A Family of Related Proteins Is Encoded by the Major *Drosophila* Heat Shock Gene Family

SAMUEL C. WADSWORTH

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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At least four proteins of 70,000 to 75,000 molecular weight (70-75K) were synthesized from mRNA which hybridized with a cloned heat shock gene previously shown to be localized to the 87A and 87C heat shock puff sites. These in vitro-synthesized proteins were indistinguishable from in vivo-synthesized heat shock-induced proteins when analyzed on sodium dodecyl sulfate-polyacrylamide gels. A comparison of the pattern of this group of proteins synthesized in vivo during a 5-min pulse or during continuous labeling indicates that the 72-75K proteins are probably not kinetic precursors to the major 70K heat shock protein. Partial digestion products generated with V8 protease indicated that the 70-75K heat shock proteins are closely related, but that there are clear differences between them. The partial digestion patterns obtained from heat shock proteins from the Kc cell line and from the Oregon R strain of *Drosophila melanogaster* are very similar. Genetic analysis of the patterns of 70-75K heat shock protein synthesis indicated that the genes encoding at least two of the three 72-75K heat shock proteins are located outside of the major 87A and 87C puff sites.

Drosophila cells undergo a dramatic shift in their pattern of gene expression within minutes of their transfer from the normal growth temperature of 25°C to a temperature of 36°C. Eight or nine loci on polytene chromosomes elaborate puffs (2, 23) and a similar number of proteins are synthesized (4, 26), while ongoing transcription (3, 24) and translation (16, 26) are largely shut off. Recombinant DNA clones representing all of the major heat shock protein (HSP) genes have been isolated, and one or more of these genes have been correlated with each of the major heat shock puff sites (6, 7, 9, 27, 28). Studies from several laboratories have demonstrated that genes for the major 70,000-molecular-weight (70K) HSP are repeated at least five times in the genome and are located at the major 87A and 87C heat shock puff sites. Three-gene tandem arrays (8, 17), two-gene tandem arrays (25), head-to-head orientations (9, 17), and single genes (8, 9, 15, 25) have all been described on the basis of analysis of cloned genomic DNA segments and Southern blot analysis of restriction endonuclease-cleaved genomic DNA. Comparison of restriction endonuclease cleavage data from the above studies clearly shows that differences exist at the nucleotide sequence level between the coding region of different cloned 70K HSP gene copies.

Genetic analysis of the 70K HSP genes encoded at the 87A and 87C loci has also been performed. This has led to the conclusion that two 70K HSP genes are located at the 87A puff

site and three 70K HSP genes are located at the 87C puff site, and that there are clear differences between the genes at the two sites (9, 12). That is to say, there are "87A type" and "87C type" 70K HSP genes.

Despite the differences in the 70K HSP genes, the proteins encoded by this gene family seem to be relatively uniform. Ish-Horowicz et al. (11) have shown that the 70K HSP from embryos carrying deficiencies for either the 87A or 87C puff site alone yields tryptic peptide maps nearly identical to those from wild-type 70K HSP. They concluded that the 87A and 87C puff sites code for polypeptides that, though not identical, are very similar.

In contrast, however, Craig et al. (8) showed through hybridization-arrested translation and mRNA hybridization selection experiments that cloned 70K HSP gene sequences are complementary to mRNA species that translate in a cell-free system into one or more slightly larger proteins as well as the major 70K HSP. Since they obtained this result with two cDNA clones from different coding regions of the gene, the simplest interpretation was that a family of related genes encoded these proteins. Since multiple 70K HSP genes exist at widely separated genetic loci, a reasonable hypothesis would be that some level of nucleotide sequence heterogeneity among the genes accounts for the heterogeneity in the proteins encoded by this group or family of genes. The work described in this report was undertaken to determine the relationship be-

tween the major 70K HSP and the slightly larger HSPs when synthesized *in vivo* and *in vitro* and to attempt to determine whether the genes for these proteins are located at the major 87A or 87C puff sites where the 70K HSP genes reside.

MATERIALS AND METHODS

Preparation of heat shock mRNA and cell-free translation. The Kc line of *Drosophila* cells was the source of heat shock mRNA which was prepared exactly as described before (8). The mRNA-dependent reticulocyte lysate of Pelham and Jackson (21) was employed for cell-free translation experiments. The labeled amino acid was [35 S] methionine. To purify mRNA species complementary to 70K HSP sequences, DNA from the G3 70K HSP clone was used (8). This clone contains a 14.5-kilobase genomic *Hind*III fragment which carries three tandem repeats of the 70K HSP gene as well as flanking sequences on either side of the gene cluster. The results of the hybridization selection experiment were identical when 70K HSP cDNA clones were used. The G3 clone was used in these studies for convenience. The DNA was cleaved with the *Hind*III restriction endonuclease, denatured, and hybridized with heat shock mRNA under the conditions specified by Paterson et al. (20). The hydroxyapatite column fractionation procedure of Lewis et al. (14) was used to purify the mRNA-DNA hybrids. To synthesize high-specific-activity HSPs for the partial protease mapping experiments shown in Fig. 3, 250 μ Ci of [35 S]methionine was dried down and added to a 25- μ l cell-free translation reaction.

In vivo labeling of HSPs. Kc cells, salivary glands plus brain-imaginal disk complexes from third-instar larvae, and testes or ovaries from adult flies were heat shocked for 20 to 30 min at 36°C in unlabeled D20 medium lacking serum, lactalbumin hydrolysate, and Yeastolate (Difco) (medium D20-M). A sample of [35 S]methionine was then added directly to the medium, and cultures were held at 36°C for various lengths of time. Routinely, cultures were labeled for 30 to 60 min. The *in vivo*-labeled HSPs used in the partial protease mapping experiments shown in Fig. 4 and 5 were prepared by adding 200 μ Ci of [35 S]methionine to 5×10^6 Kc cells in 1 ml of D20-M or to two pairs of salivary glands from third-instar larvae, strain Oregon R. Cells and glands were incubated for 1 h in labeled medium.

Gel electrophoresis. The sodium dodecyl sulfate (SDS)-polyacrylamide gel system of Laemmli (13) was used for gel electrophoresis. The partial V8 protease digests were electrophoresed on 12% gels; all other samples were electrophoresed on 8 or 9% gels.

Partial protease digestion. The basic procedure of Cleveland et al. (5) using V8 protease (Miles) was used with the modifications as noted below. The sample slots for accepting the longitudinal gel slices were 30 by 1.5 mm. An 80- μ l volume of the appropriate dilution of V8 protease was applied over the gel slice. The bromophenol blue marker dye was run to the bottom of the stacking gel, and the current was shut off for 30 min before electrophoresis was continued.

Drosophila strains. Adult flies homozygous for the Kar^{3J} deficiency (9) were identified as Sb^{+},st,th individuals arising from random matings of a stock with the following genotype: $Dp(3;2)w^{+70hR}/In(2LR)Cyo$;

$Df(3R)Kar^{3J},st,th Kar^{3J}/Tp(3) MRS$. This strain was obtained from K. Livak. Embryos heterozygous and homozygous for the Kar^{1w} 87A plus 87C heat shock puff site deficiency (10) were collected from random matings between individuals of genotype, $ru,h,th,st Df(3R) Kar^{1w}/MKRS$. This strain was obtained from A. Chovnick. Single embryos were placed in 20 μ l of D20-M in microtiter wells and incubated for 30 min at 36°C. The embryos were then pierced with a sharpened tungsten needle, [35 S]methionine was added, and the incubation at 36°C was continued for an additional hour.

RESULTS

70K HSP gene sequences are complementary to multiple mRNA species. As noted above, the original observation that prompted this study was that hybridization-arrested translation or mRNA selection experiments demonstrated a relationship between mRNA encoding the 70K HSP and one or more larger proteins (8). In Fig. 1, the cell-free translation products of heat-shocked Kc cell mRNA purified by hybridization to a cloned 70K HSP gene (lane a) are shown in comparison to *in vivo*-synthesized HSPs from Kc cells (lane b) and salivary glands from Oregon R larvae (lane c). The results of this experiment confirm the original observation that cloned 70K HSP gene sequences hybridize to mRNA that encodes several proteins, 70K and larger.

Several other important features of the results exemplified in Fig. 1 and results of similar experiments are worthy of note. From this series of experiments, I conclude that the selected mRNA directs the synthesis of three proteins larger than the 70K HSP and smaller than the 84K HSP. By assuming these values for the

FIG. 1. SDS-gel electrophoresis of cell-free-synthesized and cell-synthesized HSPs. (a) Cell-free translation products of mRNA purified by hybridization to a 70K HSP clone, the G3 genomic clone of Craig et al. (8). (b) Heat shock protein synthesized in Kc cells. (c) Heat shock proteins synthesized in Oregon R salivary gland. All lanes are from the same gel, which was exposed for varying lengths of time.

prominent HSPs, the relative molecular weights of the selected HSPs were estimated to be 72,000, 73,000, and 75,000. In addition, the relative mobility of these proteins is independent of whether they were synthesized *in vitro*, in Kc cells, or in salivary glands.

By comparing the cell-free translation products of mRNA selected by the 70K HSP gene clone to HSPs synthesized in Kc cells, it can be seen that with one exception, the *in vivo*-synthesized HSPs are represented in the translation products of the selected mRNA. The appearance of the protein band not seen in the translation products of selected mRNA is variable, and its origin is unknown. It could be a normal cell protein whose synthesis is variably inhibited by heat shock or a heat shock-induced protein not related to the 70-75K HSP family.

It should also be noted that in the particular experiment shown in Fig. 1, the selected mRNA also encodes the 68K HSP. Holmgren et al. (9) have recently shown that cloned 68K HSP genes and 70K HSP genes bear sequence homology.

There are several potential origins of the 72K, 73K, and 75K HSPs. The fact that these proteins are of indistinguishable size when synthesized *in vivo* or *in vitro* strongly suggests that these proteins are synthesized from discrete mRNA species and therefore that the 72-75K HSPs observed as cell-free translation products are not precursors to the major 70K HSP. To make certain that the *in vivo*-labeled 72-75K HSPs were not precursors to the 70K HSP, the labeling kinetics of the 70-75K HSPs were investigated. If the 72-75K HSPs were 70K HSP precursors, they should be more heavily labeled relative to the 70K HSP during a short pulse than during a long labeling period. The result of such an experiment is shown in Fig. 2. The 72-75K HSPs were synthesized at similar levels relative to the 70K HSP during the 5-min pulse or 60-min continuous labeling periods.

Partial protease mapping of the 70-75K HSPs. To provide further evidence for the relatedness of the 70-75K HSPs, partial protease digestion experiments were performed using a modification of the method of Cleveland et al. (5). Labeled HSPs were electrophoresed through an 8 or 9% SDS-acrylamide gel, a region of the gel including the 68K through 84K HSPs was excised, and longitudinal slices of the preparative lanes were placed in wide sample slots and overlaid with different amounts of protease for partial digestion. Ordinarily, homology between different proteins analyzed by partial protease digestion is revealed by the generation of similar banding patterns. The method used here yields a series of spots that will align if the proteins are related. An analogous procedure has been published previously (4). Three types of labeled

FIG. 2. SDS-gel electrophoresis of *in vivo*-labeled HSPs. Salivary glands from Oregon R third-instar larvae were heat shocked at 36°C for 20 min, and three to four pairs of glands were labeled for various intervals. Left lane, 5-min pulse label. Right lane, 90-min continuous label. Both lanes are from the same gel.

HSPs were tested in this experimental series: those from Kc cells synthesized *in vitro* or *in vivo* and those from Oregon R salivary glands.

In the first experiments, the substrates for the generation of partial protease digestion products were HSPs synthesized in a cell-free translation system using Kc cell mRNA. Cell-free-synthesized HSPs were chosen because a higher specific activity can be attained as compared to *in vivo*-synthesized proteins, and relative to the major 70K HSP, the 72-75K HSPs are more highly labeled when synthesized *in vitro* than in

FIG. 3. Partial protease digestion of cell-free-synthesized HSPs. V8 protease samples of 8, 0.8, or 0.08 μ g were used to generate the digests shown in a, b, and c, respectively. The positions corresponding to the 70K HSP and 84K HSP bands in the first-dimension gel are shown at the top of each panel. The numbered arrows indicate the spots referred to in the text.

vivo. The partial products resulting from digestion of longitudinal slices of the same gel lane with 8, 0.8, or 0.08 μ g of V8 protease are shown in Fig. 3. It can clearly be seen that the patterns generated by the 84K and 70K HSPs are different from each other and change as a function of the amount of protease used. It is also clear that most of the major spots resulting from cleavage of the 70K HSP align with spots resulting from cleavage of the 72-75K HSPs. Some of these alignments are indicated by the arrowheads in Fig. 3. This result is indicative of a high level of homology between the 70-75K HSPs. However, the data presented in Fig. 3 also tend to indicate some level of nonhomology between the members of this protein family. The two clearest examples are indicated by the arrows in Fig. 3b. Note that spot 1, a major partial product of the 70K HSP, does not seem to be a partial product of any of the 72-75K HSPs whether digested with 10 times more (Fig. 3a) or 10 times less (Fig. 3c) V8 protease. Similarly, spot 2 seems to be a major partial protease product of only one of the 72-75K HSPs and is apparently not a partial product of the 70K HSP.

To make certain that the examples of apparent homology and nonhomology were not artifacts of cell-free-synthesized HSPs nor restricted to Kc cell HSPs, partial protease digestion experiments were performed on *in vivo*-synthesized

HSPs from Kc cells and Oregon R salivary glands. The results of these experiments are shown in Fig. 4 and 5, respectively. The samples displayed in panels a and b in each figure were derived from the same gel lane and were digested with 8 and 0.8 μ g of V8 protease, respectively. Some of the peptide spot alignments in the digestion patterns of the 70-75K HSPs are designated by the arrowheads. Although the V8 partial protease patterns from *in vivo*-labeled HSPs are not as clear as those shown in Fig. 3, they are very similar and indicate a high level of homology between the 70-75K HSPs from Kc cells or from Oregon R salivary glands. The apparent nonalignments of certain spots in these digests are indicated by the arrows and are numbered like those shown in Fig. 3.

Genetic analysis of the 70-75K HSP family. It is known that the 70K HSP genes are repeated at two chromosomal loci, 87A and 87C, and therefore, genes for one or more of the 72-75K HSPs could be unique to one or the other of these loci. In an attempt to deduce the location of the 72-75K HSP genes, the patterns of HSP synthesis were examined in flies homozygous for an 87C deficiency, *Ka*^{3J}, and flies homozygous for an 87A plus 87C deficiency, *Kar*^{1W}. The homozygous 87C deficiency flies synthesized all 70-75K HSP family members, as do Oregon R flies (data not shown). Homozygotes for the *Kar*^{3J} deficiency can survive to the adult stage and can be easily recognized by visible genetic traits, and

FIG. 4. Partial protease digestion of Kc cell HSPs. V8 protease at 8 and 0.8 μ g was used to generate the digests shown in a and b, respectively. The positions corresponding to the 70K HSP and 84K HSP bands in the first-dimension gel are shown at the top of each panel. The numbered arrows indicate the spots referred to in the text.

FIG. 5. Partial protease digestion of Oregon R salivary gland HSPs. V8 protease at 8 and 0.8 μ g was used to generate the digests shown in a and b, respectively. The positions corresponding to the 70K and 84K HSP bands in the first-dimension gel are shown at the top of each panel. The numbered arrows indicate the spots referred to in the text.

thus ample material can be obtained for analysis. In contrast, the Kar^{1w} deficiency is late embryolethal when homozygous and is not easily recognized. Therefore, the patterns of HSP synthesis were examined using single embryos from random matings of $Kar^{1w/+}$ individuals. Examples of single embryo HSP preparations are shown in Fig. 6. The individuals homozygous for the 87A plus 87C deficiency can be easily recognized by the absence of the major 70K HSP (lanes a and d). However, it can also be readily observed that two of the 72-75K HSPs remain as intense bands in the HSP profiles from individuals heterozygous (lanes b and c) and homozygous (lanes a and d) for the Kar^{1w} deficiency. This result shows that the genes encoding at least two of the proteins in the 72-75K HSP group are located outside of the Kar^{1w} deficiency. In the third larval instar and adult stages of all other strains tested, three HSPs are found between 70K and 84K. To learn whether the $Kar^{1w/+}$ stock differed in this respect, the HSP patterns of an adult $Kar^{1w/+}$ fly and an adult Oregon R fly were compared. The results (Fig. 7) show that, like all other strains tested, the $Kar^{1w/+}$ stock synthesizes three HSPs between 70K and 84K. Thus, no conclusion about the chromosomal location of the gene for the single 72-75K HSP missing in embryos can be made, nor do the existing data identify exactly which member of the group is missing. However, it is clear that whereas embryos do not synthesize one of the

members of this group, adults and third-instar larvae synthesize all three.

DISCUSSION

The 70-75K HSPs are members of a family of proteins. The principal conclusion of this work is that the 72-75K HSPs and the major 70K HSP constitute a family of closely related proteins. I conclude that there are a minimum of three HSPs larger than the 70K HSP and smaller than the 84K, with relative molecular weights of 72K, 73K, and 75K. The mRNA species encoding these proteins are related at the nucleotide sequence level since they all hybridize with the same cloned gene sequences. This is the case when a genomic clone carrying the entire 70K HSP coding region is used (Fig. 1) and when either one of two cDNA clones carrying only a fraction of the coding region is used (8). The 70-75K HSPs themselves are closely related since they yield very similar partial protease digestion products. The observation that all members of this protein family appear in cell-free-synthesized HSPs clearly shows that the 72-75K HSPs are distinct from the 70K HSP. As well, the simplest interpretation of the labeling kinetics of the *in vivo*-synthesized 72-75K HSPs is that they are not kinetic precursors to the major 70K HSP. As noted above, the partial protease digestion patterns showed that the 70-75K HSPs are

FIG. 6. SDS-gel electrophoresis of HSPs from individual embryos. Lanes a and d are HSPs from Kar^{1w}/Kar^{1w} embryos. Lanes b and c are HSPs from $Kar^{1w}/+$ embryos. Lanes a and b are from the same gel, as are lanes c and d. The separate lanes were cut from the autoradiogram and placed next to each other. Only the upper portion of the autoradiogram is shown.

FIG. 7. SDS-gel electrophoresis of HSPs from $Kar^{1w/+}$ (lane b) and Oregon R (lane d) adults. Lanes a and c represent the proteins labeled at 25°C in the same tissue of $Kar^{1w/+}$ and Oregon R adults, respectively.

related. The same patterns also provided some evidence of differences between these proteins.

Origin of the 72-75K HSP mRNA species. As shown by the data presented here, the 70-75K HSPs are discrete protein species. Since the 70K HSP genes are repeated and are located at two puff sites, the simplest hypothesis would be that the several repeated genes encode the different members of the 70-75K HSP family. Multiple copies of the 70K HSP gene sequences have been cloned from embryonic and cultured cell DNA. These cloned gene sequences have been extensively mapped through restriction endonuclease cleavage and have been employed to deduce the genomic arrangement of these genes. Although not all studies employed the same set of restriction endonucleases, an examination of the published data reveals that certain features of these genes are conserved, but also reveals that there is not absolute conservation of the coding sequences. Two or three alleles have been found in Oregon R embryonic DNA (1, 15, 19) in Kc cell DNA (17), and in Schneider cell DNA (8).

In more detailed studies, Holmgren et al. (9) and Ish-Horowicz et al. (11) concluded from restriction endonuclease mapping of DNA from *Drosophila* strains carrying certain deficiencies that there are two genes of one type at the 87A puff site and three genes of another type at the

87C puff site. In their report, Holmgren et al. (9) show more specifically that the major difference between the 87A and 87C gene types is sequence divergence in the 5'-proximal gene region to the extent of approximately 15%. As well, the bulk of the in situ hybridization data from several laboratories would support the assignment of the majority of the 70K HSP coding sequences to the 87A and 87C puff sites.

In genetic analyses of the HSPs encoded by the 87A and 87C puff sites, Ish-Horowicz et al. (10, 11) have shown that deletion of either puff site alone leaves an active 70K HSP gene, but deletion of both puff sites deletes all active 70K HSP genes. The HSP profiles from individuals with the same 87A plus 87C deletion shown in this study are basically in agreement with Ish-Horowicz et al. (11) in that no trace of the major 70K HSP remains. However, at least two members of the 72-75K HSP group are still synthesized by individuals homozygous for the double deficiency. This result clearly indicates that the genes encoding two of these HSPs are not included in either of the major 87A or 87C puff sites. There are, as well, other data to support this conclusion. Craig et al. (8) reported hybridization of sequences from 70K HSP-specific cDNA clones to restriction endonuclease-digested DNA fragments that could not be accounted for in the cleavage maps of the major cloned 70K HSP genes. Thus it is known that 70K HSP gene sequences are represented in the *Drosophila* genome in arrangements different from the major 70K HSP genes represented in recombinant DNA clones and that these different gene arrangements are not characteristic of the 70K HSP genes at either the 87A or 87C puff sites. Holmgren et al. (9) have shown that sequences complementary to 70K HSP gene sequences are present at at least one additional locus, 87D 10-12, a site not identified as a heat shock puff site. However, Ish-Horowicz et al. (10) reported that the distal breakpoint of the Kar^{1w} deficiency is 87D 12-13, and thus the 87D 10-12 sequences complementary to the 70K HSP gene have no bearing on the results reported here. It should be noted that three loci identified by Ashburner (2) as heat shock puff sites have not yet been related to any of the major HSPs.

There is one interesting aspect of the HSP pattern from the 87A plus 87C deficiency embryos presented in Fig. 6 that was revealed in the current study. Note that the HSP pattern obtained from a $Kar^{1w/+}$ adult (Fig. 7) shows the major 70K HSP plus the three 72-75K HSPs, essentially identical to that obtained from adults and larvae of all *Drosophila* strains tested. However, only two members of the 72-75K HSP group are synthesized in $Kar^{1w/+}$ embryos, and these two members are still synthesized in

Kar^{1w}/Kar^{1w} embryos. One explanation for this observation is that synthesis of one of the 72-75K HSPs is restricted in embryonic, but not larval or adult tissues. Experiments comparing the embryonic and adult HSPs will identify which member of this group is missing in the embryonic HSP pattern. An analysis of the 72-75K HSP patterns as a function of development will identify the developmental stage at which the expression of this HSP can be induced.

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