Proteins related to the mouse L-cell major heat shock protein are synthesized in the absence of heat shock gene expression

(DNA transfer blot analysis/heterologous hybridization selection/cell-free protein synthesis/gene family)

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ABSTRACT Heat shock of mouse L cells induces the synthesis of two polypeptides of Mr 68,000 and 89,000. Using a fragment of the cloned gene encoding the Drosophila melanogaster Mr 70,000 heat shock protein (hsp70), we have shown that this protein has been highly conserved during eukaryotic evolution. We extended this observation by probing at low stringency for the expression in mouse L cells of RNA homologous to the Drosophila hsp70 gene. In addition to the RNA encoding the inducible Mr 68,000 heat shock protein (hsp68), there are mouse mRNAs encoding proteins of Mr 70,000 and 74,000 that are homologous to the Drosophila hsp70 gene. The Mr 70,000 and 74,000 proteins and their mRNAs are abundant components of unstressed mouse L cells. These constitutively expressed proteins are unique polypeptides in contrast to the several isolectric point variants of the inducible hsp68. We do not detect hsp68 or its mRNA in unstressed L cells. In addition to the mRNAs corresponding to hsp68 and the Mr 70,000 and 74,000 proteins, we detect a fourth RNA homologous to the Drosophila hsp70 gene but whose protein product has not been identified. Our results suggest that the hsp68 gene of mouse L cells is a member of a multigene family and that the individual family members are distinguishable by their degree of similarity but show differences in the regulation of their expression.

All organisms examined to date synthesize new proteins in response to heat shock or other forms of stress. A common feature of this response is the induced synthesis of a protein in the range of Mr 70,000 (hsp70). Kelley and Schlesinger (1) have demonstrated immunological crossreactivity between anti-chicken hsp70 and yeast, slime mold, dinoflagellate, nematode, Drosophila, Xenopus, mouse, and human heat shock proteins of similar sizes. We have shown that the mouse genome contains multiple copies of a sequence related to the Drosophila hsp70 gene (2) and that the restriction fragments carrying the homologous sequences (>70% sequence similarity) contain the gene(s) encoding the major mouse heat shock protein, hsp68 (3). In yeast, Ignolia et al. (4) have shown that the hsp70 genes share significant sequence homology with the Drosophila hsp70 genes. These results establish the selective conservation of at least one stress-inducible gene throughout eukaryotic evolution. The induction of homologous proteins by the same stress in different organisms implies that these proteins are functionally related in terms of their cellular role in the homeostatic response to environmental stress.

Unstressed Drosophila, Xenopus, mouse, and human cells all contain some antigen that crossreacts with anti-chicken hsp70 (1). It is not clear whether this reflects the low-level synthesis of heat-inducible protein or the presence of related but distinct protein that crossreacts with the antibody. In unstressed Drosophila cells there are normally very few transcripts of the inducible hsp70 genes and no hsp70 is detected. However, Drosophila cells do contain genes, termed cognate genes (hsc70), whose sequence is at least partially related to the inducible hsp70 genes, and RNA homologous to the 5' end of these genes is present in unstressed cells (5). The existence of polypeptides encoded by these hsc70 genes has not yet been established. A similar situation exists in yeast, where some members of the hsp70 gene family are specifically transcribed in unstressed cells but the protein products have not been characterized (ref. 4; unpublished data).

To further identify proteins encoded by the mouse genomic DNA sequences that are homologous to the Drosophila hsp70 gene (2, 3), we have performed nucleic acid hybridization experiments at reduced stringencies. RNA transfer blot hybridization analysis shows that there are at least four different poly(A)* RNAs derived from sequences in the mouse genome that are homologous to the Drosophila hsp70 gene. The mature transcripts show differing degrees of homology to the Drosophila hsp70 gene and display a marked difference in the regulation of their expression. We demonstrate that two of the transcripts encode abundant mouse proteins of Mr 74,000 (hsc74) and 70,000 (hsc70), which are present in unstressed mouse L cells in the absence of detectable hsp68 gene expression. After heat shock, the rapid rate of de novo hsp68 synthesis is a consequence of the new high steady-state concentration of hsp68 mRNA.

MATERIALS AND METHODS

Cell Culture and Heat Shock Conditions. Mouse L cells (WT-4) were grown in alpha Eagle minimal essential medium with 10% fetal calf serum. Drosophila melanogaster Schneider line 2 (SN-2) cells were grown at 25°C in D2 medium containing 2% fetal calf serum. Mouse L cells were heat shocked at 44°C for 10 min, allowed to recover for 2 hr at 37°C, and labeled for 60 min with [35S]methionine. Drosophila SN-2 cells were heat shocked for 3 hr at 37°C (6).

Blot Analysis of RNA. RNA was extracted from mouse L cells and SN-2 cells with phenol/NaDodSO4 (7), and poly(A)* RNA was purified (8). Poly(A)* RNA was electrophoresed through a 1.5% agarose/6% formaldehyde gel (9) and blotted to nitrocellulose (BA85 Schleicher & Schuell) in 1.5 M NaCl/0.15 M trisodium citrate (CIP) (10). The probe was a 2.2-kilobase (kb) Drosophila hsp70 gene Sal I fragment isolated from the hybrid plasmid 56H8 (11) and subcloned into pBR322 (plasmid pRN301). The Sal I fragment was purified by centrifugation of digested pRN301 through a 5-20% sucrose gradient, and the isolated fragment was labeled with P by nick-translaction (2, 12). Blots were prehybridized (without glycine) and hybridized as described (13), except 1× concentrated instead of 5× Denhardt's solution (14) was

Abbreviations: hsp, heat shock protein; hsc, heat shock cognate; kb, kilobase(s).
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used in the hybridizations. Filters were washed with 0.75 M NaCl/0.075 M Cit/0.1% NaDodSO₄ for 90 min at 42°C.

Hybridization Selection and Cell-Free Protein Synthesis. Hybridization selection of mRNA (15) was performed at 42°C in a 50-μl reaction volume with 40% formamide/10 mM Pipes pH 6.4/0.78 M NaCl/0.5% NaDodSO₄/100 μg of tRNA per ml/100 μg (mouse) or 30 μg (Drosophila) of poly(A)⁺ RNA per ml/2 μg of denatured 56H8 Sal I fragment immobilized on nitrocellulose. Hybrid-selected RNA or 0.5 μg of poly(A)⁺ RNA was treated with methylmercury hydroxide (16) and translated in a rabbit reticulocyte lysate (17, 18) as described by Thompson and Lane (19).

Gel Electrophoresis of Proteins. Samples of labeled proteins were analyzed by electrophoresis through 10% NaDodSO₄/polyacrylamide slab gels (20, 21). Gels were stained with Coomassie blue and dried for autoradiography. For fluorography, gels were impregnated with 2.5-diphenyloxazole before drying (22). For comparative two-dimensional analysis (pH range, 5–7) (23), samples were vacuum dried and resuspended in 20 μl of O’Farrell’s lysis buffer (23) to give a maximal final NaDodSO₄ concentration of 0.5% (24). Two-dimensional analysis was in the Pharmacia GE-4 apparatus.

RESULTS

Heat Shock Protein Induction. The autoradiogram shown in Fig. 1A compares the proteins that have been pulse labeled in untreated cells (C) with those labeled in cells during the third hour after a 10-min heat shock treatment (HS) at 44°C. The general profile of proteins synthesized in unstressed and stressed cells is very similar, except that a protein of M₄ 68,000 (hsps68), one of the most intensely labeled proteins from the heat-shocked cells, is not synthesized in unstressed cells. The rapid rate of synthesis of hsps68 after heat shock allows it to be visualized by staining within 3 hr of induction (Fig. 1B, HS). Although not evident in this analysis, the synthesis of a protein of M₄ 89,000 is also induced by heat shock. This protein can be resolved from one of similar size synthesized in unstressed cells by two-dimensional analysis or by electrophoresis through a 7.5% NaDodSO₄/polyacrylamide gel (unpublished data).

Identification of RNAs Homologous to the Drosophila hsp70 Gene. To assay directly for the expression of sequences homologous to the Drosophila hsp70 gene in mouse L cells, we probed RNA transfer blots of mouse poly(A)⁺ RNA isolated from control and heat-shocked cells. The probe DNA was a 2.2-kb Sal I fragment, containing the entire amino acid coding region of a hsp70 gene found at D. melanogaster chromosomal locus 87A7 (see Materials and Methods). Probe hybridization to Drosophila heat shock and control mRNA is a positive control (Fig. 2A and B, lanes 1 and 2) and shows the induction of hsp70 mRNA by heat shock. Only 50 ng of Drosophila poly(A)⁺ RNA was loaded onto the gels used in these blots to give a comparable intensity of probe hybridization to Drosophila and mouse hsp mRNAs, and this results in very little probe hybridization to homologous sequences in control Drosophila RNA, as shown (Fig. 2A and B, lane 2).

The filter in Fig. 2A was hybridized to ³²P-labeled probe under conditions that will detect homologous sequences with a minimum of 67% similarity (calculated as described in ref. 25; see also ref. 2). A transcript labeled "a" of ~2.7 kb is present in mouse heat shock RNA but not in control RNA (Fig. 2A, lanes 3 and 4). This inducible RNA can be assigned to hsps68 (3). Additionally, in unstressed mouse cells there are two RNAs of ~2.5 and ~1.9 kb in size, RNAs "b" and "c" respectively, which hybridize to the Drosophila hsp70 gene probe (Fig. 2A, lane 4).

Lower stringency hybridization to RNA transfer blots reveals two additional bands. The high molecular weight RNA species in Fig. 2B, lanes 3 and 4, is due to nonspecific probe binding to 28S rRNA contamination of the poly(A)⁺ RNA, but band "d" is due to specific hybridization to a 1.6-kb RNA present in both heat shock and control mouse RNA samples (Fig. 2B, lanes 3 and 4). The hybridization conditions employed with this RNA blot allow the detection of sequences with a further 10% base mismatch to the probe.

These experiments identify the mRNA for mouse hsps68 and three additional homologous RNAs that are present in unstressed cells and that display different degrees of relatedness. Unstressed mouse L cells contain RNAs b–d but we do not detect any RNA a corresponding to hsps68 (3). With a
high specific activity probe (>10^6 cpm/µg) such as that used in these experiments, the limit of detection is around 50 pg of a specific RNA sequence (26). Thus, the results from probing RNA transfer blots of mouse RNA with the Drosophila hsp70 gene indicate that, if present, mouse hsp68 mRNA is <0.005% by mass of the poly(A)^+ RNA in unstrressed cells.

**Identification of Proteins Homologous to the Drosophila hsp70 Gene.** Using Southern blot analysis, we have previously shown that the mouse genome contains sequences that are homologous to the D. melanogaster hsp70 gene (2). At least part of these sequences encode the major mouse heat shock protein hsp68 (3). To determine whether mouse RNAs b and c (Fig. 2) correspond to additional proteins homologous to hsp68, we performed a heterologous hybridization selection experiment at low stringency.

Fig. 3, lanes 1 and 2, shows the L-[35S]methionine-labeled in vitro translation products from control and heat shock mouse L-cell RNAs, respectively. The complement of proteins synthesized from each mRNA population is very similar, except that the induction of hsp68 is evident in the heat shock mRNA translation products. For hybridization selection the control and heat shock mouse mRNA populations were incubated with the Drosophila hsp70 gene denatured and immobilized on nitrocellulose. The hybridization stringency was equivalent to that used to detect RNAs a–c (Fig. 2A). The selected RNA was eluted and translated in vitro to produce two proteins from control RNA (Fig. 3, lane 3) and three proteins from heat shock RNA (Fig. 3, lane 4). The proteins synthesized from hybrid-selected control mouse RNA have the same one-dimensional mobility as two abundant proteins of Ms 74,000 and 70,000 that are present in unstrressed mouse L cells. We refer to these heat shock cognate proteins as hsc70 and hsc74. The proteins made in vitro from hybrid-selected heat shock RNA correspond to hsc74, hsc70, and hsp68 (Fig. 3, lanes 4). Fig. 3, lane 5, is a positive control showing the hybrid selection in vitro translation product (hsp70) from Drosophila heat shock RNA, and lane 6 is the minus RNA in vitro translation control.

These data show that the mouse genomic sequences homologous to the Drosophila hsp70 gene are expressed to produce different but related proteins and suggest that there is a family of homologous genes encoding these proteins.

**Two-Dimensional Analysis of Proteins.** Comparative two-dimensional gel electrophoresis of L-[35S]methionine-labeled proteins was performed to identify the hsc and hsp proteins and to determine if these proteins are distinct polypeptides. Isoelectric focusing was in the pH range of 5–7, with the acidic proteins on the left of the autoradiograms shown in Fig. 4. The two-dimensional pattern of labeled proteins synthesized in control (C) and heat-shocked (HS) mouse L cells is shown in Fig. 4A. The arrows indicate two abundant proteins of Ms 70,000 and 74,000 (more acidic) found in the control and heat shock samples. The arrowhead indicates the induced hsp68 along with several more basic isoelectric point variants (Fig. 4A, HS). This region of the gel is devoid of labeled protein in the sample from control cells. Fig. 4B shows the reticulocyte lysate in vitro translation products from control (C) and heat shock (HS) mouse mRNA populations. The same relative mobilities of the abundant proteins of Ms 70,000 and 74,000 are observed in relation to hsp68. Microheterogeneity in two dimensions is characteristic of the major heat shock protein in several species, and we observe here qualitatively the same microheterogeneity in hsp68 proteins synthesized in cells or in vitro (compare HS in Fig. 4A and B).

The hybrid selection in vitro translation products shown in Fig. 3, lanes 3 and 4, were analyzed in two dimensions (Fig. 4C, C and HS). The arrows indicate the more acidic hsc74 on the left and hsc70, both of which have the same relative mobility compared to the labeled proteins from cells and in vitro translations. The arrowhead in Fig. 4C, HS indicates hsp68 along with the more basic isoelectric point variants. This two-dimensional analysis identifies hsc74 and hsc70 as distinct polypeptides. The induced hsp68 displays isoelectric point microheterogeneity and has a pI similar to hsc70.

**DISCUSSION**

The structure of the major stress-induced protein (hsp70) has been highly conserved throughout evolution, as shown by the cross-reaction of antibody to chicken hsp70 with similar proteins from yeasts, slime molds, insects, and mammals (1). The yeast and mouse genomes contain sequences related to the Drosophila hsp70 gene (2) and the homologous mouse sequences have been shown to encode the mouse hsp68 (3). In the experiments reported here we have utilized a probe consisting of the amino acid coding region of the Drosophila hsp70 gene to extend the observation of evolutionary conservation of mouse hsp68 to a set of related proteins synthesized in unstrressed cells. Corces et al. (27) also observed homologous mouse RNAs in noninduced cells.

The hsc70 and hsc74 proteins are not induced de novo by heat shock and are abundant components of normal mouse cells, as seen by one- and two-dimensional polyacrylamide gel electrophoresis. Two-dimensional analysis shows that hsc70 and hsc74 are distinct proteins in contrast to the several isoelectric point variants of hsp68. In some mammalian systems, published data (28, 29) suggest that the rate of syn-
 Autoradiogram.

Analysis of the proteins were performed by autoradiogram. Unstressed (C) and heat-shocked (HS) mouse L cells were pulse labeled with L-[35S]methionine and equal amounts of protein were analyzed (A). The in vitro translation products from mouse C and HS RNAs are shown in B. The M, 70,000 region of the gels showing the hybrid selection in vitro translation products from mouse C and HS RNAs is shown in C. The apparent molecular weights (×10^3) of several proteins are indicated to the left of A and B.

The synthesis of a protein that could be hsc70 is increased following heat shock. In our experiments with L cells in culture (see Materials and Methods) we usually do not detect obvious changes in the rate of synthesis of hsc70 following heat shock (see Fig. 1). However, a comparison of the proteins synthesized in vitro from control and heat shock mouse RNAs does show an increase in the rate of hsc70 synthesis (compare Fig. 3, lanes 1 and 2). This difference is also reflected in the hybrid selection experiments (compare Fig. 3, lanes 3 and 4; Fig. 4C, C and HS). This may be a reflection of an increased abundance of hsc70 mRNA in heat shocked cells, but whether this is due to an increased rate of transcription of the relevant hsc70 gene or an increase in stability of the RNA remains to be determined.

Other genes related to the hsp70 gene, termed "cognate genes," have been identified in Drosophila; they are transcribed in unstressed cells and transcription is not increased by heat shock (5). It has not been demonstrated that the cognate genes actually give rise to a protein product, although it has been known for some time that there are several hsp70-related proteins present in Drosophila cells (30, 31). In Drosophila, the gene for the M, 68,000 heat shock protein (hsp68) has been shown to be 85% homologous to the hsp70 gene at 87C1 (32), although we have consistently failed to detect hsp68 mRNA in Drosophila cells by the techniques employed here (unpublished data). Thus, in flies, the hsp70 gene family consists of five or six copies of the hsp70 gene (33), one hsp68 gene, and several cognate or hsc70 genes, which may be pseudo genes. In yeast, the homologous gene family consists of up to 10 members, although the number of stress-inducible, cognate, or pseudo genes is unknown (4).

Our data suggest that the homologous family of functional genes in mouse may consist of at least one inducible hsp68 gene, two noninducible genes hsc70 and hsc74, and a fourth gene giving rise to RNA d in Fig. 4B, but whose protein product we have not identified. The different members of this gene family show varying degrees of relatedness and display a marked difference in the regulation of their expression. We suggest that this difference will be reflected in the structure of the upstream control elements of these genes. Molecular cloning and characterization of these mouse genes will allow determination of the evolutionary relationship between the structural and regulatory sequences of the cognate and inducible genes. Recent information from a cDNA cloning experiment indicates that the 3' 1500 nucleotides of hsp68 mRNA are <67% related to the mRNAs for hsc70 and hsc74 (unpublished data). This suggests that the hsp68 and hsc proteins have an uneven distribution of related nucleotide sequence. We have also determined that band a (see Fig. 2) consists of at least four different transcripts of similar size.

So far no biochemical role has been assigned to any of the heat shock proteins. However, a direct correlation does exist between the induction of heat shock proteins and the induction of thermotolerance in yeast (34), Dictyostelium (35), Drosophila (36), and Chinese hamster fibroblasts (37). In mammalian and avian cells, hsp70 has been identified in association with brain microtubules, tissue culture cell intermediate filaments, and myofibrils (38). hsp70 has also been found in immunoprecipitates of a M, 90,000 cell surface glycoprotein from NIH 3T3 cells (39). The data from studies with Drosophila species suggest that hsp70 is associated with chromatin following a heat shock (40–42), although a substantial portion may be found at the cell surface (42). In studies on the function and localization of the major heat shock protein in higher eukaryotes, care should be taken to identify and distinguish the induced protein(s) from similar constitutively expressed proteins, which, as we have shown for mouse L cells, can be closely related. By making this distinction it should be possible to determine whether the cognate and induced proteins have different, overlapping, or the same functional role(s) in the cell.

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