Stress protein systems of mammalian cells

SUBJECK, JOHN R., AND THUNG-TAI SHYY. Stress protein systems of mammalian cells. Am J. Physiol. 250 (Cell Physiol. 19): C1-C17, 1986.—Living organisms are known to react to a heat stress by the selective induction in the synthesis of several polypeptides. In this review we list the major stress proteins of mammalian cells that are induced by heat shock and other environments and categorize these proteins into specific subgroups: 1) the major heat shock proteins, 2) the glucose-regulated proteins, and 3) the low-molecular-weight heat shock proteins. Characteristics of the localization and expression of proteins in each of these subgroups are presented. Specifically, the nuclear/nucleolar locale of certain of the major heat shock proteins is considered with respect to their association with RNA and the recovery of cells after a heat exposure. The induction of these major heat shock proteins and the repression of the glucose-regulated proteins as a result of reoxygenation of anoxic cells or by the addition of glucose to glucose-deprived cultures is described. Changes in the expression of these protein systems during embryogenesis and differentiation in mammalian and nonmammalian systems is summarized, and the protective role that some of these proteins appear to play in protecting the animal against the lethal effects of a severe heat treatment and against teratogenesis is critically examined.

heat shock; glucose; oxygen; anoxia; thermotolerance

IT IS WELL KNOWN THAT HEAT SHOCK induces the synthesis of a set of cellular proteins or heat shock proteins (HSPs). It is evident that certain HSPs examined in mammalian cells, Drosophila, yeast, and even Escherichia coli (10, 77) are closely related. These heat shock genes have been implicated in several major biological phenomena: embryogenesis and differentiation, viral infection, growth state and metabolism, and protection from phenocopy induction and thermotolerance. The present work reviews our present knowledge on the localization and behavior of the major heat shock or stress proteins and surveys these areas of mammalian “heat shock” biology. The focus is on the function and role of the mammalian stress proteins in cell biology/physiology, although relevant studies in Drosophila and other organisms are also considered. Aspects of the structure and regulation of stress genes are not considered here. For additional information on this and other points, the reader is referred to other reviews on this topic (8, 28, 123, 127, 139, 145).

Definition of Stress Protein Subsets

Although HSPs were initially studied in Drosophila (168), it soon became evident that a heat shock response also occurred in avian and mammalian cells (76). Since then several mammalian stress proteins of various molecular sizes have been described, some of which are more strongly induced and/or widely occurring than others. Heat shock, glucose, and oxygen deprivation and restoration studies in this laboratory, employing polyacrylamide gel electrophoresis, have collectively shown in Chinese hamster ovary (CHO) cells that principal stress proteins are observed at approximately 28, 34, 46, 56, 68, 70, 76, 89, 97, 110, and 174 kDa. Although this list covers the major stress proteins usually considered in the literature, comparison of molecular sizes from different gel systems in different laboratories is difficult, and this list should therefore be considered extendible.1 In addition, at each of these approximate molecular sizes, anywhere from one to several stress proteins may be observed. Calcium ionophores (185), glucose analogues (141), sodium arsenite (73), amino acid analogues (76, 96), tran-

1It is possible, with a reasonable certainty, to identify the same stress proteins from different studies, despite the fact that somewhat different molecular masses are used by different authors. We will therefore adopt a unified system of molecular sizes for those stress proteins that have repeatedly appeared in a variety of mammalian (and in some cases, nonmammalian) studies. In previously published studies from this laboratory we referred to major stress proteins in the 70-kDa range with sizes of 66 and 68 kDa. In this review these proteins are referred to by the molecular sizes of 68 and 70 kDa. Other laboratories have attributed other sizes to these proteins, the most common being 68/69, 71/73, or 72/73 kDa. The 68/70 designation used here represents the most widely used and recognized. Similarly, the HSP of molecular size 83–90 kDa is designated 89 kDa, and the HSP of 106-112 kDa is designated 110 kDa. The glucose-regulated proteins of 75–83 and 95–100 kDa are designated 76 and 97 kDa, respectively.
transition series metals (92), ethanol (93), and certain other agents in addition to heat are also recognized to induce some, but not all, of these stress proteins. This complex situation can be better interpreted by subdividing the stress proteins into subsets. Such a division follows. Characteristics of certain of these proteins is then considered in further detail in the subsequent section (Stress Protein Localization and Expression).

The most widely recognized stress proteins occur at 68, 70, 89, and 110 kDa (subgroup 1; Fig. 1). Of these, the 68- and 70-kDa components are usually the most strongly induced HSPs in most organisms, and HSP 70 appears to exhibit a high degree of conservation between Drosophila and mammals and Drosophila and E. coli (10, 77, 119). The 89-kDa HSP species is also widely recognized in a variety of organisms (77), whereas the 110 kDa protein has been principally studied and observed only at the mammalian level. A 100-kDa HSP is recognized in yeast (107), but its relationship to the mammalian 110-kDa protein is unclear. Although HSP 110 is not strongly induced in all cells (and can therefore go unrecognized), we have observed its induction with HSP 70 and HSP 89 in all cells examined (see Fig. 1). Among this group, HSP 89 is distinct since it is clearly a soluble cytoplasmic protein, while HSP 68, 70, and 110 all share a nucleolar and/or nuclear locale (described below). HSP 70, 89, and 110 are also expressed at 37°C in the absence of heat shock.

The stress proteins observed at 76 and 97 kDa have been referred to as minor HSPs (subgroup 2). However, they are better recognized because of their induction under glucose deprivation as the major glucose-regulated proteins (GRPs). Although some laboratories have reported that these proteins are slightly heat inducible, we have not observed their induction in well-fed proliferating cultures by moderate heat exposures. It is possible that their induction by heat may be determined by the nutrient environment or state of confluency of the cells at the time of shock. In addition to GRP 76 and 97, two other glucose-regulated proteins, with molecular sizes of 34 and 174 kDa (148), are induced under glucose deprivation.

The low-molecular-weight HSP (subgroup 3) have been well studied in Drosophila where they may be induced independently of the high-molecular-weight HSPs with the molting hormone ecdysterone (see below). The Drosophila HSPs 22–23 are structurally similar to mammalian α-crystalline (69). Though mammalian low-molecular-weight proteins are also observed, they have only recently begun to be well studied. The radiographic visualization of these proteins in mammalian cells is poor when [³⁵S]methionine is used as label.

This leaves other proteins at 46 and 56 kDa in the above list that have not been mentioned (subgroup 4). Although we have observed these two proteins to be heat inducible in CHO cells, proteins of the same approximate size are also inducible by glucose deprivation (or anoxia).
Stress Protein Localization and Expression

Conservation of the heat shock response and the induction of HSPs throughout all living organisms would appear to suggest that a common intracellular localization and functional role for these proteins might exist. However, the association of HSPs with distinct subcellular structures has been found to be highly variable. Information on the localization and behavior of certain stress proteins is considered here.

Major heat shock proteins: nuclear/nucleolar or cytoplasmic (68–70, 89, and 110 kDa)? Several years ago Mitchell and Lipps (113) demonstrated that proteins synthesized in the cytoplasm after heating in Drosophila salivary gland were rapidly transported back into the nucleus where they appeared to associate with puff regions of chromosomes. Other studies in Drosophila salivary gland and tissue culture cells indicated that constituents of the HSP 70 family specifically were found to be distributed equally between cytoplasmic and nuclear compartments (7, 157, 167, 173, 174). In the cytoplasm of Drosophila, HSPs appear to associate in part with the cytoskeleton (37). However, it appears in this and other organisms that during heat shock these proteins tend to accumulate and concentrate in the cell nucleus, with an apparent association with chromosomes and nucleoli (7, 102, 113, 173–175). The extent to which the nucleoskeletal association is a result of the presence of residual nucleoli in such preparations is unclear (181). An accumulation of proteins after heat shock in nucleolar structures has been reported in cultured plant cells (124, 129).

Likewise, HSP have been observed to occupy both nuclear and cytoplasmic locations in higher organisms. In avian cells, HSP 70 has been found to be cytoplasmic and associated with components of the cytoskeleton (77, 147). An association between a HSP 70-like protein and microtubules was also observed in HeLa cells (178). HSP 70 is also a methylated protein in avian and mammalian cells (87, 176). LaThangue (87) has recently reported that a monoclonal antibody against what appears to be HSP 70 stains microfilaments as well as exhibiting an affinity for the cell nucleus. He reports that the nuclear affinity is enhanced in proliferating cells. In addition, Hughes and August (65) have shown in mouse fibroblasts that a small fraction of cellular HSP 70 coprecipitates with a cell surface glycoprotein. In this latter study a linkage between plasma membrane and cytoskeleton was suggested. Other studies in several mammalian tissue culture cells (9, 140, 181) have shown that HSP 70 is also present in the nuclear matrix. However, it is not clear whether the broad distribution that this protein exhibits is functional or a by-product of its “sticky” nature (see the immunoprecipitation study in Ref. 166) and its great abundance in the heat-shocked cell. A rapid purification procedure has recently been reported for HSPs 68 and 70, which should facilitate future functional and localization studies with these proteins (183).

An important property of the heat shock response is the multiplicity of structurally related proteins in a particular molecular weight region, a characteristic best studied with HSP 70. In Drosophila it is established that multiple genes encode the 70-kDa HSP (reviewed in Refs. 8 and 28). Although these genes are heat inducible, closely related genes have been found that are not heat inducible but that are transcribed at normal temperatures (so-called heat shock “cognate” genes) (29). The reason for this multiplicity of heat shock and related cognate proteins is unclear, but the cognate genes are known to be regulated during development (29). By gel electrophoretic criteria, mammalian HSP 70 also expresses multiple isoforms, and Welch et al. (184, 185) have previously shown that mammalian HSPs 68 and 70 share identical solubility properties. In Drosophila it is established that the 68- and 70-kDa proteins share a significant degree of homology at the gene level (63). Drosophila HSP 70 and mammalian HSP 70 are also homologous (119), although less so than Drosophila HSPs 68 and 70. It is unknown to what extent (if any) Drosophila HSP 68 and mammalian HSP 68 resemble one another. However, peptide mapping of the mammalian 68- and 70-kDa proteins (Fig. 2) indicates that some degree of homology exists at this level as well (although the maps of these proteins are clearly distinct). This concurs with the recent report of Welch and Feramisco (181).

By using a rabbit antiserum, Welch and Feramisco (181) have demonstrated that HSP 68 is transiently found in the nucleolus of mammalian cells after heat treatment. This affinity of HSP 68 for nucleoli correlates with the synthesis of HSP 68, and it is not associated with nucleoli after HSP 68 synthesis has ended, at which time it appears to occupy a nuclear, but nonnucleolar,

![FIG. 2. Fluorogram of one-dimensional peptide mapping of heat shock proteins 70 (1) and 68 (2). Radiolabeled gel pieces from heat-shocked mouse 10T1/2 cells were digested with Staphylococcus aureus protease V8. Enzyme concentration used: A, 0 μg/ml; B, 0.005; C, 0.05; and D, 0.1. Equal masses of protein were loaded.](http://ajpcell.physiology.org/Downloadedfrom.Http://ajpcell.physiology.org)
site. These authors have recently reported that the antigen is released from the nucleus after digestion with ribonuclease (182). Pelham (136), using an antibody against Drosophila HSP 70 and a corresponding plasmid, transfected mouse L cells and monkey COS cells and showed that this Drosophila HSP also concentrates in their nucleoli. Although not entirely clear, it appears that this Drosophila protein more closely resembles the mammalian 68-kDa HSP. Pelham suggests that the presence of high levels of this protein in unstressed COS cells correlates with a more rapid recovery of nucleolar morphology after heat shock. In this study, nucleolar staining was again observed only after heat shock. Therefore the apparent cytoplasmic (synthesis)-to-nucleolus-to-nucleoplasm movement characteristic of mammalian HSP 68 was also observed in this study.

Although HSP 70 is a major cellular protein in mammalian cells at 37°C in all cell lines and mouse tissues examined in this laboratory, HSP 68 is either not visibly synthesized or is so in only very small quantities at 37°C (165, 181). In rat tissues (31) and mouse skeletal muscle labeled in situ (165), no HSP 68 is observed in the absence of heat, and its slight expression in some cells (e.g., 10T1/2) at 37°C may indicate a stress response to some element of the culture environment. Mild or moderate heat exposures do not induce HSP 68 in CHO or FM3A cell lines, but strongly express this HSP in 10T1/2, MCA, EMT6, MDCK, and ts85 FM3A cell lines and in skeletal muscle in situ. HSP 68 is only inducible in the CHO and FM3A lines after severe heat exposures. The temperature-exposure threshold for HSP 68/70 induction in animal tissues in situ appears to be less than that in cultured cells (165). In addition Morange et al. (120) have shown that HSP 68 expression after heat shock is restricted to differentiated cells and is not observed in undifferentiated cells and that its expression is also absent in the early embryo (see below, Embryogenesis and Differentiation).

In addition to the nucleolar locale of HSP 68, we have previously reported that another major mammalian heat shock protein, at 110 kDa, is also observed in the nucleolus (see Fig. 3) as distinguished using a rabbit antiseraum (166). Unlike HSP 68, HSP 110 is observed in the nucleolus of control cells (as well as in shock cells). In addition to a specific nucleolar binding, a secondary affinity for the nucleoplasm was also observed. HSP 110 is released from the nucleolus by treatment with ribonuclease, indicating that it is bound to RNA or to an ensemble of proteins that binds RNA (in which case the whole ensemble is released). In addition, when contact-inhibited 10T1/2 or 3T3 mouse fibroblasts reach a confluent state, nucleoli concurrently become smaller, and HSP 110 is observed to separate from the phase-dense component of the nucleolus. At the same time, general nucleoplasmic staining is significantly diminished. In this case immuno-electronmicroscopy indicates that HSP 110 is seen to associate with the nucleolar component, which, based on morphology and electron density, appears to be the fibrillar segment (the locale of rDNA) of these segmented nucleoli. Heat shock was seen to transiently reverse this nucleolar segmentation and increase nucleoplasmic fluorescence. This change correlates with the synthesis of HSP 110 and disappears (i.e., nucleoli resegment and nucleoplasmic staining subsides) when HSP 110 synthesis is restored to control levels (T. Shyy et al., unpublished data). It is not clear in this case whether the apparent transient increase in HSP 110 in the nucleus is due to the synthesis and transport of new HSP 110 into (and later, out of) the nucleus or to exposure of preexisting antigen during the heat shock response. However, with a monoclonal antibody, it has been shown that the 70-kDa HSP of Drosophila (174) also appears to enter and leave the nucleus in correspondence with HSP induction. This protein does not appear to associate with nucleoli, but the absence of this association may also be a result of the highly specific character of many monoclonal antibodies. In addition, these results would tend to agree with previous fractionation analysis in Drosophila (7, 91) regarding the movement of HSPs, as well as with recent studies in mammalian cells (122).

Therefore HSPs 68 and 110 exhibit (in part) a nuclear/nucleolar locale with an affinity for RNA, specifically...
BRIEF REVIEW

Plasmic, and they demonstrate a high level of HSP 89 in proteins by newly synthesized proteins may be a major factor in the expression of thermotolerance (discussed in further detail in Protection from Phenocopy Induction and Thermotolerance).

Furthermore, this resiliency appears to parallel the lethality of the heat treatment. Heat-shocked thermotolerant CHO cells express the ability to restore normal protein synthesis patterns much more rapidly when challenged by a second more severe heat exposure than do control cells subject to the same challenge heat exposure. Furthermore, this resiliency appears to parallel the expression of thermotolerance (discussed in further detail in Protection from Phenocopy Induction and Thermotolerance).

Glucose-regulated proteins: their relationship with major heat shock system I (34, 76, 97, and 174 kDa). The depletion of glucose in a cell's growth medium imposes an energy source stress to which the cell must accommodate itself, and it is well known that glucose deprivation also induces a set of cellular proteins (62, 141, 153) in mammalian cells. These GRPs, which are also induced by calcium ionophores (185) or glucose analogues (141), differ on one- and two-dimensional polyacrylamide gels from the major HSPs (at 68, 70, 89, and 110 kDa) discussed above (Fig. 4). Although these GRPs are clearly an independent set of stress proteins, GRPs 76 and 97 can in some instances also be heat inducible. This has led to their being referred to as “minor” HSPs. However, these GRPs are not always induced by heat. This variable inducibility by heat may suggest a role for the nutrient/hormonal environment of the cell or state of confluency at the time of heat shock in the control of their expression. In addition, certain methods of stress protein induction (e.g., amino acid analogues) may be sufficiently extensive in their ability to alter cell function to simultaneously trigger the induction of both the HSP and GRP systems.

Like the HSPs at 70 and 89 kDa, the GRP system appears to express a significant degree of conservation between avian and mammalian cells (88). Further studies are required to determine if this conservation extends to lower organisms, as is the case with the major HSPs. While their function is a mystery, several reports suggest...
that GRPs ultimately insert into the cell membranes, specifically the plasma membrane (141, 142, 153, 185). However, it has been demonstrated that these GRPs are not involved in glucose transport (22, 189). GRP 76 has been observed to be ribosylated and to undergo a rapid decrease in ribosylation during glucose deprivation (21). This GRP appears to be associated with intracellular membranes, probably the endoplasmic reticulum (185). GRP 97 is recognized as a major cellular glycoprotein, and a monoclonal antibody against GRP 97 has been observed to associate with the Golgi (99, 185). However, GRP 97 also partially resides in the nucleus in some cells, and heat shock increases this association (185). McCormick et al. have examined the distribution of GRP 97 in plasma membranes of the developing mouse embryo (109) and in cultured cells (108) and suggest a regulatory role for this protein in controlling ectodermal versus endodermal differentiation.

An important area in the stress protein field concerns the relationship between the various stress protein subsets. An interesting observation regarding a possible connection between the GRP and HSP systems has been noted in studies of CHO cells (148). When glucose is added to glucose-deprived GRP-induced cells, the GRPs are slowly repressed, as expected. However, during repression of the GRPs, the major HSPs are induced. The pattern of induction is identical to that observed after a mild heat shock of these cells (149, 165). Thus addition of glucose to glucose-starved GRP-induced cells elicits a heat shock response (Fig. 5). In addition, unlike most treatments with heat and chemicals, this “glucose shock” results in no cellular damage (it is entirely non-lethal). In these studies the repression of the most strongly induced GRP at 76 kDa closely parallels the induction of the major HSP seen at 70 kDa. If the repression of the 76-kDa GRP is accelerated by adding complete fresh media (rather than by simply adding glucose), the corresponding induction of the major HSP is accelerated by an exact amount. Further information is needed on this approach to HSP induction and on the apparent interaction between these two stress protein systems, which occur under these conditions. In addition, it has been reported that glucocorticoids can elicit a heat shock response in glucose-deprived cells (73a). A stimulation of gluconeogenesis in this instance could result in an increase in intracellular glucose levels. In a recent paper these authors report that insulin, even in the presence of glucose, induces GRPs and that this effect is antagonized by dexamethasone (74).

Anderson and co-workers (5) have previously examined the effects of chronic anoxia on the protein synthesis patterns in mammalian cells and have shown a prominent induction of proteins at approximately 34, 75, and 95 kDa. The similarity with the heat shock response was noted. Later studies of others also suggested that anoxia may alter HSP synthesis (98). Furthermore, early studies in lower organisms (50, reviewed in Ref. 8) have also demonstrated that release from anoxia can elicit a heat shock response and that GRPs ultimately insert into the cell membranes, specifically the plasma membrane (141, 142, 153, 185). However, it has been demonstrated that these GRPs are not involved in glucose transport (22, 189). GRP 76 has been observed to be ribosylated and to undergo a rapid decrease in ribosylation during glucose deprivation (21). This GRP appears to be associated with intracellular membranes, probably the endoplasmic reticulum (185). GRP 97 is recognized as a major cellular glycoprotein, and a monoclonal antibody against GRP 97 has been observed to associate with the Golgi (99, 185). However, GRP 97 also partially resides in the nucleus in some cells, and heat shock increases this association (185). McCormick et al. have examined the distribution of GRP 97 in plasma membranes of the developing mouse embryo (109) and in cultured cells (108) and suggest a regulatory role for this protein in controlling ectodermal versus endodermal differentiation.

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These are not HSPs. It is reported that the initial transition to anoxia in maize roots induces "transition proteins," and one of the major transition proteins appears to be a HSP. The kinetic relationship between the transition proteins and the chronically induced anaerobic stress proteins and the induction of HSPs and GRPs just described in mammalian cells is intriguing.

It is well documented that HSPs are inducible in vivo in several mammalian tissues (26, 31, 62, 165) by heat shock. In addition, certain studies indicate that tissues are capable of mounting a heat shock response under ischemic conditions (31, 53). These studies specifically emphasize the induction of HSPs near 70 kDa. However, it has been reported that GRPs are also induced in the presence of glucose under conditions that may be related to ischemia (62). It has been recently shown in cardiac muscle made hypoxic by hypobaric decompression that a protein which appears to be HSP 70 is transiently induced early in the treatment, after which other polypeptides, with molecular sizes resembling GRPs, are expressed (64).

One important area in which this natural staggered expression of these GRPs and HSPs is of particular interest is in the area of tumor biology. It has long been recognized that oxygen deprivation and/or nutrient deprivation can occur during tumor development (172). Therefore the conditions and corresponding responses just described may have applications in this area. Furthermore, transformed cells have been observed to induce GRPs more rapidly than nontransformed cells when glucose deprivation occurs (141). All of this may be important in tumors where a potentially restricted nutrient supply could result in the differential induction of the GRPs between normal and tumor tissue. Induction of proteins under chronic anoxia further enhances this possibility. Conversely, tumor reoxygenation (as may occur during a course of cancer therapy, e.g., radiation) or glucose restoration may result in a differential in HSP levels between normal and tumor tissues.

Stress proteins may also be related to resistance to certain drugs. Chronic anoxia (a GRP inducer) is known to lead to a cellular resistance to adriamycin (16, 106, 159), and 2-deoxyglucose [also a known GRP inducer (141)] is also able to induce a resistance to adriamycin (25). Therefore the GRP system may confer a tolerance to cells against certain drugs, including adriamycin. In addition, it has been shown that heat shock can confer a tolerance to adriamycin (95). However, none of these studies examined cell protein synthesis, and the relationship of these observations to the presence of stress proteins is only speculative.

Low-molecular-weight HSPs. With respect to the low-molecular-weight HSPs (15–32 kDa), the intracellular distribution of these small proteins also appears to vary among different organisms or cell types. In Dictyostelium, Loomis and Wheeler (102, 103) have shown that eight small HSPs (26–32 kDa) are preferentially localized in nuclei and can be recognized as chromatin-associated proteins by virtue of their extractability from purified nuclei with high salt. In Drosophila, on the other hand, the nuclear accumulation of four small HSPs (22–26

FIG. 5. Addition of glucose to glucose-deprived and glucose-regulated protein (GRP) induced cells, repressed GRPs, and concurrently induced major heat shock proteins (HSPs). Right margin: lines, 97- and 76-kDa GRPs; arrowheads, 89- and 70-kDa HSPs. C, control; 0–24 hours after addition of glucose. Autoradiogram of gel is shown. Equal masses of protein were loaded. See Refs. 148 and 151 for additional information.
kDa) was found to be primarily associated with nuclear scaffold rather than the chromatin (91, 157). It has also been demonstrated that the four small Drosophila HSPs are related to each other and to \( \alpha \)-crystallines (69). In avian cells, only one low-molecular-weight protein (HSP 24) was observed to be induced at a high level by heat shock, and it appeared in this case to be a component of the cytoskeleton (77, 102). Small HSPs were found to be present in a soluble form in hamster fibroblasts and human KB cells (9). Kim et al. (78) described the induction of at least three stress proteins in rat myoblasts after treatment with sodium arsenite or sodium arsenate or after heat shock. In rat embryo fibroblasts Welch (180) has shown the induction of four isoelectric variants at 28 kDa by heat. He further reports that two of these isoforms are phosphorylated in quiescent cells by phorbol esters, calcium ionophores, or fresh serum. Interestingly, >20 low-molecular-weight proteins (15–25 kDa) have been recognized in plants [e.g., in tomato cell cultures (128)]. A possible property of these small \( \alpha \)-crystalline-like HSPs may be their capacity to form higher order aggregates that can be visualized as newly formed cytoplasmic granules (heat shock granules) during the heat shock response in plant cultures (124, 129). At normal temperature (25°C) these small proteins accumulate exclusively in soluble form.

While all the studies described above have indicated a rather extensive distribution for different HSP species, surprisingly, no association between HSPs and mitochondria has been demonstrated, despite a great deal of evidence linking the heat shock response to respiratory stress (reviewed in Ref. 8). Because mitochondria are known to share a molecular biology with procaryotes, it is interesting to speculate as to whether mitochondria possess their own procaryotic heat shock genes and whether these genes are heat inducible.

**Embryogenesis and Differentiation**

In the very early mouse embryo, the activation of the embryonic genome occurs at the late two-cell stage (39) and results in a marked alteration in the pattern of protein synthesis. This activation is preceded by a decrease in the overall synthesis of proteins at the end of the one-cell stage and the appearance of a set of new polypeptides (mol wt ~70 kDa) at the early two-cell stage. Bensaude et al. (11) have shown that these early two-cell-specific 70-kDa proteins are identical to two of the major mouse HSPs at 70 and 68 kDa. In addition, Bensaude and Morange (12) have shown that HSPs 89 and 70 are constitutively expressed at high levels in mouse embryonic ectoderm cells and in embryonic carcinoma cells. The specific expression of heat shock genes at this very early embryonic stage may play a role in the activation of other cellular genes. Moreover, Wittig et al.
itiation by retinoic acid. It is possible that reduced con-
differentiation. Immunological studies of the undifferentiated carcinoma cell lines (i.e., HSP 68 inducibil-
and early embryos before blastoderm. They also found that the preblastoderm embryos failed to induce
ese heat shock messages even if subjected to temperature shock. All of these results indicate that the expres-
HSPs can be dramatically altered during embryonic development.

Buzin and Bournia-Vardiabasis (20) have investigated the effects of a number of drugs that are known to be teratogens in mammalian systems on the differentiation of Drosophila embryonic cell cultures. They discovered that drugs which inhibit differentiation also induce the synthesis of the three small proteins, identified as low-molecular-weight Drosophila HSPs (HSPs 23, 22a, and 22b). Conversely, drugs that do not inhibit differentiation to a significant degree also do not induce HSPs 23, 22a, and 22b in any appreciable amount. Furthermore, they found that the steroid hormone ecdysterone inhibits muscle and neuron differentiation in primary embryonic cell cultures and increases the synthesis of HSPs 23, 22a, and 22b. Although these findings strongly suggest that this induced synthesis of HSPs 23, 22a, and 22b may be important in the regulation of embryonic differentiation, it is also interesting that the synthesis of these three small proteins by mild heat pretreatment may protect cell differentiation otherwise inhibited by teratogens or high temperature (see below, Protection from Phenocopy Induction and Thermotolerance).

While the real functional role for the small HSPs mentioned above is unknown, it has been shown that at least one protein (HSPs 23) of this family is developmentally regulated in Drosophila imaginal wing disc under nonheat shock conditions (23). During mid third instar, the level of synthesis of HSP 23 is low; at the end of the third instar, however, synthesis of this protein dramatically increases. The increased synthesis of HSP 23 at the end of the third instar coincides with the rise in ecdysterone titer, which occurs in this period of development. These results indicate that it is probable that ecdysterone triggers the synthesis of HSP 23, since the induction of HSP 23 and the other low-molecular-weight HSPs are ecdysterone inducible (20, 70, 71).

Protection from Phenocopy Induction and Thermotolerance

For about half a century, temperature shock has been used to induce defects in developing Drosophila. The term "phenocopy" was first used by Goldschmidt (48) to denote heat shock-induced developmental abnormalities that closely resembled genetically determined traits. Because a high temperature shuts down both transcriptional and translational activities, it is thought that the induction of phenocopies may result from the interruption of the developmental time table via the interruption of normal protein synthesis. Indeed, evidence (114) suggests that a particular phenocopy may result from the absence of a particular translational product required at a specific time in the developmental sequence.

Early studies (110, 111) indicated that subjecting Dro-
sophila pupae to a mild heat treatment before a heat exposure that would be lethal (if applied alone) could protect the animals from the lethal exposure. In addition to the mild pretreatment protecting the survival of the animals, it also was shown to protect larvae against the induction of phenocopies induced during the later exposure. A connection between IISPs synthesis and protection from phenocopy induction came many years later. Mitchell et al. (115) demonstrated that the synthesis of HSPs induced by the mild heat treatment correlated with increased survival and protection against pheno
copy induction. They also showed that the optimal condition for the prevention of phenocopy induction is a preliminary mild heat pretreatment, which induces the synthesis of HSPs but does not inhibit normal protein synthesis. Thus it appears that this concomitant synthesis capacity for HSPs and normal proteins both contribute to the protection phenomenon. They further indicate that the recovery of protein synthesis after a severe heat shock is enhanced by the mild pretreatment. The same laboratory has more recently examined the molecular events involved in the induction of the phenocopy “multihair” (116, 138). These studies suggest that at the heat-induced inhibition of protein synthesis, phenocopy induction may result from the noncoordinate recovery in the synthesis of different gene products required at that stage in the developmental sequence. Although a role for HSPs in mediating protection from phenocopy induction and protection of survival (thermotolerance) is indicated, it cannot be assumed that the mechanisms through which IISPs presumably mediate these two protective phenomena are identical.

A specific role for HSPs in conferring heat resistance was also suggested in the work of others. Early work in Drosophila embryos (49) and developing sea urchins (144) noted a thermal protective effect that correlated with the onset of HSP (and protein) synthesis. McAllister and Finkelstein (107) found in yeast a correlation between acquired thermal resistance and the induction of a 100-kDa HSP and demonstrated that this resistance was dependent on mRNA transport across the nuclear envelope. In Dictyostelium, Loomis and Wheeler (102, 103) also recognized a connection between HSPs and thermal protection. They further demonstrated in heat shock-impaired mutants that the loss of the induction of several low-molecular-weight HSPs (26-32 kDa) correlated with the inhibition of thermal protection, suggesting a linkage between the thermal protection and the capacity for synthesis of these low-molecular-weight proteins. In apparent agreement with these observations, Berger and Woodward (13) reported that the selective induction of the low-molecular-weight HSPs in Drosophila cultured cells induced thermotolerance.

Independent of these studies on thermal protection and HSP synthesis, a significant amount of research has accumulated in several other areas of biology on analogous thermal protective phenomena. A thermotolerance phenomenon has been observed in E. coli (reviewed in Ref. 123). In vertebrate ectotherms, investigators have measured the effects of the thermal environment on the lethal temperature for animals (LD$_{50}$) and on the ability of animals to withstand a sudden severe heat shock, as measured using the “critical thermal maximum” (for a review of this area see Ref. 67). A thermotolerance to a sudden heating, also referred to as “heat hardening,” has been clearly demonstrated (36, 105).

It has been suggested that the ability to develop thermotolerance may determine the presence or absence of a species in a particular geographic area (66, 160). A heat hardening phenomenon is also known to occur in plants where an initial heat shock induces resistance to a later heat stress (55, 188). Alexandrov (1) studied the concept of heat hardening in plants and amoebas and showed that the temperature at which a cessation in protoplasmic streaming occurs could be raised by a brief exposure to a lethal temperature. Although it is not clear whether all of these observations are attributable to HSP induction, some of them may be.

Several early studies described what appeared to be a tolerance phenomenon at the mammalian level (30, 45, 54, 135, 152). In these studies an initial heat exposure resulted in a resistance to a later heat treatment. However, it was difficult to rule out the selection of a resistant population in many of these studies. The work of Gerner and co-workers (41, 42) and of Henle and Leeper (58) first clearly defined this thermotolerance phenomenon from a cell survival standpoint. Several studies from other laboratories have also contributed to the characterization of this phenomenon (reviewed in Ref. 57). Thermotolerance differed from a repair or recovery pheno

denomenon (where damaged cells regain, after a time, their control level of resistance) in the important respect that not only do cells recover their control levels of resistance, but they proceed to develop additional resistance, which may be of three, four, or five logs in magnitude (Fig. 7). The cellular basis for this dramatic change in cellular resistance was clearly not a result of clonal selection but was a result of a transient change in the cell itself. Several factors have been studied that can significantly modify a cell’s sensitivity to hyperthermia [e.g., membrane composition and structure (80, 90), polyhydroxy compounds (61), polyamines (40), and pH (46)]. However, Henle and Leeper (50, 60) initially suggested that some aspect of protein synthesis was responsible for mammalian thermotolerance. These authors demonstrated that thermotolerance developed in coincidence with the recovery of total cell protein synthesis and that inhibitors of protein synthesis such as cycloheximide inhibited the development of thermotolerance. These studies and the close resemblance of mammalian thermotolerance to the protective effect observed in lower organisms led other laboratories to investigate the possible role of HSPs in mammalian (thermotolerance (83, 85, 97, 98, 146, 149, 162-164, 170).

In our laboratory (149, 162-164) the development of thermotolerance in CHO cells was studied after several different inducing heat exposures (41°C for 1-6 h; 45°C for 5, 12, and 22 min). All of these treatment protocols indicated that a good overall agreement existed between the selective expression HSPs and thermotolerance. In addition, it was demonstrated that in the case of the 45°C, 12-min treatment, an approximate dose effect or
FIG. 7. Effects of heat fractionation on Chinese hamster ovary (CHO) cell survival. Survival curve resulting from continuous exposures at 45°C is shown together with 1) a discontinuous 45°C exposure (interrupted after 5 min at 45°C by incubation at 37°C for 6 h, after which 45°C treatment is continued) and 2) a 2nd discontinuous 45°C exposure (interrupted after 12 min by incubation at 37°C for 12 h). Both interruptions result in significant resistance to subsequent heat exposures (thermotolerance). Also shown are 1) effect of initial treatment at 45°C for 22 min separated by 18 h at 37°C from a subsequent exposure to 45°C for 10 min (dashed line); and 2) effect of initial treatment at 41°C for 4 h immediately preceding exposure to 45°C for 27 min (41°C incubation itself is totally nonlethal). The 37°C interruption times (Δt) are also times to development of maximal thermotolerance and maximal heat shock protein (HSP) synthesis, and times required for recovery of normal protein synthesis, in each case. These survival data represent composite of data published in Refs. 149, 163-165, where related details of HSP and thermotolerance induction for these treatments are presented.

A logarithmic relationship existed between the level of each of the major HSPs (HSPs 70, 89, and 110) and thermotolerance during the induction phase. However, one discrepancy in the correlation was noted in all of these studies: at the moment that thermotolerance leveled off, the quantity of each of the HSPs continued to increase in the cell for several hours. Conversely, all the data available suggest that the expression of full tolerance predicts the time at which the expression of the heat shock response is curtailed, suggesting a possible role for the tolerant condition in the regulation of the heat shock response. Because it has been proposed previously that at least one HSP appears to be self-regulated (33), perhaps the role for HSPs in self-regulation and thermotolerance are interrelated.

Similar studies were performed by others (83, 85, 97, 98) with generally similar conclusions: the induction of HSP synthesis closely parallels the expression of thermotolerance. In addition, these laboratories also examined HSP and thermotolerance decay and again demonstrated a good correlation between these phenomena. Li (94) has recently examined this question in further detail and has demonstrated a logarithmic relationship between levels of HSP 70 and thermotolerance during the decay period. Furthermore, this group also has shown that arsenite and ethanolate induce HSPs and thermotolerance (93). A different approach to this problem was presented by Heikkila et al. (56). These authors utilized the fact that HSPs are not heat inducible in the early Xenopus laevis embryos and demonstrated that these embryos also failed to express thermotolerance. Furthermore, it was shown that thermotolerance becomes inducible at the same time at which the embryo develops the ability to express HSPs and other genes. Finally, the mammalian thermotolerance studies discussed here suggest that tolerance is related to the expression of the high- and/or low-molecular-weight HSPs (subgroups 1 and 3), but not GRPs (subgroup 2), which may conversely thermosensitive proliferating cells (148).

Although several studies in a variety of organisms have collectively enforced the suggestion that HSPs are directly responsible for thermotolerance, there is at this time no direct evidence for a causal relationship between these two phenomena. In addition, several studies have indicated that certain discrepancies exist in this general hypothesis. We have already described our studies which show that the full expression of thermotolerance appears to predict the time at which HSPs begin to be repressed, i.e., total cell levels of the major HSPs (subgroup 1) continue to increase after tolerance has plateaued. Landry and Chretien (84) report that arsenite, an HSP inducer, does not induce thermotolerance. The basis for the discrepancy between this work and that of Li (93) (who has shown that arsenite induces thermotolerance) is unclear, but it may be related to the concentration of arsenite used. In addition, Tanguay and Vincent (167)
report that HSPs induced in response to arsenite do not become associated with the nucleus as they do during heat shock. Hall (51) has reported that yeasts develop thermostolerance without the synthesis of HSPs (which was blocked by cycloheximide before heating). However, two other similar studies demonstrate an inhibition of tolerance under these conditions (107, 118). Two possible explanations for the discrepancies involving the effects of cycloheximide on thermostolerance development have been suggested by Hallberg et al. (52). These authors show that whereas the addition of cycloheximide to *Tetrahymena* initially inhibits protein synthesis, this inhibitory effect is lost after continued incubation in the presence of moderate concentrations of this drug. Therefore incubation in the presence of cycloheximide before an increase in temperature may allow some HSP synthesis to occur. These authors also show that whereas extended survival (thermotolerance) at an elevated temperature requires HSP synthesis, a resistance to a brief heat exposure follows conditions inhibitory to total cell protein synthesis (including HSPs). The latter observation reflects earlier studies at the mammalian level (60, 84). Altschuler and Mascarenhas (2, 3) have reported that protection against heat damage in growing pollen tubes of *Tradescantia* can develop without detectable synthesis of HSPs when an increase in temperature occurs gradually. Tomosovic et al. (170) have demonstrated that the degree of HSP synthesis is greater when mammalian cells are rapidly transferred to 42°C as opposed to a slow transition to the same temperature. However, these authors showed that the slow transition results in greater thermostolerance. These results were based on equivalent radioactivity (cpm) as a method of gel loading, and Tomosovic et al. (169) have recently suggested that loading of gels to equivalent radioactivity would tend to overemphasize the amount of HSP present in those treatments that generally inhibit total cell protein synthesis. Finally, other cellular changes can lead to significant alterations in the cell’s resistance to heat, the most obvious being cell cycle redistribution (186). Therefore drugs and conditions that alter the distribution of cells in the cell cycle can also be expected to alter thermostolerance.

In addition to differences in HSP measurement that may result from different methods of gel loading between different studies, methods of measuring thermostolerance also vary significantly. Single cell colony survival as usually employed in mammalian studies is likely to differ from whole embryo survival. Thermostolerance studies in various ectotherms utilize the principle of critical thermal maximum, and studies in *Drosophila* have employed trypan blue exclusion. Only recently has the much more sensitive technique of single cell survival been applied to *Drosophila* cell cultures (171). It is necessary to keep all of these factors in mind when comparing different HSP/thermostolerance studies. In addition to those problems, the term heat shock conjures up the impression of a specific experimental condition, when indeed a variety of significantly different heat shocks with significantly different cellular responses are possible (e.g., examine the heating protocols presented in Ref. 149; also see Ref. 52). Finally, cells can yield different responses at different times after heat shock when using a higher temperature-inducing shock with a subsequent incubation at physiological temperature. In this respect a continuous exposure at a nonlethal, physiological temperature (e.g., 41°C in mammalian cells) may be preferable in studies of the heat shock response.

It appears at the moment that two general hypotheses exist concerning the mechanism for thermostolerance. 1) HSPs are responsible for thermostolerance (either directly or indirectly). They may bind directly to cellular structures to stabilize them (112). Alternatively, they may facilitate the (coordinate) recovery of normal protein synthesis patterns, as suggested by Mitchell and coworkers (115, discussed above). In this case the replacement of the proteins altered by the heat shock-inducing treatment would be responsible for thermostolerance. The fact that hyperthermia in the range considered here can lead to changes in enzymatic activity, protein unfolding, and denaturation is well documented (6, 17, 81). In support of the latter hypothesis, we have observed that the period of delay to recovery of normal protein synthesis after heat shock is an indication of the likelihood of cell death in a specific cell line. In Fig. 7, \( \Delta t \) corresponds to the time required for the recovery of normal protein synthesis (as well as for the expression of maximal thermostolerance). Increasing time is required for the recovery of normal protein synthesis as the inducing exposure becomes increasingly lethal. In addition, in thermotolerant (preheated) cells, the recovery of normal protein synthesis patterns occurs more rapidly, in a manner that appears to be approximately consistent with the likelihood of survival in the thermostolerant population [i.e., a 45°C, 22-min exposure of control CHO cells significantly inhibits protein synthesis for 16 h (both HSPs and other cell proteins) and kills >99% of the population; a 45°C, 22-min treatment of a thermostolerant population inhibits the recovery of normal protein synthesis for only 4 h; a 45°C, 5 min exposure of control cells also inhibits recovery for 4 h; neither of the latter two conditions notably reduce survival]. In addition, this facilitated recovery of protein synthesis develops after an inducing heat shock in a manner kinetically similar to the expression of thermostolerance. Restoration of normal cell function would also be expected to encompass the removal and degradation of the proteins altered during the inducing treatment. It has been shown that the protease La of *E. coli* (a product of the lon gene), which plays an important role in the degradation of abnormal proteins, is under heat shock control (reviewed in Ref. 123). It has been recently shown that this and other *E. coli* HSPs are induced in the presence of aberrant proteins (47). In addition, it has been reported that a mammalian cell mutant, with a reduced capacity for protein degradation due to a temperature-sensitive defect in ubiquitin-protein conjugation, shows an enhanced synthesis of HSPs (38) and an increased sensitivity to heat (165). Bond and Schlesinger (15) have recently reported that ubiquitin itself is a HSP in avian cells. 2) HSPs are not responsible for thermostolerance. Whereas there can be little doubt that HSP induction and ther-
motolerance represent two aspects of the heat shock response and therefore share some connection, HSPs may not play a causative role. In this case some parallel aspect of the heat shock response might result in thermotolerance. The level of this hypothetical mediator might, conversely, act to repress HSP induction. It has been demonstrated that glutathione levels are increased in thermotolerant CHO cells when tolerance is induced by either heat or ethanol (117). Conversely, this study shows that glutathione depletion results in thermosensitivity. A role for adenylated nucleotides in HSP induction has been suggested (89). It is recognized that the level of these molecules changes dramatically (by orders of magnitude) in the cell in a manner that closely parallels DNA synthesis. What effect do increased levels of these molecules have on cellular resistance to heat? One apparently strong piece of evidence directly linking HSPs and thermotolerance is the coincidence of their decay. Li (94) and Landry et al. (83) have reported that during decay the level of HSPs predicts tolerance. However Gerweck (43) has shown that the decay of HSPs occurs only slightly faster than would be expected by dilution by cell division. Therefore thermotolerance decay appears to be a reflection of cell division and not a unique property of HSPs. Any other stable cellular molecules would decay in a similar manner. A similar decay experiment in postconfluent cells, where cell division is restricted and the decay of heat shock proteins and thermotolerance (44) is significantly lengthened, would be useful.

Finally, in addition to protection of an organism from a lethal heat exposure, moderate hyperthermia is also known to result in an increased survival in fish subject to infection (27). Studies at the mammalian level (72, 143, 158) have also demonstrated that heat can lead to an enhancement of immune function (e.g., enhanced activity of specific lymphoid cell classes). Although it is clear that hyperthermia has a significant effect on the immune system, it is not clear whether this activation involves any aspect of the heat shock response. However, Morimoto and Fodor (121) have recently demonstrated that avian lymphocytes rapidly induce HSP 70 after small changes in temperature (2–3°C). In addition, the relatively low temperature threshold for induction of HSPs in animal tissues (26, 165) suggests that a febrile response may lead to a heat shock response. In this regard, the interaction of HSPs with virally encoded proteins, discussed in an earlier section, may be relevant. In summary, there is a rapidly increasing literature concerning the involvement of stress proteins in several cellular functions. Questions arise as to whether molecular effects contribute to the physiological phenomenon of thermotolerance. Is the protective role of the stress response its principal function, or is it secondary to some more fundamental role? Does the expression of heat shock proteins as the initial zygotic gene products suggest a more basic role? Perhaps the answers to these questions are inseparable; stress protein expression in the early embryo may represent a stress response (e.g., as a result of the transition to anoxia or some other undetermined environmental change). In the last few years several studies have emphasized the remarkable conservation of this response in all living systems (reviewed in Refs. 28, 123). A thermotolerance phenomenon also appears to be exhibited by all transcriptionally competent organisms yet examined (discussed above). As expressed by Neidhardt et al. (123), “The central problem of heat shock is to discover the meaning of its conservation throughout the evolution of all living systems.”

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