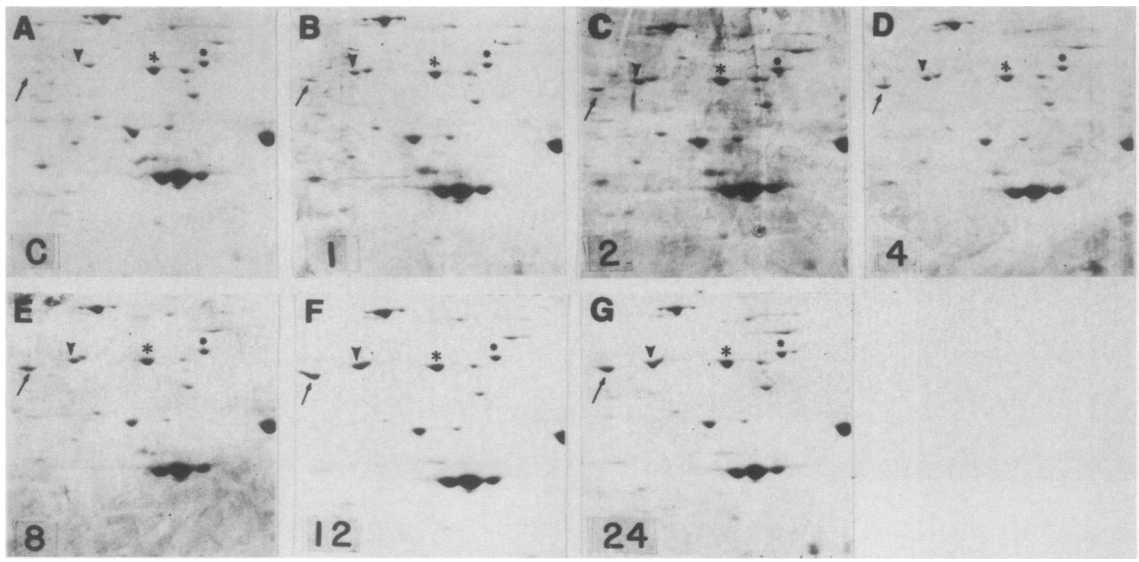


FIG. 5. Levels of hsp70-related proteins during prolonged heat shock. Adult male flies were heat shocked at 37°C for 1, 2, 4, 8, 12, or 24 h as indicated (A through G, respectively). Protein samples were prepared for each time point, separated by two-dimensional gel electrophoresis, and stained with Coomassie blue. Asterisks indicate positions of hsc70 and hsc72; arrowhead, hsp70; and arrow, hsp68. A steady-state level of hsp70 is reached between 1 and 2 h, and a steady-state level of hsp68 is reached between 4 and 8 h. Levels of hsc70 and hsc72 remain unchanged during heat shock.

turned over with a half-life of about 1 to 2 h, assuming there was no new hsp70 synthesis during recovery. In contrast, hsp68 levels increased during the recovery period. They reached a steady-state level by 6 h after the temperature downshift and were maintained for at least 12 h (the last time point examined). A previous report of pulse-labeling experiments in *D. melanogaster* tissue culture cells indicated that hsp70 was stable for up to 20 h of recovery from heat shock

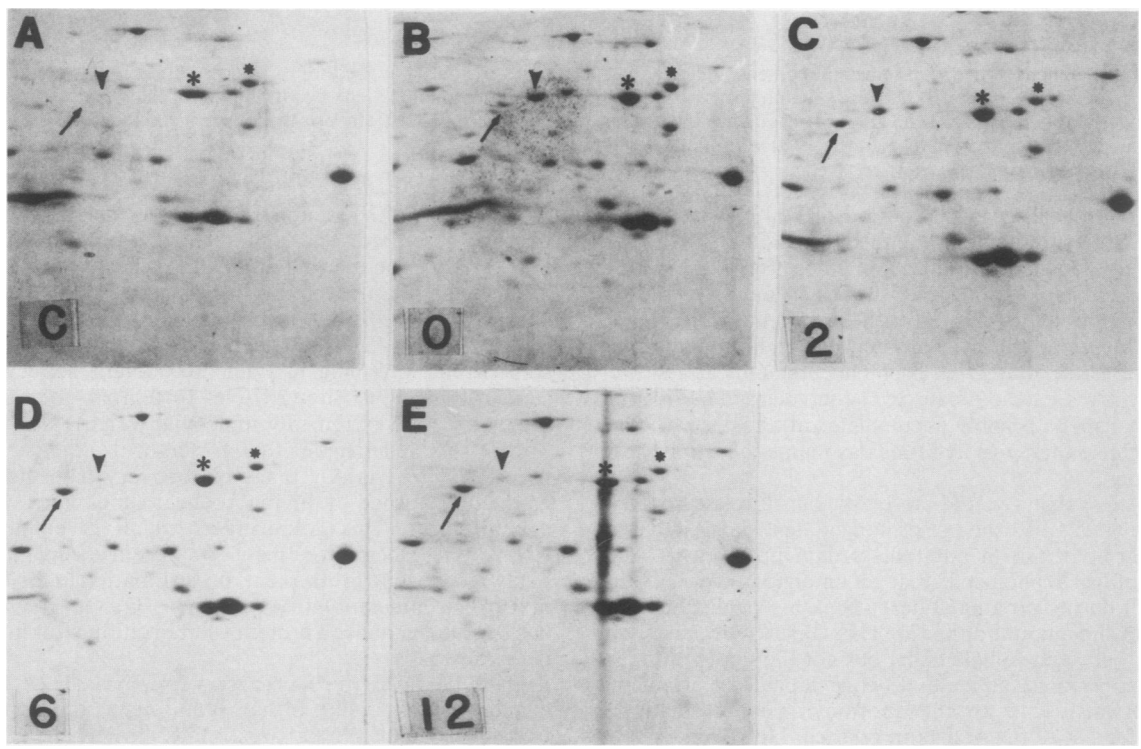


FIG. 6. Levels of hsp70-related proteins during recovery from heat shock. Adult female flies were heat shocked for 60 min at 37°C (B) and allowed to recover at 23°C for 2, 6, and 12 h (C, D, and E, respectively). (A) Non-heat-shocked control. Identification of hsp70-related proteins is as in Fig. 5. hsp68 is stable during the recovery period, whereas hsp70 is not.

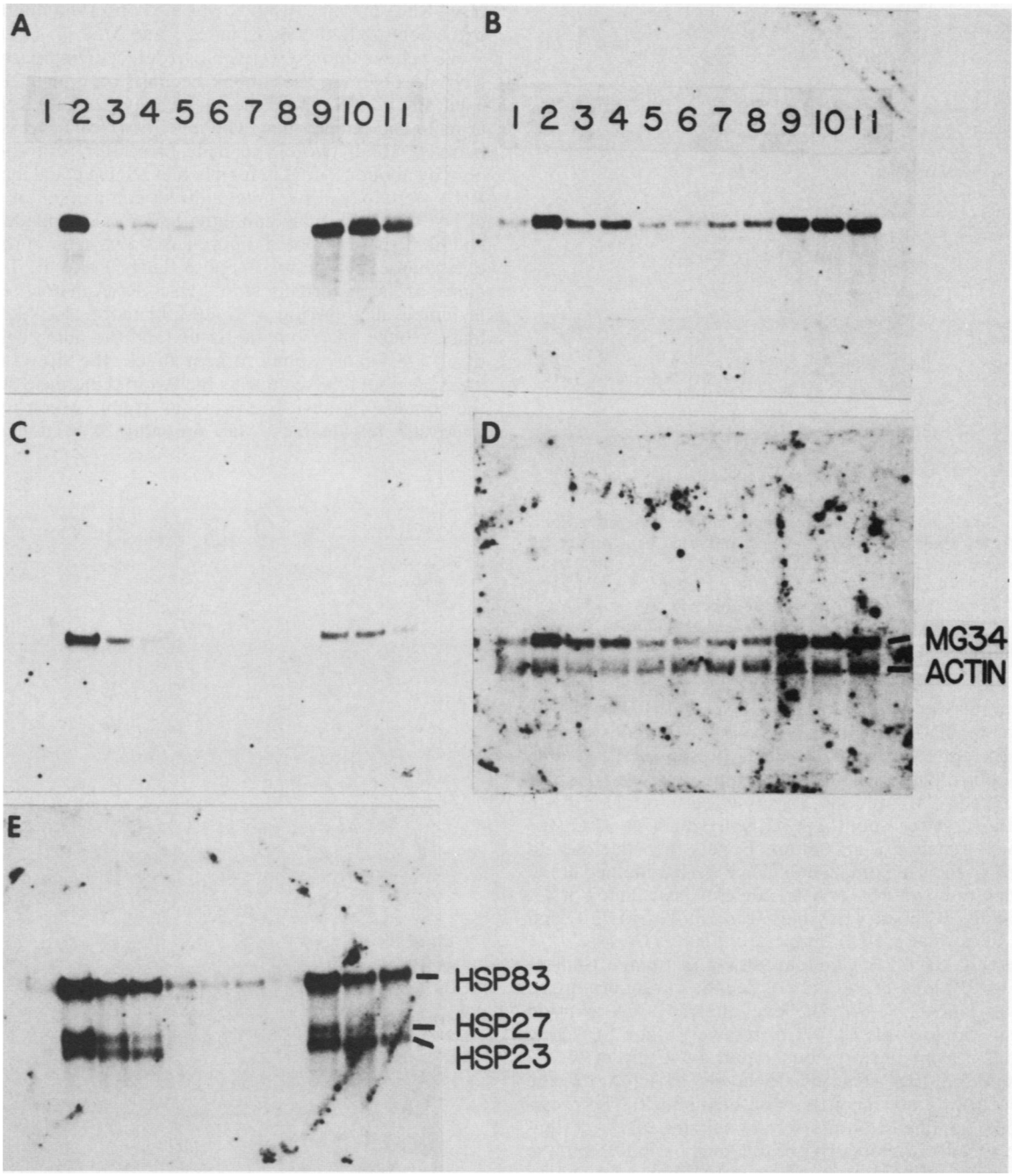


FIG. 7. Levels of heat shock and cognate RNA during heat shock and recovery. Adult male flies were heat shocked at 37°C from 1 to 8 h or heat shocked for 1 h and allowed to recover at 23°C for up to 24 h. Fly RNA was isolated and separated by gel electrophoresis through formaldehyde-agarose gels and subsequently blotted to nitrocellulose. Blots were hybridized with probes for Hsp70 (A), Hsp68 (C), Hsc4 (B), or a mixture of Hsp83, Hsp27, and Hsp23 (E). Blot B was rehybridized with an actin probe to normalize the amount of RNA per lane (D). Lane 1, control; 2, 1-h heat shock; 3, 1-h recovery; 4, 2-h recovery; 5, 4-h recovery; 6, 8-h recovery; 7, 12-h recovery; 8, 24-h recovery, 9, 2-h heat shock; 10, 4-h heat shock; 11, 8-h heat shock.

(37). However, N. Petersen (personal communication) has concluded, on the basis of pulse-labeling experiments with larvae, that hsp70 is unstable during recovery, while hsp68 is very stable. The difference in stability of hsp70 reported for tissue culture cells may be a consequence of label actually being present in cognate proteins, since analysis was carried out by one-dimensional gel electrophoresis. Alternatively, in vivo regulation may differ from regulation in tissue culture cells.

Regulation of heat shock and cognate RNA levels during heat shock and recovery. Using Northern blot analysis, we have examined the levels of RNA transcribed from various heat shock and cognate genes in adult male flies that were heat shocked at 37°C for 1 to 8 h or heat shocked for 1 h and

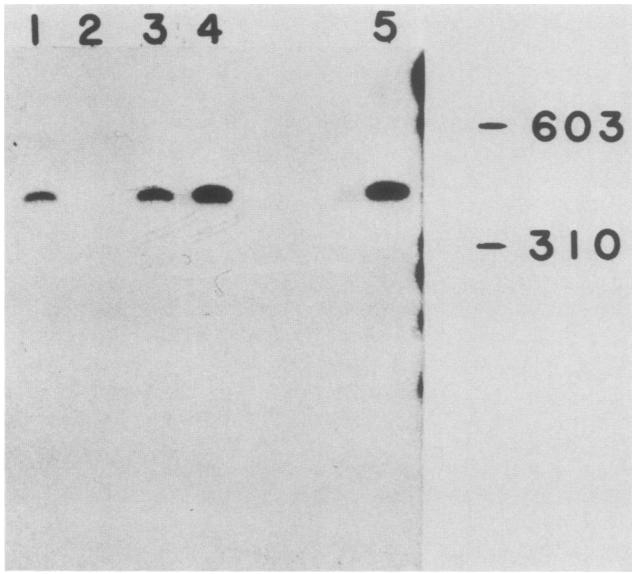


FIG. 8. Analysis of RNA transcribed from Hsc4. A 76-bp *Hae*II fragment from Mg34 was 5'-end labeled and used as a primer for cDNA extension. Lanes: Hybridization to 10 μg of control male RNA (1); no RNA (2); 10 μg of heat-shocked male RNA (3); 10 μg of control female RNA (4); 10 μg of heat-shocked female RNA (5). *Hae*III-cleaved φX174 DNA was used as marker. Numbers indicate sizes in bases.

allowed to recover at 23°C for up to 24 h (Fig. 7). The overall pattern of regulation for the five heat shock genes is very similar. Except for Hsp83, which is present at detectable levels in control flies, the heat shock genes analyzed (Hsp70, -68, -27, and -23) are not expressed at normal growth temperatures. After about 1 h of heat shock at 37°C, the RNA levels reached a maximum. Levels then declined to about 30% of their maxima when flies were maintained at the high temperature (lanes 2, 8 to 10). All heat shock RNA levels rapidly declined when flies were shifted to 23°C and returned to normal growth temperature levels by 4 h.

The level of Hsc4 RNA increased twofold upon a 1-h heat shock (Fig. 7B, lane 2) and did not decline during prolonged heat shock (lanes 8, 9, 10). The level of Hsc4 RNA returned to preheat shock levels by 4 h of recovery (lane 5). These results differ from the previous report by Craig et al. (9) which reported that steady-state levels of RNA for the cognate genes do not increase upon heat shock. The previous report examined total fly RNA, utilizing cDNA extension with specific primers. To exclude the possibility that the twofold apparent increase in Hsc4 RNA upon heat shock, observed on Northern blots, was due to cross-hybridization to Hsp68 or Hsp70 RNA, RNA levels were also examined by quantitative cDNA extension experiments (Fig. 8). Each of the members of the Hsp70 gene family has a 5' noncoding region of unique length, so cross-hybridization would result in extension products of different sizes. A ^{32}P-5'-labeled DNA primer, prepared from the amino-terminal end of the protein-coding region of Hsc4, was denatured and hybridized to the same control and heat-shocked RNA samples as those used in the experiments presented in Fig. 7 and 9. The primer DNA was elongated by reverse transcriptase and the size of the cDNA synthesized was determined by polyacrylamide gel electrophoresis. A single band with the expected size of about 400 b predicted for the Hsc4 RNA was observed. For adult males, a twofold increase in level of Hsc4 RNA upon heat shock was also observed by the cDNA extension method (Fig. 8, lanes 1 and 3).

We believe that we were able to detect an increase in Hsc4 RNA levels after heat shock because only male flies were used. Figure 9 compares heat shock and cognate RNA levels in male and female flies. The level of Hsc4 RNA is seen to increase about twofold in male flies after heat shock, but only by about 1.3-fold in female flies. Since equal amounts of RNA were loaded per lane, a direct comparison of the level of Hsc4 RNA in male and female flies can be made (lanes 1 and 6). This sex-related difference is also seen in the cDNA extension experiments of Fig. 8 (lanes 1 and 4). The abundance of the maternally stored Hsc4 RNA in oocytes results in female flies having 4- to 10-fold more Hsc4 RNA than males. Since only somatic tissue (and the nurse cells of the ovary [76]) can respond to heat shock, the stored maternal message pool of Hsc4 masks the twofold increase of Hsc4 in the somatic tissues. The previous study, which used both male and female flies, was probably skewed even more

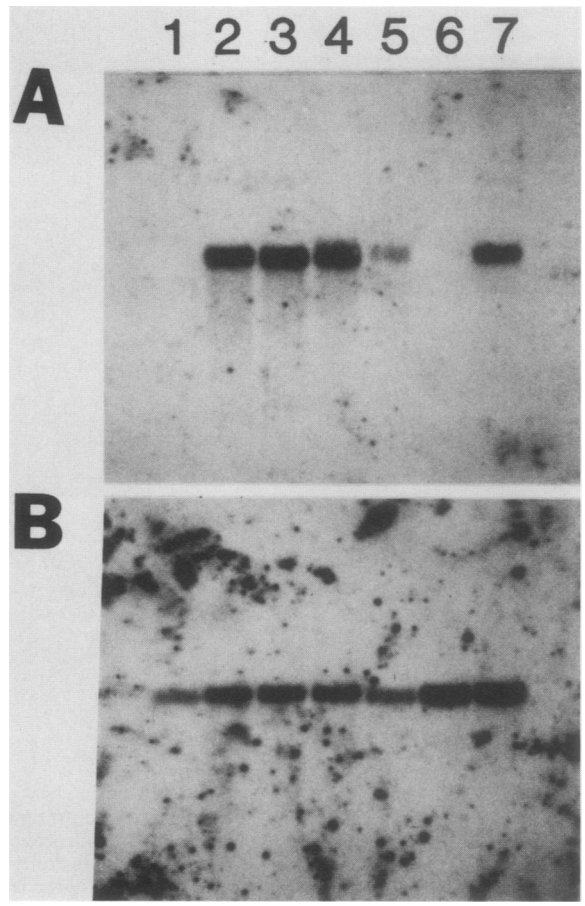


FIG. 9. Cognate RNA enhanced after heat shock. Adult male or female flies were heat shocked at 37°C or allowed to recover at 23°C for various times. Fly RNA was isolated and analyzed by Northern blots. Blots were hybridized with probes for Hsp70 (A) or Hsc4 (B). Lane 1, Control male; 2, 30 min at 37°C, male; 3, 60 min at 37°C, male; 4, 4 h at 37°C, male; 5, 4 h of recovery, male; 6, control female; 7, 4 h at 37°C, female. Note that males show an approximately twofold increase in the level of Hsc4 RNA upon heat shock, whereas females do not. The level of Hsc4 RNA in control females is fourfold that of control males due to the stored message pool in the ovaries.

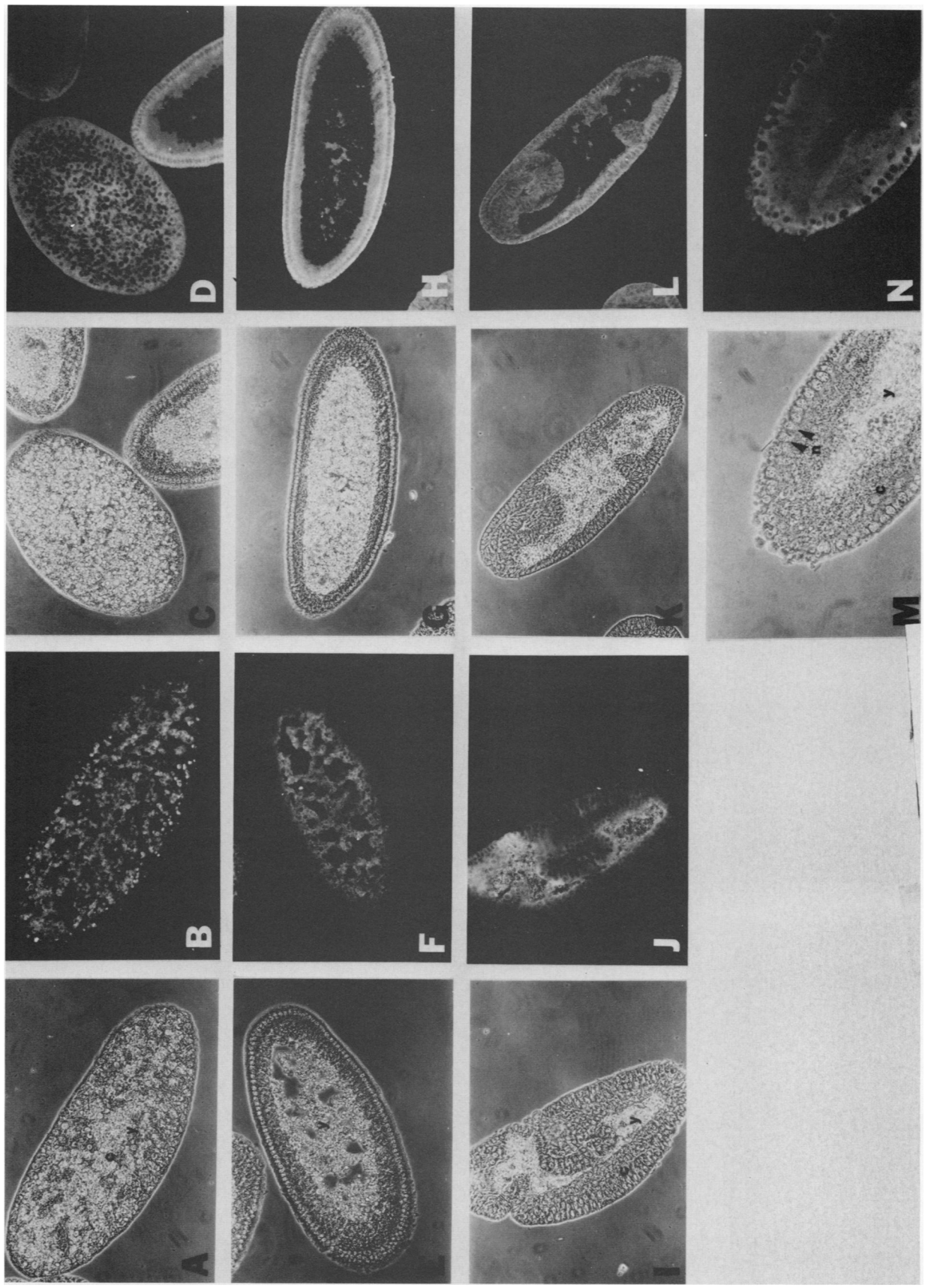
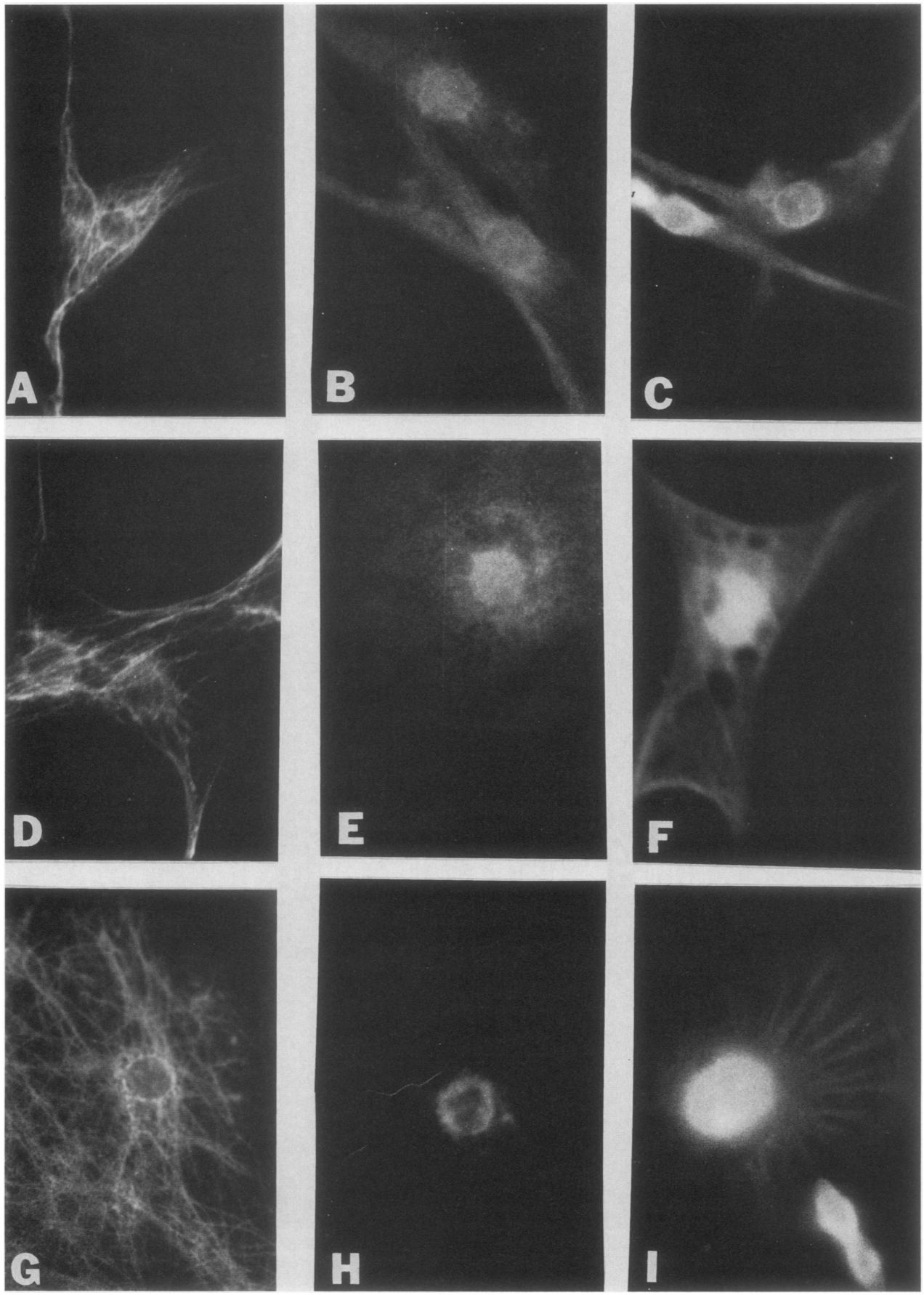


FIG. 10. Localization of cognate protein, hsc70, in *D. melanogaster* embryos. Embryos of different stages were fixed and cryostat sectioned in preparation for indirect immunofluorescence. Panels on left were stained with antiserum to vitellogenin and those on the right were stained with monoclonal antibody IPC4, which recognizes only hsc70 in control tissue. Post-fertilization stage, vitellogenin: (A) phase, (B) fluorescence. Post-fertilization stage; cognate: (C) phase, (D) fluorescence. Blastoderm stage, vitellogenin: (E) phase, (F) fluorescence. Blastoderm stage, cognate: (G) phase, (H) fluorescence. Gastrula stage, vitellogenin: (I) phase, (J) fluorescence. Gastrula stage, cognate: (K) phase, (L) fluorescence. Enlargement of region of blastoderm embryo showing nuclei, cognate: (M) phase, (N) fluorescence. Note the heavy labeling of cognate protein in the area surrounding the nuclei.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towards females because recovery of RNA is greater in females than in males (unpublished observation). Consistent with Hsc4 being a heat-responsive gene, Kirby and Bonner (29) have reported that two of the polytene chromosome bands to which cognate genes have been localized (87D and 88E) are sites of RNA polymerase II binding after heat shock. The significance of enhanced levels of Hsc4 RNA after heat shock is not clear, since the level of protein does not increase (Fig. 5). Perhaps increased levels of RNA are necessary to compensate for diminished processing or translational efficiency.

The cognate protein is localized to cytoplasmic fibers that concentrate around the nucleus. All monoclonal antibodies to hsc70 that have been characterized so far cross-react to varying degrees with hsp70 (Fig. 3). However, since hsp70 is virtually absent from normally growing cells, the antibodies can be used to localize hsc70 in nonstressed cells and embryos. Figure 10 shows the localization by indirect immunofluorescence of hsc70 in *D. melanogaster* embryos of different stages. The panels on the left show the patterns of embryos stained with antiserum to vitellogenin as a control, and the panels on the right show comparably staged embryos stained with monoclonal IPC4, which recognizes hsc70 (Fig. 3F). The distribution of hsc70 is exclusively cytoplasmic, being uniformly dispersed throughout the egg after fertilization (panels C and D) and concentrating in the egg cortex during blastoderm formation (panels G and H). Once cell formation is complete, hsc70 is found in the cytoplasm of each cell (panels K and L). An enlargement of the nuclear region, which shows that hsc70 is heavily concentrated around each nucleus, is shown in Fig. 10M and N.

Based on the extreme abundance of hsc70, one might expect it to be a structural protein. We have therefore examined the localization of hsc70 under conditions typically used for visualizing cytoskeletal proteins. Primary cultures of embryonic *D. melanogaster* cells differentiate into a wide variety of cell types (55). Muscle and fat body cells have a flattened morphology that is especially suited to visualizing cytoskeletal structures. Antibodies to tubulin and actin stain these cells in filamentous patterns characteristic of such cytoskeletal structures in mammalian cell lines (43). Figure 11 displays the staining patterns of three distinctive cell types with antibodies to hsc70 (panels C, F, H, and I), tubulin (panels A, D, and G), and intermediate filaments (panels B and E) as controls. hsc70 stains as a fibrous meshwork throughout the cytoplasm, with heavy localization in or around the nucleus. The less flattened cells show a ringlike staining around the nucleus (panels C and H), which is no longer visible in highly flattened cells (panels F and I). The pattern is strikingly similar to that obtained when cultures are stained with a monoclonal antibody specific for a 46,000-dalton *D. melanogaster* intermediate filament protein (panels B and E). *D. melanogaster* intermediate filament proteins stain these cells as a latticework of fine fibers instead of the thick fiber bundles characteristic of mammalian cell lines (16). The monoclonal antibodies to hsc70 and the 46,000-dalton intermediate filament protein are specific only for the 70,000- and 46,000-dalton proteins (data not shown), respectively, so the similarity in staining patterns is not due to cross-reactivity of the antibodies. Determining whether this similarity of staining patterns indicates a true co-localization of the two proteins requires examination by double immunofluorescence, where fluorescence patterns from the two antibodies can be superimposed in the same cell.

Visualizaton of cytoskeletal elements can sometimes be improved if cells are converted into cytoskeletons by treatment with a nonionic detergent and then fixed. Triton X-100 treatment of cells results in the extraction of most soluble proteins, leaving behind cytoskeletal proteins (43). When *D. melanogaster* cells are so treated prior to antibody staining for hsc70, a staining pattern identical to that of untreated cells is obtained, except that the latticework in the cytoplasm is more distinct (data not shown). The heavy localization of staining around the nucleus is also more apparent. *D. melanogaster* cells stained with monoclonal antibodies to hsp83 show diffuse staining throughout the cytoplasm, and this staining is eliminated by Triton treatment of cells (unpublished data).

Since hsp70 is reported to localize to the nucleus upon heat shock (3, 53, 58, 66, 67), we used antibody IPC4, which recognizes both hsc70 and hsp70, to examine their distribution after heat shock and have noted little change in intensity or distribution of fluorescence. Nuclear localization would be difficult to detect in embryonic cultured cells, as the staining is already intense over the nuclear region.

DISCUSSION

hscs are abundant in normal development. We have demonstrated that proteins related to hsp70 are present throughout *D. melanogaster* development. Three cognate proteins are present in adult flies; the most abundant (hsc70) is shown to be encoded by the previously identified gene Hsc4. hsc70 is a major protein at all stages of development, but it is especially enriched in ovaries and embryos, where it greatly exceeds the amounts of actin and tubulin. Cognate genes Hsc1 and Hsc2 are primarily expressed in adults, and either could encode the minor 70,000-dalton cognate protein. A 72,000-dalton cognate protein, hsc72, is present at all stages of development and therefore does not fit the pattern of expression of either Hsc1 or Hsc2. There may exist additional cognate genes and proteins.

Regulation of hsp70-related proteins. We have examined the levels of accumulation of all major hsp70-related proteins in larvae and adult flies to observe biologically significant regulation and also the physiological effects of various treatments. We do not detect hsp70 or hsp68 in nonstressed tissues by either immunoblot or Northern blot analysis, in agreement with a previous report (68). The most significant finding to come out of these studies is that the steady-state levels of hsp70 and hsp68 at 37°C are only 30% that of hsc70. If one sums the amount of all hsp70-related protein in flies at 23 and 37°C, the level would increase less than twofold. The maximal level of accumulation of hsps in adult flies occurs at

FIG. 11. Localizaton of a cognate protein to cytoplasmic fibers that concentrate around the nucleus. Primary cultures of *D. melanogaster* embryonic cells were fixed and stained with monoclonal antibodies specific for intermediate filament proteins (B, E), monoclonal antibody IPC4, which is specific for hsc70 in control cells (C, F, H, I), and tubulin (A, D, G). Note the similarity in staining patterns of cognate proteins and intermediate filament proteins in *D. melanogaster* cells. Antibodies IPC4 and α-int-fil are specific for the 70,000- and 46,000-dalton proteins, respectively, as determined by immunoblots of one- and two-dimensional NEPHGE gels. The *D. melanogaster* 46,000-dalton protein focuses outside of the pH range of the gels shown in Fig. 1. In NEPHGE gels it focuses directly above the yolk protein, YP3.

37°C, since above this temperature, flies will not survive for more than a few minutes.

Careful studies of *D. melanogaster* tissue culture cells that have been heat shocked at various temperatures have shown a reproducible positive correlation between the level of accumulation of hsps and the severity of the stress (12). hsps are synthesized to varying extents over a wide range of temperatures, starting from 27°C. As flies are fertile up to 29°C, hsps are induced at temperatures that can be considered normal growth temperatures. A similar pattern of rapid increase in rates of transcription of hsps to achieve a new steady-state level of proteins is found in *E. coli* (19). DnaK, which is 48% homologous to hsp70, is the seventh most abundant protein at 30°C, and its steady-state level is increased about twofold at 42°C and fivefold at 46°C (19). This increase is equivalent to the increase in all hsp70-related proteins in *D. melanogaster* over the same temperature interval. In *E. coli*, a single Hsp70-related gene suffices for both normal growth and growth at elevated temperature.

In higher organisms there are both constitutive and inducible Hsp70-related genes. If both classes of genes perform similar functions, then the purpose of the heat shock response in higher organisms might also be to modulate the amount of Hsp70-related proteins. On the other hand, heat-inducible and cognate proteins may perform different, although related, functions. In yeasts, there are eight members of the Hsp70 family, which can be grouped into four classes by their regulation (11a). Two members, YG100 and YG102, are 96% homologous and have been shown genetically to have the same function. However, YG100 has a low basal level, shows significant induction upon heat shock, and may be categorized as a heat shock gene, while YG102 has a high basal level, shows negligible induction upon heat shock, and may be categorized as a cognate gene (14). In strains in which both YG100 and YG102 are deleted, another gene, YG107, is constitutively expressed at low temperatures (11a). Although YG107 is expressed only at high temperatures in wild-type strains, it is able to partially compensate for the absence of the YG100 and YG102 products when expressed at low temperatures (M. Werner-Washburne and E. Craig, unpublished results). This suggests that the products of these genes have similar functions. In *D. melanogaster*, as well, the question of whether all hsps and cognates of the 70,000-dalton family perform the same or different functions has not been resolved.

The major cognate protein has an organized structure around the nucleus. One criterion for evaluating whether hsp70 and hsp68 have functions similar to those of the cognate proteins is whether they have the same subcellular location. We have shown by indirect immunofluorescence microscopy of embryo sections that hsc70 is localized to the cytoplasm of syncytial embryos and individual cells. The staining is especially intense around each nucleus, giving the impression that hsc70 is forming a basket around each nucleus that is contiguous with fibers that extend into the cytoplasm. Similar staining of primary cultures of embryonic *D. melanogaster* cells is observed. The staining is so intense over the nuclear region that it is often difficult to distinguish whether staining is intra- or extranuclear. We believe that the staining of tissue culture cells is extranuclear for two reasons. The staining is clearly extranuclear in sections of gastrula staged embryos and adult tissues (unpublished results). Therefore, hsc70 would have to translocate into the nucleus as a consequence of growth in culture, which we think unlikely. When cells are first plated and are round, the staining looks cytoplasmic and only begins to look nuclear as the cells flatten. Second, the staining over the nucleus never has a defined boundary but is always contiguous with fibers extending well into the cytoplasm (Fig. 11). We have also shown that the staining pattern remains unchanged if cells are extracted with Triton X-100 before fixation and subsequent staining (unpublished results). Detergent treatment of cells results in extraction of most soluble proteins and leaves behind cytoskeleton-associated proteins.

The pattern of immunofluorescent staining for hsc70 is remarkably similar to that of *D. melanogaster* intermediate filament proteins in both cultured cells and embryos (Fig. 11) (6, 70). In *D. melanogaster*, a 46,000-dalton protein that is related to vimentin forms structures similar to the 10-nm filaments of vertebrates, but in tissue culture cells fails to form the thick bundles that give rise to the characteristic vertebrate vimentin-staining pattern (70). Other workers have shown that, although monoclonal antibodies specific for this 46,000-dalton protein give immunofluorescent staining patterns that appear to show nuclear staining in cultured cells, immunoelectron microscopy clearly shows no intranuclear staining, as expected for a cytoskeletal protein (70). In collaboration with M. Walter and H. Biessmann, we have examined the staining patterns of whole *D. melanogaster* embryos, using either intermediate filament- or cognate-specific antibodies. Both proteins clearly localize to cytoplasmic fibers that are concentrated around the nucleus (manuscript in preparation). We do not yet know whether the similarity in staining patterns for hsc70 and intermediate filament proteins represents true co-localization of the proteins. A monoclonal antibody that seems to recognize a determinant common to all intermediate filament classes in all species tested (47) fails to cross-react with hsc70, and therefore it seems unlikely that hsc70 is itself an intermediate filament type of protein. Studies utilizing double immunofluorescence with monospecific antibodies for each protein are currently in progress. The significant observation is that one can obtain immunofluorescent staining patterns in cultured cells that appear to show nuclear staining even for proteins that are cytoskeletal such as intermediate filament proteins and, we believe, hsc70.

In *D. melanogaster* and vertebrate cells, localization by indirect immunofluorescence and cell fractionation techniques places hsp70 in both the nucleus and the cytoplasm with decreasing nuclear localization on recovery from heat shock (66, 67). Nuclear hsp70 has been reported to be chromatin (3, 67), nucleolus (45, 73), and nuclear matrix associated (32, 57). A fraction of the cytoplasmic hsp70 of chicken and vertebrate cells is reported to be cytoskeleton associated (22, 71, 73). Velazquez and Lindquist (67) have shown that the relative distribution of hsp70 between cytoplasm and nucleus is temperature or stress dependent. If hsp70 and hsc70 perform similar functions, one explanation for the apparent paradox in their different cellular localization would be that both proteins actually have the same cellular distribution, such that they would be primarily cytoplasmic at low temperature and nuclear at elevated temperature. It is clearly necessary to examine sectioned material utilizing double immunofluorescence with monospecific antibodies for hsp70 and hsc70 to determine if the two proteins have the same or different locations after heat shock and to distinguish intra- from extranuclear staining. In preliminary experiments, immunofluorescent staining of sections of heat-shocked gut cells with monoclonal antibody IPC35 (Fig. 3) indicates that hsc70 also redistributes to the nucleus after heat shock (K. B. P. and G. Gorbsky, unpublished results).

Function of Hsp70-related proteins in normal and stressed cells. Although many different theories have been put forth to explain the functions of hsps, they have failed to take into account the presence of hsps and the related cognate proteins in normal development. Genetic analysis has shown *E. coli* DnaK to be necessary for growth, at least at elevated temperature (50). DnaK has also been shown to possess a weak ATPase and kinase activities and seems to play a role in lambda DNA replication (77). In yeasts, an hsp70-related protein product (YG100 or YG102) has been shown to be necessary to attain normal growth rates at the optimal growth temperature of 30°C and is essential for colony formation at 37°C. While heat-inducible members of the 70,000-dalton family are not present in *D. melanogaster* under normal conditions, as reported here, related proteins are present in all stages of development. Also, preblastoderm staged embryos contain hsp83, hsp27, and hsp26 (76). The cognate and hsps probably perform functions during normal development that are not stress specific, but may be especially useful during stress conditions. hsp83 and hsc70, which are always present under normal growth conditions, are especially abundant in embyros. High levels of these proteins may be related to the high metabolic rate of embryos.

When organisms are given a moderate heat treatment at temperatures that induce hsps but do not interfere with normal protein synthesis, they exhibit enhanced survival when challenged by more severe heat treatment. The protective effects of hsps extend to other stresses so that in *D. melanogaster* pretreatment with mild anoxia or heat will mutually protect the cell from the effects of the other (67). Investigators have tried to correlate the presence of a particular hsp with the cell's acquired thermotolerance, but results have been inconclusive and must await mutational analysis. Mutations in two yeast hsp70-related genes (YG100 and YG102) result in cells that are unable to grow continuously at 37°C, but that still exhibit normal thermoprotection when briefly challenged at 52°C (10). Simultaneously, mutating both of the highly inducible hsp70-related genes (YG106 and YG107) results in no detectable phenotype (M. Werner-Washburne and E. Craig, unpublished results). Thus, hsp70 may not be involved in thermoprotection, but in the ability to maintain growth at high temperature. There are at least two types of mechanisms that can be proposed to explain the protective effects of hsps. In the first, hsps either directly prevent damage to cellular structures or help repair damage if it occurs. In the second type of mechanism, hsps alter the cell's metabolic efficiency in such a way that it is better able to meet the challenge of a stress. The latter mechanism is consistent with the observation that a multitude of stimuli can elicit the stress response and also with the presence of hsps and hscs in normal development.

ACKNOWLEDGMENTS

K.B.P. is indebted to John Sedat and Alan Blumenthal for instruction on monoclonal antibody technology and to Tom Cotner and Juliet Fuhrman-Wolf for helpful advice on immunological techniques. We thank Susan Lindquist, Jose Velazquez, and Margaret Fuller for providing some of the monoclonal antibodies used in this study and Jose Bonner for providing the first extract of heat-shocked cells. We thank Griffith Parks for performing the cell-free translations. We are grateful to Kit Tilly, Lynn Manseau, and Margaret Werner-Washburne for useful discussions and criticism of the manuscript.

This research was supported in part by a grant from the National Science Foundation awarded to A.P.M. and a grant from the Public Health Service National Institutes of Health awarded to E.A.C.. K.B.P. was supported by a National Institutes of Health postdoctoral fellowship, and M.W. was supported by an American Cancer Society postdoctoral fellowship.

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