Is the Major *Drosophila* Heat Shock Protein Present in Cells That Have Not Been Heat Shocked?

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ABSTRACT When eukaryotic cells are exposed to elevated temperatures they respond by vigorously synthesizing a small group of proteins called the heat shock proteins. An essential element in defining the role of these proteins is determining whether they are unique to a stressed state or are also found in healthy, rapidly growing cells at normal temperatures. To date, there have been conflicting reports concerning the major heat-induced protein of *Drosophila* cells, HSP 70.

We report the development of monoclonal antibodies specific for this protein. These antibodies were used to assay HSP 70 in cells incubated under different culture conditions. The protein was detectable in cells maintained at normal temperatures, but only when immunological techniques were pushed to the limits of their sensitivity. To test for the possibility that these cells contain a reservoir of protein in a cryptic antigenic state (i.e., waiting posttranslational modification for use at high temperature), we treated cells with cycloheximide or actinomycin D immediately before heat shock. HSP 70 was not detected in these cells. Finally, we tested for the presence of a reservoir of inactive messages by using a high stringency hybridization of ^{32}P-labeled cloned gene sequences to electrophoretically separated RNAs. Although HSP 70 mRNA was detectable in rapidly growing cells, it was present at less than 1/1,000th the level achieved after induction.

The heat shock response was first discovered in the fruit fly *Drosophila*. When cells of this organism are shifted from 25° to 37°C, protein synthesis is rapidly redirected from the broad spectrum characteristic of normal growth to a small group of proteins of as yet unknown function, the so-called heat shock proteins (HSPs) (see reference 2 for review). Remarkably similar responses exist in virtually every eukaryote examined to date, from chickens and hamsters (16) to slime molds (25) and soybean seedlings (4, 18). The response is induced not only by heat but also by a bewildering variety of other agents (e.g. treatment with respiratory inhibitors, heavy metal ions, and amino acid analogues) and may well be a general response to stress (see reference 32). In every species the major protein has a molecular weight close to 70,000 daltons. This protein, designated HSP 70, has been shown to be highly conserved by a variety of immunological (17) and biochemical criteria (25, 34). The cloned gene for the *Drosophila* protein cross-hydridizes with DNA sequences from humans, mice, chickens, lizards, yeast, and corn (R. Morimoto, personal communication). Finally, the amino acid coding sequence of yeast HSP 70 (deduced from nucleotide sequencing) shows 72% homology with the *Drosophila* protein (14).

That this response is so rapid, ubiquitous and highly conserved suggests that these proteins are of fundamental importance to the biology of eukaryotic cells. To the point, recent evidence from several laboratories suggests that the proteins provide protection from developmental anomalies and lethality at elevated temperatures (11, 25, 26, 29; 1979; see also reference 32). It is therefore extremely frustrating that, despite the intensive investigation this response has received, we still do not know how these proteins function at a molecular level. In ascertaining their function, a key question is whether they are unique to a stressed state or are also found in normal cells under optimal growth conditions.

At present, the literature contains contradictory reports. Several authors have noted that actinomycin D completely blocks the appearance of HSP 70 when administered just before heat shock. This strongly suggests that the protein is synthesized on new, heat-induced messages (21, 27). On the other hand, other authors have observed the protein as a major stainable spot on two-dimensional gels of protein from non-heat shocked cells (28, 30, 31). And, using a rabbit serum prepared against chicken HSP 70, Kelley and Schlesinger (17) found substantial quantities of cross-reacting material in 25°C *Drosophila* embryos. Furthermore, Findly and Pederson (9) found modest quantities of HSP 70 RNA in 25°C cells (0.045% of total nuclear RNA in 25°C cells was complementary to HSP 70 DNA).

A possible explanation for these seeming contradictions is provided by the recent discovery of Ignolia and Craig (13) that

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the *Drosophila* genome contains a family of unlinked DNA sequences with a high degree of homology with respect to those coding for HSP 70. These heat shock "cognate" genes are abundantly transcribed into RNA at normal temperatures but are not induced by heat. The RNAs contain long open reading frames that would code for proteins closely related to HSP 70 (13). Recent evidence from Wadsworth's laboratory strongly suggests that they are translated into protein (36). Against this background of "cognate" synthesis, determining whether HSP 70 and its RNA are also present at normal temperatures requires more rigorous criteria than have been previously employed.

MATERIALS AND METHODS

Cell Culture: Cells of Schneiders *Drosophila* tissue culture cell line #2 were grown in powdered Shields and Sang medium (24) supplemented with 12% fetal calf serum. With volumes of no more than 2.5 ml in a 25 cm^2 falcon flask, cell densities plateaued at greater than 3×10^7/ml. Log phase cells at densities of $1.0–0.5 \times 10^7$/ml were used for all experiments.

Immunological Procedures: Antigenic specificity of rat sera was tested on nitrocellulose blots of electrophoretically separated ^{3}H-proteins from heat-shocked and control cells (22). Gel blots (5) were incubated with 1% BSA and 10% serum in 0.85% NaCl for 2 h, washed in 0.85% NaCl, then reacted with a 1:100 dilution of immune serum for 2 h. After several washes in saline the blot was incubated with FITC-conjugated rabbit anti rat IgG, rinsed, and photographed under UV illumination. The blot was then dipped in a solution of 7% PPO in CC14 for fluorographic analysis of labeled proteins.

The specificity of monoclonal antibodies was tested by immunoadsorption. Ammonium sulfate-precipitated immunoglobins from hybridoma culture supernatants were mixed and bound to CNBr-activated filter paper as described by Clarke et al. (7). Cells washed with ice-cold 0.85% saline were lysed in ice-cold 10 mM Tris-HCl pH 7.4, 0.5% NP40, and 1 mM PMSF by passage through a 25-gauge syringe. Filters with bound antibody were washed extensively in 140 mM NaCl, 10 mM Na_2HPO_4 (pH 7.4) and incubated with cell lysates overnight at 4°C. The filters were washed extensively with 0.85% saline followed by detergent solution (140 mM NaCl, 10 mM Na_2HPO_4 pH 7.4, 1% NP40, 1% Na deoxycholate, 0.1% SDS, and 1 mM PMSF). Proteins were eluted with 2% SDS sample buffer (2% SDS, 2.5% β-mercaptoethanol, 10 mM Tris pH 6.8) heated to 100°C for 5 min, and analyzed by electrophoresis (19, 20).

For enzyme-linked immunoabsorbence assays (ELISA), cell lysates were prepared by sonication. The protein concentration of each lysate was adjusted to 25 μg/ml in 50 mM sodium carbonate pH 9.6 with 3 mM sodium azide. Aliquots of lysate (100 μl) were added to individual wells of polyvinyl chloride microtiter plates and incubated overnight at 4°C. The plates were washed extensively with WB (140 mM NaCl, 10 mM PO_4 pH 7.4, 0.05% Tween 20). Culture supernatants from hybridoma clones (diluted 1:2 with WB) or rat sera (diluted 1:100 with WB) were added to the wells and incubated at 37°C for 2 h. Plates were washed with WB, incubated with rabbit anti-rat IgG, washed again, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h at 37°C. Plates were washed again with WB before the addition of phosphatase substrate (p-nitrophenol phosphate) in diethanolamine buffer (35). Optical densities were read at 405 nm after 30 min at room temperature. Wells treated with antigen, rabbit anti-rat antibody, enzyme, and substrate (minus the hybridoma supernatants or rat sera) were used to correct for background.

RNA Analysis: Cells were gently removed from falcon flasks, collected by centrifugation (50 *g* for 90 s), and resuspended in Shields and Sang medium minus serum at a density of 10^7/ml. Aliquots of 100 μl were distributed to 10 × 75 mm glass tubes, incubated for 30 min at 25°C, then shifted to the appropriate temperatures for one hour. Incubations were terminated by the addition of 1 ml of ice-cold isotonic saline. Cells were immediately collected by centrifugation, the supernatants discarded, and the insides of the tubes wiped to remove remaining medium. A 200-μl aliquot of ice-cold lysis buffer (100 μg/ml Proteinase K, 0.5% SDS, 50 mM Tris-HCl, pH 7.3) was added to each tube. After digestion at 37°C for 1 h, 10 μl of 1M NaCl was added and nucleic acids were precipitated with 800 μl of absolute ethanol. The precipitate was collected by centrifugation, rinsed once with 70% ETOH (buffered with 0.01 mM HEPES, pH 6), and dried in vacuo.

Samples were resuspended in 50 μl of gel loading buffer (0.1 × E buffer, 10% glycerol (3). Bromphenol blue and xylene cyanole were added to 0.05% and SDS to 0.2%. Samples were placed at 60°C for 2–3 min, and 10 μl (~2 μg) were layered on 1.5% agarose- 10 mM CH_3HgOH gels (1.5-mm thick).

Gels were stained in 10 mM Tris-HCl, pH 7.3, 5 mM 2-mercaptoethanol, and 2 μg/ml ethidium bromide for 30 min. Three 10-min washes of a 7 mM Na iodoacetate, 10 mM $NaPO_4$, pH 6.8 solution were used to destain the gels, after which they were photographed and the RNA was transferred to DMB paper (1). Prehybridized filters (1) were hybridized with nick-translated plasmids in 50% formamide, 10% dextran sulfate, 1 × Dernhardt's solution and 1 × SSC for 20 h at 42°C, extensively washed at 50°C in 50% formamide, 0.1% SDS, 1 × SSC, followed by a wash in 0.1 × SSC and 0.5% SDS at 52°C. Filters were air dried and exposed to x-ray film at −86°C with a Dupont cronex lighting plus screen. Films were quantified with a Joyce-Loebl densitometer. A serial dilution of ribosomal RNA, blotted to nitrocellulose and hybridized with a nick-translated plasmid probe, was used as a standard for quantification.

RESULTS

The Production of Monoclonal Antibodies

Antigen was prepared from *Drosophila* tissue culture cells heat shocked at 36.5°C for 3 h. Proteins were isolated from the 70,000-dalton region of SDS polyacrylamide gels and injected into two Lewis rats. To increase the likelihood of obtaining IgG producing hybridomas, we gave booster injections over a 2½ month period. Throughout this period, the antigenic response of each rat was monitored. Given the extremely high evolutionary conservation of HSP 70, we were surprised to find that the protein was very antigenic. Strong responses against HSP 70 were detected in both rats although the sera also contained antibodies against other, non–heat-induced proteins. Fig. 1 displays nitrocellulose blots of electrophoretically separated proteins from heat shock and control cells. The fluorogram (on the left) displays the distribution of total labeled proteins in these cells; the immunofluorescence (on the right) displays the antigenic specificity of serum from one rat. Two bands are visible in the 70,000-dalton region in the heat-shocked sample. One of these bands is also present in the 25°C sample.

To obtain hybridomas, we fused spleen cells from this rat

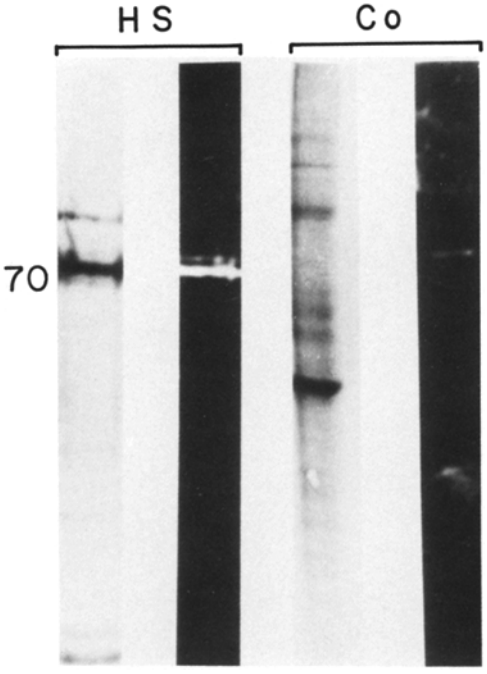

FIGURE 1 Antigenic specificity of rat sera. Proteins from heat-shocked and control cells were electrophoretically separated and blotted onto nitrocellulose. Labeled proteins were visualized by fluorography (*left*). Proteins reacting with rat sera were visualized by indirect immunofluorescence using an FITC conjugated rabbit anti-rat IgG (*right*).

with mouse myeloma cells (Sp2/0-Ag14) and plated them in HAT medium to select for hybrids (Goding 1980). Over 90% of the 672 wells plated were positive for growth. Supernatants from each of the positive wells were tested for immunological specificity in enzyme-linked immunosorbence assays (ELISA) (see Materials and Methods for details). Twenty-one supernatants contained antibodies against *Drosophila* proteins; of these, twelve were specific for proteins from heat-shocked cells. The 10 highest positive clones were chosen for replating at limiting dilutions. As the hybrids shed chromosomes and became stable for rapid growth, some clones lost their antigenic specificity. After repeated subculturing, three clones established stable, fast-growing lines with high rates of antibody production.

Protein Assays

The specificity of the monoclonal antibodies is demonstrated in Fig. 2. Antibodies from the three clones were mixed and bound to CNBr-activated filter paper (7). These filters were used to select protein from three types of cell lysates: (#1) labeled during heat shock at 36.5°C; (#2) labeled first at 25°C (normal growing temperature), then shifted to 36.5°C halfway through the labeling period; (#3) labeled at 25°C. After several stringent washes, bound proteins were eluted from the filters with SDS sample buffer and analyzed by electrophoresis and fluorography. Total labeled proteins from the original lysates (T) are displayed next to proteins selected from them by the antibodies (S).

The only protein selected was HSP 70. Note that HSP 68, whose nucleotide coding sequence shares 85% homology with that of HSP 70, was not bound. Large quantities of HSP 70 were obtained from cells labeled at 36.5°C (#1). Significant quantities were obtained from cells shifted to 36.5°C midway through the incubation (#2). At this level of exposure (2 wk), no HSP 70 was detected in cells labeled and maintained at 25°C (#3). An extremely light band was observed at this position with a two-month exposure, but it was much too faint to be quantified.

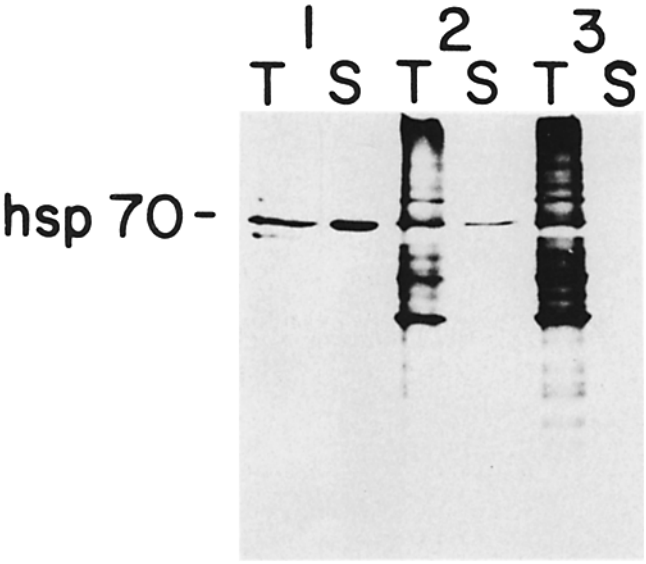

FIGURE 2 Immunoabsorption of *Drosophila* HSP 70 with monoclonal antibodies. Antibodies were covalently coupled to an inert support and used to select proteins from total cell lysates. Bound proteins were eluted with sample buffer and analyzed on SDS polyacrylamide gels on aliquots of the original lysate run on the same gel. Labeled proteins were visualized by fluorography. *T*, total protein; *S*, protein selected by immunoabsorption. Tissue culture cells were labeled with [^{3}H]leucine as follows: 1. heat shocked for 30 min 36.5°C, then labeled at 36.5°C for 2 h. 2. labeled at 25°C for 1 h, then shifted to 36.5°C for 1 h. 3. labeled at 25°C for 2 h.

TABLE I

ELISA Assays of Drosophila Cell Lysates with Rat Sera and Hybridoma Supernatants

	Hybridoma			Rat serum	
Lysate	7.1	25	2	1	2
25°C control	0.05	0.01	0.00	0.34	0.59
36.5°C heat shock	1.03	0.81	0.97	0.73	0.85
Heat shock + actinomycin t = 0	0.03	0.03	0.01	NT	NT
Heat shock + cycloheximide t = 0	0.05	0.03	0.01	NT	NT

Cells were heat shocked for 1 h at 36.5°C. Actinomycin (1 μg/ml) and cycloheximide (10 μg/ml) were added 2 min before temperature elevation. The second antibody (rabbit anti-rat) was coupled to alkaline phosphatase and optical densities were read at 405 nm. *NT*, not tested.

These data suggest that HSP 70 is not present in substantial quantities in healthy, rapidly growing cells. It is possible, however, that the protein is present but in a cryptic antigenic state. Although we used three different hybridomas, the specificity of monoclonal antibodies is extremely narrow. They might be directed against new antigenic determinants formed by heat-induced post-translational modifications of pre-existing protein. To investigate this possibility, we treated cells with cycloheximide or actinomycin D immediately before heat shock. These treatments will block synthesis of new HSP 70 but should allow posttranslational modification of pre-existing protein. Cell lysates were tested in ELISA assays with antibodies produced by each of the three hybridomas. (The sensitivity of this assay is very high, comparable to that of radioimmunoassays; reference 35). Data from a typical experiment are presented in Table I. Both inhibitors blocked the appearance of HSP 70 antigen. Thus, cells that have never been exposed to heat do not contain a reservoir of HSP 70 awaiting modification for use at high temperatures.

RNA Assays

The antibody data do not address the question of whether or not HSP 70 mRNAs are present in 25°C cells. A pool of message might exist in these cells, blocked from translation until activated by heat. We therefore tested for the presence of RNAs of discrete message size which would hybridize to HSP 70 DNA sequences.

Tissue culture cells grown at 25°C were incubated at various temperatures for 1 h. RNA was isolated, electrophoretically separated on methyl mercury gels, transferred to nitrocellulose, and hybridized with a nick-translated plasmid containing the HSP 70 gene. The stringency of hybridization was sufficient to prevent cross hybridization with "cognate" RNAs.

As shown in Fig. 3, massive quantities of message were produced with incubation at 35°C or 37°C. No induction was apparent at 27°C, and only a slight induction was observed at 29°C, in agreement with studies of protein synthesis (22). An extremely small quantity of message-size RNA was detected in cells maintained at 25°C or 27°C, but only with very long autoradiographic exposures. Densitometric scans of a series of exposures of this blot indicate that the RNA is present at 25°C in ~1/1,000th the concentration attained at 35°C.

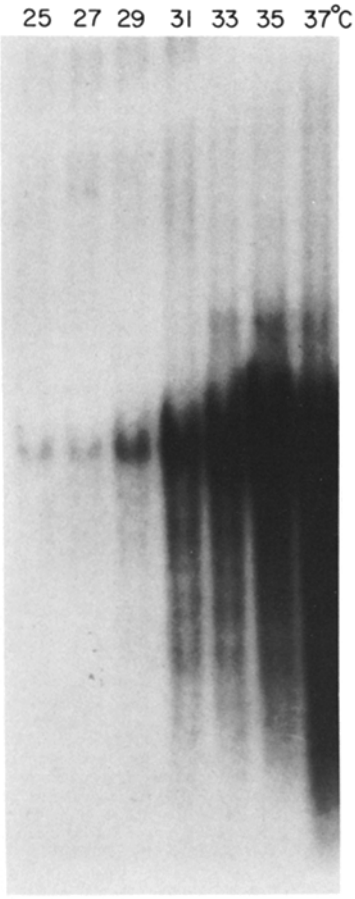

FIGURE 3 Assay for HSP 70 mRNA at various temperatures. RNA was extracted from cells incubated at the indicated temperatures for 1 h. After electrophoretic separation, ethidium bromide staining showed that each well contained an equal quantity of total cellular RNA. Following transfer to nitrocellulose, the RNA was hybridized with nick-translated HSP 70 DNA at 42°C in 50% formamide with 1 × Dernhardt's solution and 1 × SSC. Filters were extensively washed at 50°C in 50% formamide and then at 52°C in 0.1 × SSC and 0.1% SDS prior to autoradiography.

Independent of the antibody analysis, data from the Northern blots allow us to calculate an upper limit for the number of HSP 70 molecules that could be present in our cells at 25°C. Under maximum induction conditions, there are several thousand HSP 70 mRNAs per cell (23). The induction shown in Fig. 3 was not produced under maximum conditions (36.5°C with this medium) (22), yet a 1,000-fold increase in message-sized RNA was observed. Thus, there can be no more than two or three molecules of HSP 70 mRNA per cell at normal growing temperatures. The rate of synthesis of HSP 70 was previously determined to be 9–14 molecules per message per minute at 37°C (23), equivalent to the fastest known rates in other eukaryotic systems at this temperature. Using the Arrhenius equation and assuming an activation energy of −26 kcal for translation, production should slow to two to three molecules per message per minute at 25°C (Spirin, 1979; Conconi et al., 1966; Hunt et al., 1969). (This rate is consistent with experimentally determined values in other insect systems at 25°C (15). With a cell doubling time of 20 h and an infinite protein half-life, there could be at the very most 10^4 molecules of HSP 70 per cell at 25°C. Under maximum induction conditions, when the protein appears as a major stainable band on SDS gels, there are at least 2×10^7 molecules per cell (23).

DISCUSSION

Our data demonstrate that *Drosophila* cells growing at normal temperatures do not contain substantial quantities of HSP 70. We cannot eliminate the possibility that the protein is nonrandomly distributed in a small fraction of cells, in S phase for example. We were not able to test this possibility because methods employed to synchronize *Drosophila* cells are also likely to stress them. Nevertheless, it seems clear that the primary function of HSP 70 is specific to the stressed state.

There are several reasonable explanations for previous reports of high concentrations of HSP 70 in 25°C cells. One worth mentioning is a difference in culture conditions. We find (data not shown) that cells grown in suspension culture, or in Falcon flasks with large volumes of media, have higher constitutive levels of HSP 70 mRNA. This is most likely due to slight anoxia produced by low surface-to-volume ratios. (Indeed, we find that partial anoxia is a very good inducer. J. M. Velazquez, manuscript in preparation). While we have not surveyed other cell lines, we find generally that conditions that lead to suboptimal growth rates in our cells also lead to higher constitutive levels of heat-shock synthesis. Should this prove a general rule, immunological assays for HSP 70 might provide a fast and convenient method for optimizing cell culture conditions.

A related issue is the possibility of inadvertent induction during experimental manipulation. Indeed, we find that a transient induction of HSP 70 mRNA occurs when cells are passed too vigorously through a pipette or pelleted too hard during centrifugation. These findings demonstrate that to provide zero induction controls for the heat-shock response one must use healthy, rapidly growing cells which have been exposed to a minimum of stress.

These types of problems certainly cannot explain all previous reports of HS mRNA and protein in 25°C cells. As mentioned in the Introduction, a most intriguing possibility is that the "cognate" genes of Ignolia and Craig (13) produce substantial quantities of protein at 25°C (see also reference 36). This raises several interesting questions. Do the cognate proteins play an entirely independent role in the cell, or do they provide the same function at low temperature as the heat shock proteins do at high temperature? If the latter, what is different about their form and function that would account for such different induction characteristics?

Another intriguing question is whether the extremely low basal level of HSP 70 synthesis we observe at 25°C is quantitatively regulated. The coding sequence for this protein is present in five copies per haploid genome. At 25°C the genes are in an open chromatin configuration with hypersensitive sites at their 5′ ends (37, 38). A low level of transcription may well be an inescapable and insignificant physical consequence of this arrangement. Alternatively, it might be an essential part of some regulatory mechanism that keeps the genes ready for immediate activation. In other systems, genes that are able to shift rapidly to high levels of synthesis in response to an environmental signal also exhibit a low level of expression in the repressed state (6). In the lactose operon, this limited transcription provides a low concentration of lactose permease, which permits the cell to respond immediately when lactose is introduced into its environment. Clearly, basal transcription of HSP 70 does not play an analogous role, but it might possibly serve some specific purpose. The answer to this question must await a more detailed understanding of the specific molecular functions of these proteins. In this paper, monoclonal antibodies were used to demonstrate that HSP 70 is not present in substantial quantities in healthy cells at normal temperatures. They should also provide a valuable tool in investigating the intracellular distribution and intramolecular associations of this protein.

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