THE HEAT SHOCK RESPONSE

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I. INTRODUCTION

The exposure of cells from a wide variety of species to an increase in temperature results in the enhanced synthesis of several proteins, which have been referred to as heat shock proteins (hsps). This phenomenon has been called the heat shock response even though recovery from anoxia, ethanol, inhibitors of oxidative phosphorylation, and a number of other chemicals have been shown to induce the synthesis of the same proteins. Therefore, the response should, perhaps, more appropriately be referred to as a stress response. Induction of these stress proteins has been observed in bacteria, yeast, soybeans, maize, tobacco, *Dictyostelium*, *Tetrahymena*, and in cells of a wide variety of higher eukaryotes, including *Drosophila*, nematodes, chicken, rat, mouse, and man. In fact, this response has been observed in every species examined. The response is not specific for a particular tissue for it has been seen in a wide variety of tissues studied, as well as in many tissue culture lines.

Although the stress response is universal, it was first observed in *Drosophila melanogaster*, since in the fruit fly it can be observed with the aid of only the light microscope. About 22 years ago, Ritossa observed that upon a shift from 20 to 37°C, as well as treatment with dinitrophenol or sodium salicylate, several new puffs appeared in the salivary gland polytene chromosomes.[1,2] In 1974 the induction of a set of proteins upon heat shock was reported by Tissieres and co-workers.[3] Over the next several years it became clear that the puffs were the sites of vigorous RNA transcription and that a number of these RNAs were translated into the heat shock proteins. In the past few years, the genes encoding the hsps have been isolated, thus, allowing by *in situ* hybridization the assignment of genes to puffs and through sequence analysis the grouping of the hsps into three gene families. Also, genes related to members of the three families of *Drosophila* heat shock genes have been identified in distantly related species, thus attesting to the conservation of the hsps through evolution.

Recently, a number of findings and technical advances have opened up new avenues of study of the heat shock response. Mutations of some heat shock genes, as well as genes involved in the regulation of their expression, have been isolated in *Saccharomyces cerevisiae* or *Escherichia coli*, thus allowing a genetic analysis of the regulation of expression and function of hsps. Sequences important for the induction of heat shock genes have been identified in both eukaryotes and prokaryotes. Polyclonal and monoclonal antibodies directed against hsps have been generated and used to determine the cellular distribution of hsps and to identify and characterize related proteins. Analysis in a number of species has revealed that heat shock or related proteins are commonly present during normal growth and various developmental stages. Although substantial progress has been made in the analysis of the heat shock response in many

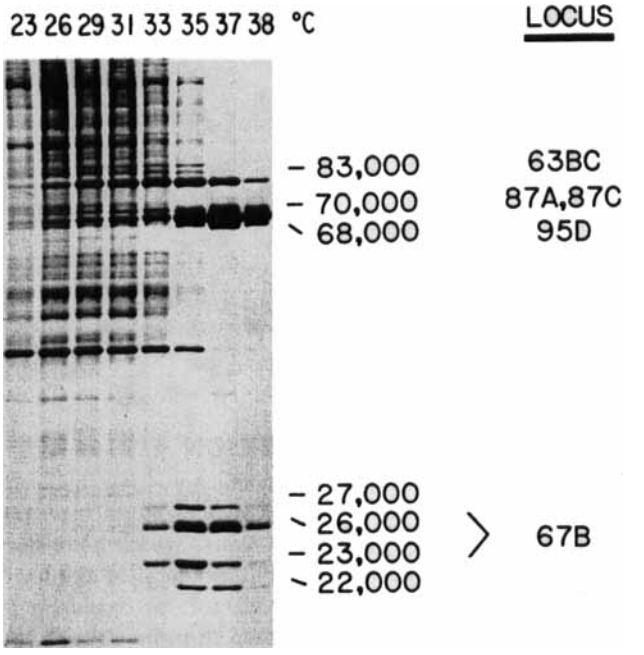

FIGURE 1. Synthesis of *Drosophila* hsps. Cells of Schneider's line 2 were grown at 23°C and incubated for 1 hr at the indicated temperatures. ^{3}H-leucine was then added, and incubation was continued for an additional hour. The proteins were electrophoresed in an acrylamide gel. The apparent molecular weights of the hsps are indicated. The chromosomal location of the genes encoding the proteins are shown at the right. (Adapted from Lindquist, S., *Dev. Biol.*, 77, 463, 1980. With permission.)

organisms, the *Drosophila* system still remains the best characterized. Therefore, I will compare available information about the heat shock/stress response in other species to what is known of the *Drosophila* response. This review is divided into four sections following this introduction:

- The identification and characterization of the heat shock genes and proteins
- The physiological role of the heat shock proteins
- The expression of heat shock and related proteins during normal growth
- The regulation of the heat shock response

II. IDENTIFICATION AND CHARACTERIZATION OF HEAT SHOCK GENES AND PROTEINS

The seven major heat-induced proteins of *D. melanogaster* are designated hsp83, hsp70, hsp68, hsp27, hsp26, hsp23, and hsp22 (Figure 1). The proteins are named according to their apparent molecular weights on SDS polyacrylamide gels, i.e., hsp83 = 83,000 daltons. These seven proteins have been placed into three families based upon structural homologies. Hsp83, encoded by a single gene at cytological locus 63BC, is the sole member of the first group. The second group is composed of hsp70 and hsp68. The *D. melanogaster* genome usually contains five genes which encode hsp70, three of these at 87C and two at 87A. The hsp70 genes at the two loci are closely related, showing 96% identity at the nucleotide level. Hsp68, encoded at 95D, is related to

hsp70 and, therefore, considered a member of the 70K family. The third group includes the four related small hsps: hsp27, hsp26, hsp23, and hsp22.

The heat shock proteins show a remarkable conservation throughout evolution. Nearly all species induce the synthesis of proteins in the size ranges of 80 to 90K, 68 to 74K, and 18 to 30K. The larger proteins appear to be more highly conserved than the smaller. Proteins and/or genes related to both *Drosophila* hsp83 and hsp70 have been found in both a prokaryote, *E. coli*, as well as in higher and lower eukaryotes. After more extensive analyses, it may be found that the small hsps are, in fact, conserved over a vast range of species as well. Recently, genes isolated from *X. laevis* and *C. elegans* which encode small hsps have been shown to be related to the *Drosophila* genes.

A. HSP83

The gene encoding hsp83 of *Drosophila* was shown to reside at the cytological locus 63B by *in situ* hybridization of a recombinant clone to polytene chromosomes.[4] This location had been predicted earlier because RNA, which encoded hsp83, hybridized to 63B.[5] The existence of an intron in hsp83 was first suggested by the presence in nuclear RNA prepared from heat-shocked cells of transcripts about 0.7 kb larger than the predominantly cytoplasmic 3-kb RNA. The presence of the intron was confirmed by S1 nuclease and exonuclease VII digestion experiments[6] and mapped to the 5′ nonprotein coding region. The 5′ splice junction occurs 151 bases from the site encoding the 5′ end of the mRNA. The intron is 1.13 kb in length, and the 3′ splice site occurs immediately prior to the ATG which is the beginning of a long (>1122 nucleotide) open reading frame.[7,8] The amino acid sequence of the *D. melanogaster* hsp83 has been deduced from the DNA sequence.[8] In the amino terminal 20% of the protein is generally nonpolar in nature and the first 21 acids are particularly hydrophobic. The remainder of the protein is generally hydrophilic, although there are some short nonpolar stretches of amino acids. The region between aa210 and aa307 is particularly rich in acidic and basic residues. Within this 98 amino acid domain, 65 amino acids are polar, including 19 lysines, 17 aspartic acids, and 22 glutamic acids.

The complete DNA sequence of the hsp83 genes of three other *Drosophila* species has also been determined.[8] The *simulans* gene shows only 1.2% divergence and the encoded protein is identical to that of *melanogaster*. The *pseudoobscura* and *virilis* genes are 10% divergent from the *melanogaster* gene; the proteins have 12 (3.2% divergence) and 44 (1.1% divergence) amino acids differences, respectively. Six of these changes in the *pseudoobscura* sequence and two in the *virilis* sequence occur in a 15-amino-acid stretch (aa223 to 237) contained within the hydrophilic region described above. Furthermore, the *virilis* protein is missing aa223.

A gene which encodes a 90K-heat-inducible protein has been isolated from *Saccharomyces cerevisiae*.[9] Comparison of the predicted sequence of the yeast hsp90 and the *Drosophila* hsp83 protein revealed about a 60% identity. Long regions (38 to 103 aa in length) showed between 60 and 90% identity. The first stretch having this high degree of homology begins at aa6 of the *Drosophila* open reading frame.[10]

Recently, a heat-inducible gene which hybridizes with the *Drosphila* hsp83 gene has been isolated from *E. coli*. Limited DNA sequence analysis has shown ~45% identity of amino acids in an open reading frame between the codons of aa 1 and aa 180 of the *Drosophila* protein. Further analysis is needed to determine the size of the protein encoded, the location on the *E. coli* genetic map, and the extent of the homology.[11]

Evidence suggests that other eukaryotes also contain an hsp83-related protein. However, as in yeast, varying mobilities on acrylamide gels protein have resulted in differing size designations, hsp89 in chicken and hsp90 in human cells. A polyclonal antibody prepared against gel-purified chicken hsp89 was found to react with proteins of

similar mobilities in human, rodent, frog, and *Drosophila* cells.[12] A gene from *C. elegans* containing sequences homologous to *Drosophila* hsp83 has been isolated.[13] Also, a few of the tryptic peptides of *Drosophila*, chicken, and human proteins comigrate during two-dimensional thin-layer chromatography suggesting a similarity of the proteins from the three species.[14] It should be noted that in the case of mammalian cells, the protein referred to as hsp90 by many workers, not the heat-inducible 80K protein, is the protein related to *Drosophila* hsp83.

B. HSP70

Hsp70 genes and the encoded proteins are present in a wide variety of species. Polyclonal antibodies prepared against purified chicken hsp70 react with a protein of ~70K from yeast, dinoflagellates, slime molds, corn seedlings, worms, frogs, flies, rodents, and humans.[12,15] Genes related to *Drosophila* hsp70 has been isolated from yeast, *E. coli*, maize, frog, nematode, chicken, and *Dictyostelium* and either the complete or partial DNA sequence determined (Figure 2). A high degree of homology, ranging between 60 and 78%, has been found among the eukaryotic proteins. The *E. coli dna* K protein shows 40 to 50% identity with the related proteins from eukaryotes. While homology is found throughout the protein, certain regions such as the segments aa3 to 13, aa140 to 155, aa175 to 185, aa200 to 213, aa325 to 335, and aa390 to 398 maintain a high degree of homology in all species analyzed (Figure 3). These highly conserved segments most likely represent functionally important regions of the proteins. On the other hand, the similarity decreases in the carboxyl terminal one sixth of the protein.

1. Drosophila HSP70 and HSP68

The existence of multiple genes encoding the 70K hsps of *Drosophila* became evident when it was shown that RNA which encoded hsp70 hybridized to two cytological loci, 87A and 87C.[5,16] Subsequently, genomic clones containing the hsp70 genes were isolated and analyzed extensively in several laboratories to determine the organization and sequence of the genes at the two loci.[17-21] Each chromosomal locus contains hsp70 genes which can be distinguished from those at the other locus by characteristic restriction endonuclease sites. The overall organization of the genes at 87A and 87C was determined using strains containing a series of overlapping deficiencies covering either the 87A7 or 87C1 hsp70 genes.[22-24] All of the genes encoding hsp70 are found at these two loci, since embryos lacking 87A and 87C fail to induce hsp70 synthesis after heat shock. However, both loci do contain active genes since embryos retaining either 87A7 or 87C1 synthesize hsp70.

The organization within each locus is complex. The 87A7 locus contains two genes of opposite polarity, usually separated by 1.7 kb of DNA;[25,26] however, a number of variants have been found which contain large deletions or insertions, particularly within the spacer region. One variant contains an insertion of a middle repetitive sequence near the 5′ end of the distal gene.[21] Two deletion variants of the 87A locus have been identified. One contains a deletion of most of the spacer DNA and the 5′ portion of the centromere proximal gene.[26,27] A second contains a deletion of the 3′ portion of the centromere proximal gene which results in the synthesis of a heat-inducible 40K hsp70 NH_2 terminal fragment.[28] The organization of the genes at the 87C1 locus is more complex than that at 87A7. This locus of most strains contains three hsp70 genes; a single gene located proximal to the centromere is separated by approximately 40 kb from two genes arranged in tandem, approximately 0.8 kb apart. Some strains and Schneider and Kc tissue culture cells contain a tandem repeat of three, rather than two, genes.[19,23,29]

A comparison of the DNA sequence of the protein coding region of an 87C1[30] and an 87A7[31] variant reveals a 4.2% divergence. A long uninterrupted reading frame with

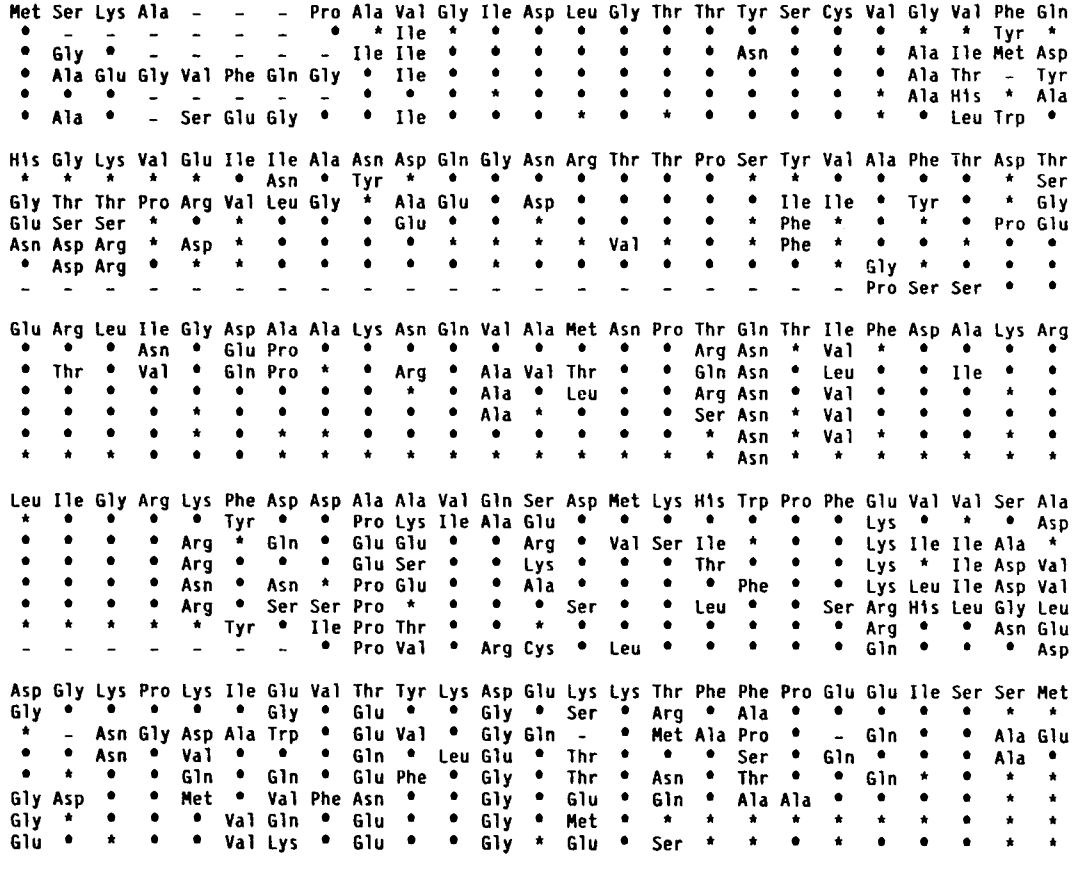

FIGURE 2. Comparison of sequences of hsp70 proteins. The amino terminal sequences, predicted from DNA sequences, of the *Drosophila* heat shock 70K cognate encoded at 88E (hsc70-4) is displayed on the top line. The other sequences are aligned below, with an asterisk indicating that the same amino acid is present as is found in hsc70-4. The sequences are from the following sources: *Drosophila* hsc 70-4, Craig et al.,[148,153] *Drosophila* hsp70, Ingolia et al.,[30] *S. cerevisiae* hsc70 (YG101), Ingolia et al.[33,153] *S. cerevisiae* hsp70 (YG100), Ingolia et al [34,153] *E. coli dnaK*, Bardwell and Craig;[41] maize, Shah et al.,[495] *Xenopus*, Bienz;[61] chicken, Morimoto et al.[169]

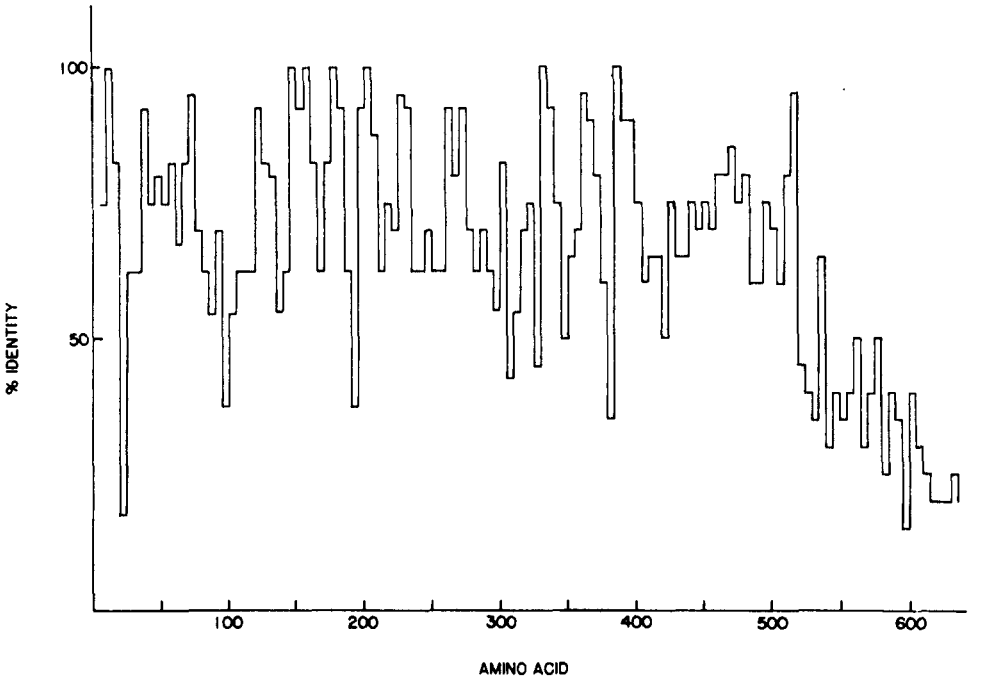

FIGURE 3. Distribution of homology throughout hsp70 proteins. The aligned hsp70 sequences described in the legend of Figure 2 (with the exception of the *Xenopus* and chicken sequences) were separated into five amino acid segments and the percent homology within those segments displayed.

two insertions (or deletions) of triplets is conserved. Most of the substitutions occur in the third position of the codons resulting in only 16 substitutions out of 643 amino acids, a 2.7% divergence of the amino acid sequence. As expected from the maps of restriction endonuclease maps, limited sequence data shows even less divergence between genes within the same locus.[30]

Another heat-inducible gene, hsp68, which resides at cytological locus 95D, is related to hsp70.[4] Melting studies of duplexes formed between isolated hsp70 and hsp68 genes indicated about a 15% sequence divergence between the genes. However, comparison of limited DNA sequence of hsp68 with hsp70 revealed only 73%[32,33] identity between the two genes. The discrepancy may, in part, be due to the limited sequence data available for hsp68, and more homologous sequences may exist in regions of the gene not analyzed. A number of other genes related to hsp70 are present in the *Drosophila* genome. These genes whose transcription is not inducible by heat are discussed in detail in Section IV.B.1.

2. S. cerevisiae Hsp70

The hsp70 genes of yeast have been studied in more detail than those of any eukaryote other than *Drosophila*. *Saccharomyces cerevisiae* contains a family of genes related to the hsp70 of *Drosophila*.[34] Eight genes have been isolated; two genes (YG100 and YG102) have been analyzed most extensively. These two genes are very similar, possessing 97% identity of nucleotides in the protein coding region. RNA isolated from yeast harvested 30 min after a shift from 23 to 38°C and labeled in vitro gave a stronger signal after hybridization to YG100 or YG102, than RNA from cells growing at 23°C. This result indicated that transcripts of either YG100 or YG102, or both, were more abundant after heat shock than before . However, because of the high degree of homology between the genes, this hybridization technique could not distinguish between

YG100 and YG102 transcripts. S1 nuclease and primer extension experiments suggested that transcripts of YG100 were increased dramatically after heat shock, while those from YG102 were not. To allow an unambiguous identification of transcripts from the two genes, fusions between the promoters of the two genes and the *E. coli* β-galactosidase structural gene were constructed.[35] Results of the analysis of β-galactosidase activity and fusion transcripts were in agreement with those described above. In cells containing the YG100 fusion, the β-galactosidase levels increased at least tenfold after heat shock; in cells containing the YG102 fusion enzyme, levels increased only slightly after heat shock. Cells containing the two fusions showed different basal levels of expression during logarithmic growth at 23°C. Cells containing the YG102 fusion have a fivefold higher level of β-galactosidase activity than YG100-containing cells.

The presence of a gene (YG102) highly homologous to the heat-inducible gene (YG100) but with a different pattern of expression is in contrast to what is found in *Drosophila*. All of the inducible members of the *Drosophila* hsp70 family (the five hsp70 and one hsp68 genes) are expressed at very low levels during nonstress situations and induced dramatically upon heat shock. In the protein coding regions, YG100 and YG102 are about as homologous to each other as the copies of the *Drosophila* hsp70 at 87A and 87C are to one another. However, while the *Drosophila* genes are homologous in the 5′ nonprotein coding and flanking regions, the yeast genes show no conservation in these regions. This lack of conservation is consistent with the differentially regulated expression of the two yeast genes.

3. HSP70 of Other Eukaryotes

Although study of the genes encoding mammalian hsp70 genes is preliminary in nature, a good deal of analysis of the proteins has been carried out. The 72K and 73K hsps of HeLa cells copurify during column chromatography and cosediment during equilibrium sucrose sedimentation. A mixture of the two proteins has a Stokes radius of 69 Å and a sedimentation coefficient of 5.8[36] It is likely that the 72K and 73K proteins are related even though it is not clear from peptide mapping that they contain any identical peptides, nor do antibodies isolated, thus far, show cross reactivity between the two proteins.[37] In both baby hamster kidney cells and chicken cells hsp70 is methylated.[38]

Genes homologous to the *Drosophila* hsp70 genes have been isolated from *C. elegans*. These genes have been grouped into three classes based upon their expression. Transcription of one is stimulated by heat shock. A second is developmentally regulated, being highly expressed in larval stages, but not enhanced by heat shock. Transcripts of members of the third class have yet to be detected. Using restriction site polymorphisms, the heat-inducible 70K gene has been mapped to the far right end of linkage group IV.[13] Comparison of these genes in two closely related strains shows a much greater sequence divergence (~7.5%) in the flanking regions of the inducible genes compared to the constitutively expressed genes (0.4%).

A gene related to the *Drosophila* hsp70 gene has been isolated from *Dictyostelium* based upon an increase in hybridization to RNA isolated from heat-shocked cells compared to RNA from control cells.[39] Comparison of a partial sequence of the *Dictyostelium* gene with the inducible hsp70 yeast and *Drosophila* genes reveals 70 and 55% identity of bases, respectively. A probe from within the *Dictyostelium* hsp70 protein coding region hybridizes to two mRNAs of about 2.5 kb that are distinguishable by electrophoresis in agarose gels. The larger message is present at 22°C but is induced approximately 50-fold after a heat shock. The smaller message is also present at 22°C but it decreases in abundance after the temperature shift. Therefore, the hsp70 gene may produce two different transcripts after the temperature shift. However, at this time it is not known whether other genes related to the one isolated exist in the *Dic-*

tyostelium genome. If so, the two RNAs may be transcription products of different genes. In any case, the RNA results are in agreement with the conclusion that substantial hsp70 (or related protein) is present in control cells.[40]

4. E. coli HSP70

The *dna*K gene product has recently been shown to be 48% identical to the hsp70 protein of yeast and *Drosophila*.[41] The *dna*K protein has been purified to near homogeneity and a partial sequence of the amino terminus determined.[42] This sequence agrees with that predicted from the DNA sequence except, as is commonly found with bacterial proteins, the amino terminal methionine is absent. The purified protein was found to hydrolyze ATP to ADP and Pi.[43] The DNA-independent ATPase activity is weak; only 15 to 20 nmol of ATP is hydrolyzed per milligram of protein per minute at 30°C, representing a turnover of only one ATP molecule per minute. The ATPase activity is relatively heat stable, with 10% of the activity remaining after a 10-min incubation of *dna*K protein at 95°C. The DNA (native or denatured) independent nature of the ATPase activity was somewhat unexpected since a number of proteins involved in DNA replication, such as *dna*B protein, protein m, helicase 1, and helicase 2, which possess ATPase activity show a DNA dependency of the reaction. The purified *dna*K protein is phosphorylated on threonine residues in vitro in the presence of [γ^{32}P]ATP. It is not clear whether this is due to autophosphorylation or due to a contaminating kinase. Two results argue in favor of autophosphorylation. First, the phosphorylation occurs in precipitates formed between the purified protein and anti-*dna*K protein antibody. Secondly, the phosphorylation is quite specific; phosphorylation of other proteins does not occur when they are added to the purified *dna*K protein. Phosphorylation of *dna*K also occurs in vivo. About 5% of the total cellular *dna*K protein is present in the phosphorylated form after labeling with ^{32}Pi. Phosphorylation of threonine residues of the *Dictyostelium discoideum* hsp70 has been observed,[44] although phosphorylation of hsp70 of a number of other species has not. It would be of interest to know whether the site of the phosphorylated threonine is the same in the two related proteins.

Since the isolation of the first *dna*K mutations,[45-48] it has been known that *dna*K protein is necessary for λ phage DNA replication. The *dna*K protein probably interacts with the P protein of λ, since mutations in λ phage that enable it to grow on *dna*K$^-$ hosts map in the P gene.[45,47] Recently, it has been shown that *dna*K protein is needed for the conversion of M13 single-stranded DNA to a double-stranded form in an unusual replication system.[43] This system does not rely on the usual in vivo mechanism of M13 single-stranded replication, which is known to require the host *E. coli* RNA polymerase. Instead, DNA strand synthesis is initiated in the presence of rifampicin provided λ replication proteins O and P are present (as well as a crude *E. coli* extract). Addition of anti-*dna*K antibody to this system results in an inhibition of M13 replication; however, activity can be restored by the addition of an excess of purified *dna*K protein.

Although this biochemical, as well as the earlier genetic, data indicate a role for *dna*K in λ DNA replication, the role of *dna*K in bacterial functions is not clear. The gene was named *dna*K because of the inhibition of DNA synthesis after the shift of a temperature-sensitive mutation (*dna*K756) to the nonpermissive temperature.[48] However, an analysis of nucleic acid synthesis after the temperature upshift of *dna*K756 and another *dna*K mutation (*dna*K7) showed that RNA synthesis was inhibited more rapidly than DNA synthesis after the temperature shift.[49] Also, unlike mutations in a number of other *dna* genes, 50% inhibition of DNA synthesis was not reached until 1 hr after shift to the nonpermissive temperature, and synthesis actually continued to increase for the first 10 min (*dna*K7) to 15 min (*dna*K756) after the shift. Therefore, *dna*K mutations appear to affect RNA as well as DNA synthesis, and whether the

primary effect is on DNA synthesis is not known. Also, the effect on host and phage can be separated; mutations in *dnaK* have been isolated which prohibit λ propagation but do not affect growth of the host.[48] Therefore, it is possible that the role of *dnaK* in λ replication is not functionally similar to its role in host metabolism. However, the *dnaK* protein does appear to be essential for bacterial growth, at least at high temperatures, since a number of *dnaK* mutations block colony formation at 42°C.

C. The Small HSPS

1. Drosophila Small HSPS

In early studies, it was shown by in vitro translation that 12S RNA isolated from polysomes of heat-shocked *D. melanogaster* tissue culture cells encoded the small hsps (hsp27, 26 , 23, and 22).[50-52] This RNA hybridized *in situ* to the major heat shock puff on 3L, locus 67B.[5,48] By analyzing the mobility of proteins isolated from progeny of crosses between strains containing electrophoretic variants of hsp27, 26, and 23, these hsps were mapped to a small region of 3L, which included the 67B heat shock puff.[53] Subsequently, isolation of cDNA and genomic clones have allowed a detailed description of the organization, expression, and structure of the genes encoding the small hsps.[54-57] The four genes are closely linked, located in a 12-kb DNA segment. Three of the genes are transcribed in the same direction, a fourth (hsp26) in the opposite (Figure 5). The hsp27 transcript is about 1.3 kb, while the transcripts for the other three are about 1.0 kb in size. No evidence for a precursor has been found, suggesting that each gene has its own promoter and contains no intervening sequences.

Hybridization-selection experiments performed under reduced stringency suggested that hsp27, 26, and 23 transcripts were partially homologous, since each could select mRNA which translated into the other two.[56] DNA sequence analysis of the genes revealed extensive homologies among the four genes[58,59] over a portion of their length. Each transcribed region includes a single, long, open reading frame which would encode proteins of 23,620, 22,997, 20,603, and 19,705 daltons for hsp27, 26, 23, 22, respectively. Comparison of the protein sequences which could be encoded from these open reading frames also reveals homologies within a stretch of 108 amino acids; the same amino acid is present in all four proteins at 35% of the positions, and three out of four of the proteins at 71% of the positions. Hsp22 shows less homology within the conserved region than the other three. A schematic diagram of the relationships between the four small hsps is shown in Figure 4. The homologous segment is located within a central region, skewed toward the carboxyl end, surrounded on the amino terminal side by a 58-84 amino acid nonhomologous region and on the carboxyl side by a short 7-21 nonhomologous amino acid segment.

A second region of weak homology was also observed among the first 14 amino acids of the proteins of hsp27, hsp26, and hsp23, but not hsp22. A resemblance between these 14 amino acids and part of the signal peptide from human preproinsulin and residues 20 to 29 in bacteriorhodopsin, a bacterial transmembrane protein, has been noted.[59] Although in vitro translation and in vivo experiments indicate that the N terminus is not cleaved, it is possible that this sequence is involved in some interaction with membranes.

Analysis of the DNA sequence of the transcribed nonprotein coding regions and the flanking regions reveals no extensive homologies. However, CAP sites and TATAATA boxes at the 5′ end and poly A addition sites at the 3′ end which are commonly found in many genes and short sequences thought to be involved in the regulation of inducibility of these genes (see Section V.D. 1) were found in the hsp sequences. The linkage of these four partially homologous genes in a 12-kb DNA segment suggests that the genes arose by duplication and inversion of a single gene.

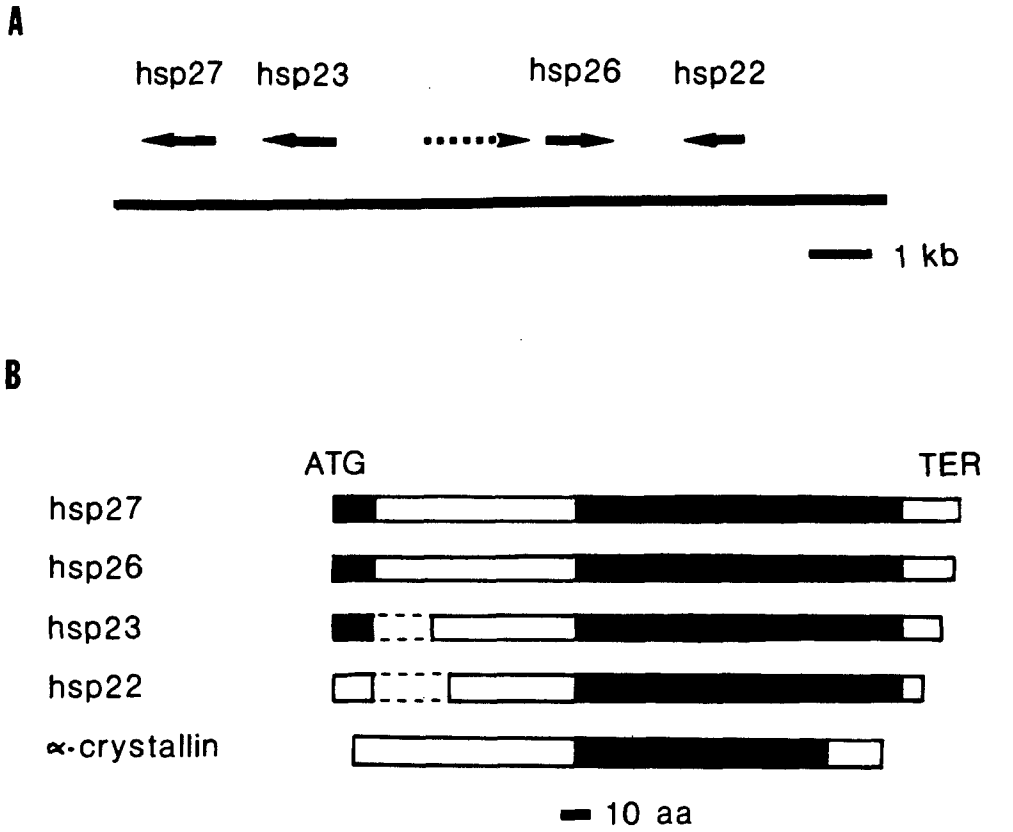

FIGURE 4. The genome organization of the genes encoding the four small *Drosophila* hsps and a schematic diagram of the homologies among the four genes and bovine α-crystallin. In (A) the direction of transcription is indicated by the arrow whose length represents the transcribed region. The dashed line indicates the position of a developmentally regulated gene as described by Sirotkin and Davidson.[159] The organization of the heat shock genes was taken from Corces et al.,[56] Craig and McCarthy,[55] and Voellmy et al.[57] In (B) the genes are drawn so that their homologous regions are aligned. The solid regions represent those segments showing homology among the *Drosophila* hsps and between the *Drosophila* proteins and bovine α-crystallin. Open bars indicate regions which show no obvious homology.

2. Small HSPS of Other Eukaryotes

Genes encoding hsps in the size range of 15 to 25K have been isolated from the genomes of nematodes[60] and frogs,[61] and DNA sequence analysis reveals homologies to the *Drosophila* heat shock genes (Figure 5). Not surprisingly, these homologies occur in the region of conservation found among the four *Drosophila* genes.

In the nematode, *C. elegans*, three proteins of differing molecular weight under 29K have been identified.[60,62] In adult worms these proteins, hsp25, hsp18, and hsp16, appear not to be synthesized in control cells in detectable amounts. cDNA clones encoding a portion of the protein coding region of hsp16 have been isolated.[60] Two types of related sequences were isolated. In a portion of the protein coding region, corresponding to aa83 of the *Drosophila* hsp27 protein to the carboxyl terminus, the two predicted nematode proteins are 91% homologous. In a short segment outside this region extending toward the amino terminus, none of the amino acids encoded are the same. Preliminary results indicate that this junction between homologous and nonhomologous sequences in the cDNA clone represent an intron-exon boundary in an isolated genomic clone. The analysis performed, thus far, on the genes of *C. elegans* has revealed some differences between the organization and structure of partially homologous genes of nematodes and fruit flies. First, the two genes isolated from nematodes

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D. mel. hsp27      Val Gly Lys Asp Gly Phe Gln Val Cys Ile Asp Val Ser Gln Phe Lys Pro Asn Glu Leu Thr Val Lys Val Val Asp Asn Thr Val Val  -
D. mel. hsp26       •   •   •   •   *   •   •   *   *  Met   *   •  Ala   •   •   •   •  Ser   •   *  Asn   •   •   •   •   •  Ala Ser Ile Leu  -
D. mel. hsp23      Ile  *   •   •   •   •   •   •   *  Met   *   *   •  His   •  Glu   •  Ser   •   •  Val   •   •   *  Gln   •   •  Ser   •  Leu  -
D. mel. hsp22       •  Asn  •   •   •  Tyr Lys Leu Thr Leu   •   •  Lys Asp Tyr   -   -  Ser   •   •  Lys   *   *   *  Leu   •  Gly Ser   •   •  Leu
Xenopus hsp30      ----------*  His   •  Glu Leu Thr Leu   •   •  Arg Asp   •  Ser   •  His   •   •   *   *   *  Thr Gln Gly Arg Arg   •  Ile  -
C. elegans hsp16   Asn Asp Glu Ser Lys   •  Ser   •  Gln Leu   •   *   •  His   •   •   •  Glu Asn   •  Lys Ile   •  Leu Asp Gly Arg Glu Leu Lys  -
αcryst β2          Leu Glu   •   *  Arg   •  Ser   •  Asn Leu Asn   •  Lys His   *  Ser   •  Glu   •   •  Lys   *   *   •  Leu Gly Asp Val Ile Glu  -
αcryst A2          Ser Asp Arg   •  Lys   •  Val Ile Phe Leu   •   •  Lys His   •  Ser   •  Glu Asp   •   *   *   •   •  Gln Glu Asp Phe   •  Glu  -

D. mel. hsp27      Val Glu Gly Lys His Glu Glu Arg Gly Asp Gly His Gly Met Ile  -  Gln Arg His Phe Val Arg Lys Tyr Thr  -   -   -  Leu Pro Lys
D. mel. hsp26       •   •   •   *   •   •   •   •  Gln   •  Asp   •   *  His   •   -  Met   •   *   •   *   •  Arg   •  Lys   -   -   -  Val   •  Asp
D. mel. hsp23       •   •   •  Asn   •   *   *   •  Glu   •  Asp   •   *  Phe   •   -  Thr   •   •   *   *   •  Arg   •  Ala   -   -   -   *   •  Pro
D. mel. hsp22       •  Gly  •   •  Ser   •  Gln Gln Glu Ala Glu Gln   *  Gly Tyr Ser Ser   •   •   *  Leu   •  Arg Phe Val   -   -   -   •   •  Glu
Xenopus hsp30       •  Thr  •   •   •   •  Arg Lys Ser   •  Thr Lys Asp Gly Ser   -  Tyr Phe   •  Glu Tyr   •  Glu Arg Lys Arg Glu Ala   *   •  Glu
C. elegans hsp16   Ile   •   •  Ile Gln   •  Thr Lys Ser   -  Glu   •   *  Tyr Phe   -  Lys   •  Ser   •  Ser Lys Met Ile Leu   -   -   -   *   •  Glu
αcryst β2           •  His  •   •   •   *   •   •  Gln   •  Glu   *   *  Phe   *   -  Ser   •  Glu   •  His   •   •   •  Arg   -   -   -  Ile   •  Ala
αcryst A2          Ile His  *   •   •  Asn   •   •  Gln   •  Asp   •   *  Tyr   -   -  Ser   •  Glu   •  His   •  Arg   •  Arg   -   -   -   *   *  Ser

D. mel. hsp27      Gly Leu Thr Pro Thr Lys Val Val Ser Thr Val Ser Ser Asp Gly Val Leu Thr Leu Arg Ala Pro Pro Pro Pro Gly Arg Glu Arg Ala Arg
D. mel. hsp26       •  Tyr Lys Ala Glu Gln   •   •   •  Gln Leu   *   *   *   •   •   *   •  Val Ser Ile   •  Lys   •  Gln Ala Val   •  Asp Lys Ser
D. mel. hsp23       •  Tyr Glu Ala Asp   •   •  Ala   *   *  Leu   *   *   *   •   •   *   •  Ile Lys Val   *  Lys   •   *  Ala Ile   •  Asp Lys Gly
D. mel. hsp22       •  Tyr Glu Ala Asp   •   *  Thr   •   *  Leu   •   •   •   *   •   •   •  Ile Ser Val   •  Asn   •   •   •  Val Gln Glu Thr Leu
Xenopus hsp30       •  Val Asn   •  Glu Gln   •   •  Cys Ser Leu   •  Lys   •   •  His   •  His Ile Gln   •   •  Arg Leu Ala Leu Pro Pro Ala Pro Glu
C. elegans hsp16   Asp Ala Asp Leu Pro Ser   *  Lys   •  Ala Ile   •  Asn Glu   •  Lys   •  Gln Ile Glu   •   •  Lys Lys Thr Asn Ser Ser   •  Ser Ile
αcryst β2          Asp Val Asp   •  Leu Ala Ile Thr   •  Ser Leu   •   *   *   *   *   •   •  Val Asn Gly   *  Arg Lys Gln Ala Ser Gly Pro Glu   •
αcryst A2          Asn Val Asp Gln Ser Ala Leu Ser Cys Ser Leu   •  Ala   •   *  Met   •   •  Phe Ser Gly   •  Lys Ile   •  Ala Gly His Ser Glu   *
                                                                                                                    Ser Gly Val Asp
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FIGURE 5. Comparison of the conserved stretch of amino acids among *Drosophila*, *Xenopus*, and nematode small hsps and the A_2 and B_2 bovine α-crystallins. The *Drosophila* sequences were from Ingolia and Craig,[58] the *Xenopus* from Bienz,[61] the nematode from Russnak et al.,[60] and the α-crystallin sequences from Vander Ouderaa et al.[66] The top line in each section shows the amino acid sequence of hsp27. An asterisk indicates the presence of the same amino acid at that position as hsp27. The long dashes indicate gaps introduced to obtain an optimal alignment of the sequences. Short dashes indicate that the sequence had not been determined. The sequences presented begin at aa85, hsp27; aa86, hsp20; aa66, hsp23; aa59, hsp22 of *Drosophila;* aa70, B_2 α-crystallin; aa66, A_2 α-crystallin. The amino terminus of the protein coding regions of the nematode and *Xenopus* genes has not been determined yet.

are more homologous to each other than the *Drosophila* genes are to one another. Also, the presence of the intron in the hsp16 gene is the first intron identified in genes for small hsps.

One major small hsp, hsp30, is induced in *Xenopus* cells after heat shock.[63] cDNA clones encoding a portion of hsp30 have been isolated and the DNA sequence determined. These clones derived from the 3′ end of the mRNA and encompassing about 60% of the protein coding region are between 35 and 50% identical to the *Drosophila* genes encoding small hsps.

Small hsps have been identified in many organisms. While the number varies, most organisms synthesize one to five different proteins. However, plants seem to have a greater number of small hsps. For example, the soybean small hsps (15,000 to 23,000 daltons) are resolved into at least 15 spots on two-dimensional gels. Since proteins synthesized in vitro using heat shock mRNA as a template also resolved into about 15 spots, it is likely that the multiple spots represent unique proteins translated from different mRNAs rather than postsynthetic modifications. Some of these proteins are related to one another, since RNA isolated by hybridization to cDNA clones translates into more than one hsp. One clone selects RNA encoding 13 proteins of 13,000 to 18,000 daltons; another selects mRNA for 3 proteins in the range of 21,000 to 23,000 daltons.[65]

3. Homology between Small HSPS and Mammalian α-Crystallin

After completion of the sequence of the genes encoding the small *Drosophila* hsp, homology between the small hsps and bovine α-crystallin chain was observed.[58] This region of homology includes the first 83 of the 108 amino acid stretch conserved among the *Drosophila* proteins which was described above. This homologous region includes amino acids 70 to 152 of α-crystallin, out of a total of 175 aa.[66] The *Xenopus* and nematode proteins are also homologous to α-crystallin in the same region. Hsp24 of chicken is also probably related to lens-crystallin since anti-hsp24 react with crystallin purified from 11-day embryonic chicken lens.[67]

The functional significance of the homology between the small hsps and mammalian α-crystallin is not clear. However, it is intriguing to compare some of the physical characteristics of the two types of proteins. Mammalian α-crystallins are a major component of the vertebrate eye lens, comprising about 35% of the protein of the lens. α-Crystallins are highly polymeric proteins, forming large aggregates with an average molecular weight of 800,000.[68,69] These aggregates perform a major structural role in determining the unique properties of the eye lens. It is interesting that *Drosophila* hsp27, 26, and 23 have been observed in large complexes in the cytoplasm of *Drosophila* tissue culture cells.[70] The conserved domain may well facilitate specific aggregation of these proteins. An analysis of the hydrophobic/hydrophilic characteristics of the *Drosophila* small hsps has been reported.[59] Due to the amino acid homology between the small hsps and α-crystallins, the five proteins exhibit a similar hydropathic profile in their shared regions. A prominent hydrophilic peak is found in the hydropathic profile of the conserved region and probably represents a major surface domain common to all five proteins.

D. Other HSPS of *E. coli*

One heat shock protein of *E. coli* which has no identified counterpart in eukaryotes, is a lysl tRNA synthetase, polypeptide D60.5.[71] The gene encoding the heat-inducible synthetase, *lysU*, maps near the gene for lysine decarboxylase, *cad*A, at 92 min on the *E. coli* genetic map. There are two unlinked genes encoding lysl tRNA synthetases; one, *lysU*, is heat inducible, the other, *lysS*, is not. Under most growth conditions, only the products of the *lysS* gene are made. However, cells grown in the presence of

alanine, leucine, fructose, or glycyl-leucine express the *lysU* as well as the *lysS* gene. Inaddition, a *metK* mutation that reduces S-adenosylmethionine synthetase activity to low levels also results in an increase in synthesis of the *lysU* gene product.[72] The existence of two genes encoding proteins with the same activity is unusual, but not unprecedented in *E. coli*. For instance, there are two genes encoding elongation factor Tu.[73]

Two other *E. coli* hsps are the products of the closely linked *groEL* and *groES* genes.[74,75] *groE* mutants were isolated as hosts in which bacteriophage λ was unable to undergo productive infection. Subsequently, it was shown that *groEL* mutations had pleiotropic effects; growth of phages T4 and T5, as well as the bacterial host, is affected. The effects on phage growth are at the level of morphogenesis. T4 and λ are unable to make functional heads, while in T5-infected cells, tail assembly is defective.[76] The *groEL* protein product is a 65K protein, B65.5.[77] In its native state, *groEL* is found as a decatetramer with its 14 subunits arranged in a double ring with sevenfold symmetry. The complex sediments at 25S and possesses a weak ATPase activity.[78] *groES* is a 15K protein, C15.4.[79] Genetic experiments suggest that the *groES* and *groEL* interact in vivo. Mutations in the *groEL* gene have been isolated that suppress defects in the *groES* gene for both bacterial growth and phage morphogenesis.[80] However, the role of *groEL* or *groES* in bacterial growth is unknown.

The synthesis of the sigma subunit of RNA polymerase is enhanced after heat shock. *E. coli* RNA polymerase is a multisubunit enzyme composed of α, β, β', and δ subunits. The enzyme is found in two forms: as holoenzyme ($\alpha_2\beta\beta'\delta$), capable of selective DNA binding on initiation, and as core polymerase ($\alpha_2\beta\beta'$), capable of RNA chain elongation but not selectivity.[81] Therefore, the δ subunit is necessary for selective DNA binding and may well play a role in the regulation of transcription initiation in *E. coli*. The gene for δ, *rpoD*, located at 66 min on the *E. coli* chromosome, has been isolated on a recombinant clone and its sequence determined.[82] *rpoD* is cotranscribed with *rpsU* and *dnaG* which encode ribosomal protein S21 and DNA primase, respectively. After temperature upshift, a promoter located within *dnaG* is transiently induced (see Section V.D.2).

An hsp, H94.0, has been recently identified as the product of the *lon* gene,[83] a protease.[84] This protease degrades foreign proteins and protein fragments, as well as some normal cellular proteins.[85]

E. Other HSPS of Mammalian Cells

Mammalian cells induce the synthesis of three hsps which are not related to members of the three groups of *Drosophila* hsps. These proteins are approximately 80K, 100K, and 110K, and have been referred to as minor hsps due to their relatively low level of synthesis after heat shock compared to other hsps. The two smaller proteins have been identified as glucose-regulated proteins whose synthesis is increased upon glucose deprivation.[86,87] Hsp80 contains a modification, an ADP-ribosylation. Heat shock and glucose starvation of cells induce a rapid decrease in the incorporation of ADP-ribose into hsp80.[87] Both hsp80 and hsp100 have been shown to be phosphorylated in at least some rat and mouse cells. Also, hsp100 was shown to be a glycoprotein, incorporating [^{3}H]mannose.[86]

F. RNAs Which May Not Encode Proteins

1. αβ Sequences of Drosophila

A set of tandemly repeated sequences, called $\alpha\beta$ sequences, are present at cytological locus 87C of *D. melanogaster*.[88] Transcription of these sequences is heat inducible, while similar sequences found at the chromocenter are not.[89] Similar sequences are also found in the genome of a sibling species, *D. simulans*, but only at the chromocenter,

and are not transcribed. RNAs transcribed from these sequences in *D. melanogaster* are found in three size classes, the longest being a 3-kb transcript of an entire $\alpha\beta\alpha$ unit. The 40 kb of DNA separating the 70K genes at 87C contain between 5 and 14 $\alpha\beta$ repeats, the number differing between strains. Adjacent to a number of $\alpha\beta$ repeats are sequences highly homologous (98%) to sequences at the 5′ end of hsp70 genes. This homologous region includes 64 b of the protein coding region as well as 406 bases upstream.[90,91] This region contains the sequences necessary for heat-inducible transcription (see Section V.D.1). It is likely that $\alpha\beta$ sequences recently moved to 87C and became juxtaposed to the hsp70 regulatory sequences. The $\alpha\beta$ sequences probably do not encode proteins and it is quite possible that they serve no function.

2. 93D Locus of Drosophila

93D is the site of one of the six major heat-inducible puffs and is a major site of transcription after heat shock. However, 93D does not appear to encode any of the major hsps. Unlike other heat-inducible RNAs, much of the 93D RNA is nuclear and that found in the cytoplasm is polyA$^-$. The nuclear RNA, on the other hand, is a mixture of polyA$^+$ and polyA$^-$.[92] The puff at 93D differs from other heat-inducible puffs in several regards. It can be induced independently of the other puffs by benzamide[93] and its kinetics and duration of induction vary depending upon the environmental stimuli.[94]

Sequences of 93D have not been isolated, but a genetic analysis of this locus has been carried out.[95,96] Strains having overlapping deletions of the 93D locus are viable, although unable to form a puff in this region. No heat-inducible RNA from this region can be detected, suggesting that all inducible sequences have been deleted. A test of the heat shock response in flies carrying these deletions demonstrated that all other puffs as well as the set of major hsps were inducible. In addition, they possessed the same degree of thermoresistance after a mild 33°C heat shock as flies having a wild-type 93D locus. These deletions were used to screen for mutations in the region of the locus. Although 62 mutations falling into 20 complementation groups in the 3 subdivisions of 93 C-E were isolated, no mutations within the heat shock locus were found. It is proposed that the 93D locus may contain repeated sequences, thus, making isolation of a mutation unlikely. Therefore, the function of this locus remains completely unknown.

3. Dictyostelium Repetitive Sequences

Members of a repeated family of sequences of the *Dictyostelium* genome whose transcription is enhanced after heat shock have been isolated.[39,97] The genome contains about 50 highly conserved copies as well as 100 more divergent sequences. The repeated sequence contains 313 base inverted repeats suggestive of a transposable element. The pattern of hybridization of sequences flanking the repetitive DNA are different in various wild-type and laboratory strains, a result also suggestive of a mobile genetic element. It is not known whether the 0.9-kb transcript encoded by the repetitive sequences could encode a protein.

III. PHYSIOLOGICAL ROLE OF HEAT SHOCK PROTEINS

Hsps are abundant proteins, well characterized at the level of expression and structure, but their function remains unknown. Finding a function or functions for these proteins is proving to be a difficult task. Three areas of study which may provide clues to their function will be discussed. First, the identification of other cellular proteins of known function with which the hsps associate may give a hint as to their function. Similarly, a clue to function may come from the identification of the cellular localiza-

tion of the hsps. Thirdly, the positive correlation between the presence of hsps in cells and the resistance of cells to heat and other conditions suggests a role in protection of cells against the agents which enhance their synthesis.

A. Association with Other Proteins

In Rous sarcoma virus-infected chicken cells, a small fraction of hsp90 is associated with newly synthesized $pp60^{v\text{-}src}$, the virus encoded transforming protein.[98-100] This association was first observed because of the coprecipitation of hsp90 and a 50K cellular protein, with $pp60^{v\text{-}src}$ after incubation with $pp60^{v\text{-}src}$ specific antibody. $pp60^{v\text{-}src}$ Is synthesized on free polyribosomes, released into the cytosol, and reaches the plasma membrane 5 to 15 min later. The bulk of $pp60^{v\text{-}src}$ is found in the cytoplasmic face of the plasma membrane. The sequences responsible for this attachment to the plasma membrane appear to be located in the NH_2 terminal fourth of the protein. Lipid, which is thought to be responsible for the association with the plasma membrane, is bound to this amino terminal portion of the protein,[101,102] and it has been proposed that the complex between the two cellular proteins and $pp60^{v\text{-}src}$ is the vehicle by which $pp60^{v\text{-}src}$ reaches the plasma membrane.[103,104] This complex is short-lived, with a half-life from 9 to 15 min in cells transformed by nondefective RSV. Behavior of this complex is altered when cells are infected with virus containing a temperature-sensitive defect in the src gene. At the nonpermissive temperature, $pp60^{v\text{-}src}$ is synthesized in normal amounts, but little is found in the plasma membrane. Under these conditions, more than 90% of $pp60^{v\text{-}src}$ is found associated with hsp90 and the 50K cellular protein. The $pp60^{v\text{-}src}$ synthesized under permissive conditions seems to dissociate from the membrane and return to the cytoplasmic complex.

The role of hsp90 in this complex or in the transport of $pp60^{v\text{-}src}$ to the plasma membrane is not clear. However, the association with viral-encoded proteins appears not to be restricted to RSV-infected cells. Chicken cells infected with other retroviruses, Fujinami, and Y73 avian sarcoma viruses have been shown to contain complexes between kinases and hsp90.[105] The association of hsp90 with other proteins has been studied in cells infected with Sindbis, an α virus, and vesticular stomatitis virus (VSV), a rhabdovirus,[106] by determining what other proteins were coprecipitated when hsp90 was immunoprecipitated with hsp90 antibody. In Sindbis and VSV-infected cells, C (capsid) protein and N (nucleocapsid) protein, respectively, were found to coprecipitate with hsp90.

Based on coprecipitation with monoclonal antibodies, it has been concluded that two other hsps (hsp70 and hsp72) of mammalian cells are associated with a 90K cell surface glycoprotein.[107] The amount of hsp 70/72 precipitated with the 90K glycoprotein was only a fraction of that found in the cell. Hsp72 is also found in normal cells,while hsp70 synthesis was only observed after a heat shock. Hsp70/72 isolated by these workers was similar, based upon tryptic peptide maps, to a protein previously identified in other cells which appeared to be associated with microtubules. It was proposed that hsp70/72 serves to mediate an association between the plasma membrane and the cytoskeleton.

A possible clue to the role(s) of hsp70 may be provided by experiments which link hsp70 to uncoating activity of clathrin-coated vesicles in both *Drosophila* and mammalian cells.[108] In *Drosophila* cells, the amount of uncoating activity which is ATP dependent increases 10 to 20 times after heat shock and the increase is blocked by the addition of cycloheximide. Furthermore, hsp70 as well as an uninduced 70K protein shows ATP-dependent binding to coated vesicles and has an ATP-binding site.

B. Cellular Location

Determination of the cellular localization of the hsps is of great importance because

it may provide a clue to their function. Several questions arise when attempting to interpret the results of localization experiments. First, the proteins are often found to be distributed among more than one cellular compartment. Are both locations physiologically relevant? Also, localization of induced proteins can differ depending on the inducer. Are the different localizations physiologically relevant in each case, or are only certain inducers "natural" and only in these cases do the proteins function?

1. Localization of Drosophila Proteins

Initial[109] as well as more recent localization experiments in *Drosophila*[70] and Chironomous[110] were performed to determine the distribution of hsps during the fractionation of cellular compartments. These experiments indicated that hsp70, 68, 27, 26, 23, and 22 were approximately equally distributed between the nucleus and the cytoplasm. In general, a higher proportion of the small hsps than hsp70 and hsp68 were found in nuclei. Hsp83 was found almost exclusively in the cytoplasm. The hsps found to partially fractionate in the nucleus after induction by heat shock are not found in the nucleus after induction by arsenite, but these preexisting hsps do enter the nucleus after a subsequent heat shock.[111] Cell fractionation experiments could potentially be misleading due to possible leakage of protein from cellular organelles or spurious binding to structures with which they do not normally associate in the living cell.

In more recent experiments, localization of hsps has been studied by direct autoradiographic analysis using light and electron microscope.[70,112] Cells were labeled with ^{3}H-amino acids, then chased with cold amino acids. It was estimated from analysis of the proteins synthesized that 80% of the ^{3}H was incorporated into hsp70. A major portion of the radioactivity was found in the nucleus. Concentration was observed in nucleoli and bound to chromosomes, with little associating with heterochromatin, chromocenters, or highly condensed polytene bands. More recently, monoclonal antibodies specific for hsp70 have been used in immunofluorescence studies of cellular localization.[113] After both recovery from anoxia and heat shock the immunofluorescence was concentrated in the nucleus. However, unlike the observation in the autoradiographic studies, no concentration in nucleoli was observed. After removal of the stress, the hsp70 detected returned slowly to the cytoplasm. With a second heat shock, fluorescence was again concentrated in the nucleus. The amount of the total cellular hsp70 localized to the nucleus was greater at higher temperatures.

Antibodies directed against hsp23 have also been used in immunofluorescence studies of salivary gland nuclei.[114] The staining was concentrated in the nucleus, and as with hsp70-specific antibodies, fluorescence was then observed in the cytoplasm after a recovery time at 25°C.

A possible complicating factor in the localization studies is the fact that the 10-n*M* (intermediate) filament network collapses upon the nucleus after heat shock or treatment with valinomycin,[115-117] and some proteins which may appear to be inside the nucleus may be in the collapsed network structure surrounding the nucleus. It is not known whether intermediate filaments are collapsed around the nucleus during recovery from anoxia when hsp70 is localized to the nucleus or after sodium arsenite treatment when they are not.

Several studies using fractionation have been directed toward determinining the localization within the isolated nuclei fraction of the hsps. Two such studies[118,119] suggest that the nuclear-associated proteins are in a nuclease-resistant and high-salt-insoluble nuclear fraction, perhaps in the nuclear scaffold. However, in another study[70] the hsps seemed to be associated with chromatin and were released as free polypeptides by DNase digestion. In this same study, fractionation experiments indicated that 30 to 40% of the nuclear content of hsp70, 68, 26, 27, and 23 and 50 to 60% of hsp22 were present in nucleoli, a result in agreement with direct autoradiography, but not with immunofluorescence data.

2. Localization of Mammalian Proteins

Localization studies have also been carried out in mammalian systems. A polyclonal sera directed against hamster hsp110 has been used in immunofluorescence studies. A comparison of phase contrast and indirect immunofluorescence shows that hsp110 is localized at or near the nucleolus in cultured cells and cells of a number of murine tissues. Treatment of cultured cells with deoxyribonuclease destroyed the organization of staining within the nucleus. Ribonuclease appeared to completely release the antigen from the nucleus, implying an association with RNA or with structures associated with RNA. Staining was not restricted to the nucleus; secondary staining of cytoplasmic structures was observed.[120]

The localization of hsp100 has also been studied by immunofluorescence using both monoclonal antibodies and a polyclonal rabbit sera. In normally growing cells, bright fluorescence with a lamellar-like structure was observed near the perinuclear region of the cells. In addition, a nuclear fluorescence was found in a subpopulation ($\sim$15%) of the cells. It could not be concluded whether the nuclear localization was intra- or extranuclear. The perinuclear location found in most cells was shown to be the Golgi apparatus based on several criteria, including enzyme markers and the use of drugs known to disrupt the Golgi apparatus. After heat shock, the proportion of the cells showing nuclear staining increased and the Golgi staining appeared to be somewhat disrupted. These experiments were carried out in mouse, rat, gerbil, and human cells, with similar results, suggesting the cellular location of hsp100 is the same in a wide variety of mammalian species.

Mammalian cells contain two hsps of approximately 70K called hsp72 and hsp73. Hsp72 is not normally present, while hsp73 is present in nonheat-shocked cells, and its synthesis increases after heat shock. Polyclonal sera which reacted with hsp72, but not hsp73, has been used in indirect immunofluorescence studies.[121] Diffuse cytoplasmic and nuclear staining was observed in cells grown at 37°C. In heat-shocked cells, the staining was found to increase in both the cytoplasm and the nucleus. The staining in the nucleus occurred in discrete regions of the nucleus, many of which were coincident with nucleoli. This discrete nuclear staining was observed in many cell types, but not in 100% of cells examined. Between 70 and 90% of cells were found to have discrete regions of fluorescence. Cells exposed to other inducers of hsps, such as sodium arsenite or an amino acid analog of proline, showed increased nuclear fluorescence but no localized regions of staining. Fractionation experiments were also performed by the same investigators. They found about 30 to 40% of the total hsp72 protein was present in the nuclear fraction of heat-treated cells, while 60 to 70% was in the cytoplasm. Of the nuclear-associated hsp72 (or hsp73), a portion was associated with the nuclear matrix, resistant to nuclease digestion. Comparison of these results with those for the *Drosophila* hsp70 reveal many similarities including a similar distribution between nucleus and cytoplasm, concentration in nucleoli, and association with the nuclear matrix. Also, heat shock and addition of amino acid analogs led to the collapse of the intermediate filament network around the nucleus in mammalian cells, as in *Drosophila* cells.

The location of the cytoplasmic portion of hsp72 and hsp73 is not clear. Several workers have reported that both can be found in preparations of cycled microtubules.[37,107] This association, as well as an association with a plasma membrane glycoprotein (see Section III.A), led Hughes and August[107] to suggest that hsp72 and 73 were involved in an attachment of microtubules to the plasma membrane. The association between hsp72 and hsp73 with other cytoskeletal components has led to the proposal that these hsps are involved as a cross-linker between microtubules and 10-n*M* filaments. However, it should be kept in mind that only a small fraction of the total hsp72/73 in the cytoplasm has been found in these associations. It is not clear whether or not some associations are physiologically relevant, since the association of a few

percent of an abundant protein with recycled microtubules may be due to nonspecific contamination rather than physiologically important interactions.

C. Thermotolerance

Experiments in a number of plant and animal species have indicated a correlation between the presence of hsps and the resistance of cells to a severe heat shock. Initial experiments demonstrated that an initial mild, nonlethal heat treatment caused cells to be resistant to a brief shift to higher temperatures which were normally lethal.[122-126] The exact conditions of the two shifts varied with the species examined, as do optimal growth conditions and normal susceptibility to heat. For instance, experiments with *Drosophila* involved growth at 20°C, a pretreatment for 50 min at 34°C, and a challenge at 41°C for 20 to 30 min. With yeast, cells growing at 23°C are often shifted to 37°C for 20 min and then to a normally lethal temperature of 51°C for 10 min. Experiments with cells from thermoregulating animals such as mammalian tissue culture cells are usually performed in a narrower temperature range. Cells growing at 37°C are either pretreated for a short time (6 min) at 46°C or a longer time (1 to 4 hr) at 41°C, prior to an incubation for several hours at 37°C, and then a challenge at 45°C for 45 min. The idea that inducible proteins were responsible for the increase in thermoresistance was furthered by the results of experiments indicating that in yeast[125,127] and *Dictyostelium*[124] protein synthesis was necessary during the incubation at the intermediate temperature for the acquisition of resistance. These experiments showed that incubation at the intermediate temperature in the presence of cycloheximide prevented thermoresistance. Also, it was soon shown that other conditions which resulted in the synthesis of hsps also conferred thermoresistance. These treatments included incubation of yeast with 6% ethanol at normal temperatures,[128] incubation of mammalian cells with 100 μM arsenite, 100 μM cadmium chloride, and 6% ethanol, as well as recovery from anoxia.[129-131] Exposure of yeast to ionizing radiation (50 krad) followed by a short incubation at low temperature also induces resistance to killing by heat.[127] Recovery from anoxia induces thermotolerance as well as hsp synthesis in *Drosophila*.[110] The reciprocal effect, the induction of resistance to other stresses, by a heat treatment has been observed in several cases. After an initial exposure to heat, arsenite, or ethanol, Chinese hamster cells acquired a tolerance to adriamycin, an oxidizing quinone.[131] Exposure to heat provides resistance of *Drosophila* larvae to recovery from anoxia[113] and resistance of yeast to ionizing radiation.[127] Certain chemicals which are known teratogens in mammalian systems inhibit muscle and/or neuron differentiation in cultures of post-gastrula embryonic *Drosophila* cells. These chemicals, including coumarin and diphenylhydantoin, also induce the synthesis of hsp23 and hsp22, but not the other hsps.[132] A pretreatment of these embryonic cultures at 35°C for 30 min allowed differentiation of the embryonic cells to continue even in the presence of the drugs.

A recent publication reports that protein synthesis in yeast is not necessary for the induction of thermoresistance in yeast.[133] The addition of cycloheximide prior to the pretreatment at the intermediate temperature did not diminish the increase in thermoresistance. These results are in direct conflict with earlier results,[125] as well as recent experiments.[127,134] The reason for the discrepancy is not clear; perhaps, the drug did not totally inhibit protein synthesis or, perhaps, thermotolerance can be achieved by more than one mechanism depending upon the physiological state of the cells. These inhibition experiments are of importance because they are the only direct link between the synthesis of protein and the induction of thermotolerance.

The increase in thermotolerance generated with the various pretreatments described above is transient, the kinetics of appearance and disappearance depending upon the species studied and the nature of the pretreatment. For example, in yeast the increase in thermotolerance occurs very quickly after a shift from 23 to 37°C, reaching a max-

imum by 45 min after the shift.[133] Two hours after the shift, thermoresistance has decreased to 50% of the maximal level. The acquisition of maximal thermotolerance in mammalian tissue cultures occurs several hours after the pretreatment. However, the thermoresistance persists for many hours. In Chinese hamster cells, thermotolerance decreased only threefold between 8 and 36 hr after pretreatment.[131]

Recently, mutants have been isolated which represent the beginning of a genetic analysis of the heat shock response and thermotolerance which may lead to a better understanding of the phenomenon. A mutant strain of *Dictyostelium* (HL122) which fails to become thermally resistant following a mild heat shock has been isolated.[135,136] The mutation is recessive since a heterozygous diploid showed normal thermoprotection. Haploids resulting from segregation of a heterozygous diploid show linkage of the thermosensitive phenotype to a mutation on linkage group III. The pattern of protein synthesis following heat shock in mutant and wild-type strains were compared. In the mutant, synthesis of hsp70, which is also synthesized during normal growth, is reduced as is synthesis its mRNA.[39] No synthesis of the low molecular weight hsps was observed. Although it is clear the mutation affects the synthesis of proteins after heat shock, how it exerts its effect is not. The mutation may affect a component necessary for the induction of hsps. Alternatively, there might be a temperature-sensitive mutation in some component necessary for all mRNA synthesis or maturation. In either case, the phenotype of this mutant strongly suggests a need for hsp synthesis for acquisition of thermoresistance.

Yeast strains containing in vitro-constructed mutations in two genes encoding 70K proteins synthesized after heat shock have been studied (see Section IV.B.2 for details). A double mutant containing mutations in both genes is temperature sensitive for growth.[137] The double mutant grows about 2.2 times slower than the wild type at 30°C. Several hours after a shift up to 37°C, growth stops. Consistant with this data is the inability of this mutant to form colonies at 37°C. These results indicate that the gene products are necessary for growth at 37°C, a temperature at which wild-type yeast grow logarithmically and form colonies efficiently. Mutant cells growing at 23°C were tested for the ability to acquire thermoresistance after a 20-min incubation at 37°C. The double mutant was as thermoresistant as the wild-type strain after the pretreatment, demonstrating that the wild-type hsp70 proteins were not needed for thermoprotection. It cannot be stated at this time that no protein of the hsp70 family is needed for thermoresistance, because at least six unaltered genes of the family are present in the double mutant, their transcription in the double mutant has not been analyzed. However, the behavior of this mutant does suggest that hsp70 is not involved in the acquisition of resistance to a short exposure to a high temperature. The temperature-sensitive phenotype of the double mutants does suggest that these proteins are needed for continued growth at higher temperatures. Hsps may serve different roles in regard to growth and survival at different temperatures. Some may be involved in the resistance to short exposures to normally lethal temperatures, which is commonly measured in studies of thermotolerance, while others may be important for sustained growth at intermediate temperatures. Hsp70 appears to fall into the second category.

Because of the results of the genetic experiments reported to date, small hsps or minor proteins synthesized after heat shock may be the best candidates for involvement in thermoresistance. The yeast mutants suggest hsp70 is not involved. Hsp83 is known to be present in substantial amounts in a number of species during normal growth, and one would not expect a vast increase in thermoresistance to be due to only a doubling or less of a particular protein. Also, the *Dictyostelium* mutant which does not acquire resistance fails to induce the small hsps, but continues to synthesize moderate amounts of hsp70.

IV. EXPRESSION OF HEAT SHOCK AND RELATED PROTEINS DURING NORMAL GROWTH

Evidence exists for the presence of members of each of the three families of hsps, hsp83, hsp70, hsp22 to 26, or a related protein, in some stage of development of *Drosophila*. Although data are less complete for other species, in general, the picture is similar. The exception may be the small hsps. In *Dictyostelium*, the small hsps were not detected under nonstress conditions. The presence of hsps or related proteins with possibly related functions indicates that these proteins do not function only under stress conditions, but also during normal growth and development.

A. HSP83 Proteins

Hsp83 has been found in *Drosophila* cells under normal growth conditions. Many of the studies have been done with cell lines[138,139] and, thus, are somewhat questionable in the sense that unless very carefully maintained, tissue culture cells are often stressed and, thus, apt to be mounting at least a minimal stress response. However, hsp83 RNA has been found in the ovary of nonheat-shocked females and in preblastoderm embryos.[140] The hsp83 RNA appears to be maternally derived, synthesized in the nurse cells, and transported into the oocyte. The protein also appears to be present in early embryos since monoclonal antibodies specific for hsp83 were derived after immunization using total protein from 2-hr embryos as an immunogen.[141] Hsp83 RNA has also been found in larval salivary glands in the absence of stress. However, hsp83 may not be expressed at a high level in all cell types. Total RNA isolated from whole flies after a heat shock has 20 times more hsp83 transcripts than RNA from control flies, while only a 20% increase in hsp83 transcripts is found in RNA from heat-shocked Kc tissue culture cells compared to control RNA.[139] In tissue culture cells, the hsp83 mRNA found under normal conditions appears mainly in the polyA$^-$ fraction. After heat shock, the hsp83 RNA is distributed between the polyA$^+$ and polyA$^-$ fractions.[142] What relationship, if any, the absence or presence of a polyA tail on the mRNA has to its ability to be translated is unclear. The reports described above represent a portion of the accumulating evidence that at least in *Drosophila* cells hsp83 is found under normal growth conditions in a number of cell types, and may well be present in most cells.

The situation in other species is less clear. The presence of the related protein, hsp89, in normal chick[15] and hsp90 in human cells[36] has been reported. Two nearly identical genes, which are related to *Drosophila* hsp83, have been isolated from yeast.[10] While one is heat inducible, the second is transcribed extensively under normal growth conditions.

B. HSP70 and Related Proteins

1. Drosophila melanogaster

In *Drosophila*, hsp70 and its mRNA are present at very low levels during normal growth.[144,145] Earlier reports of the moderate abundance during normal growth[146,147] were derived from experiments involving tissue culture cells, and, probably, were due either to the fact that the cells were mildly stressed or to the presence of related proteins and their mRNAs. For, although the 70K hsp is not normally present, related proteins, referred to as heat shock cognates (hscs), have been found in all cell types examined. The first indication of the presence of cognates was the isolation of a number of genes related to the hsp70 genes but found at cytological locations 70C, 87D, and 88E, sites distinct from heat shock puffs.[145,148] These cognate genes are about 75% identical in DNA sequence in the protein coding region to each other and to the hsp70 and hsp68 genes. Transcripts of these genes are present in flies grown at 25°C, and unlike the hsp70 transcripts, do not increase in amount after heat shock. While transcripts of

Drosophila	Locus	Embryo	Larva	Fly 25°-normal	Fly 37°-heat shock
Hsp70	87A (2 copies) 87C (3-4 copies)	N.D.	N.D.	+	+++++
Hsc1	70C	+	-	++	++
Hsc2	87D	-	-	+	+
Hsc4	88E	++++	++++	++++	++++

FIGURE 6. The cytological location and abundance of transcripts of *Drosophila* heat shock cognate genes. The cytological locations of heat shock and heat shock cognate genes were determined by *in situ* hybridization. The abundance of RNA transcripts of the genes was determined by cDNA primer extension assays. The table was compiled from data represented by Ingolia and Craig[148] and Craig et al.[146] N.D. indicated that the levels of transcripts were not determined.

two of the cognates, hsc1 (70C) and hsc2 (87D), are not abundant, transcripts from hsc4 (88E) are abundant in embryos, larvae, and adults (Figure 6). In adults the abundance of the hsc4 transcripts is close to that of the 87E actin gene transcripts, a major adult actin mRNA.[149] RNA hybridization selection and translation experiments have demonstrated that RNA homologous to hsc4 encodes a protein of 70 kd with a similar, though distinguishable, electrophoretic mobility from hsp70.

The isolation of monoclonal antibodies which cross react with hsp70 and cognates has allowed the identification of cognates on two-dimensional gels.[150,151] Two abundant cognate proteins have been identified in this manner. One (hsc70) with a very slightly slower mobility than hsp70 in SDS-polyacrylamide has a pI of 5.75 compared to 6.1 of hsp70 and 6.3 of hsp68. A second (hsc72) is slower in mobility and has a more acidic pI of 5.6. Although the incorporation of ^{35}S-methionine into both appears to decrease after heat shock, the decrease is less than that seen with many other proteins. The continued synthesis of hsc72 is particularly apparent. Both cognates are found in protein preparations from different developmental stages, although the exact abundance in stages or tissues may vary. For example, hsc70 is more abundant in the ovary than in other tissues of the adult.

At this time, it is not known which protein is encoded by which cognate gene. However, because of the abundance, it is likely that hsc4 encodes one of the two identified proteins. Probably at least one cognate gene remains to be isolated, since neither of the other two isolated cognates, hsc1 or hsc2, is transcribed in the appropriate abundance or in a developmental timetable compatible with expression of the identified proteins. The abundance of hsc1 transcripts in adults is approximately 30 times less than that of hsc4, while hsc2 transcripts are approximately 2 to 3 times less abundant than those of hsc1. Hsc1 and hsc2 transcripts were not detected in larval RNA preparations, and only very low amounts, at least 10 times less than in adults, of hsc1 were detected in embryos (Figure 6).

In earlier studies, it was shown through hybridization-arrested translation and mRNA hybridization-selection experiments that cloned hsp70 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.[19] More recently, [152] it has been reported that at least two of these related proteins are encoded by genes residing outside of the region encoding hsp70. Embryos homozygous for a deficiency in the interval 87A7-9 to 87D12-13 still synthesize these proteins, but not hsp70.

Because of the identification of cognate proteins on two-dimensional gels, it has been possible to compare the amount of cognate in cells to the amount of hsp70 and hsp68 after a heat shock. During either a prolonged heat shock or an hour treatment at 37°C followed by recovery at 23°C, the amount of hsp70 and hsp68 accumulating

	Control	Hours at 37°C						Hours of Recovery at 23°C after 1 hr at 37°C		
	23°C	1	2	4	8	12	24	2	6	12
hsp70	-	++	+++	+++	+++	+++	+++	++	+/-	+/--
hsp68	-	+/-	+	++	+++	+++	+++	++	+++	+++
hsc70	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
hsc72	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

FIGURE 7. The abundance of *Drosophila* heat shock and cognate proteins during heat shock and recovery. The abundance of heat shock and cognate proteins was estimated from two-dimensional gels stained with Coomassie Blue. Protein samples were obtained from adult males grown at 23°C and shifted to 37°C for various lengths of time or shifted to 37°C for 1 hr and then returned to 23°C. − indicates no protein spot visible. +/−− indicates a spot which is barely visible. The steady-state level of hsp70 during prolonged heat shock at 31°C is about 60% of the level of hsc70.[150]

```
                                    eucaryotic consensus C/A AGGT A/G AGT
                  aa56
                   Val Ala Met Asn Pro Asn Asn Thr Ile Phe
Drosophila Hsc70-1 CTG GCC ATG AAT CCC AAC AAC ACG ATC TTTGgtgagtctcgttcgcgca--(~1700b)
Maize              G.C ... ... ..C ... .C. ... ..G G.. ..C...ac.cgctcactt...c--(~678b)
                    *   *   *   *   *  Thr  •   *  Val  •
                   aa61

          eucaryotic consensus   T/C N C/T AGG

                                    Asp Ala Lys Arg Leu Ile Gly Arg Arg Phe Asp
Drosophila Hsc70-1 ---agtgattctgacttgcagAT  GCC AAG CGG CTG ATT GGC CGC CGT TTC GAC
Maize              ---tcattcat..taa.....  ... ... ... T.. ..C ... A.G A.G ... TCT
                                          *   *   *   *   *   *   *   *   *   *  Ser
```

FIGURE 8. Comparison of the nucleotide and proposed amino acid sequence surrounding the intervening sequence of a maize and a *Drosophila* hsp70-related gene. A dot represents a base in the maize gene which is identical to the *Drosophila* gene. The proposed amino acid sequence of the *Drosophila* protein is displayed above the *Drosophila* nucleotide sequence. The proposed maize sequence is displayed below the maize nucleotide sequence. An asterisk represents an amino acid in the maize protein identical to the *Drosophila*. The eukaryotic consensus sequence found at splice junctions is taken from Mount.[143] The *Drosophila* and maize sequences are from Ingolia and Craig[148] and Shah et al.,[498] respectively.

in cells of the fly does not equal that of the normally present related protein (Figure 7). Therefore, although the amount of the inducible proteins in cells after stress is substantial, becoming one of the most abundant proteins of the cell, less than a doubling of the total amount of hsp70-related protein in the cell occurs.[150]

In contrast to hsp70 genes, intervening sequences are found in the genes encoding the cognates. Hsc1 and hsc2 are interrupted in the codons for aa66 and aa55, respectively, while hsc4 contains an intervening sequence in the 5′ noncoding region.[145,153] The sequences at the 5′ and 3′ junctions of the insertions are similar to those found in intervening sequences of *Drosophila* and of many other organisms. Recently, a gene has been isolated from maize which is related to members of the *Drosophila* hsp70 family and contains an insertion at exactly the position found in hsc1[49] (Figure 8). The maize gene also contains the typical consensus sequence at the insertion site. The origin of these identically placed intervening sequences in evolution is an interesting point for speculation.

2. *Saccharomyces cerevisiae*

S. cerevisiae contains a multigene family of hsp70 and hsp70-related genes. Eight of

the genes have been isolated as recombinant clones.[34,153] Two of these (YG100 and YG102) hybridize to RNA which is more abundant after a 30-min heat shock than before. These two genes are 97% identical in the protein coding region, but promoter fusion studies indicate that they are not regulated identically. YG100 is normally expressed at a relatively low level; however, like *dna*K (see Section IV. B.3), the amount of RNA present increases with increasing growth temperature. YG102 is transcribed substantially at 30°C, the optimal growth temperature for yeast. At this temperature, its transcripts are 5 to 10 times more abundant than those of YG100.[35] Yeast strains have been constructed in which the wild-type YG100 and YG102 genes have been replaced by in vitro constructed mutations.[137] Strains lacking either a normal YG100 or a YG102 are not distinguishable from wild-type strains; the strains grow at the same rate at diverse growth temperatures, are able to mate and sporulate, and are no more sensitive to a semilethal heat shock than the wild-type strain. However, a strain containing both the YG100 and YG102 mutations grows at a reduced rate at 30°C with a doubling time of 4 1/2 hr compared to the 2-hr doubling time of the wild type. At 37°C, a temperature at which wild-type yeast grow with a doubling time of 2 1/2 hr, the double mutant is not capable of colony formation or sustained growth in liquid after a gradual transfer from 30 to 37°C. These genetic studies suggest that this hsp70 protein is required for growth at higher growth temperature and is necessary to achieve wild-type growth rates even at an optimal growth temperature (30°C).

Hybridization experiments indicate that four other genes are expressed during normal growth, but that this expression is not enhanced after a heat shock. Whether the proteins encoded by these four genes perform the same or different functions than the YG100 and YG102 gene products remains to be determined. However, mutations of two of these cognate genes have been constructed in vitro and used to replace the wild-type gene as described above.[134] These two genes, YG101 and YG103, are approximately 94% identical at the nucleotide level. Transcripts homologous to these two genes decrease in abundance after heat shock. Cells containing mutations in either YG101 or YG103 are indistinguishable from wild type. However, cells containing both a YG101 and YG103 mutation have a slow growth phenotype. Unlike wild-type cells which grow best at 30°C, the double mutant grows fastest at 37°C. As the temperature is lowered, the rate of growth decreases. At 23°C the doubling time is 2.6 times that of wild type, while at 37°C it is 1.2 times that of wild type. Although a growth difference is seen at all temperatures leading to the conclusion that the gene products play a role in growth at all temperatures, these hscs play a greater role at low temperatures.

The existence of hsp70-related proteins under normal conditions in cells of other species is indicated. For instance, polyclonal antibodies to the chicken hsp70 react strongly with proteins from chicken and mammalian cells grown under nonstress conditions.[12] Hsp70 or similar proteins have been detected in both *Tetrahymena* and *Dictyostelium* during normal growth.[124,154] Hsp70 has also been reported to be the first major products of zygotic gene activity in mouse embryo, occurring at the two-cell stage.[155] In this report, however, it was not considered whether the manipulations of the embryo prior to labeling might have stressed the cells, thus resulting in an induction of hsps.

3. dnaK

*dna*K protein is the most thoroughly studied hsp70 or hsp70-related protein. It has been previously identified as the B66.0 protein,[156] an abundant protein under normal growth conditions, whose abundance increases relative to other proteins with increasing temperature and growth rate. For example, if the amount of *dna*K relative to cells grown at 37°C is designated 1.0, the relative level of *dna*K at 23°C, 30°C, 42°C, and 46°C is 0.63, 0.78, 1.42, and 3.06, respectively.[157] The relative amount of *dna*K in cells

growing at different rates has also been compared. Cells were grown in minimal media with acetate, glycerol, or glucose and rich media with glucose, with increasing growth rates, respectively. If the amount in minimal glucose media is considered 1, the amount in minimal-acetate, minimal-glycerol, and rich-glucose media is 0.67, 0.89, and 1.51, respectively.[158] Therefore, *dnaK* is an abundant protein under diverse growth conditions and temperatures; in minimal glucose medium and rich medium *dnaK* makes up 0.93 and 1.4% of the weight fraction of the cellular protein, respectively.

C. Small HSPS

A number of experiments have clearly demonstrated that the small heat shock genes are transcribed at times during the development of *Drosophila melanogaster*, although a comprehensive study of expression has not been made. The initial observation identified transcripts of hsp26 and hsp22 during late larval and early pupal stages.[159] Another study has detected hsp23 synthesis during the late third instar, coincident with the rise in ecdysterone titers.[160] The synthesis of the four small hsps has been found to be induced in response to the addition of ecdysterone in some tissue culture lines and in isolated imaginal discs.[161,162] Hsp26 and hsp27 mRNA are found in the developing oocyte; these RNAs are synthesized in the nurse cells and transported into the oocyte.[140] The translation products of some of the small heat shock genes are present in 0 to 2-hr embryos, since monoclonal antibodies which cross react to the four proteins were produced after using protein from early embryos as an immunogen.[141] Therefore, the four small heat shock genes are under complex transcriptional regulation, being under developmental control as well as responsive to environmental conditions.

V. REGULATION OF THE HEAT SHOCK RESPONSE

A wide variety of inducers of hsp synthesis has been identified in a number of species. It is reasonable to suppose that the inducers act via a common mechanism. Although this mechanism has not been defined, features of the induction common to diverse species have been identified. In vitro transcription data and chromatin studies suggest that induction of the *Drosophila* gene is under positive control. A mutation of a putative regulatory gene in *E. coli* results in the loss of inducibility of a set of 17 hsps, a finding which also suggests positive regulation. Consensus sequences necessary for heat inducibility and a role of hsps in the regulation of their own synthesis have been proposed in both *Drosophila* and *E. coli*.

A. Induction

By definition, heat shock is the one universal stimulus which induces synthesis of hsps. Many other stimuli, such as recovery from anoxia, arsenate, ethanol, and other chemicals induce the synthesis of either all of the hsps or a subset of them in some species. However, differences in the response of organisms to a number of inducers have been noted. For example, ethanol has been shown to induce hsps in yeast,[128] Chinese hamster fibroblasts,[129] and *E. coli*,[163] but induction has not been observed in *Drosophila* or HeLa cells. An extensive list of inducers in a number of species has been published.[67,164] I will not attempt in this review to make a comprehensive list of all inducers for all systems. Instead, various treatments which induce or have an effect on the synthesis of a subset of the heat-inducible proteins and a possible commonality of action among different inducers will be discussed.

1. Heat and Chemicals as Inducers during Development

Heat shock genes are not susceptible to induction by heat in cells of all tissues or all developmental stages. For example, prior to the blastoderm stage of *Drosophila* early

embryonic development, hsps are not induced by heat treatment.[165,166] A similar lack of induction in early embryonic stages of sea urchin development has also been observed,[167] and hsp synthesis was not detected in pollen tubes.[168] A quantitative analysis of the inducibility of *Xenopus* hsp70 and hsp30 RNAs in development has been reported.[61] Large amounts of translationally inactive hsp70, but not hsp30 transcripts, are present in oocytes (Reference 170, see Section V.E.3). However, no transcripts of the heat shock genes were detected in the early stages of development, even after heat shock, indicating that the hsp70 RNAs present in oocytes were degraded. The first detectable heat-inducible RNAs are hsp70 transcripts found in the embryos of the late blastula stage at the level of about 1000 copies per nucleus. As noted in the report, it would be difficult to detect inducible transcripts at an earlier stage, due to the level of sensitivity of the RNA assay and the high cytoplasm/nuclei ratio. However, protein-labeling experiments also indicate lack of induction of hsps in the early developmental stages. Gastrula cells, which are clearly capable of transcribing hsp70 genes after heat shock, do not accumulate hsp30 transcripts. In the developmental analysis, hsp30 transcripts were not detected until the tadpole stage. Also, whereas all adult tissues seemed to express the same level of hsp70 mRNAs, the level of hsp30 transcripts varied . In kidney and gut, hsp30 transcripts were found to be about threefold lower than those of hsp70, whereas in all other tissues examined they are about 30-fold lower.

Some differences in inducibility during development have also been noted in mammalian and avian systems. For example, hsp70 synthesis cannot be induced in the brain of the rat until 3 weeks postpartum, while other organs tested showed no difference in expression during postpartum development.[171] Also, no small hsp was induced in 11-day-old quail embryos, whereas high amounts were induced in breast and limb bud tissue. Interestingly, 11-day-old chick embryos also do not synthesize the small hsp24. However, another protein, hsp50, not found in other tissues is synthesized. This 50K protein reacts with antibody directed against hsp24, suggesting that an hsp24-related protein is being made in response to heat shock.[67] As with the *Xenopus* study, small hsp synthesis in different tissues of avian and mammalian cells seems to be more variable than that of the larger hsps. In an analysis of hsps in tissues of the rat, it was found that the 25 to 30-Kd hsps, induced in myogenic cells and fibroblasts, were not found in hepatoma or pituitary cells.[172]

In the cases described above, the hsp usually lacking in the group of induced proteins was the small hsp. In *Drosophila*, an example of the induction of a subset of the small hsps, independent of the increased synthesis of the others, has been studied.[132] Drugs which inhibit the differentation of embryonic cells put into culture, such as coumarin, diphenylhydantoin, and pentobarbital, induce the synthesis of hsp22 and hsp23, but not hsp26, hsp27, hsp68, or hsp70. Even though these cells were shown to be capable of synthesizing the full complement of hsps in response to heat, induction with this subset of chemicals was restricted to these two proteins, which have not been induced as a pair under other conditions, either during development or in response to other stresses.

2. Glucose Regulation and Heat Shock

When mammalian cells are incubated in the absence of glucose, the rate of synthesis of several proteins is altered. Among this group are four hsps. Synthesis of two of the "major" hsps, hsp70 and hsp90 which are related to the abundant *Drosophila* hsps, hsp70 and hsp83, respectively, is diminished upon glucose deprivation in mammalian as well as avian cells.[173] However, synthesis of two "minor" hsps, hsp80 and hsp100, is greatly enhanced after glucose deprivation. These have been shown to be the previously identified "glucose regulated proteins". Calcium ionophores also induce the synthesis of these two hsps.[86,174]

3. Virus Infection as an Inducer

Infection of cells with a variety of viruses results in the increase in synthesis of at least a subset of the hsps. This induction has been observed after infection of chick cells with New Castle disease virus,[175] Sindbis, and vesicular stomatitis virus.[106] Simian cells infected with SV40, murine cells infected with polyoma virus,[176] human cells infected with adenovirus,[177] and *E. coli* infected with λ phage[178] show enhanced synthesis of hsps. The effect of viral infection upon hsp synthesis has been studied in greatest detail in the adenovirus-infected cell. The abundance of hsp70 RNA begins to increase within 5 hr after infection and by 8 hr after infection is 100 times more abundant than before infection.[179] After 8 hr, both the rate of transcription as measured in isolated nuclei and the amount of RNA present in the cytoplasm decrease dramatically. By 12 hr, the level of mRNA has decreased to levels present prior to infection. Therefore, the hsp70 RNA is quite unstable, with a half-life of approximately 40 min. Through the analysis of mutants it has been shown that the induction is dependent upon the presence of the adenovirus E1A gene product, which is required for expression of all other early adenovirus genes. This requirement for wild-type E1A gene product for early transcription can be overcome by using a high multiplicity of infection. In these cases, early adenovirus mRNA occurs, but hsp70 transcription does not. Because of this result and the fact that early transcription can occur if host protein synthesis had been inhibited prior to infection, it has been proposed that the role of the E1A gene product is to inactivate a cellular factor that inhibits transcription from the early viral gene promoters. The role of E1A in the induction of hsp70 synthesis is not clear. However, here is a case where the presence of a single gene product results in the induction of hsp synthesis and is, therefore, amenable to direct analysis, which is not possible with other inducers discussed above.

4. Possible Mechanisms of Induction

An important question is whether all the inducers of the hsps act via the same mechanism, that is via some common signal. Since the inducers affect the transcription of the same genes, it is logical that a common mechanism is involved. In the discussion above, differences in induction patterns were emphasized. However, in an attempt to propose a simple unifying scheme, it is possible to accommodate a number of the observations into a general model. For example, suppose the binding of a particular factor to specific sequences results in the enhanced transcription of the heat shock genes (see following section). This "factor" needed for induction of transcription of all the genes could be activated by a metabolite whose concentration is altered after cells interact with a variety of stresses. The affinity of this altered factor for regulatory regions of inducible genes could be different. In several cases described, hsp70 synthesis was observed at times when small hsps were not. If hsp70 had a higher affinity for the activated factor than the other hsps, and the concentration of activated factor was limiting, then the genes would be competing for the factor, and induction of hsp70, but not the other hsps, might occur. An additional assumption needs to be included in this type of model to allow for the differences in hsp synthesis found among different cell types and developmental stages. For instance, differences in the amount of a metabolite produced in different cell types in response to the same level of stimulus could result in differential expression. Similarly, factors with differing affinities for the genes could be present in various cell types and be responsible for tissue specific variations. Whether the expression of some heat shock genes during normal development (as discussed briefly above, and in more detail in Section IV) is regulated by a mechanism related to that used after heat shock is not known.

Although it is reasonable to suppose that inducers affect the action of the same regulatory factor(s), the identity of such a common signal is not known. Recently, several small molecules have been identified in *Salmonella typhimurium* and *E. coli*

which accumulate after a heat shock.[180] The five adenylated nucleotides (AppppA, AppppG, ApppA, ApppG, and ApppGpp) also accumulate after addition of ethanol, a treatment which induces the synthesis of hsps in these bacteria as well. The intracellular concentration of the nucleotides increase five- to tenfold within 5 min after temperature shift, and continue to increase for at least an additional 15 min. These adenylated nucleotides are synthesized in vitro and, presumably, in vivo by a side reaction of aminoacyl-tRNA synthetases that results in the adenylation of ATP, ppGpp, GTP, GDP, and ADP.[170] An *htp*R⁻ mutant (see Section V.D.2), which is unable to induce synthesis of hsps, still accumulates adenylated nucleotides, indicating that the accumulation of the nucleotides is not due to the synthesis of the hsps. Lee et al. [180] propose that one or more of these adenylated nucleotides are responsible for the induction of the hsps. Although a five- to tenfold increase is observed in 5 to 10 min after heat shock or ethanol treatment, the increase in the first minute was not reported. The increase in transcription after heat shift is observed within a minute and transcription has reached a maximum level and begun to decrease within 5 min of the temperature shift. However, it is intriguing that one of the identified *E. coli* hsps is a lysyl-tRNA synthetase.

Hsps are induced by a wide variety of agents which do not have an obvious common effect. However, a change in oxidation is an effect of a number of inducers.[164,181] For example, hydrogen peroxide, menadione and adriamycin — oxidizing quinones, diamide — a glutathione depleter, and the sulfhydryl reagents, iodoacetamide and cadmium, have been shown to induce hsps in eukaryotes; these oxidizing agents have also been shown to induce the accumulation of adenylated nucleotides. Exposure to oxygen after anoxia is also both an oxidative stress and an inducer of hsps in *Drosophila* and yeast. How cell oxidation by heat or ethanol, two of the most "universal" inducers, might occur is not clear. It has been proposed that these inducers may cause damage to the membrane, resulting in interference with electron transport. A number of inducers are known to disrupt electron transport. Obtaining proof that the inducers act via a common mechanism is one of the major problems confronting workers in the heat shock field. Such proof may well depend on a defined and regulated in vitro transcription system as well as physiological experiments.

B. DNA Sequences and Proteins Involved in Induction

1. Drosophila Genes

Initial studies of the sequences necessary for inducibility of the heat shock genes were performed by introduction of the genes into cells of other species. The results of these experiments showed that the mechanism of regulation of heat shock genes is similar in widely divergent species. *Drosophila* hsp70 genes were integrated into the mouse genome by cotransformation with the Herpes thymidine kinase gene,[182] introduced into monkey COS cells by transfection using the multicopy SV40-derived vectors[183,184] and introduced into *Xenopus* oocytes by injection.[185,186] In the cells of each of these three species, the *Drosophila* genes were inducible by heat shock, under the conditions normally used to induce the response in cells of the host. In one case[183] sodium arsenite was also shown to induce *Drosophila* hsp70 transcription in the heterologous system. The sequences necessary for this induction were more precisely defined by analysis of a series of deletions of the 5′ flanking region, linked to the Herpes thymidine kinase structural gene. This type of deletion, again analyzed in cells of the three species, indicated that the sequences necessary for induction were present in the 66-bp upstream from the cap site, since deletions of 5′ sequences to −66 or −68 (+1 being the start of the mRNA) maintained inducibility, while deletions to −44 did not.[183,186]

A comparison of sequences upstream of *Drosophila* hsp genes had revealed a se-

quence with dyad symmetry,[6,187] and it was suggested that these sequences which bore some homology might be responsible for rendering the hsps heat inducible.[186] The idea that this consensus sequence (5'CTNGAANNTTCNAG) is sufficient for heat inducibility was confirmed. The upstream regulatory elements of the thymidine kinase (tk) gene were replaced by synthetic oligonucleotide containing eight out of ten or ten out of ten matches with the consensus sequence. These tk genes were rendered heat inducible when placed in either *Xenopus* oocytes or COS cells.[186] Similar synthetic fragments, placed 5' of the major late adenovirus promoter, also caused transcription to be heat inducible.[188] Interestingly, a member of a heat-inducible repetitive family of *Dictyostelium* which has been shown to be inducible in yeast contains a very similar consensus sequence (14 matches out of 15) in the 391-b control region. Further confirmation of the idea that this sequence is important in the regulation of heat shock genes comes from the results of experiments in which fusions of the four small heat shock promoters were introduced into COS cells. Those genes which possess the sequences more homologous to the consensus sequence, hsp22 and hsp26, are induced to a higher level than those with a poorer match, such as hsp27 or those whose consensus sequence is displaced upstream from the promoter, such as hsp23 (Figure 9). However, since hsp23 and hsp27 are induced to high levels in *Drosophila* cells, details of the significance of sequence specificity await analysis of these sequences in the homologous *Drosophila* system. Such experiments are now possible because of the development of the P-factor transformation system.[189]

The idea that the induction of transcription of hsps involves the interaction of a regulatory protein with the consensus sequence defined above is supported by several experiments. Recently, an in vitro RNA polymerase II transcription system from *Drosophila* tissue culture cells has been developed.[190] A factor necessary for hsp70 transcription has been identified. Footprint analysis of this factor on the hsp70 gene shows binding to a 55-bp region upstream from the TATA box, which includes the consensus sequence necessary for heat inducibility of the hsp70 gene.[191]

DNase hypersensitive sites have been found in a broad region around the 5' end of hsp70 genes.[192] More precise analysis revealed that the sites in chromatin sensitive to DNaseI extend from positions +100 to −8 and from −38 to −215, with a peak at −93 (with +1 being the 5' end of the mRNA). Therefore, there is a relatively resistant 30-bp region between positions −8 and −38. Recently, a method which allows mapping of DNA sequences within nuclease-hypersensitive sites in chromatin that are protected from exhaustive exonuclease III digestion *in situ* has been developed.[193] In this method, isolated nuclei are treated lightly with DNaseI so that, on the average, a cleavage occurs every 15 kb. This creates a free end from which exonuclease III degrades 3' to 5' until a specific block is encountered (such as a protein bound to the DNA). The DNA is then purified, the single-stranded ends trimmed with S1 nuclease, and the DNA analyzed as described previously.[192] In the *Drosophila* hsp70 and hsp83 genes, 22 and 28 bp of DNA surrounding the TATA box are protected prior to induction. After induction, an additional 36 and 68 bp, respectively, are protected from digestion. The additional region protected after heat shock contains the consensus sequence described above. These results are consistent with the idea that prior to induction a protein is bound to the region encompassing the TATA box, and that after induction proteins are bound to both the TATA regions and the consensus sequence. These results are in keeping with the notion that the regulatory protein involved in the induction of hsps is a positive activator of transcription, since the removal of a repressor would have resulted in loss of a protected region after induction. The chromatin studies are also consistent with the in vitro transcription data and the idea that the consensus sequence is important in transcriptional activation.

	NUMBER OF MATCHES TO CONSENSUS	FOLD INDUCTION
CONSENSUS: CT GAA TTC AG		
Hsp83 ** **_ *** **		
CCTCTAGAAGTTTCTAGAGACTTCCAGTTCGGTCGGGTTTTTCTATAAA	9/10	N.D.
Hsp70 ** *** *** _*		
CGAGAGAGCGCGCCTCGAATGTTCGCGAAAAGAGCGCCGGAGTATAAA	9/10	20-50
Hsp68 ** *** *** __		
CTCGCAGGGAAATCTCGAATTTTCCCCTCCCGGCGACAGAGTATAAA	8/10	N.D.
Hsp27 _* __* __* **		
GTTCCGTCCCTGGTTGCCATGCACTAGTGTGTGTGAGCCCAGCGTCAGTATAAAA	5/10	10
Hsp26 *_ **_ *** **		
TTTCTGTCACTTTCCGGACTCTTCTAGAAAAGCTCCAGCGGGTATAAAA	8/10	20-50
Hsp23 *_ *** *** _*		
GATATTTTCAGCCCGAGAAGTTTCGTGTCCCTTCTCGATGTTTGTGCCCCCTAG	8/10	1
CACACAGACACGACGCGCACACACACAGCGCCGACGGGCGCACGCACACTTCGA		
CAGCAAGCGGTTGTATAAA		
Hsp22 *_ *_* *** **		
ATTCGAGAGAGTGCCGGTATTTTCTAGATTATATGGATTTCCTCTCTGTCAAGAGTATAAA	8/10	20-50

FIGURE 9. Comparison of the upstream regions of the *Drosophila* heat shock genes. An asterisk indicates the same nucleotide is present as in the consensus sequence described by Pelham[185] which is displayed above the sequences. The number of matches between this consensus sequence and the gene is shown at the right. The fold induction of transcripts from these promoters linked to the Herpes thymidine kinase structural gene in COS cells is given. (Adapted from Pelham, H. R. B. and Lewis, M., *UCLA Symp. Mol. Cell. Biol.*, 8.)

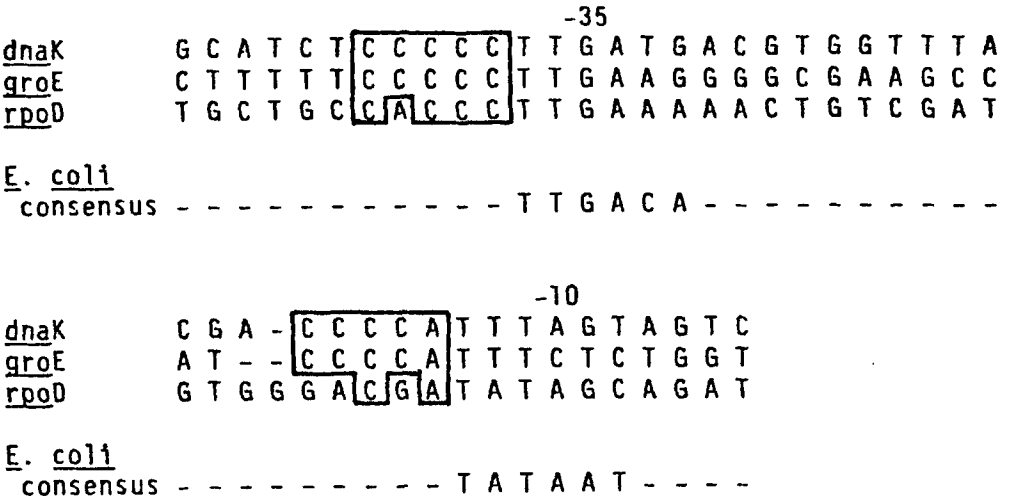

FIGURE 10. Comparison of the promoters of *E. coli* heat shock genes. The 6 bp consensus sequences for the −10 and −35 regions are shown below the heat shock gene sequences.[197] The sequences are from Taylor et al.,[196] *rpo*D; Cowing et al.,[194] *gro*E and *dna*K.

2. E. coli

The transcription of several *E. coli* heat shock genes has been studied; the DNA sequences surrounding the 5′ end have been determined and fusions of promoter-containing fragments to unrelated genes constructed. Genes analyzed to date include those encoding *dna*K,[194] *gro*E,[194] and *rpo*D, the gene encoding the sigma subunit of RNA polymerase.[196]

The regulation of the gene encoding sigma has been studied most extensively. It is cotranscribed with *rps*U and *dna*G, encoding ribosomal protein S21 and DNA primase, respectively. After a temperature upshift, a promoter located within the *dna*G is transiently induced, causing increased transcription of *rpo*D. The initiation site of this promoter has been located 362 bp upstream from the protein coding region of *rpo*D by promoter cloning and S1 nuclease mapping. Analysis of deletions of the promoter region showed that a sequence CTGCCACCC in the −44 to −36 region of this promoter is necessary for the heat inducibility. Transcription from this internal promoter is sufficient to explain the heat inducibility of sigma synthesis.

Transcription of *dna*K has also been studied by promoter cloning and S1 mapping.[194] 5′ Ends, at −115, −43, and −20 from the ATG, have been identified. The longest transcript is about fourfold more abundant than the shorter transcripts, but all increase in abundance after heat shock. Results of promoter fusion experiments suggest that at least the transcripts mapping to positions −115 and −43 initiate at these points. Comparison of the sequences near the start sites of these two genes and *gro*E has led to proposed consensus sequences for heat-inducible promoters in *E. coli* (Figure 10). Unlike promoters which are not inducible by heat or ethanol, of which at least 92 have been sequenced, inducible promoters analyzed tend to have a series of Cs followed by an A prior to the −10 sequence, as well as a series of C immediately adjacent to the −35 sequence. The later run of Cs is extremely unusual, found in none of the promoters previously analyzed,[197] and is included in the sequence described above which is adjacent to the −35 sequence of the *rpo*D heat-inducible promoter and necessary for induction.

A gene of *E. coli* has been identified, whose product is necessary for induction of 17 proteins after heat shock. A nonsense mutation in a strain (K165) carrying a tRNA that suppresses amber mutations at low temperature (28°C) but not high (42°C) tem-

perature eliminates the response of the inducible genes to a temperature increase[198,199] or ethanol.[163] The mutation is in a gene located at about 75 min on the *E. coli* chromosome and designated *htp*R (for *h*igh *t*emperature *p*roduction *r*egulating) or *hin* (for *h*eat-shock *in*ducible) by two groups studying the mutant originally isolated and analyzed by other investigators.[200] At high temperatures (42°C) mutant cells not only fail to induce the 17 proteins of the *htp*R regulon, but also lyse after about 1 hr at 42°C. A plasmid containing a segment of the chromosome around 75 min complements all of the phenotypic characteristics of the mutant strain K165. In minicells this plasmid makes a protein of 34K dalton (termed F33.4).[83]

The role of *htp*R protein product in regulation of synthesis of the hsps is not clear. It is tempting to speculate that *htp*R protein interacts directly with the inducible genes and is the positive effector responsible for enhanced transcription. This notion is supported by the results of a set of experiments which measured *gro*EL protein levels in strains containing suppressors of different efficiencies and the amber *htp*R-165 mutation.[198] Both the rate of induction and the maximum level of protein obtained at 42°C are proportional to the level of *htp*R gene expression. The maximal induction is about tenfold the rate at 30°C for the htpR$^+$ strain, and it is sevenfold, fourfold, or less than twofold for the strains with suppressor activities of approximately 100, 50, or 20%. Recent evidence indicates that htpR protein is a sigma factor for heat shock promoters.[193] This is consistent with the apparent homology between sigma factor and htpR proteins.[195a]

Strains containing the *htp*R mutation are defective in degrading normally unstable proteins.[201] The defect in proteolysis which was observed at 30°C as well as after shift to high temperature was unambiguously mapped to the *htp*R locus. Introduction of the more efficient nontemperature-sensitive nonsense suppression restores both normal heat shock and proteolysis. These results indicate that the proteolysis phenotype of *htp*R$^-$ strains results from decreased levels of the *htp*R product. It is not clear at present whether or not the decrease in proteolytic activity is a direct or indirect result of the *htp*R mutation. However, it is intriguing that protease involvement has been found in a number of cases of gene regulation of *E. coli*, including the induction of the SOS response to DNA damage.[202]

The phenotype of the *htp*R-165 mutation is not straightforward. One question that remains is whether *htp*R protein is involved in regulation of transcription at all temperatures. The abundance of a number of hsps, including *dna*K and *gro*EL proteins, increases with increasing growth temperature. Study of additional *htp*R mutations may be useful in determining the role of *htp*R protein during logarithmic growth, as well as after a temperature shift. As discussed above, the effect of the *htp*R mutation suggests that the product of that gene may be a positive regulatory protein required for heat induction. This hypothesis of positive regulation is consistent with the data of a different nature obtained studying the *Drosophila* system. It will be of interest in the future to compare the mechanism of induction of heat shock RNA synthesis in prokaryotes and eukaryotes.

C. Turn Off of the Response

1. Repression of RNA Transcription

In all systems examined, the synthesis of hsps is repressed and normal protein synthesis returns after a shift back to normal temperatures following a moderate heat shock. A set of experiments showing a positive correlation between the production of hsp70 and the degree of stress led to the proposal that hsp70 is involved in regulating its own synthesis.[203] When the production of normal hsps was disrupted by addition of cycloheximide or amino acid analogs, transcription of the hsp genes continued after

the removal of the inhibitor or analog. These experiments strongly suggested that synthesis of a protein is necessary for repression of transcription; because of the positive correlation observed between the accumulation of hsp70 and repression of its synthesis, the most obvious candidate for the protein responsible for repression is hsp70 itself.

The strongest case for a role of hsp70 in regulating its own synthesis comes from studies of mutants of the *E. coli dnaK* gene.[204,205] Cells carrying *dnaK*756, a ts mutation of the *dnaK* protein, fail to turn off the heat shock response for several hours after a shift from 30 to 43°C, whereas in wild-type cells, repression begins a few minutes after shift. It has been shown that the prolonged heat shock response is due to a mutation in *dnaK*. The *dnaK*$^-$ phenotype cotransduces with the failure to shut off heat shock protein synthesis, and ts$^+$ revertants of *dnaK*756 bacteria regain the ability to turn off the response. Strains which produce large amounts of *dnaK* proteins due to the presence of the *dnaK* gene on an overproducing plasmid fail to mount a full heat shock response at 43°C. Both these results are consistent with the idea that the *dnaK* protein is a negative regulator of the synthesis of hsps. If an excess of *dnaK* protein is present, little increase in synthesis of *dnaK*, *groEL*, or *groES* occurs. If no functional *dnaK* is present, as in the case of the *dnaK*756 at 43°C, normal repression of these hsps does not occur.

The failure of *dnaK*756 bacteria to turn off the heat shock response can be explained by two general types of models.[204] First, the *dnaK* protein might affect the activity of the *htpR* regulatory protein either directly or indirectly, inhibiting its action. Second, the *dnaK* protein might be a direct inhibitor of hsp synthesis, while *htpR* protein acts in a positive fashion by interfering with the repressive action of the *dnaK* protein. The two possibilities can be distinguished by determining the phenotype of an *htpR*$^-$*dnaK*$^-$ double mutant. If model one were correct, then the double mutant should not induce hsps. If the second is correct, the double mutant should overproduce hsps. The phenotype of the double mutant is the same as that of the *htpR*$^-$ single mutant[204] showing a lack of a heat shock response, suggesting that *dnaK* interferes with *htpR* protein activity. The manner in which *dnaK* modulates *htpR* activity is not known. The effect could be either indirect or direct. For instance, *dnaK* could directly affect *htpR* activity or it could affect the activity or concentration of another, yet unknown, molecule which is an antagonist of *htpR*.

2. HSP RNA and Protein Stability

The repression of hsp synthesis observed after return to normal temperatures appears to be caused by the destabilization of hsp RNA as well as the turn-off of hsp RNA synthesis, as described above. In *Drosophila*, hsp70 RNA disappears with a half-life of 1 hr or less after return to normal temperature.[150,203] If actinomycin D is added, thus, limiting the amount of RNA available for translation, the lifetime of the mRNA is extended, indicating that the stability of the RNA is affected by the amount of protein synthesized. This type of regulation of RNA stability, that is, destabilization of RNA after shut down of transcription, has been observed in a number of cases unrelated to heat shock. Aggregation-induced RNAs of *Dictyostelium* are stable during induction; however, when transcription declines these induced RNAs are selectively degraded.[206] Histone mRNAs in both yeast and human cells appear to be destabilized at particular times during the cell cycle when histone synthesis does not occur.[207,208]

An interesting example of regulation of heat shock mRNA stability occurs in adenovirus-infected and transformed cells.[179] In early adenovirus-infected cells where transient expression of hsps occurs, hsp70 RNA is degraded with a half-life of 40 min or less. In transformed cells where hsps are continuously expressed, the half-life of hsp70 mRNA is 4 to 5 hr. In lytically infected cells early adenovirus mRNAs are unstable

with a half-life of about 40 min. The early viral mRNAs synthesized in transformed cells are much more stable with a half-life of several hours. The rapid decay of the early viral mRNA in lytically infected cells is due to the action of an early viral gene product, the 72-kd DNA binding protein.[209] Whether the 72K viral protein affects heat shock mRNA stability or whether some other mechanism is operating is not known.

An important aspect of the heat shock response, perhaps the most important when considering the function of the hsps, is the accumulation and stability of the proteins themselves. Experiments concerning the stability of hsp70 carried out in different laboratories are not in complete agreement. The intensity of immunofluorescence staining of salivary glands with hsp70 specific antibody 17 hr after a shift down from a 1-hr heat shock was found to be nearly equal to that found immediately after heat shock.[113] This result suggests that hsp70 is very stable during recovery from heat shock. An analysis of the accumulation and stability of unlabeled hsp68 and hsp70 in *Drosophila* larvae and adults after a 1-hr heat shock at 37°C and return to 25°C has been carried out on two-dimensional gels by other investigators.[150] In these studies, hsp70 accumulated rapidly reaching a peak within 1 hr after return to the lower temperature (Figure 7). By 4 hr, the amount of hsp70 has significantly decreased in abundance and by 12 hr was at preheat shock levels, indicating a half-life of 1 to 2 hr. Accumulation of hsp68 had kinetics different from those of hsp70; its accumulation was more gradual, taking several hours of recovery to reach the level of hsp70 present 1 hr after heat shock. Therefore, although hsp70 is unstable in these studies, decreasing rapidly in amount after return to normal temperature, the increase in amount of a related protein, hsp68, results in the abundant presence of an induced hsp70-related protein from shortly after heat shock until at least 12 hr after return to 25°C. Similar results were also observed when the experiments were performed with larvae, and essentially the same conclusions about the accumulation and stability of hsp70 and hsp68 are drawn from labeling experiments performed with larvae.[210]

A similar analysis of accumulation of hsp70 in a number of organs of the rat has been carried out.[211] The temperature of rats was raised to 42°C for 15 min. In all organs analyzed, hsp70 was synthesized 30 min, 1 hr, and 2 hr after heat shock, but not 1 or 2 days after the initial temperature shift. Hsp70 accumulated rapidly, becoming a major protein constituent. In adrenal glands the concentration of hsp70 was maximum by 6 hr after heat shock. The concentration declined slowly, reaching a basal level in about 8 days. The decline in concentration to basal levels in rat organs varied between 4 days in the brain and 8 to 16 days in most other organs. Therefore, in rat, hsp70 is quite stable, with a half-life of >10 hr. In rat, no other hsp of similar molecular weight to hsp70 has been observed. To attempt to interpret any functional significance of the stabilities, it needs to be known whether the related hsp68 and hsp70 of *Drosophila* have the same function. If they do, an equivalent function, in the form of either hsp68 or hsp70, is present in *Drosophila* for many hours after downshift.

The accumulation of hsp70 and hsp68 was also analyzed during a prolonged heat shock of flies at 37°C.[150] A level slightly higher than that obtained after the 1-hr heat shock was maintained as a steady-state level for 24 hr, the duration of the experiment (Figure 7). Hsp68 accumulated more slowly than hsp70, reaching a steady-state level approximately equivalent to that of hsp70 at about 4 hr after upshift. Whether the maintenance of the steady-state level of hsp70 is due to increased stability, continued synthesis or both is not known. Synthesis of hsp70 probably continues in a prolonged heat shock, since substantial levels of hsp70 mRNA are maintained for at least 8 hr after the upshift. However, regardless of at what level the control occurs, it is clear that the concentration of hsp70 and hsp68 are tightly regulated.

3. Translational Control

Translational control is associated with the heat shock response in two ways. In one

case, hsp mRNA has been shown to be present in cells under normal conditions, but is translated only after a stress. *Xenopus* oocytes contain hsp70 RNA which is in a "masked" state and is not translated until a temperature upshift occurs.[170] Enucleated or α-amanitin-injected oocytes are capable of synthesizing normal amounts of hsp70 after heat shock. It appears that hsp70 mRNA is synthesized during oogenesis and that the entire heat shock response of the oocyte is regulated at the level of translation, with no synthesis of hsp70 mRNA occurring. The reason for this unusual regulation is thought to be the large cytoplasmic to DNA ratio; assuming the hsp70 gene is single copy, it is predicted that 10 to 100 days would be needed to accumulate enough RNA to translate into the amount of hsp70 synthesized in the oocyte in 4 hr.

Secondly, in some cases, RNAs present at the time of stress are sequestered and not translated, but maintain the capacity to be translated at a later time, after return to normal conditions. Such a discrimination among mRNAs has been observed in a number of species, including mammalian cells,[212] yeast,[213] and *Drosophila*,[214] after extreme temperature shifts. This phenomenon has been studied in most detail in *Drosophila*. The preference for translation of heat shock mRNAs does not appear to be due to the ability of those RNAs to compete for a limited translational apparatus. In in vitro translation studies, no competition between heat shock RNAs and 25°C RNAs was seen.[142,215,216] The translational selection has been reproduced in cell-free extracts of *Drosophila* tissue culture cells[142,216,217] and indicate that heat shock induces a stable (but ultimately reversible) change in the translational apparatus. These studies suggest that the altered translational machinery recognizes some feature of the mRNAs which are translated after heat shock. These RNAs include not only RNAs encoding hsps, but, also, some viral RNAs[218,219] and histone H2B RNA.[220] What structural feature that renders these RNAs preferentially translatable has not been determined.

In vitro translation studies suggest that the change involves the ribosome or something closely associated with the ribosomes and affects both the rate of initiation and elongation.[221,222] Neither hsps nor their mRNAs are involved in the initiation of this translational regulation. Treatment with actinomycin at the time of heat shock prevents heat shock mRNA and protein synthesis, but not the inhibition of translation of 25°C mRNAs.[223] However, hsps or other proteins synthesized after heat shock may be involved in the reactivation of the translational machinery, so that 25°C RNAs may again be translated.

VI. CONCLUDING REMARKS

In recent years the heat shock response has been the subject of intense study. The universality of the response and the conservation of the inducible genes throughout evolution indicates that hsps perform functions of fundamental importance. Two basic aspects of the heat shock response, the mechanism of induction and repression of synthesis of these proteins and their function, will continue to be the focus of much study. Besides learning the details of regulation of particular systems, it will be important to determine how similar the mechanisms are in diverse species. Also, although the identification of related proteins in plants, animals, and bacteria suggests related functions, it will be important to carefully compare results in several species to determine if these different proteins function identically or if differences in function have evolved as the species diverged.

The determination of the induction mechanisms and, particularly, of the functions of the hsps will be difficult. Two approaches now beginning to be used may prove particularly useful: (1) a genetic approach in lower eukaryotes and bacteria and (2) the use of antibodies specific for hsps in cells of all species. An understanding of the function of heat-induced proteins will undoubtedly lead to a better understanding of how

cells cope with less than optimal conditions, including changes of temperature, tissue damage, hypoxia, and poisoning by certain chemicals.

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