The Heat Shock Response of the Rat Embryo During Organogenesis

by

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Abstract

Mammalian embryos respond to a stressful external stimulus with a typical stress response. This response is characterized by the increased synthesis of the stress proteins and the concomitant transient depression of overall protein synthesis. The heat shock proteins are believed to be involved in protecting the embryo from the harmful effects of external agents. However, the heat shock response that occurs following exposure of the developing embryo to heat or to other teratogens such as cadmium, may alter the established program of activation and inactivation of genetic material essential for normal embryogenesis, which in turn may lead to developmental abnormalities. Little is known about the pathway leading from a stressful stimulus such as hyperthermia or cadmium to a malformation, and the possibility of the heat shock response being linked to this phenomenon prompted the studies in this thesis. In order to determine the relationship between the ability of heat or cadmium to induce a heat shock response and the ability of these stressors to induce abnormal embryogenesis, rat embryos were cultured during organogenesis and the steady-state mRNAs of two heat shock proteins were used as indicators of the stress response in these embryos. Hsp27 and hsp70 were chosen based on the ability of hyperthermia and cadmium to specifically induce these heat shock proteins in other systems. Malformations were apparent in embryos exposed either to hyperthermia (5, 10, 15 or 20 minutes at

43°C) or to 2.5 μM cadmium. When embryos were heat shocked for 5, 10, 15 or 20 minutes at 43°C, hsp27 and hsp70 mRNA accumulated in a dose-dependent manner. The time course of hsp27 and hsp70 mRNA accumulation 1, 2, 4 or 20 hours post heat shock was different for each heat shock protein. In addition, the extent of mRNA accumulation for each heat shock protein varied depending on the tissue examined. When embryos were cultured in the presence of cadmium chloride, hsp27 and hsp70 mRNAs took much longer to accumulate compared to those observed following heat shock. Thus, the time course of heat shock protein mRNA accumulation was quite different in embryos exposed to cadmium from those exposed to hyperthermia. In addition, the concentrations of hsp27 and hsp70 mRNA appears to be differentially regulated in both embryo and yolk sac tissues for embryos treated with heat or cadmium. In conclusion, mammalian embryos are able to mount a heat shock response through the accumulation of hsp27 and hsp70 mRNAs in response to hyperthermia or cadmium treatments. The extent of this response appears to correlate with the teratogenicity effects of hyperthermia but not with those of cadmium.

Résumé

L'embryon mammifère réagit à un stimulus externe stressant par une réaction de stress typique. Cette réaction est caracterisée par l'augmentation de la synthèse des protéines de stress et la dépression transitoire de la synthèse totale de protéines. On croit que les protéines de stress sont impliquées dans la protection de l'embryon des effets malfaisants des agents externes. Cependant, la réaction de stress, qui se produit à la suite de l'exposition de l'embryon à la chaleur ou à d'autres teratogènes tels que cadmium, peut altérer le programme d'activation et d'inactivation du matériel génétique qui est essentielle au développement normale de l'embryon. Très peu est connu sur la voie menant d'un stimulus stressant, tel que hyperthermie ou cadmium, aux malformations des embryons. C'est la possibilité que la réaction de stresse soit reliée à ce phénomène qui a incitée les études dans cette thèse. Afin de déterminer le lien entre l'habilité de la chaleur ou le cadmium d'induire une réaction de stress et l'habileté de ces facteurs stressantes d'induire un développement anormale, les messagers d'ARN de deux protéines de stress, hsp27 et hsp70, ont été étudiées. Ces deux protéines ont été utilisées comme indicateurs de la réaction de stress chez l'embryon du rat durant la période d'organogénèse. Hsp27 et hsp70 ont été choisis du fait que l'hyperthérmie et le cadmium sont capables d'induire ces deux protéines de stress dans des systèmes similaires. Les

malformations dans l'embryon sont apparentes lorsqu'il est exposé soit à l'hyperthermie (5, 10, 15 ou 20 minutes) ou au cadmium 2.5 μM. Quand les embryons ont été stressés par la chaleur pendant 5, 10, 15, ou 20 minutes à 43°C, on a observé que les messagers d'ARN du hsp27 et du hsp70 ont accumulé par rapport au temps d'exposition. La courrue du temps de l'accumulation du messager d'ARN des protéines de stress, à 1, 2, 4 et 20 heures après l'application de la chaleur, ont été différente parmi les deux protéines étudiées. La courrue du temps de l'accumulation des messagers d'ARN des protéines de stress diffèrent de celle exposée à l'hyperthermie lorsque les embryons sont cultivés dans la présence du cadmium. De plus, les concentrations de messager d'ARN de ces protéines de stress sont apparemment réglés de façons différentes. En conclusion, les embryons mammifères sont capables de monter une réaction de stress par l'accumulation de messager d'ARN pour le hsp27 et le hsp70. L'étendue de cette réaction paraît reliée aux effets teratogéniques de l'hyperthermie et non aux celles du cadmium.

Claims to Originality

For the first time a differential heat shock response was demonstrated for embryo and yolk sac tissues in response to hyperthermia or cadmium treatment. Rat embryos were shown to accumulate both hsp27 and hsp70 mRNAs at specific times following heat shock or cadmium treatment. Furthermore, the heat shock response was different in embryos exposed to hyperthermia when compared to those exposed to cadmium. That is, the steady-state mRNA levels of hsp27 and hsp70 were already elevated 1 hour post-heat treatment while those following cadmium treatment took as long as 6 hours to accumulate. Thus, the hyperthermia-induced stress response appears to be related to hyperthermia-induced teratogenicity, while the cadmium-induced stress response does not appear to be related to cadmium-induced teratogenicity.

CHAPTER 1

INTRODUCTION

1.1. The Heat Shock or Stress Response

The physical environment of many organisms constantly changes due to natural physiological causes or to external conditions which can be either favourable or unfavourable. When an organism is confronted with a sudden change in its environmental circumstance, it will exhibit specific homeostatic-like responses. Thus, the ability of an organism to adapt or acclimate to its new environment may be an integral driving force for survival. Not surprisingly, many organisms have evolved a number of ways to adapt to the physiological impact of environmental change. One such evolutionary adaptation is known as the "heat shock" or "stress" response (Morimoto, Tissieres and Georgopoulos, 1990). This response is characterized by the rapid and transient increase in the rate of expression of a small group of evolutionarily conserved genes (Lindquist, 1986). The products of these genes (referred to as heat shock or stress proteins) are ordinarily present under physiological conditions (Welch, Mizzen and Arrigo, 1989). However, after exposure to a physiological stress the synthesis of these heat shock proteins may reach extremely high levels. Although the precise function of the heat shock proteins is not clear, many experiments have strongly implicated that they play a role in maintaining processes essential to the lifestyle of the normal, unstressed organism (German, 1984; Bond and Schlesinger, 1987). Concomitant to the elevated amounts of the heat shock proteins, the transcription of most other genes is reduced during heat shock (Ashburner and Bonner, 1979; Schlesinger, Ashburner and Tissieres, 1982; Craig, 1985; Lindquist, 1986). Thus, heat shock or other stressful conditions result in the perturbation of normal gene expression, which, if it persists, may have drastic consequences to the organism. For example, in cultured *Drosophila* cells (Arrigo, 1980) one of the specific changes that occurs upon a 12°C elevation in temperature is the sharp reduction in the rate of transcription of the ribosomal genes. In another experiment, when the intron-containing alcohol dehydrogenase gene of *Drosophila* was placed under the control of the 70-kDa heat shock protein promoter and subject to severe heat shock temperatures, the transcripts were not spliced, thereby resulting in the accumulation of intron-containing precursors (Yost and Linquist, 1986 and 1988). The phenomenon that high temperatures disrupt the splicing of intervening sequences from mRNA precursors has been demonstrated in many organisms, including *Dictyostelium* (Maniak and Nellen, 1988), chickens (Bond and Schlesinger, 1986) and mammals (Kay et al., 1987; Bond, 1988). Thus, heat shock elicits a general block in the splicing of mRNA precursors. Other ways in which gene expression is altered upon heat shock are effects on mRNA stability and translation

(Lindquist, 1980) and transcriptional termination (Velazquez and Lindquist, 1984). In addition, mitosis and DNA synthesis are arrested (Kramhoft and Zeuthen, 1971) and several morphological changes take place. In the nuclei, filaments containing actin are affected; the nucleoli, including the granular ribonucleoprotein components, are transiently damaged and the assembly and export of ribosomes from these organelles are blocked (Pelham, 1984). The Golgi apparatus is disturbed and vimentin-containing filaments around the nucleus collapse and aggregate to form perichromatin granules (Welch and Suhan, 1985). Because heterogeneous nuclear RNA processing, and not transcription per se, appears to be compromised after a heat shock, it is likely that such granules represent accumulated aggregates of unprocessed mRNA (Mayrand and Pederson, 1983; Yost and Lindquist; 1986). Hyperthermia also results in significant changes in the integrity and morphology of the site in the nucleolus involved in ribosome biogenesis. Biochemical studies demonstrated an inhibition of proper ribosomal RNA processing and a corresponding cessation of ribosome biogenesis (Rubin and Hogness, 1975; Bouche et al., 1979).

It has been suggested that the occurrence of these disruptions and many more which were not mentioned, at critical times during gene expression, can lead to deleterious effects in the developing embryos. That is, the expression of essential genes which are necessary for normal embryogenesis processes may be inhibited, thus resulting in malformations.

1.2. The Universal Nature of the Heat Shock or Stress Response

The heat shock or stress response has been observed in almost all organisms examined to date. Whether they be prokaryotes, yeasts, plants, invertebrates, vertebrates or even humans, virtually every organism possesses a set of genes that allows the cells of that organism to be able to tolerate the damages imposed by environmental stress which would otherwise lead to irreversible injuries to the organism (Bond and Schlesinger, 1987).

Although there are differences among the various organisms, the similarities between the species are remarkable. For example, when the sequences of the respective heat shock genes from *Escherichia coli*, yeasts, plants, *Drosophila* and man, to name only a few, were compared, the homologies were found to be amazingly high. Furthermore, when antibodies were used to test for structural similarities between heat shock proteins from a number of distant species, the heat shock proteins were discovered to be among the most highly conserved proteins in nature (Bardwell and Craig, 1984, 1987; Hunt and Morimoto, 1985).

1.3. The Families of Heat Shock or Stress Proteins

The functions of the individual heat shock proteins in cells during normal cellular events, or in those subject to external stress, are still largely based on speculations and correlative studies. However, new information is rapidly accumulating about the roles of some of the heat shock proteins and their constitutively expressed relatives (known as heat shock cognate proteins) in normal cellular processes, which in turn may provide important insight into the possible functions of heat shock proteins during heat stress and thermotolerance. Below is a brief summary (Weber, 1992) of various families of heat shock proteins.

1.3.1. Hsp 110 kDa Family

Of all the major members of the stress protein family, the least is known about the properties of this family of proteins. The 110 kDa stress protein (hsp110) is constitutively expressed and increases approximately 5-fold after stress. This protein appears to be localized within the region of the nucleolus, involved in rRNA transcription and/or processing (Subjeck et al., 1983). Due to the fact that rRNA transcription is inhibited after a heat shock insult, hsp110 is speculated to be involved in the resumption of normal nucleolar transcription processes during recovery from the heat shock.

1.3.2. **Hsp 90 kDa Family**

The mammalian 90 kDa stress protein (hsp90) is very abundant in cells grown under normal conditions. Following a heat stress, the synthesis of the 90 kDa stress protein increases approximately 3- to 5-fold. Hsp90 is heavily phosphorylated with at least 12 isoforms, half of which appear to contain phosphate (Welch et al., 1983). This protein exists as a dimer when it is purified from the cytosolic fraction of cells. In addition, it copurifies with a small amount of the 100 Kda stress protein (Welch and Feramisco, 1982) and it has been shown to interact with a number of other interesting intracellular proteins. For example, hsp90 interacts transiently with a number of tyrosine kinases (Brugge, 1986). In cells transformed with the Rous sarcoma virus, newly synthesized pp60^{src} rapidly associates with the 90 kDa stress protein and another cellular protein of 50 kDa (Sefton et al., 1978; Oppermann et al., 1981). As long as the Src protein is present in this complex, it will not exhibit its normal tyrosine kinase activity nor phosphorylated tyrosine residues. As the complex containing the pp60^{src} reaches the plasma membrane, its final destination, it dissociates. Src is then deposited on the inner side of the plasma membrane and the protein now has both tyrosine kinase activity and autophosphorylated tyrosine. Subsequent studies by numerous groups have shown that many retrovirus-encoded oncogene proteins, most of which appear to be tyrosine-specific protein kinases, interact with p50 and hsp90 proteins in similar ways (Brugge, 1986). Analogously, hsp90 was discovered to associate with only the 8S/9S forms of a number of steroid hormone receptor complexes (Catelli et al., 1985; Sanchez et al., 1985). Each receptor complex consists of the steroid binding protein, hsp90 and hsp73 and is unable to bind DNA. Consequently, these complexes are incapable of activating gene expression. Following binding of the steroid to its receptor, hsp90 and hsp73 dissociate from the complex and the receptor is converted to the biologically active form. As a result, it has been suggested that the presence of hsp90 and/or hsp73 within the 8S/9S form of the steroid receptor complex prevents the binding of the receptor to its gene target in the absence of hormone. Finally, recent data has linked hsp90 with yet another protein kinase, specifically, with the heme-regulated protein kinase that is responsible for phosphorylating the α subunit of eukaryotic initiation factor 2 (eif-2α) (Rose et al., 1988). This initiation factor is involved in the regulation of translational initiation and exhibits an increased level of phosphorylation in some cells upon heat shock (Duncan and Hershey, 1984). Interestingly, the increased phosphorylation of eif-2α results in an inhibition of any new translational events. This may explain why overall translation is transiently arrested in some cells after a severe heat shock (DeBenedetti and Baglioni, 1986; Scorsone et al., 1987).

1.3.3. Hsp 70 kDa Family

Of all the stress proteins, the most is known about this family. There appear to be two major forms of the hsp70 proteins. The hsp73 member is present in abundance in the normal unstressed cell and is referred to as the "constitutive" or "cognate" hsp70 protein. In contrast, the second member, hsp72, is not obvious in most cells grown at physiological temperatures. The synthesis of hsp72, in general, occurs after an external stressful stimulus. However, the exception to this concept occurs in primate cells. To date, in all the primate cells examined (15 human and 3 monkey cell lines), both hsp72 and hsp73 were synthesized in cells grown under normal conditions (Welch et al., 1983).

Immunological, biochemical and DNA sequence analyses demonstrated that hsp72 and hsp73 are extremely homologous (Craig, 1985; Lindquist, 1986). The hsp70 class of proteins bind ATP and are believed to participate in energy-dependent processes such as the disassembly of clathrin-coated vesicles (Chappell et al., 1986), ATP-reversible binding of various abnormal proteins (Pelham, 1986) and translocation of proteins across membranes (Chirico et al., 1988; Deshaies et al., 1988). Cytoplasmic members of the hsp70 family appear to bind directly to nascent polypeptide chains associated with ribosomes during the elongation process (Beckmann et al., 1990). Following a heat shock, members of the hsp70 class of proteins accumulate in the nucleus, primarily in the region associated with ribosome assembly. It has been proposed that they interact reversibly with pre-existing proteins that are exposed as a result of thermal denaturation during heat stress and facilitate correct folding (Pelham, 1988). They may also help in the reassembly of multiprotein structures which were damaged by heat. Thus, the hsp70 class of proteins appears to protect cells from irreversible damage, as well as acting as a molecular chaperonin.

1.3.4. **Hsp 60 kDa Family**

Hsp 60 was first documented as a true heat shock protein in *Tetrahymena* (McMullin and Hallberg, 1987). The hsp60 class of heat shock proteins is homologous to the *Escherichia coli* GroEL gene product which is the major bacterial heat shock protein. The *Escherichia coli* homologue of hsp60 is required for bacteriophage assembly and host DNA replication at normal temperatures (Georgopoulos et al., 1973). There is evidence that GroEL also associates transiently with newly synthesized unfolded polypeptides (Bochkareva et al., 1988). Recently, a protein with a sequence similar to that of GroEL was discovered in the stroma of higher plant chloroplasts (Hemmingsen et al., 1988). The chloroplast homologue was termed RuBisCO subunit binding protein and it appears to facilitate the assembly of ribulose-1,5-biophosphate carboxylase-oxygenase and probably other oligomeric proteins. Hsp60 is synthesized in high levels after a stress, however it is already abundant at normal temperatures.

In mammalian cells, a protein with very high homology to that of hsp60 in *Tetrahymena* was discovered after a stress (Mizzen et al., 1989). The mammalian Hsp60 also localizes within the mitochondria, like that of *Tetrahymena*. It is synthesized in the cytoplasm as a precursor protein of approximately 60 kDa and upon import into the mitochondria it undergoes proteolytic cleavage to produce the mature protein. Due to the high homology between the mammalian hsp60 and the GroEL and RuBisCO subunit binding proteins, the proposed function for the mammalian hsp60 is to facilitate the assembly of oligomeric proteins which are synthesized in the cytoplasm and subsequently translocated into the mitochondria.

1.3.5. The Low Molecular Weight Heat Shock Protein Family

In lower organisms, the small heat shock proteins consist of a family of multiple size and charge forms (Lindquist and Craig, 1988). In contrast, mammalian cells synthesize a single protein, hsp27, which exists as multiple isoelectric forms (Hickey and Weber, 1982; Welch et al., 1983; Arrigo and Welch, 1987; Chretien and Landry, 1988). The different isoelectric forms are probably due to varying degrees of phosphorylation (Kim et al., 1984; Welch, 1985). Interestingly, within minutes after exposure of cells to growth factors, phorbol esters or to calcium ionophores, the phosphorylation of hsp27 increases considerably (Welch, 1985). These agents, unlike heat stress, do not result in the increased synthesis of the heat shock protein. Mammalian hsp27 shares sequence homology with the low molecular weight heat shock proteins from lower organisms and, surprisingly, with the vertebrate lens protein α-crystallin (Ingolia and Craig, 1982; Hickey et al., 1986). Similar to the lens α-crystallin, hsp27 also exists as large protein aggregates with molecular mass ranging from 200-400 daltons in non-stressed cells and forms even larger insoluble aggregates during hyperthermia (Arrigo and Welch, 1987). In all species examined, these aggregates are found in the cytoplasm at normal temperatures and migrate into or around the nucleus upon a heat shock (Lindquist and Craig, 1988). The small heat shock proteins have been reported to associate with mRNA during stress in plant and insect cells (Kloetzel and Bautz, 1983; Nover et al., 1989). This family of proteins shows tissue specific developmental regulation in *Drosophila* (Pauli et al., 1990; Haass et al., 1990). In addition, recently hsp27 expression was shown to be under estrogen regulation in certain mammary tumour cell lines (Fuqua et al., 1989). A member of the hsp27 gene family has been identified as an inhibitor of actin polymerization (Miron et al., 1991). Despite likely roles in both growth signal transduction and thermal resistance, the function of the small heat shock proteins still remains to be understood.

1.4. The Inducers of the Heat Shock or Stress Response

After the discovery that the heat shock response was elicited as a result of heat shock, many investigators found that the induction of the stress response can be achieved by many other stresses which were not heat shock. In this section of the thesis the different types of stress response inducers will be discussed.

1.4.1. Developmental Inducers of the Heat Shock Response

Since most of the studies on the heat shock response have been performed in *Drosophila*, it is not surprising that the best documented data on heat shock gene expression during the course of development are found in these insects. In ovarian nurse cells, messages for hsp26, hsp28 and hsp83 are induced to quite high amounts and these elevated levels are passed on to the developing oocyte (Zimmerman, Petri and Messelson, 1983). On the other hand, hsp70, hsp68, hsp23 and hsp22 messages are not produced and cannot even be induced with a heat shock. Hsp22, hsp23, hsp26 and hsp28 are also induced at the late third instar larva/prepupa stage of development (Sirotkin and Davidson, 1982; Cheney and Shern, 1983). The trigger for this induction during various stages of development is believed to be the molting hormone ecdysone, which reaches a peak in late third instar larvae. Furthermore, ecdysone has been shown to induce the synthesis of the same heat shock proteins in tissue culture cells (Ireland and Berger, 1982).

Ecdysone-resistant cell lines do not express the heat shock proteins when exposed to the molting hormone, but they are capable of producing all the heat shock proteins in response to a heat shock (Berger, Vitek and Morganelli, 1984). This suggests that the two forms of induction are independently regulated. Recently, this has been demonstrated to be the case in both ovarian and prepupal inductions via analysis of genes which contained varying lengths of 5' sequence transfected into the *Drosophila* germ line. Hsp26 sequences which were responsible for ovarian induction were located 522 to 352 bases upstream of the transcription start site, whereas the sequences responsible for heat shock induction were located 341 to 14 bases upstream (Cohen and Meselson, 1985). In similar experiments, the sequences responsible for 80% of the heat-induced synthesis of hsp28 mapped more than 1100 bases upstream of the start site. Meanwhile, the sequences responsible for prepupal induction were located within 227 bases of the gene (Hoffman and Corces, 1986).

In mouse embryos, certain heat shock proteins appear to be constitutively expressed. In an eight-day old mouse ectoderm and in embryonal carcinoma cells two prominent proteins of 70 and 89 kilodaltons are evident. Unfortunately, it is not clear whether these proteins are identical to the heat-inducible proteins or other members of the same families (Bensaude and Morange, 1983).

1.4.2. **Other Inducers of the Heat Shock Response**

The earliest studies of heat-induced puffs in *Drosophila* demonstrated that they could be induced by a variety of other stress treatments besides heat shock. The induction of the stress response can be obtained by the release from anoxia, addition of sodium azide (a poison of cellular respiration) or addition of 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation) (Ritossa, 1962; Ritossa 1964). Since the stresses above disrupted oxidative phosphorylation or electron transport, Ritossa (1962, 1964) speculated that the induction of the stress response served to protect cells from respiratory stress. However, several lines of evidence now indicate that this notion is probably not correct. First of all, a large number of agents with no specificity for respiratory processes were discovered which induced the stress response. Secondly, yeast mutants that are respiration-deficient nevertheless express heat shock proteins in response to high temperatures (Lindquist et al., 1982).

Many inducers are effective across a broad range of species. However the effectiveness often varies, depending on the species. Some of these inducers include sodium arsenite, ethanol, sulfhydryl reagents, hydrogen peroxide, transition series metals, amino acid analogs and even viral infection (Nover, 1984; Craig, 1985; Lindquist and Craig, 1988). Ethanol elicits a stress response in mammalian cells (Li, 1983), yeast (Plesset, Palm and McLaughlin, 1982) and *E. coli* (Neihardt, VanBogelen and Vaughn, 1984). Sodium arsenite induces the proteins in *Drosophila* (Tanguay and Vincent, 1982). mammalian cells (Li, 1983), soybeans (Key et al., 1985) and trout (Kothary and Candido, 1982). Cadmium induced the expression of the heat shock proteins in *Drosophila* (Courgeon, Maisonhaute and Best-Belpomme, 1984), mammalian cells (Li and Laszlo, 1985) and soybeans (Key et al., 1985). However, there are many species-specific differences. For example, ethanol, sodium arsenite and cadmium do not induce a heat shock response in *Dictyostelium* (Rosen et al., 1985).

1.5. The Phenomenon of Thermotolerance

When organisms are exposed to a severe enough heat shock or other stress, the majority will die. If, however, they undergo a mild, sublethal heat treatment prior to the fatal heat shock, a considerable fraction of them survive. The earliest studies on mammalian cells that documented that heat induces a transient state of heat resistance were those of Henle and Leeper (1976). They discovered that thermotolerance was inducible in Chinese hamster cells by a short heat treatment of 5 to 15 minutes at 45°C followed by incubation at 37°C. Resistance to a second lethal heat shock at 45°C was shown when the survival curves of cells which were pre-heated sublethally were compared to those that were not heated. Cells which were first given a sublethal heat treatment survived far better than the unheated cells. Upon examining the kinetics of induction and decay of thermotolerance, cells were maximally sensitive immediately after heating. Thermotolerance was maximal if an interval of about 8 hours existed between the first sublethal heat treatment and the second lethal heat shock. Resistance was shown to persist for at least 48 hours. Another example of thermotolerance is demonstrated in *Drosophila*. Most or all third-instar *Drosophila* larvae die following a 20 minute heat shock period at 40°C. However, if the larvae are pretreated at 33 to 35°C for 30 minutes and then exposed to the lethal heat stress, as many as 50% of them survive and even develop normally (Mitchell et al., 1979). Furthermore, heat shock proteins were induced while overall rates of RNA and protein synthesis were only slightly affected after the sublethal pretreatment at 33 to 35°C. Interestingly, this pretreatment with heat can be replaced by other inducers of heat shock protein synthesis, such as ethanol or arsenite, with similar effects. Since the induction of thermotolerance appears to correlate closely with the expression of heat shock proteins, particularly with hsp70 (Li and Laszlo, 1985), it is likely that at least one of the heat shock proteins probably plays a role in the development of thermotolerance. Observations similar to those mentioned have been reported in a wide variety of cells in culture and in organisms, including mice, soybean seedlings, slime molds, yeast, bacterial cells and *Drosophila* at most of its stages of development.

1.6. **Expression of Heat Shock or Stress Proteins During Human Disease**

The increased expression of stress proteins has been observed in cells and tissues in a broad distribution of human diseases including ischemia, cardiac hypertrophy, inflammation, fever, metabolic diseases and cell and tissue damage. The purpose of abnormal expression of stress proteins in human tissues is still unclear. It may represent an acute response to an altered physiological state or a chronic adaptation to a particular disease state. Although the primary function of the stress response is believed to be protective, the chronic abnormal expression of heat shock proteins may have deleterious effects. For example, the accumulation of stress proteins during a chronic physiological stress could lead to an arrest of biosynthetic activities or a reduced ability of the affected cells to respond to subsequent exposures to stressful conditions (Morimoto, Tissieres and Georgopoulos, 1990).

Exposure to transient ischemia has detrimental consequences on affected tissues. During postischemic reperfusion, there is a rapid activation of stress protein expression in the brain, kidney, heart and liver tissues (Currie and White, 1981; Currie, 1987; Mehta et al., 1988).

The mechanism by which ischemia and reperfusion induce stress gene expression and whether the induced synthesis of stress proteins is important during recovery from the disease state is unclear. One possible mechanism for ischemic damage that occurs during reperfusion involves the generation of oxygen free radicals (Benjamin, Kroger and Williams, 1990). Damage caused by free radicals can also occur following exposure to a wide variety of environmental agents including xenobiotics, aromatic hydrocarbons and photochemical air pollutant (Micheals and Hunt, 1978; Jenkinson, Anderson and Gangolli, 1986). Free radicals are known to have a broad range of cellular targets including lipid oxidation, DNA damage and effects on amino acid modification, cross-linking and general effects on protein conformation and activity (Lown, Joshua and Chen, 1982; Nicotera et al., 1985). Prokaryotic cells also have an adaptive response to the toxic side effects of molecular oxygen. For example, H_2O_2 induces a set of genes similar to those found in the family of heat shock proteins (Christman et al., 1985; Morgan et al., 1986).

Among the earliest molecular events detected after aortic constriction or work-overload-induced cardiac hypertrophy is the elevated expression of hsp70 (Dillman et al., 1986). Activation of the stress response could be due to the aberrant synthesis, accumulation or degradation of both existing and newly synthesized proteins. Alternatively, the hsp70 gene may be responding to cellular signals in the hypertrophic state.

Investigation of antigens involved in the immune response to tuberculosis and leprosy bacilli, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, respectively, led to the observation that stress proteins are among the major targets of the immune response (Shinnick, Vodkin and Williams, 1988; Young et al., 1988). The observations that stress proteins are important targets of the immune response to mycobacterial infection, and that the major stress proteins are conserved and abundant in other organisms, suggest that stress proteins are likely to be immune targets in many nonviral infections. This is indeed the case. Antigens from a wide variety of infectious agents have been identified as members of stress protein families (Young and Elliott, 1989). The major stress protein antigen recognized by antibodies in bacterial infections is hsp60. "Common antigen", an immunodominant protein antigen shown to be shared by most bacterial species, turns out to be hsp60 (Shinnick, Vodkin and Williams, 1988). It is not yet clear whether other stress proteins are general targets of the immune response to bacteria. Stress proteins have also been identified as immune targets in most major human parasite infections (Young et al., 1989). Antibodies to hsp70 have been identified in the sera of patients suffering from malaria (Bianco, 1986; Ardeshir et al., 1987; Yang et al., 1987; Peterson et al., 1988; Mattei, Ozaki and da Silva, 1988), trypanosomiasis (Engman, Kirchoff and Donelson, 1989), leishmaniasis (Macfarlane et al., 1989), schistosomiasis (Hedstrom et al., 1987; Hedstrom et al., 1988; Newport et al., 1988; Scallon, Bogitsh and Carter, 1987) and filariasis (Selkirk et al., 1987; Selkirk et al., 1989; Rothstein et al., 1989). Hsp90 is also a target of antibodies in trypanosomiasis (Dragon et al., 1987) and a member of the small heat shock protein family is observed in some patients with schistosomiasis (Nene et al., 1986).

Rheumatoid arthritis is characterized by a chronic inflammatory reaction in synovial membranes that is thought to involve autoimmune processes. Patients with rheumatoid arthritis were discovered to have increased levels of antibodies to the 65 kilodalton and 70 kilodalton families of heat shock proteins, particularly to the bacterial homologue of hsp65, *Mycobacterium bovis* (Bahr et al., 1990). It is not known whether these antibodies were originally induced as part of the immune response to a previous infection, or as a secondary consequence of the tissue breakdown and failure of the immune regulation occurring during autoimmune disease.

1.7. The Heat Shock Response During Normal Development

The elevated expression of stress genes during early stages of embryonic development and differentiation, in the absence of heat shock or other physiological stress, has been widely observed among diverse eukaryotic species (Craig, 1985; Heikkila et al., 1986; Lindquist and Craig, 1988). One of the first heat shock genes shown to be developmentally regulated was the small heat shock protein, hsp23, which was ecdysone-inducible in *Drosophila* tissue culture cells (Ireland and Berger, 1982) and in imaginal discs (Ireland et al., 1982). Hsp23 expression was also detected in the absence of heat treatment in early ovarian development (Zimmerman, Petri and Messelson, 1983).

Hsp68 and hsp70 are among the first proteins to be detected at the two-cell stage of postfertilization development, early in mouse embryogenesis (Bensaude et al., 1983). High levels of hsp70 and hsp89 were also discovered during the morula/blastocyst stages of preimplantion embryos (Wittig et al., 1983; Morange et al., 1984). Despite this, two to eight cell embryos cannot produce heat shock proteins after a temperature elevation and are thermosensitive (Wittig et al., 1983; Morange et al., 1984). In contrast, blastocysts are able to elicit a heat shock response following a stress and develop thermotolerance (Muller, Li and Goldstein, 1985). In 1987, Kothary et al. demonstrated that hsp68 mRNA was present in the trophectoderm and in the primitive endoderm at late fetal stages in mouse embryos.

In the developing postnatal rat, *in vivo* hyperthermia results in the synthesis of hsp71 in heart and adrenal tissues from an age of 1 day old to 25 weeks old (Currie and White, 1983). During this period, overall protein synthesis decreased, but the amounts of hsp71 remained relatively consistent in heart and lung tissues and increased approximately 50 fold in brains. The synthesis of hsp71 in 3 week old rats and in older rats coincides with the maturation of brain capillaries and the blood-brain barrier (Currie and White, 1983). High concentrations of hsp71 were discovered in fractions rich in cerebral microvessels.

1.8. The Heat Shock Response During Abnormal Development

As previously discussed in this thesis, stress or heat shock proteins are synthesized by many organisms in response to adverse environmental conditions (Hahnel et al., 1986). In this section, the focus will be on the effects of hyperthermia and cadmium on the organism and on the stress response.

1.8.1. Hyperthermia and Teratogenicity

Over the last 25 years many investigators have linked hyperthermia with the development of malformations in the embryo. When pregnant rats carrying embryos on days between 9 to 14 of gestation are exposed for 40 to 60 minutes in an incubator that raised deep body temperature from 38.1

± 0.51°C to 42.7 ± 0.62°C, increased fetal resorptions, retardation of growth and a number of other developmental defects are observed (Edwards, 1968). In addition, embryos exposed on different gestational days developed specific types of malformations. Grossly apparent fetal malformations included microphthalmia after exposure on day 9 of gestation, anencephaly after exposure on day 10 and defects of tails, limbs, toes, palate and body after exposure on days 12 to 14 of gestation. In a similar study, pregnant Sprague-Dawley rats were partially immersed in a water bath until their core body temperature reached 43.5°C and the procedure was repeated 6 hours later (Webster, Germain and Edwards, 1985). The two heatings were performed over a range of developmental stages from early gastrulation (8 days and 18 hours) to about the 12 somite stage (10 days and 18 hours). The main teratogenic effects observed were head-related malformations. Microphthalmia was the most common defect with approximately 90% of surviving fetuses having small eyes when heating occurred between 9 days and 6 hours and 10 days. Encephaloceles were induced in embryos exposed between gestational days 9 and 10, where 57% of surviving embryos were affected. Finally, maxillary hypoplasia resulted from heating between days 9.06 and 10.06 of gestation with 20% of surviving fetuses affected. The period most sensitive to hyperthermia was determined to span from days 9.06 to 10, which consists of a large proportion of the gastrulation stage of embryogenesis. A number of similar developmental abnormalities was also observed in *in vitro* heat treated rat embryos (Walsh et al., 1987). Presomite rat embryos (day 9.5) were explanted and exposed to elevated temperatures of 42.0, 42.5, 43.0 or 43.5°C for varying lengths of time (0.5 to 80 minutes). Four phenotypes were obtained following a brief exposure to an elevated temperature of 43°C for 7.5 minutes: gross retardation in the forebrain region, microcephaly, microphthalmia and open neural tubes. The severity of the malformations were dose-dependent. Embryos which were treated with a mild, nonteratogenic exposure to heat were protected against a subsequent exposure which would otherwise cause severe craniofacial defects (Walsh et al., 1987). This induction of thermotolerance correlated well with the synthesis of heat shock proteins (Walsh et al., 1987).

Exposure of postimplantation rat embryos on days 9, 10, 11 and 12 of gestation to an *in vitro* heat shock of 43°C for 30 minutes results in the induction of heat shock proteins in day 9 and 10 embryos, a severely attenuated response in day 11 embryos and no detectable response in day 12 embryos (Mirkes, Grace and Little, 1991). The heat shock response is characterized by the synthesis of heat shock proteins with molecular weights of 28 to 78 kilodaltons as visualized by a two-dimensional gel electrophoresis.

1.8.2. **Cadmium and Teratogenicity**

Cadmium has been found to be extremely toxic in many systems. In one study, the effects of heavy metals on chick embryogenesis was examined (Gilani and Alibhai, 1990). Of all the metals studied, cadmium was discovered to be the most embryotoxic with an LD_{50} value of 3 μg/egg. Some of the malformations observed were micromelia, abnormal beaks, reduced body size and hemorrhage.

Klein et al. (1990) cultured rat embryos for 48 hours *in vitro* with serum taken from rats injected intraperitoneally with cadmium to evaluate the teratogenic activity of this heavy metal. Susceptible embryos were hemmorrhagic and exencephalic and lacked forelimb buds. They also possessed fewer somites than control embryos (14-15 compared to 21-26). Furthermore, the protein and DNA contents of these abnormal embryos were significantly reduced compared to those of control.

The exposure of cultured CD-1 mouse embryos to 1.75 μM cadmium was lethal to 55% of the embryos and of the surviving embryos, 87% were malformed (Kapron-Bras and Hales, 1991). However, when the embryos were exposed to a sublethal heat shock prior to the addition of cadmium, a significant reduction of dead and malformed embryos were observed. Some of the malformations obtained from only the cadmium treatment included growth retardation and abnormalities of the eye and branchial arches.

1.9. Rational for the Experiments in this Thesis

Little is known about the pathway leading from the stressful stimulus to a malformation and the possibility of the heat shock response being linked to this phenomenon which therefore prompted the studies in this thesis. As previously described in this chapter, the treatment of embryos with hyperthermia or cadmium was found to be embryolethal. In addition, those embryos that survived were malformed. In order to determine the ability of heat or cadmium to induce a heat shock response, the steady-state mRNAs of two heat shock proteins were used as indicators of the stress response in the rat embryos during organogenesis. Hsp27 and hsp70 were chosen based on the ability of hyperthermia and cadmium to specifically induce these heat shock proteins in similar systems (mentioned previously). Therefore, to understand this response in the rat embryo during the critical period of gestation, rat embryos on day 10 of gestation were exposed to two forms of environmental stress, a 43°C heat shock for varying lengths of time and a 2.5 μM dose of cadmium chloride, and subsequently cultured for varying lengths of time. The results from these experiments were utilized to see whether there was a relationship between the duration of heat shock and the degree to which the mRNA levels of hsp27 and hsp70 accumulated. In addition, collecting embryos at different time points after hyperthermia and cadmium treatment allowed the time course of the heat shock response to be examined through the accumulation of hsp27 and hsp70 mRNAs. Finally, the results obtained in this thesis will allow the formulation of a possible connection between the ability of hyperthermia or cadmium treatments to lead to malformations and the ability of the embryos to mount a heat shock response.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

Timed pregnant Sprague-Dawley rats (200-250 g) were purchased from Charles River Canada (St. Constant, Québec). The day on which spermatozoa were found in the vaginal smear was considered day zero of pregnancy. Rats were housed in the McIntyre Animal Centre (McGill University, Montreal, Québec) at 22-25°C with a 12-hour light-dark schedule. Rats were supplied with Purina rat chow and tap water ad libitum.

2.2. Embryo Culture

Rat embryos were cultured using the method described by New (1978). Explantation of embryos from the dam took place in Hank's Balanced Salt Solution (HBSS) (Gibco Laboratories, Burlington, Ontario). Pregnant rats were given an overdose of ether to kill them. The uteri of etherized pregnant rats were explanted on the morning of day 10 of gestation. The embryos were then dissected free of maternal tissue and Reichert's membrane while the visceral yolk sac and the ectoplacental cone were left intact. Embryos from each litter were split between control and experimental groups. A minimum of three embryos were placed in each sterile 60 ml glass bottle containing 4.8 ml of filter-sterilized (0.45 μM pore diameter filters) medium, made up of 90% heat-inactivated rat serum and 10% Tyrode's saline (Gibco Laboratories). Immediately before culture, 10 U/ml penicillin G (Gibco Laboratories) and 10 μg/ml streptomycin (Gibco Laboratories) were added to the medium. The contents of the bottles were gassed with a mixture of 20% oxygen, 5% carbon dioxide and 75% nitrogen (Medigas Inc., St. Laurent, Québec). The bottles were placed in a 37°C incubator and rotated at 30 rpm.

2.3. **Heat and Cadmium Treatments**

The contents of the bottles were equilibrated at 37°C for 2 hours. The bottles containing the heat-treated embryos were placed upright in a 43°C waterbath for 5, 10, 15 or 20 minutes, while those containing the control embryos were left in the 37°C incubator. The medium in the heat-treated bottles reached the designated temperature within 2 minutes and remained within 0.2°C of that temperature for the duration of the treatment time. After the different lengths of heat shock, embryos were returned to the 37°C incubator and cultured for an additional 1, 2, 4 or 20 hours. *In vitro* control and heat-treated embryos and yolk sacs were then collected separately for Northern blot analysis. *In vivo* control embryos and yolk sacs were also collected at the end of each heat treatment experiment.

In a separate experiment, cadmium was added to the medium 1 hour after the start of culture in the form of cadmium chloride (Aldrich Chemical Company Inc., Milwaukee, Wisconsin) in distilled water. The final concentration of cadmium was 2.5 μM; the metal remained in the medium throughout culture periods of 2, 6 or 20 hours. Each cadmium group had an *in vitro* control cultured simultaneously. Embryos and yolk sacs were collected separately for Northern blot analysis.

At least three replicates were done for each of the different heat and cadmium treatment groups.

2.4. Northern Blot Analysis

2.4.1. cDNA Probes

Hsp27 rat cDNA probe. The specific rat cDNA probe for hsp27 was kindly provided by Dr. M.J. Welsh (University of Michigan, Ann Arbor, Michigan). The 0.85 kilobase hsp27 cDNA insert (GeneBank Accession number M86389) was cloned into the EcoRI cut site of a Bluescript SK vector. JM101 bacteria was transformed with the plasmid containing the DNA insert by the calcium chloride procedure (Sambrook et al., 1989). An overnight culture of JM101 bacteria was prepared using 50 μl of glycerol bacteria stock in 5 ml LB (Luria-Bertani) broth. The bacteria were grown overnight at 37°C in a shaking water bath. In a 500 ml flask, 1 ml of overnight culture was added to 50 ml of prewarmed LB broth. The cells were grown at 37°C with vigorous shaking for 1-2 hours or until the optical density reached 0.5 at a wavelength of 550 nm. The bacteria were transferred to a 50 ml sterile centrifuge tube, spun at 3000 rpm for 10 minutes at room temperature and left on ice. The supernatant was discarded and the pellet was resuspended in 20 ml ice-cold 50 mM calcium chloride (filter-sterilized) and left on ice for 20 minutes. The cells were spun again at 3000 rpm for 10 minutes at room temperature. The pellet was resuspended in 2 ml 50 mM calcium chloride and left on ice for 1 hour. In prechilled 15 ml test tubes, the following transformation reactions were prepared: 2 sets each of 0.2 ml host cells (negative controls) and 0.2 ml host cells with 2 μl of 50 ng/μl Bluescript vector containing the hsp27 insert. The transformation reactions were placed on ice for 40 minutes, heat shocked in a 42°C waterbath for 2 minutes and returned to the ice. LB broth, 0.4 ml, was added to one set of control and cells containing the vector, while 0.8 ml LB broth was added to the other set. The tubes were incubated for 1 hour in a shaking 37°C waterbath. Cells in 150 and 300 μl aliquots were taken from the tubes containing 0.4 ml LB broth and spread onto LB plates which contained 1 ml of 50 μg/ml ampicillin. Cells, 150, 300 and 400 μl aliquots, were also taken from the set of tubes containing 0.8 ml LB broth and spread onto another set of LB plates. LB broth only, 300 μl, was spread on two plates as negative controls. The plates were allowed to dry for 15-20 minutes before incubating them overnight at 37°C. The next day 5 ml of LB broth and 10 μl of stock ampicillin were added to each of 6 sterile tubes. Individual colonies from the plates were picked and added to each tube. The tubes were placed in a 37°C shaking waterbath overnight.

A mini-preparation was performed on the plasmid DNA according to the alkaline lysis method (Sambrook et al., 1989). A 1.5 ml aliquot of the culture was poured from each of 6 tubes into 6 Eppendorf tubes and centrifuged for 1 minute at 10,000 *g*. The remainder of the overnight culture was stored at 4°C. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended by vortexing in 100 μl of an ice-cold solution of 50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl (pH 8.0). An aliquot of 200 μl freshly prepared 0.2 N NaOH/1% SDS was added. The contents of the tubes were mixed by inverting the tubes rapidly 3 to 5 times and then stored on ice for 5 minutes. An aliquot of 150 μl of an ice-cold solution of 5 M potassium acetate was added to each tube. The contents were vortexed gently and stored on ice for 5 minutes. The tubes were spun at 10,000 *g* for 10 minutes at 4°C and the supernatants were transferred to 6 fresh tubes. An equal volume of phenol:chloroform was added to each tube and the contents mixed by vortexing. After centrifuging for 5 minutes at 10,000 *g*, the supernatant of each sample was transferred to a fresh tube. The double-stranded DNA was then precipitated with two volumes of 100% ethanol. The contents of the tubes were vortexed and allowed to sit at room temperature for 2 minutes. The samples were spun for 5 minutes at 4°C at 10,000 *g*. The supernatants were poured out and the tubes were inverted for 1 minute to air dry samples. The pellets were washed with 1 ml of 70% ethanol, the supernatants poured off and the pellets briefly vacuum dried. The DNA was then dissolved in 20 μl tris-EDTA, 0.5 μl RNAse A, DNAse free, and incubated at 37°C for 30 minutes. In order to cut out the hsp27 insert, a restriction digest of the 6 DNA samples were performed using EcoRI (Pharmacia LKB, Montreal, Québec). A mixture of 20 μl of DNA, 2 μl EcoRI and 3 μl One-Phor-All Buffer *PLUS* (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate) was incubated at 37°C for 1 hour. The DNA fragments in the restriction digests were analyzed by gel electrophoresis. A 1 kilobase DNA ladder was run simultaneously with the samples in order to confirm that the size of the insert was indeed 850 base pairs. The samples were fractionated on a low-melting agarose gel and bands around the size of 850 base pairs were visualized on the gel under ultraviolet light. The bands were cut out and the amount of DNA was estimated to be about 10 μg.

Gelase (Cedarlane Laboratories Ltd., Hornby, Ontario) was used to digest the low-melting agarose containing the hsp27 CDNA probe. First, the tubes containing the low-melting agarose and the cDNA probe were incubated at 65 °C for 30 minutes. The tubes were subsequently incubated at 40°C for 40 minutes. GELase, 8 units was added to each tube. The contents of the tubes were vortexed and incubated at 40°C for 1 hour. The DNA was stored at -20°C.

Hsp70 mouse cDNA probe. The specific mouse cDNA probe for hsp70 was kindly provided by Dr. R.I. Morimoto (Northwestern University, Evanston, Illinois). The 4.3 kilobase segment of hsp70 mouse DNA was selected from a lambda phage library and subcloned into plasmid PBR322 at the BAMHI site within a tetracycline-resistant gene. Therefore, the plasmid is tetracycline-sensitive and ampicillin-resistant. The protocol for preparing the hsp70 cDNA probe was similar to that for hsp27 cDNA probe. The hsp70 cDNA was also transformed into JM101 bacteria. However, the restriction enzymes used were BamHI, EcoRI and PstI (Pharmacia LKB). Utilizing these restriction enzymes, an insert of 3.8 kilobases was excised from the PBR322 plasmid. This insert contained part of the murine coding sequence for hsp70.

2.4.2. Oligolabelling of both hsp27 and hsp70 cDNA Probes with ^{32}P

Both hsp27 and hsp70 cDNA probes were labelled with ^{32}P by random priming using an oligolabelling kit (Pharmacia LKB). Following the protocol below, 50-100 ng of DNA (cDNA probe) was labelled to a specific activity of 1-5 X 10^8 dpm/µg using [α-^{32}P]dCTP at 3000 Ci/mmol. The appropriate amount of DNA was suspended in sterile deionized water up to a volume of 34 µl. The DNA to be labelled was first denatured in a water bath at 95-100°C for 5 minutes. The DNA was placed immediately on ice for 5 minutes. Then 10 µl of a reagent mix (buffered aqueous solution containing dATP, dGTP, dTTP and random hexadeoxyribonucleotides), 5 µl of [α-^{32}P]dCTP (3000 Ci/mmol) and 1 µl of Klenow fragment (buffered glycerol solution, 5-10 units/µl) were added to the DNA. The reaction mixture was gently mixed, then incubated at 37°C for 1-2 hours. The random hexamers present in the reagent mix annealled to random sites on the DNA and served as primers for DNA synthesis by the Klenow fragment of *E. coli* DNA polymerase I. The labelled nucleotide present during the synthesis allowed for the generation of highly labelled DNA. The labelled fragments were separated from the free nucleotides by purifying the DNA on a Sephadex G-50 column. These fragments were denatured from the template cDNA probe in a water bath at 95-100°C for 5 minutes and then immediately cooled on ice for 5 minutes. The labelled probe was added to the hybridization solution along with the membrane at 10^6 cpm/ml.

2.4.3. Isolation of RNA

RNA was extracted from the collected embryos and yolk sacs of each experimental group using the acid guanidinium-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The following protocol describes isolation of RNA for 100 mg of tissue. First, 1.0 ml of solution A (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol) was added to frozen embryo and yolk sac samples. Samples were vortexed until tissues were suspended in the solution A. Then 0.1 ml of 0.2 M sodium acetate pH 4.0, 1 ml of phenol (saturated with water), 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added sequentially to the samples and vortexed thoroughly after the addition of each reagent. Samples were centrifuged at 4°C for 20 minutes at 10,000 *g* after having been placed on ice for 20 minutes. After centrifugation, the upper aqueous phase was transferred to a sterile tube, mixed with 1 ml of isopropanol and placed at -20°C for a minimum of 1 hour to allow the RNA to precipitate. Samples were centrifuged again for 20 minutes at 10,000 *g*. The supernatant was discarded while the RNA pellet was dissolved in 0.3 ml solution A and precipitated with 0.3 ml isopropanol for a minimum of 1 hour at -20°C. After centrifuging the samples for 15 minutes at 4°C, the pellets were washed with 0.5 ml of 75% ethanol. Samples were centrifuged again for 15 minutes and vacuum dried for 15 minutes. The RNA pellets were dissolved in 10-20 μl diethyl pyrocarbonate-treated water (ICN Biomedicals Canada, Mississauga, Ontario) at 65°C for 10 minutes. The RNA preparations were stored at -70°C for further use. The concentration of total cellular RNA was measured by the absorbance at 260 nm using a Beckman DU-7 spectrophotometer.

2.4.4. **Fractionation of RNA and transfer onto a nylon membrane**

Total RNA (10 μg per sample) was denatured by adding formaldehyde, formamide and borate buffer (0.1 M sodium borate, 0.1 M boric acid, and 0.001 M EDTA, pH 8.0) to a final concentration of 16%, 40% and 0.02 M, respectively, and heating the samples for 5 minutes at 65°C. The denatured RNA samples were separated in a 1% agarose gel containing 0.02 M borate buffer and 16% formaldehyde (BDH Inc., St. Laurent, Québec). The gel was electrophoresed in recirculated 0.02 M borate buffer at 20 volts for 20 hours at 25°C (Pharmacia LKB). The separated RNA was transferred onto a GeneScreen Plus nylon membrane (Du Pont Canada, Mississauga, Ontario) by vacuum blotting for 2 hours using a Vacugene blotting system (Pharmacia LKB Biotechnology, Uppsala, Sweden). To facilitate the vacuum transfer, the agarose gel was pretreated with 50 mM NaOH/10 mM NaCl for 20 minutes and then neutralized with 0.1 M Tris (pH 7.4) for 20 minutes. The final transfer buffer added was 20 X SSC (1 X SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 80 minutes. The membrane was rinsed in transfer buffer and baked in a vacuum oven at 80°C for 2 hours before hybridization.

2.4.5. Hybridization of cDNA probes to RNA

The membrane was prehybridized in 50% deionized formamide, 1% sodium dodecyl sulfate (Mallinckrodt Canada Inc, Pointe-Claire, Québec), 1 M NaCl, 5 X Denhardt's solution (100 X Denhardt's solution consists of 2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone), 100 μg/ml yeast tRNA and 5 μg/ml poly A RNA at 42°C for 2 hours. The labelled hsp27 and hsp70 cDNA probes were then added separately to the hybridization solution and allowed to incubate overnight with the membrane. After hybridization, the membrane was washed with 2 X SSC at room temperature twice for 10 minutes and with 2 X SSC and 1% SDS at 60°C twice for 30 minutes. The membrane was then exposed to Kodak Scientific Imaging Film X-OMAT AR (Eastman Kodak Company, Rochester, New York) at -80°C using intensifying screens. The film was developed from 1 day to 1 week later. Between each hybridization, the previous probe was removed by boiling the membrane twice in 0.01 X SSC/0.1% SDS for 30 minutes each time. A 0.24-9.5 kilobase RNA ladder purchased from Bethesda Research Laboratories (Burlington, Ontario) was used to calibrate the gels.

2.4.6. 18S ribosomal RNA Probe

To normalize for the amount of RNA present in each lane, the membrane was hybridized to a ^{32}P-end-labelled synthetic oligonucleotide 24-mer probe (Sheldon Biotechnology Centre, Montreal, Québec) recognizing the 18S ribosomal RNA sequence (Szyf et al., 1990). Hybridization conditions were 6 X SSC, 5 X Denhardt's solution, 1% SDS, 100 μg/ml denatured salmon sperm DNA and 18S probe (10^6 cpm/ml) at 42°C for 20 hours. The membrane was subsequently washed twice with 6 X SSC at room temperature for 30 minutes each wash and twice with 6 X SSC/1% SDS at 55°C for 30 minutes each wash. The membrane was exposed to Kodak film X-OMAT AR at -80°C using intensifying screens. The film was developed a few hours to 1 day after it was packed with the blot.

2.5. Quantitation of mRNA

Autoradiographs were scanned using an LKB laser densitometer (Pharmacia LKB Biotechnology). The integrated areas obtained for heat shock protein mRNA signals were normalized to the areas obtained for the 18S ribosomal RNA oligonucleotide probe and adjusted to a reference point. At least three independent autoradiograghs for each treatment group were scanned; the values represent the means ± standard errors of the mean. The resolution of the UltroScan XL laser densitometer is better than 55 μm and the linear range is from 0 to 4 OD units: all absorbances were within the linear range.

2.6. Statistics

Statistical analysis was carried out using the Complete Statistical System (CSS) computer program (Statsoft Inc., Tulsa, Oklahoma). The relative absorbances of control groups and treatment groups were compared by analysis of variance (ANOVA) and by the Newman-Keuls test (Zar, 1974). In all cases value of $P \leq 0.05$ was considered significant.

CHAPTER 3

RESULTS

3.1. Heat Treatments

3.1.1. Morphology of Embryo Exposed to Heat Shock

Exposure of embryos to a temperature of 43°C for 5 or 10 minutes was not embryolethal, whereas after exposure for 15 or 20 minutes approximately 50% and 90%, respectively, of the embryos died. The incidence of malformations increased in proportion to the duration of heat shock; the greatest incidence of malformations was seen after a 20 minute exposure at 43°C and virtually no malformations were observed in embryos exposed for only 5 minutes. Figure 1B shows an embryo cultured for 20 hours following a 20 minute heat shock, the most severe dose of heat used in this experiment. An overall retardation in growth is observed when compared to the control embryo (Figure 1A). The main teratogenic effects of hyperthermia are head-related defects, such as microphthalmia, small forebrain and abnormal branchial arches. In addition, the heart was affected and in some cases the limbs were malformed. Turning of the embryos was also retarded. The yolk sac appeared relatively normal morphologically, however blood clotting was observed in some heat treated

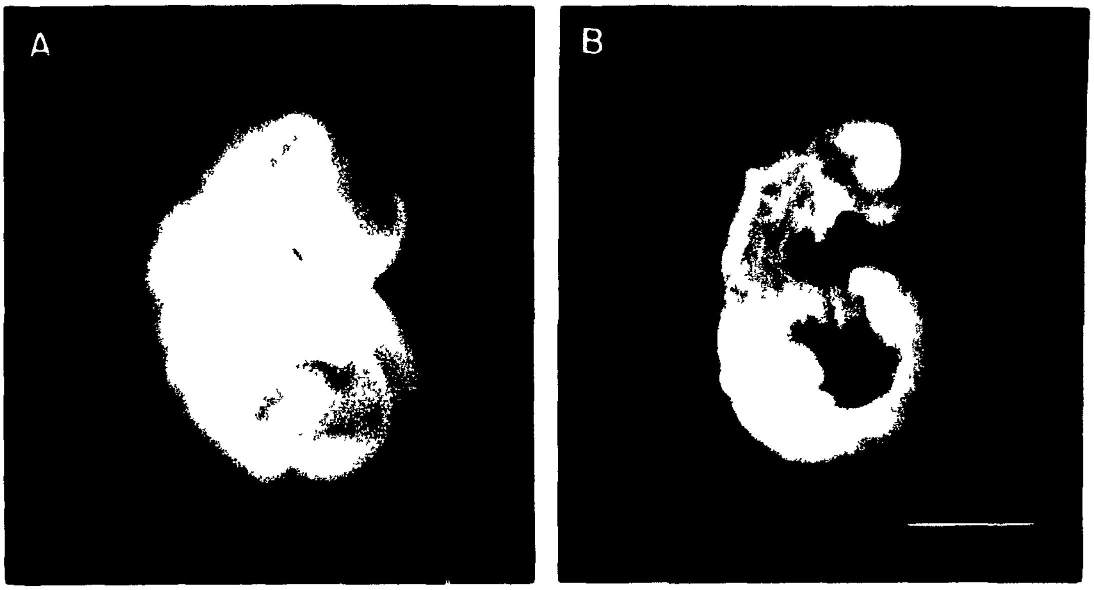


Figure 1. Postimplantation rat embryo explanted on day 10 of gestation and cultured for 20 hours at 37°C *in vitro* (A). Postimplantation rat embryo explanted on day 10 of gestation and heat shocked for 20 minutes at 43°C then returned to 37°C and cultured for an additional 20 hours *in vitro* (B). Bar represents 1 mm.

embryos (not shown).

3.1.2. Size of Hsp27 and Hsp70 Transcripts

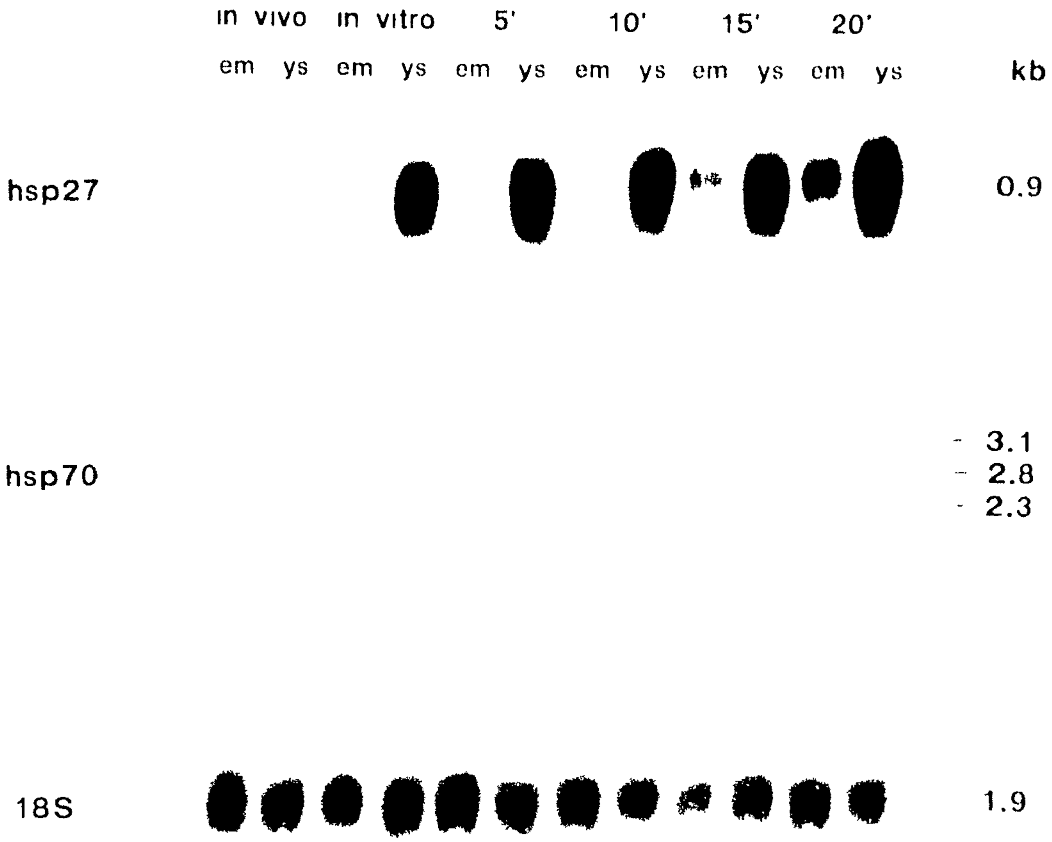
Upon Northern blot analysis, the steady-state mRNA concentrations of HSP27 and HSP70 were analyzed in both embryos and their yolk sacs. The cDNA probe coding for the 27-kDa heat shock protein hybridized to an mRNA species of 0.9 kilobases in both embryos and yolk sacs under control and heat treatment conditions (Figures 2, 4, 6 and 8, top panels). However, the signal in control embryo tissue was very weak. In contrast, the 70-kDa heat shock protein probe hybridized to three mRNA species; a constitutive mRNA species of 2.3 kilobases and two inducible mRNA species of 2.8 and 3.1 kilobases (Figures 2, 4, 6 and 8, middle panels). The 0.9 kilobase HSP27 mRNA species and the two inducible transcripts of HSP70 were quantitated by laser densitometry. Values from three independent Northern blots were standardized with the 18 S rRNA (Figures 2, 4, 6 and 8, bottom panels) and the means are shown on the graphs in Figures 3, 5, 7 and 9.

3.1.3. Dose-dependent Response to Heat Shock

To examine the dose-dependence of the response to heat shock, cultured embryos on day 10 of gestation were heated at 43°C for 5, 10, 15 or 20 minutes. Two control groups were used in all heat shock experiments; the *in vivo* control group consisted of a set of embryos collected directly from the dams at the same time that the heat treated embryos were collected and the *in vitro* control group consisted of embryos cultured alongside those that were heat-treated.

Figure 2 shows the steady-state mRNA concentrations of both hsp27 and hsp70 seen in the embryos and their respective yolk sacs 1 hour after the different durations of heat shock. The steady-state concentrations of hsp27 mRNA (Figure 2, top panel) and hsp70 mRNA (Figure 2, middle panel) present in embryos and their yolk sacs *in vivo* (Figure 2, lanes 1 and 2) did not appear to be significantly different from those *in vitro* (Figure 2, lanes 3 and 4), thus the process of culturing the embryos did not affect the mRNA levels of either heat shock protein. The constitutive levels of hsp27 mRNA in yolk sac tissues were higher than those in embryo tissues. The mRNAs for hsp27 appeared to increase in a dose-dependent manner in both tissues, with maximum response occurring after 20 minutes of heat treatment. Similarly, the mRNA levels of hsp 70 increased in a dose-dependent manner with each increment of heat shock. In addition, there was a very pronounced increase of the inducible transcripts following all heat shock treatments compared to control. After quantifying the average of three independent Northern blots, the means ± standard errors of the mean were plotted on the graph shown in Figure 3.

Figure 2. Northern blot analysis of hsp27 mRNA (top panel), hsp70 mRNA (middle panel) and 18s rRNA (bottom panel) in embryos (em) and yolk sacs (ys) heat shocked at 43°C for 5, 10, 15 or 20 minutes and subsequently cultured for 1 hour.


in vivo
in vitro
5'
10'
15'
20'
em
ys
em
ys
em
ys
em
ys
em
ys
em
ys
kb
hsp27
0.9
hsp70
3.1
2.8
2.3
18S
1.9

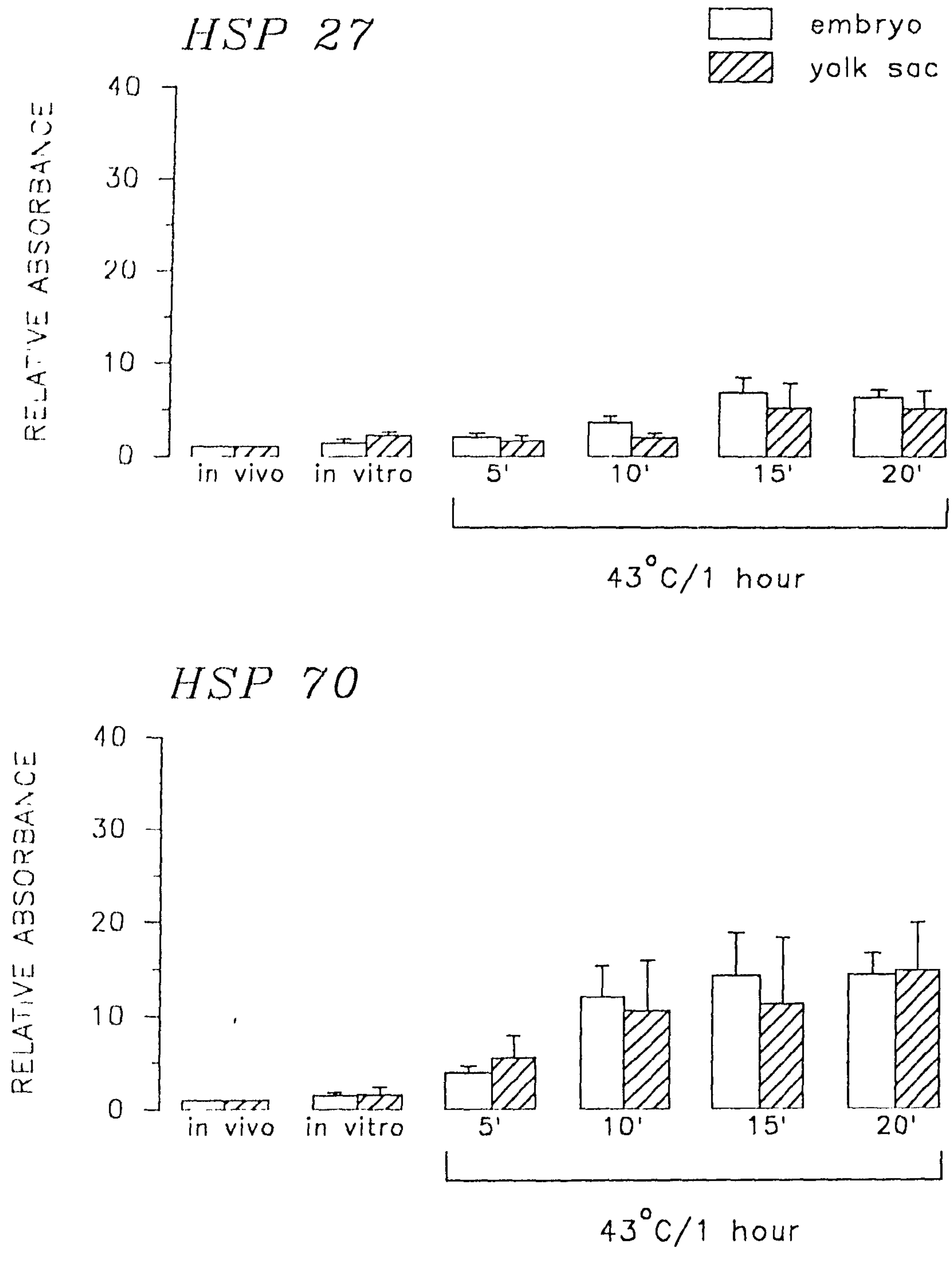
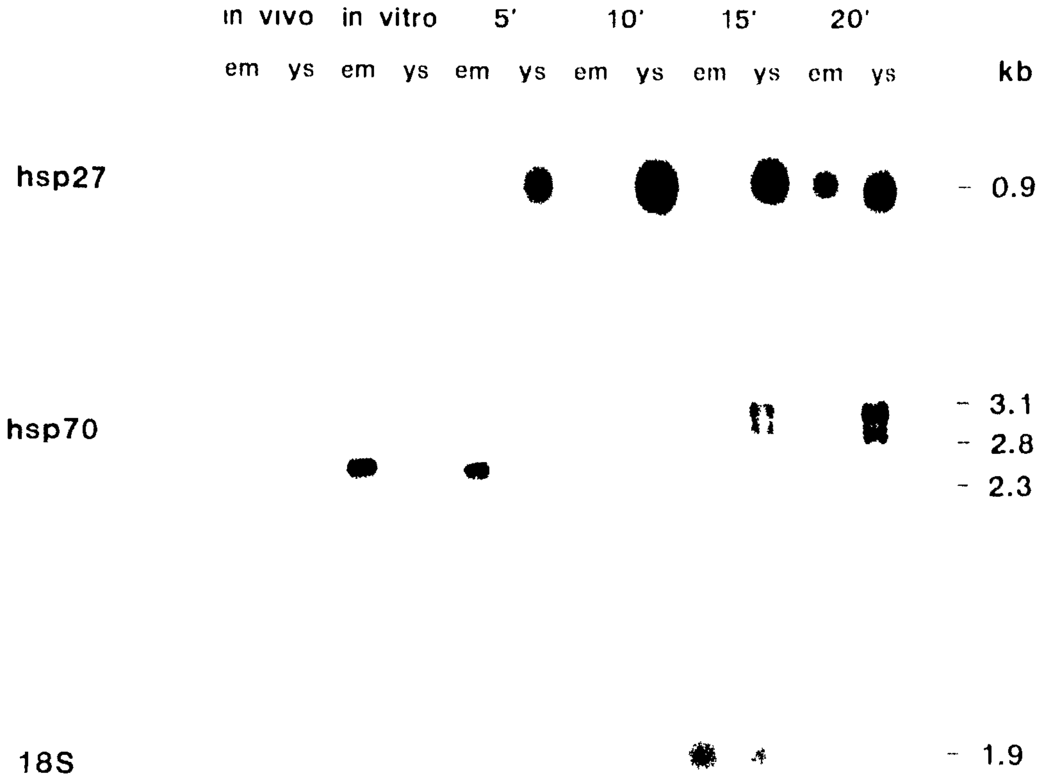


Figure 3. Densitometric analyses of hsp27 and hsp70 hybridization signals after standardization against the 18S rRNA signals in control and heat treatment groups for both embryo and yolk sac tissues. Whole embryos were heat shocked at 43°C for 5, 10, 15 or 20 mins and subsequently cultured for 1 hour at 37°C. Bars represent the means ± standard errors of the means, N=3.

Relative to control levels, the fold increases in the concentrations of hsp27 mRNA present in the embryos after heat treatment for 5, 10, 15 and 20 minutes were 2-, 3-, 6- and 6-fold, respectively (Figure 3, top panel). The fold increases in yolk sac tissues were similar to those of embryos at 1.5-, 2-, 5- and 6-fold, respectively (Figure 3, top panel). In contrast, hsp70 mRNA concentrations were induced to a much greater extent in both embryos and yolk sacs (Figure 3, bottom panel). Compared to *in vivo* control levels, embryos demonstrated a 4-, 12- and 14-fold induction after a heat shock of 5, 10 and 15 minutes, respectively. Meanwhile, the increase in the yolk sac was 5.5-, 7.5- and 11-fold following 5, 10 and 15 minutes respectively, at 43°C. Compared to the induction of hsp27 after exposure to 20 minutes of heat shock, hsp70 fold inductions were also more dramatic at 14-fold in embryos and 15-fold in yolk sacs.

The dose-dependent response observed 1 hour after heat treatment was also observed 2 hours following hyperthermia. The mRNA levels for hsp27 increased to a greater extent in the embryos compared to the yolk sacs, as seen in Figure 4. The steady-state mRNA levels of hsp27 (Figure 4, top panel) and hsp70 (Figure 4, bottom panel) in both embryos and yolk sacs increased in a dose-dependent manner as the duration of heat shock was lengthened. Again, there was more of the hsp27 message present in yolk sac tissues when compared to that found in embryo tissues. The inducible forms of hsp70 were clearly increased upon heat treatment. Three

Figure 4. Northern blot analysis of hsp27 mRNA (top panel), hsp70 mRNA (middle panel) and 18s rRNA (bottom panel) in embryos (em) and yolk sacs (ys) heat shocked at 43°C for 5, 10, 15 or 20 minutes and subsequently cultured for 2 hours.


in vivo
in vitro
5'
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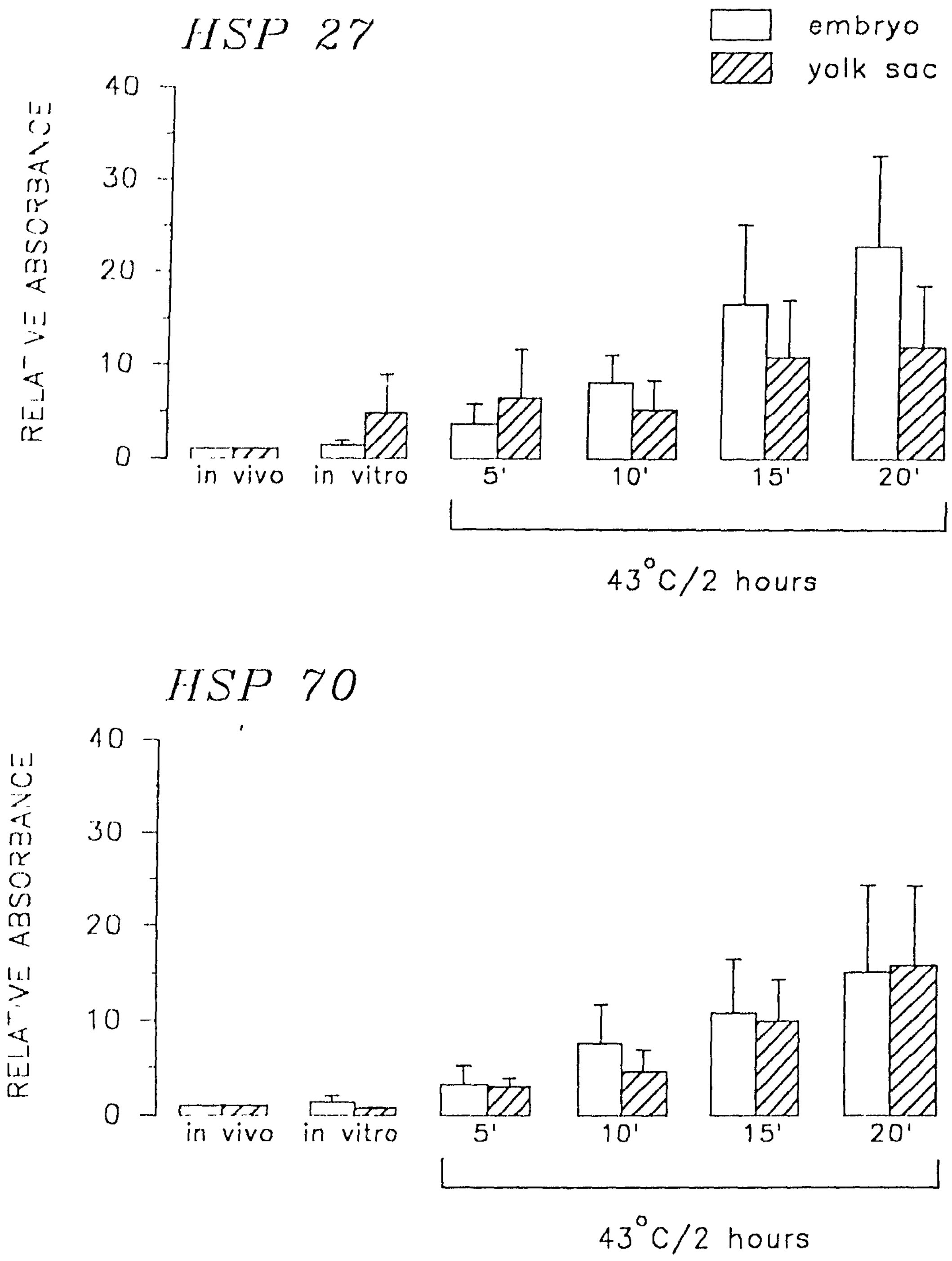
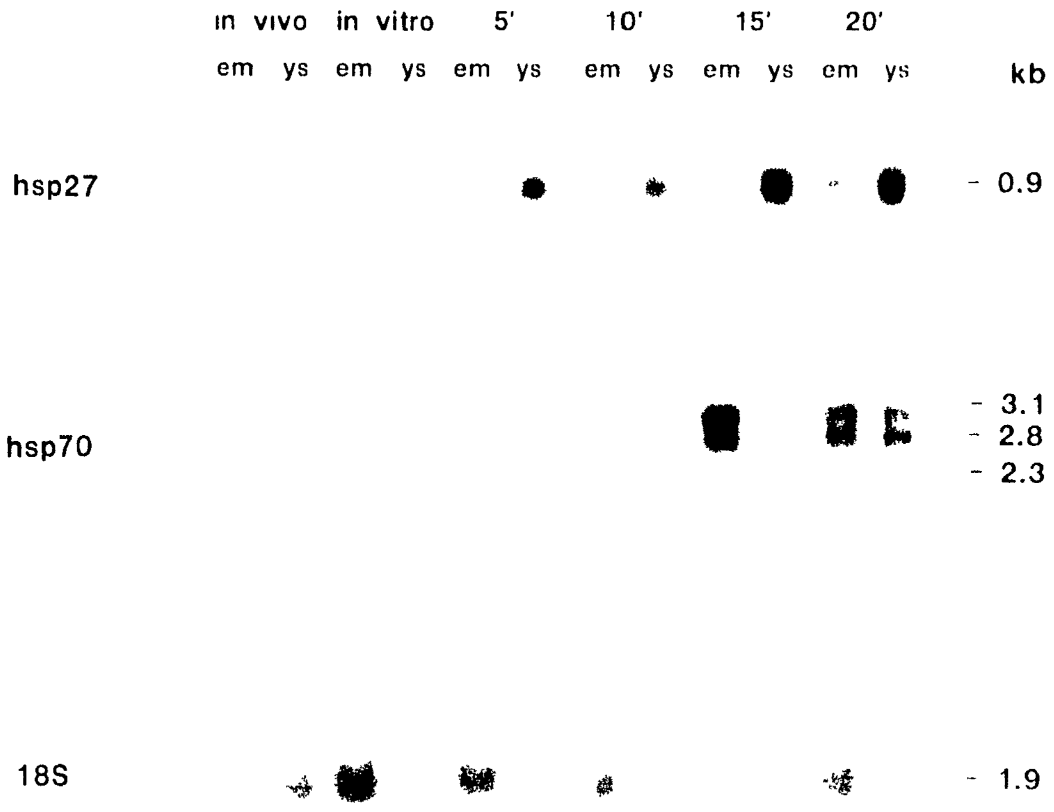


Figure 5. Densitometric analyses of hsp27 and hsp70 hybridization signals after standardization against the 18S rRNA signals in control and heat treatment groups for both embryo and yolk sac tissues. Whole embryos were heat shocked at 43°C for 5, 10, 15 or 20 mins and subsequently cultured for 2 hours at 37°C. Bars represent the means ± standard errors of the means, N=3.

independent Northern blots were scanned by laser densitometry and the means ± standard errors of the mean were plotted on the graph shown in Figure 5. As the dose of heat increased from 5 to 20 minutes in increments of 5 minutes, the fold increases in embryos of hsp 27 mRNA (Figure 5, top panel) over the control levels went from 4- to 8- to 17- to 23-fold. In the yolk sacs the fold increases were less dramatic at 6 to 5- to 11- to 12-fold (Figure 5, top panel). Thus after 20 minutes of heat treatment the steady-state mRNA levels of hsp27 were increased 23 fold in embryos and 12-fold in yolk sacs relative to control levels. In contrast, the steady-state mRNA levels for hsp70 were induced to a similar extent in both embryos and yolk sacs with fold increases ranging between 3 and 16 (Figure 5, bottom panel). Therefore 2 hours post heat treatment, a dose dependent response for both hsp27 and hsp70 is evident as the dose of heat is augmented.

In Figure 6, the Northern blots of hsp27 and hsp70 mRNA signals 4 hours after heat treatment are shown. A dose-dependent response is very apparent for both heat shock proteins. In Figure 7, it is evident that there is a marked difference for both hsp27 and hsp70 in the response of the embryos compared to the yolk sacs after 20 minutes of heat shock at 43°C. Relative to control embryo levels, the steady state mRNA concentrations of hsp27 were increased by 3-, 7-, 12 and 20-fold at heat shock durations of 5, 10, 15 and 20 minutes (Figure 7, top panel). In

Figure 6. Northern blot analysis of hsp27 mRNA (top panel), hsp70 mRNA (middle panel) and 18s rRNA (bottom panel) in embryos (em) and yolk sacs (ys) heat shocked at 43°C for 5, 10, 15 or 20 minutes and subsequently cultured for 4 hours.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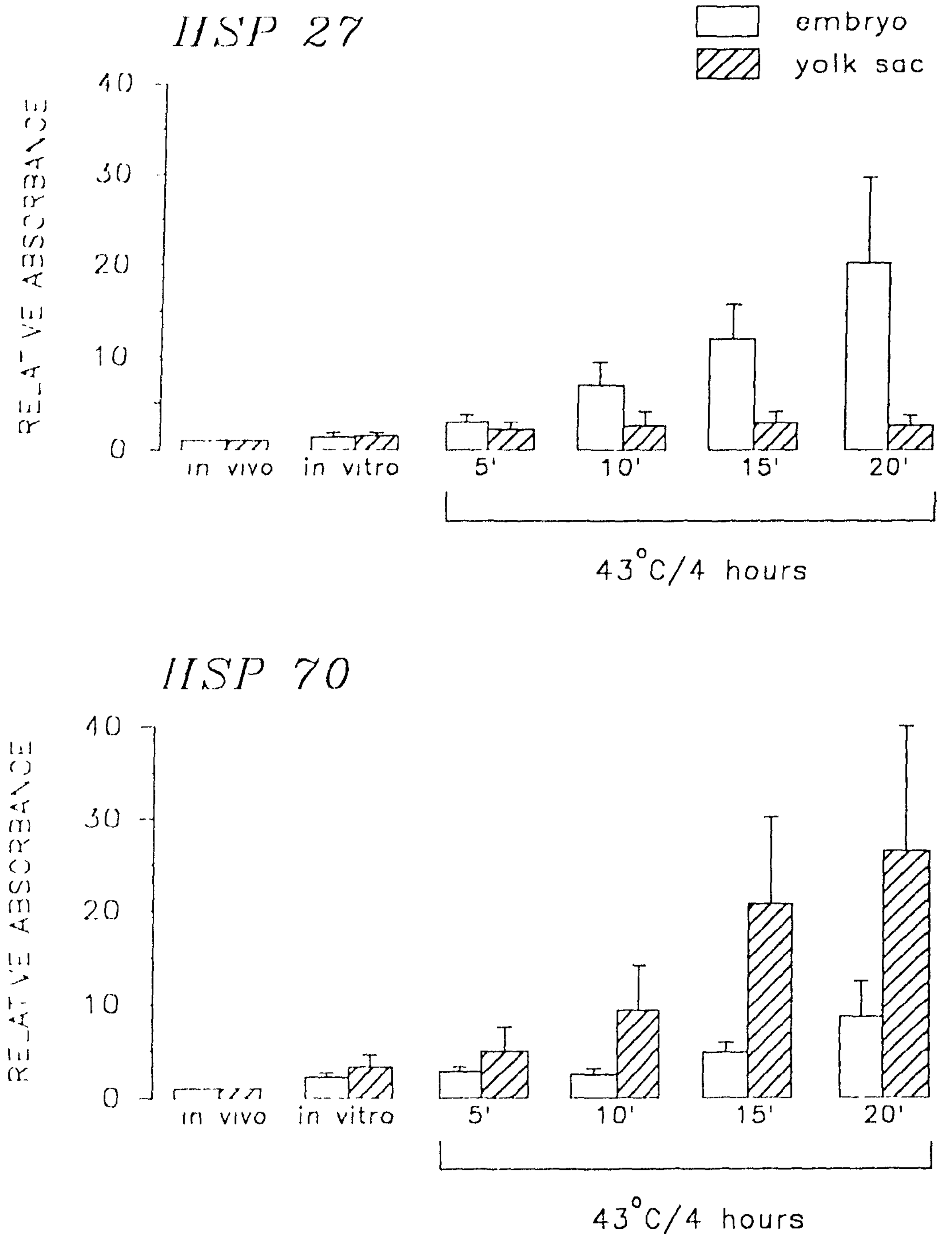


Figure 7. Densitometric analyses of hsp27 and hsp70 hybridization signals after standardization against the 18S rRNA signals in control and heat treatment groups for both embryo and yolk sac tissues. Whole embryos were heat shocked at 43°C for 5, 10, 15 or 20 mins and subsequently cultured for 4 hours at 37°C. Bars represent the means ± standard errors of the means, N=3.

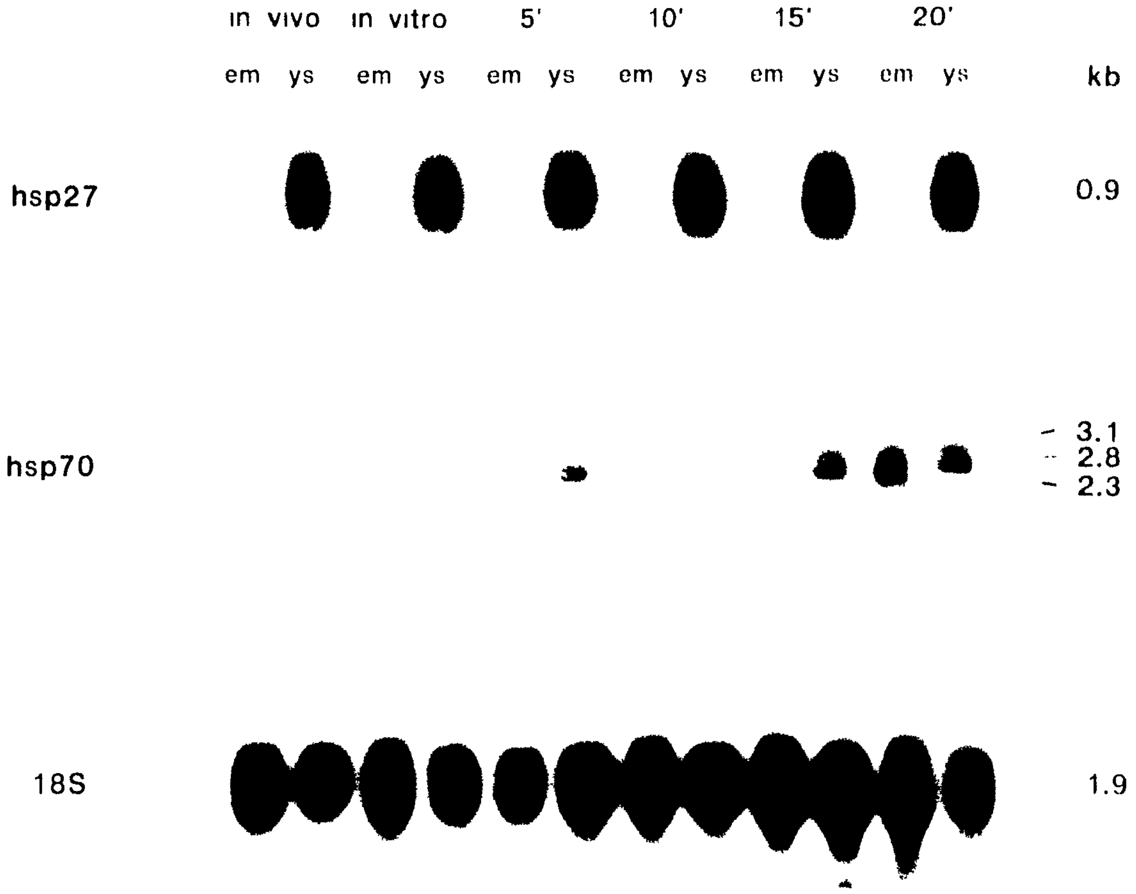
contrast, the fold increases in hsp27 mRNA concentrations in yolk sacs were not as elevated, at 2-, 2.5-, 3 and 3-fold relative to control following 5, 10, 15 and 20 minutes of heat shock, respectively (Figure 7, top panel). Interestingly, the fold increase of the levels of hsp70 was greater for the yolk sacs compared to that of the embryos; 26 fold in the yolk sacs and 9-fold in the embryos after a 20 minute dose of heat. For the lower doses of heat, the fold inductions were 3, 2.5 and 5 in the embryos and 5, 9 and 21 in the yolk sacs (Figure 7, bottom panel).

The steady-state mRNA concentrations for hsp27 (Figure 8, top panel) and hsp70 (Figure 8, bottom panel) following 20 hours of heat treatment did not appear to be different from those of control tissues. Upon quantitation of the data, the amounts of hsp27 (Figure 9, top panel) and hsp70 mRNA (Figure 9, bottom panel) in control embryos and yolk sac were not significantly different from those cultured at an elevated temperature of 43°C. Thus, 20 hours following specific doses of heat treatments, the dose-dependent relationship observed at the previous post heat treatment time points is non-existent.

3.1.4. **Time Course of the Accumulation of Hsp27 mRNA**

Whole rat embryos were cultured for 1, 2, 4 or 20 hours after heat treatment to determine the time course of hsp27 accumulation in both

Figure 8. Northern blot analysis of hsp27 mRNA (top panel), hsp70 mRNA (middle panel) and 18s rRNA (bottom panel) in embryos (em) and yolk sacs (ys) heat shocked at 43°C for 5, 10, 15 or 20 minutes and subsequently cultured for 20 hours.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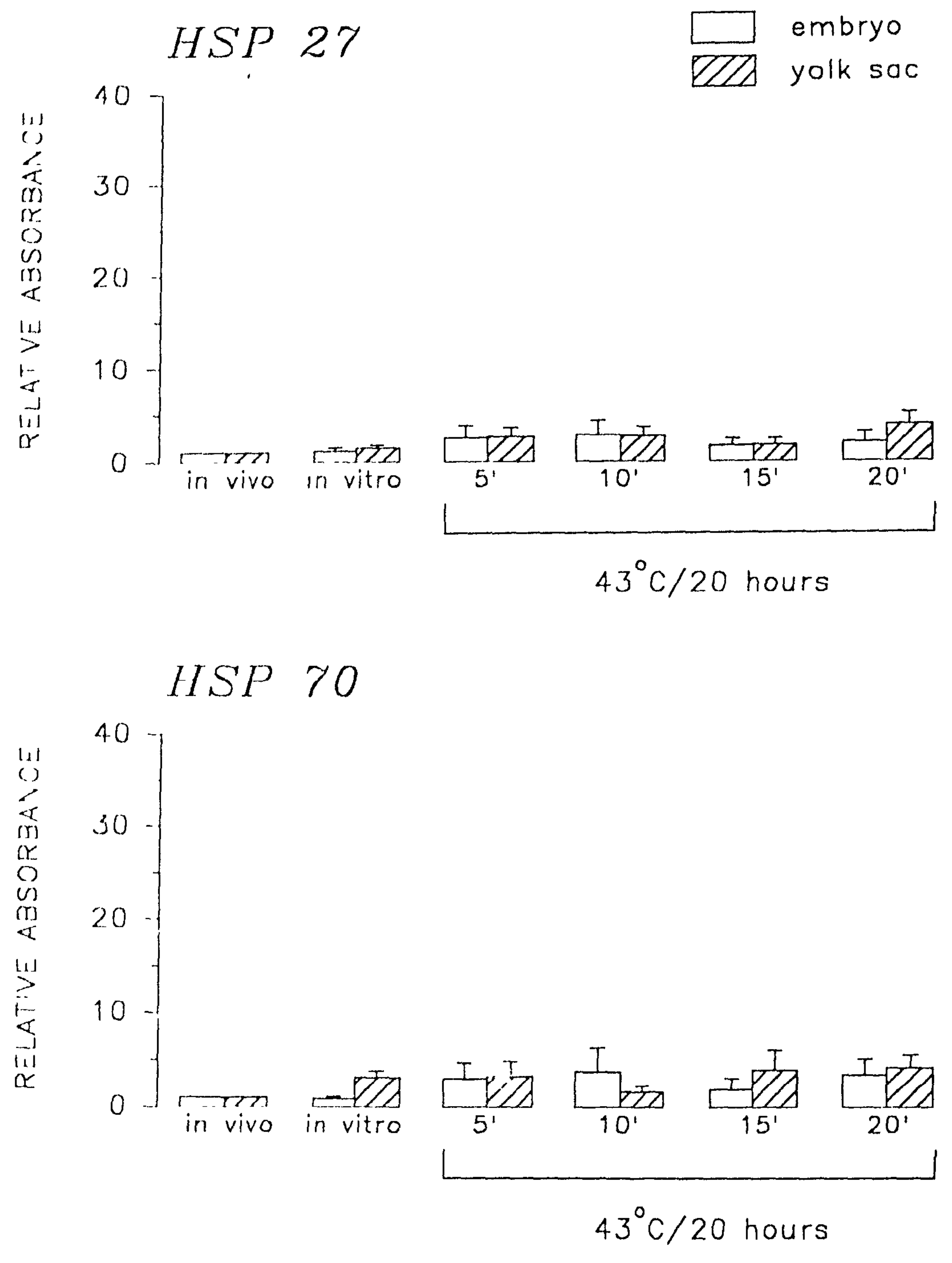


Figure 9 Densitometric analyses of hsp27 and hsp70 hybridization signals after standardization against the 18S rRNA signals in control and heat treatment groups for both embryo and yolk sac tissues. Whole embryos were heat shocked at 43°C for 5, 10, 15 or 20 mins and subsequently cultured for 20 hrs at 37°C. Bars represent the means ± standard errors of the means, N=3.

embryos and yolk sacs. In the diagrammatic representation of the time course of hsp27 accumulation (Figure 13, top panel), the fold increase representing each time point was that of the 20 minutes of heat shock.

The steady-state mRNA concentrations of hsp27 in embryos were already elevated 1 hour after all durations of heat shock (Figure 13, top panel). Hsp27 messages in embryos peaked 2 hours post-heat treatment and were already starting to decline 4 hours following heat shock. By 20 hours following heat treatment, the steady-state mRNA concentrations of hsp27 had returned to control levels.

Hsp27 concentrations in yolk sacs were already elevated approximately 5-fold over those of control only 1 hour after a 20 minute heat shock (Figure 13, top panel). The messages for hsp27 in the yolk sacs also peaked 2 hours post-heat treatment. However, the magnitude of increase at this time point in the yolk sacs is considerably less than 3 fold induction observed in embryos. The steady state amount of hsp27 appear to return to basal levels by 4 hours post-heat treatment.

3.1.5. Time Course of the Accumulation of hsp70 mRNA

Similar to the diagrammatic representation of the accumulation of hsp27, that of hsp70 also utilized the fold inductions for 20 minutes of

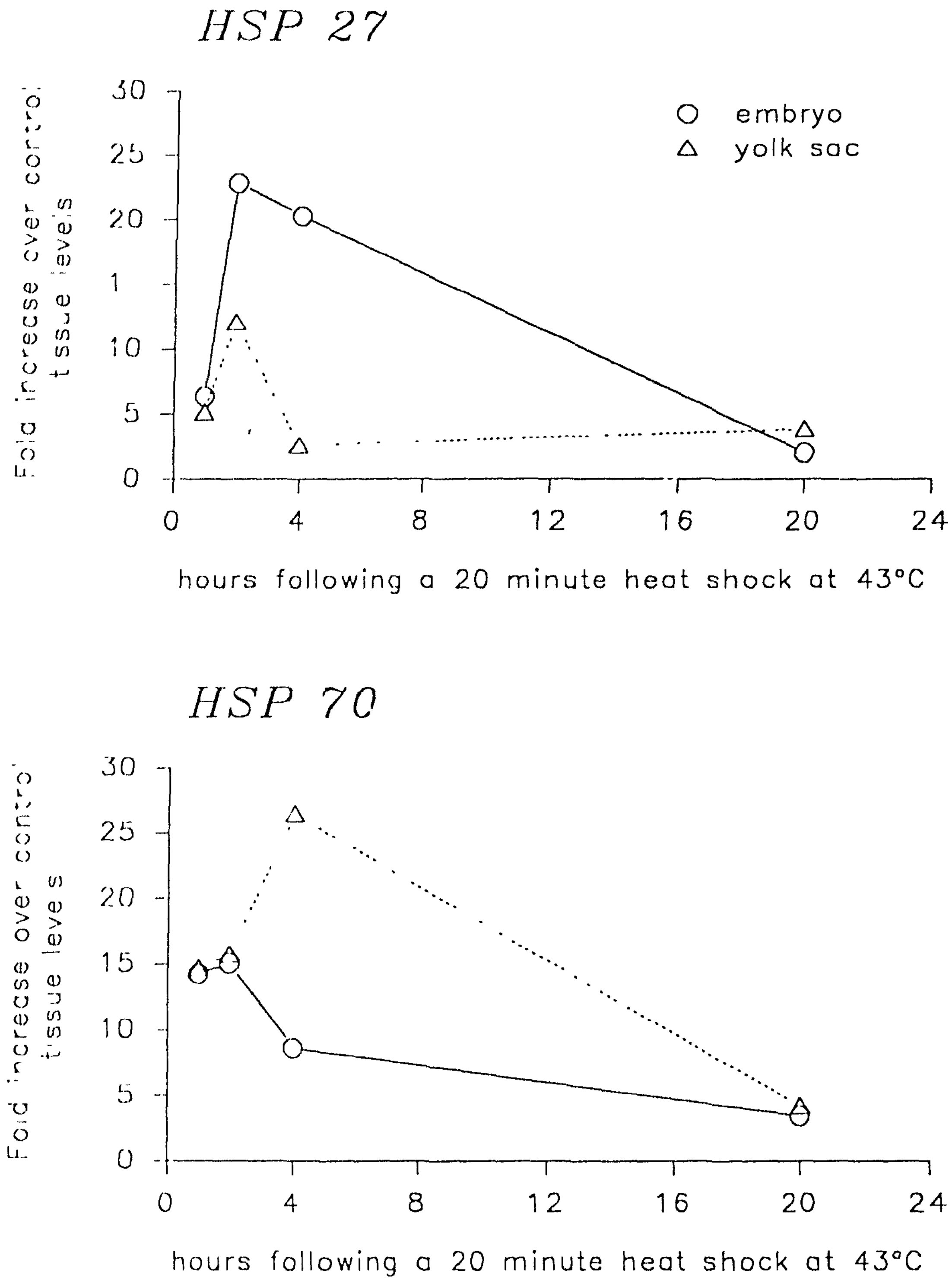


Figure 13. Diagrammatic representation of time course of hsp27 (top panel) and hsp70 (bottom panel) mRNA accumulation in both embryos and yolk sacs 1, 2, 4 and 20 hours post heat shock.

heat shock at each time point (Figure 13, bottom panel). The time course of the accumulation of the steady-state mRNA levels of hsp70 was different from that of hsp27 mRNA levels for both embryos and yolk sacs.

The amounts of hsp70 mRNA in embryos were already dramatically increased 1 hour after hyperthermia for all treatment groups (Figure 13, bottom panel). Two hours after the various doses of 43°C heat shock, the levels of hsp70 mRNA in embryos were maximal at 15 fold induction over control amounts. By 4 hours post-heat treatment the concentrations of hsp70 were attenuated. Finally, 20 hours following heat treatment, hsp70 mRNA levels returned to constitutive levels in the embryos.

One hour post-heat shock, hsp70 mRNA levels in yolk sacs were already increased to 15-fold over control concentrations (Figure 13, bottom panel). The fold increase of hsp70 mRNA in yolk sacs was slightly elevated 2 hours subsequent to heat shock compared to the increase observed 1 hour after heat treatment. However, 4 hours after embryos were heat shocked, the concentrations of hsp70 mRNA in yolk sac was dramatically elevated to a fold induction of 26. By 20 hours post heat shock, the levels of hsp70 returned to those detected in control yolk sacs.

3.2. Cadmium Treatments

3.2.1. Morphology of Embryo Exposed to Cadmium

In the second part of the study, the presence of cadmium in the culture medium also caused embryo deaths and malformations. When the embryos were cultured for a period of 1 day with 2.5 μM cadmium chloride present in the medium, 90% of the embryos died, and of the remaining embryos 100% were malformed (Figure 10). Following cadmium treatment, embryos were severely retarded in growth. In addition, abnormalities of the eyes and branchial arches were observed. Yolk sacs were reduced in size compared to the control yolk sacs.

3.2.2. Size of Hsp27 and Hsp70 Transcripts

Upon Northern blot analysis, the steady-state mRNA concentrations of HSP27 and HSP70 were analyzed in both embryos and their respective yolk sacs. The 27-kDa heat shock protein cDNA probe hybridized to a single mRNA species of 0.9 kilobases in both control tissues and cadmium-treated tissues (Figure 11, top panel). As for induction with heat, the 70-kDa heat shock protein cDNA probe hybridized to three mRNA species; a constitutive mRNA species of approximately 2.3 kilobases and two inducible mRNA species of 2.8 and 3.1 kilobases (Figure 11, middle panel). The 0.9

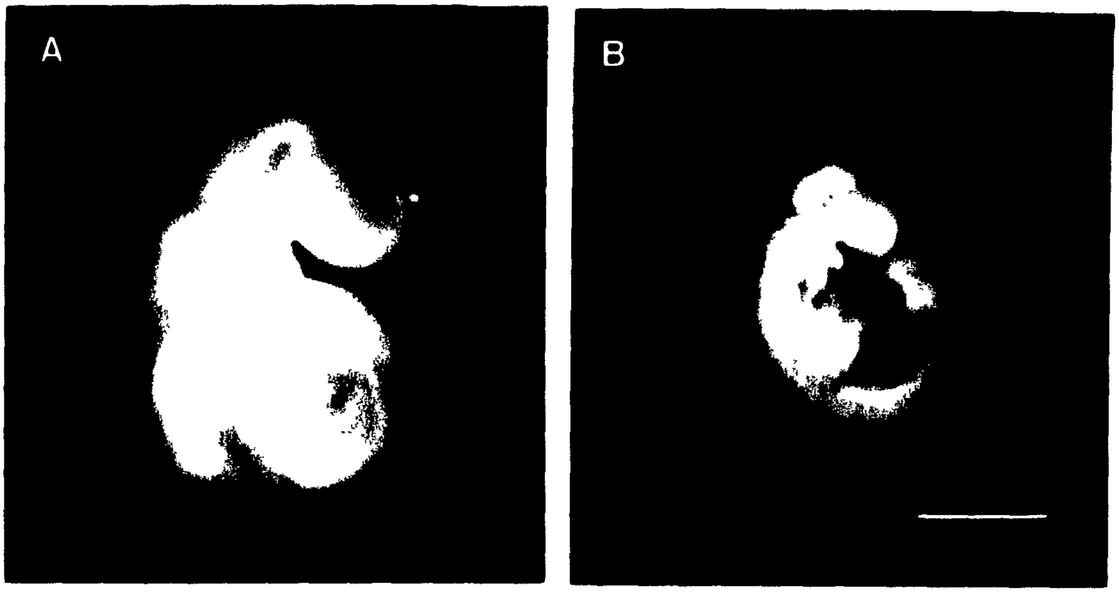


Figure 10. Postimplanation rat embryo explanted on day 10 of gestation and cultured for 20 hours at 37°C *in vitro* (A). Postimplantation rat embryo explanted on day 10 of gestation and cultured for 20 hours in the presence of 2.5 μM cadmium chloride (B). Bar represents 1 mm.

kilobase HSP27 mRNA species and the two inducible transcripts of HSP70 were quantitated by laser densitometry. Values from three independent Northern blots were standardized with the 18 S rRNA (Figure 11, bottom panel) and the means are shown in the graph in Figure 12.

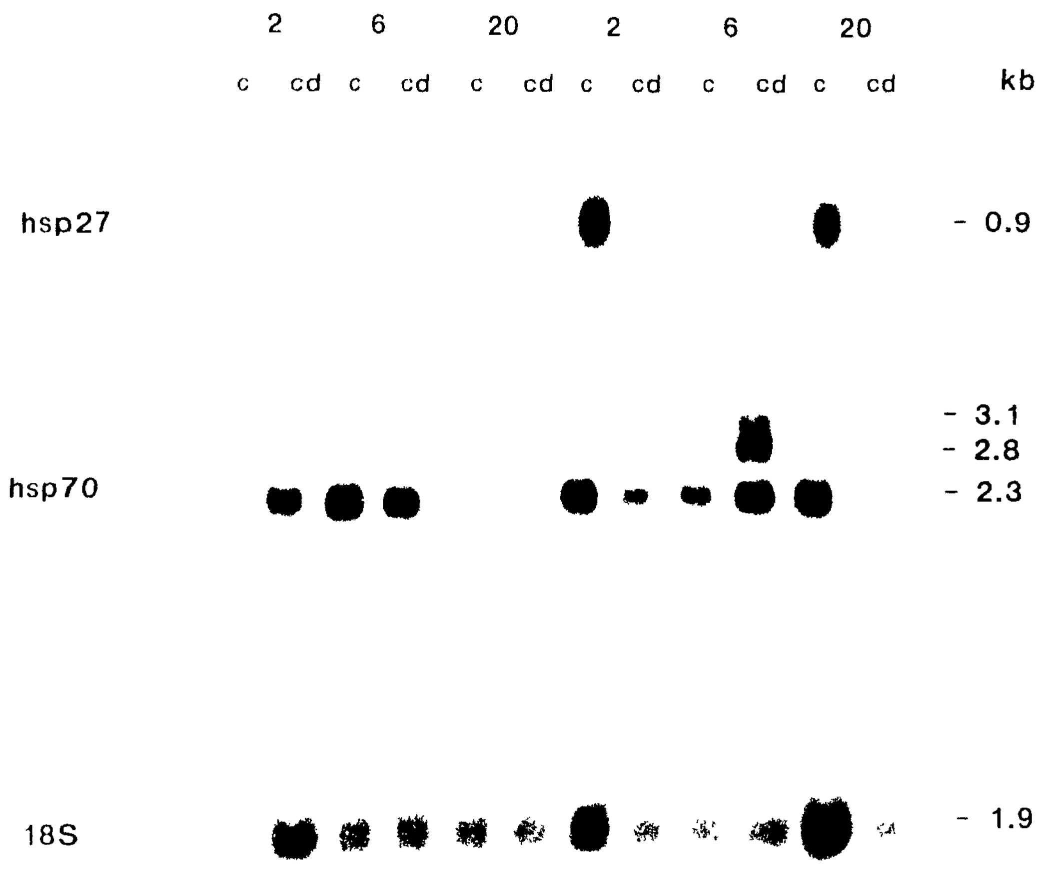
3.2.3. The Effects of Cadmium on the Steady-state mRNA Levels of Hsp27 and Hsp70

A Northern blot of embryos and yolk sacs treated with 2.5 μM cadmium is shown in Figure 11. Changes in the steady-state mRNA concentrations of either hsp27 or hsp70 in cadmium-treated embryos were not as obvious as those in heat treated embryos. However, there was clearly an increase of hsp70 mRNA levels in the drug-treated yolk sac group compared to the control group.

3.2.4. Time Course of the Accumulation of Hsp27 mRNA

When the steady-state mRNA concentrations of hsp27 was examined 2 hours after cadmium treatment (Figure 12, top panel), the levels of mRNA in the treated embryos were similar to those in control embryos. Six hours after drug-treatment, there appeared to be an increase in the hsp27 mRNA concentrations in treated embryos; 1.7-fold increase over control. Twenty hours after the addition of cadmium, the fold increase of the drug-

Figure 11. Northern blot analysis of hsp27 mRNA (top panel), hsp70 mRNA (middle panel) and 18s rRNA (bottom panel) in embryo (first 6 lanes) and yolk sac (last 6 lanes) tissues from control (c) embryos or embryos cultured in the presence of 2.5 μM cadmium chloride (cd) for 2, 6 or 20 hours.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- 0.9
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- 1.9

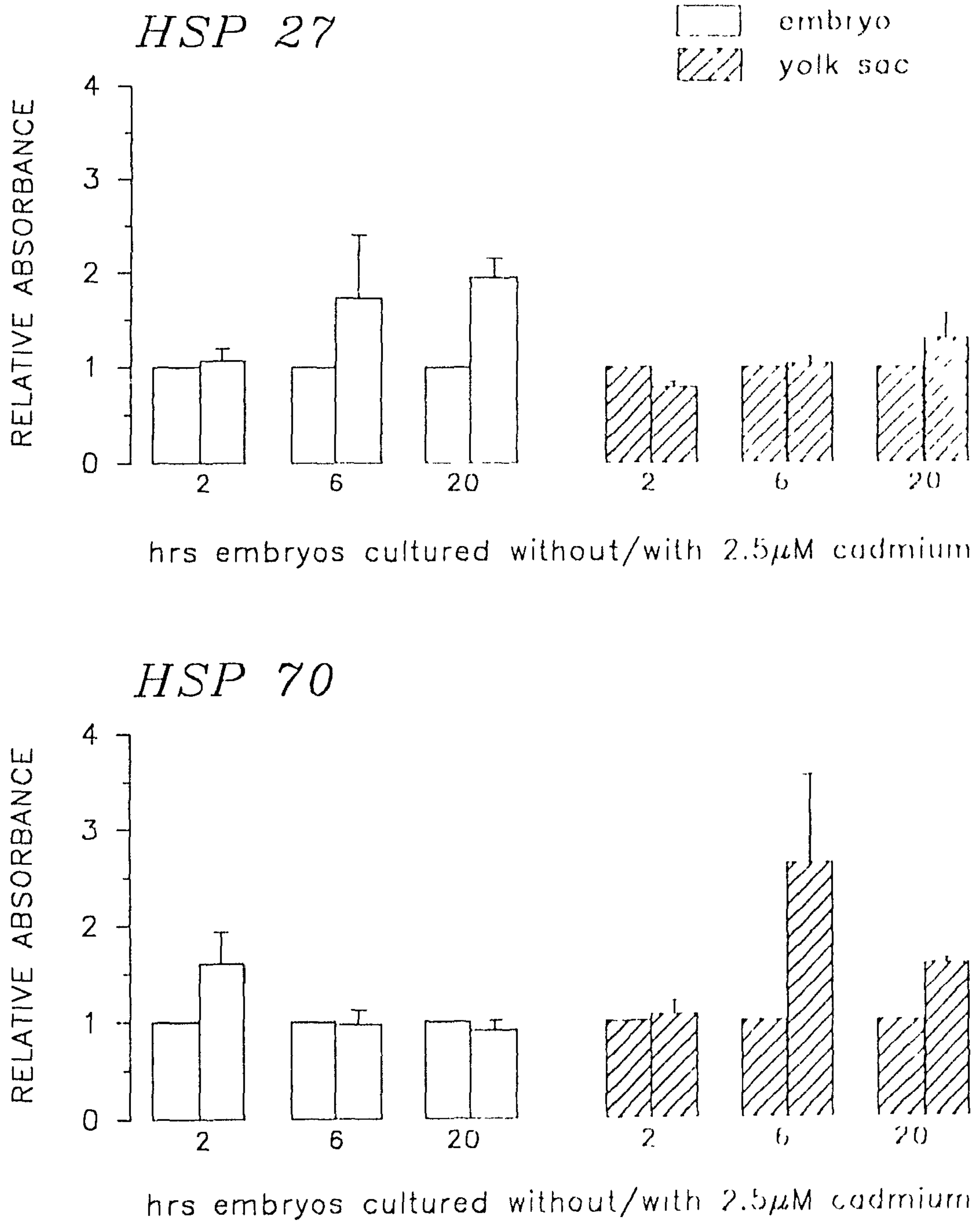

Figure 12. Densitometric analyses of hsp27 and hsp70 hybridization signals after standardization against the 18S rRNA signals in control and drug treatment groups for both embryo and yolk sac tissues. Control values are on the left of each time point while the treated tissue values are on the right. Bars represent the means ± standard errors of the means, N=3.

treated embryos was significantly increased to 2-fold ($P<0.05$).

In contrast, there were no significant changes in the hsp27 mRNA levels when the control yolk sac levels were compared to the cadmium-treated yolk sacs (Figure 12, top panel).

3.2.5. Time Course of the Accumulation of Hsp70 mRNA

Relative to control levels, the steady-state mRNA concentrations of hsp70 appeared to be increased by about 1.6-fold in the drug-treated embryos 2 hours after the addition of the drug (Figure 12, bottom panel). However, the concentrations of hsp70 mRNA returned to constitutive levels 6 and 20 hours subsequent to the addition of the drug.

In contrast, hsp70 mRNA levels in yolk sacs peaked at 6 hours after cadmium addition, at a fold increase of 2.6. By 20 hours after drug treatment the increase had decreased to 1.6-fold over control amounts.

CHAPTER 4

DISCUSSION

4.1. Overview of Discussion

The purpose of this thesis is to establish a relationship between teratogenicity and the heat shock response. However, first an interpretation of the results obtained in this thesis will be discussed by postulating various mechanisms for the differential regulation of hsp27 and hsp70 during organogenesis. This will be followed by a discussion of the relationship between the two teratogens used in this thesis and the heat shock response.

4.2. Size of Hsp27 and Hsp70 Transcripts

Blake et al. (1990) studied the expression of two heat shock protein families, *in vivo*. Upon heating whole male Wistar rats in a forced air incubator for varying times and temperatures, the expression of both hsp27 and hsp70 mRNA levels were examined in brain, liver, lung and skin of the rats. The sizes of the hsp27 transcripts obtained in the Northern blot analysis were 1.0 and/or 2.0 kilobases, depending on the tissue under investigation. Appreciable levels of the 1.0 kilobase transcript were present in lung and skin of control animals. Similarly, the hsp27 transcript observed in this thesis is also present under control conditions and is approximately the same size, 0.9 kilobases. In heat stressed rats, the 1.0 kilobase transcript in the experiments of Blake et al. (1990) was elevated. The hsp27 mRNA concentrations were also found to accumulate upon temperature elevation in this thesis. The 1.0 kilobase species corresponds to a similar species identified in human and rodent cells (Hickey et al., 1986). The presence of the 2.0 kilobase transcript was puzzling and interesting, but did not correlate with the heat treatment of the animal. Blake et al. (1990) did not detect this species in three different cell lines in other experiments conducted in the same laboratory. Therefore, hsp27 being a member of a large multigene family and sharing considerable homology with the lens α-crystallin gene, it is possible that the 2.0 kilobase transcript represents hybridization to one of these hsp27 related genes. For hsp70, the story is less complex. That is, the sizes of the hsp70 transcripts obtained in both my experiments and those of Blake et al. (1990) were identical: a 2.3 kilobase constitutive transcript and two heat-inducible transcripts of 2.8 and 3.1 kilobases. This is consistent with the results obtained from rodent cell lines (Lindquist and Craig, 1988), where three hsp70 transcripts were identified using a cDNA probe derived from a Chinese hamster ovary cell line; a constitutively expressed transcript of 2.3 kilobases and two induced transcripts of 2.8 and 3.3 kilobases. In contrast, Mirkes, Grace and

Little (1991), only obtained one hsp70 transcript of 3.0 kilobases. This inconsistency with the above data could not be explained by the use of different hsp70 probes since the hsp70 probe utilized in each experiment was obtained from the same laboratory, that of Dr. Morimoto. The only obvious difference was the temperature at which the filter was washed following hybridization of the mouse-specific probe to the filter. The temperature at which the blots in my experiments were washed was lower than the washing temperature used in Mirkes, Grace and Little's experiments. This difference may result in a decreased stringency, which could explain why they only obtained one transcript.

4.3. Possible Mechanisms for the Differential Regulation of Hsp27 and Hsp70 During Organogenesis

A heat shock response element has been identified in the promoter region of the heat shock protein genes which is responsible for the transcriptional regulation in response to heat (Pelham, 1982; Wu, Kingston and Morimoto, 1986). This same element also regulates induction by metabolic stressors such as metal ions and dinitrophenone (Morimoto et al., 1989). In addition, factors have been identified that bind to the heat shock element sequence following heat shock or treatment with specific chemicals (Sorger, Lewis and Pelham, 1987; Zimarino and Wu, 1987). Initial evidence for a factor that interacts with the heat shock element came from studies of protein-DNA interactions in *Drosophila* cell nuclei (Wu, 1984). The discovery of new eukaryotic heat shock factors was a major breakthrough. Hightower (1991) described the cloning and characterization of two murine heat shock factors, mHSF1 and mHSF2, which respectively displayed inducible and constitutive binding to DNA. The human heat shock factor, hHSF1, also displayed DNA-binding properties (Morimoto, Tissieres and Georgopoulos, 1990). The isolation of a second human factor, hHSF2, was very exciting (Hightower, 1991). Whether hHSF2 binds constitutively or inducibly to DNA is not clear, but it appeared to translocate from the cytoplasm to the nucleus upon heat shock. The presence of multiple heat shock elements (Morimoto, Tissieres and Georgopoulos, 1990) may explain the differential response of hsp27 and hsp70 to heat shock and cadmium which was observed in my experiments. In addition, the discovery of at least two heat shock factors may explain the discordance in the accumulation of hsp27 and hsp70 mRNAs in rat embryos.

The complex transcriptional regulation of the human hsp70 gene is mediated through multiple promoter elements. For example, activation by heat shock and heavy metals utilizes distinct cis-acting sequences from those required for growth regulated expression (Wu, Kingston and Morimoto, 1986). The sequences necessary for heat shock and metal ion induction mapped between bases -107 and -68 upstream of the start site (Wu, Kingston and Morimoto, 1986). Within these boundaries are two overlapping heat shock elements and a sequence analogous to the metal responsive elements of the metallothionein genes (Karin et al., 1984; Stuart et al., 1984). Using mutant promoters and reconstructing the hsp70 promoter onto the HSV TK gene, studies from the laboratory of Morimoto et al. (1989) revealed that the kinetics of hsp70 gene transcription are different depending on whether cells were heat shocked or treated with heavy metal ions. Transcription rates were measured in isolated nuclei using in vitro transcription in the presence of ^{32}P-UTP. The nascent radiolabelled RNAs were isolated and allowed to hybridize to cloned gene probes bound to nitrocellulose. The results were quantitated by scanning densitometry. During continuous heat shock the rate of hsp70 gene transcription rapidly increased to maximal levels by 30 minutes and declined to near background levels by 120 minutes. In contrast, cadmium treatment resulted in a delayed response, requiring approximately 60 minutes before transcription rates increased. The kinetics of the accumulation of hsp27 and hsp70 mRNA in response to hyperthermia or cadmium treatment obtained in this thesis were similar to those of Morimoto et al. (1989). When embryos were heat shocked for 5 to 20 minutes, the concentrations of both hsp27 and hsp70 were already elevated 1 hour post heat shock. On the other hand, embryos treated with cadmium mounted a heat shock response 6 hours after drug addition. Thus, like the situation of Morimoto et al. (1989), cadmium treatment results in a delayed heat shock response.

In outbred male Wistar rats, the *in vivo* expression of two heat shock proteins, hsp27 and hsp70, were investigated (Blake et al., 1990). The mRNA expression was examined in brain, liver, lung and skin of rats exposed to elevated ambient temperatures. The time course and relative magnitude of the heat-induced expression for the 27 and 70 kDa heat shock proteins differed between tissues of the same animal. Even within the same tissue, hsp27 and hsp70 displayed differential kinetics of induction. In my experiments a similar pattern was observed in the heat shock response of embryos and yolk sacs to elevated temperatures. That is, the kinetics of hsp27 and hsp70 mRNA accumulation were different in each embryonic tissue. Unlike cell culture systems, the induction of different heat shock proteins *in vivo* is not coordinated. Similarly, the kinetics of the accumulation of hsp27 and hsp70 mRNAs observed in the experiments of this thesis were not coordinated (Figure 13).

Very little is known about the regulation of expression of the genes encoding the vertebrate small heat shock proteins (20 to 30 kilodaltons). Like all heat shock genes studied to date, this gene has characteristic heat shock promoter elements in its 5' regulatory region and, therefore, is presumed to be transcriptionally activated upon heat shock (Lindquist and Craig, 1988). Transcription is a major site of regulation of expression of the small heat shock proteins of *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Drosophila melanogaster*; however additional control mechanisms have not been ruled out, particularly for developmental regulation (Jones et al., 1989). Several observations suggest that the expression of small heat shock proteins might be regulated differently from that of other heat shock proteins. Heat shock stimulated transcription of hsp71 and hsp88 genes is blocked by either glycerol or deuterium oxide, but hsp23 gene (chicken equivalent of human hsp27 gene) transcription is unchanged (Edington, Whelan and Hightower, 1989). In general, small heat shock proteins are regulated differently during normal development than the other major heat shock proteins (Rossi and Lindquist, 1989), indicating that they possess additional regulatory elements or levels of metabolic regulation of expression. To further support this, Edington and Hightower (1990) reported potential levels of regulation of the induction of chicken hsp23 by heat. Two levels of regulation were discovered. Induction of hsp23 by heat resulted from increases in the half-life of the hsp23 protein and from higher levels of cytoplasmic mRNA. Whereas, transcriptional activation played a major role in the increase in RNA transcripts encoding hsp71 and hsp88 in cultured chicken embryo cells (Edington, Whelan and Hightower, 1989), increases in RNA transcripts encoding hsp23 resulted from posttranslational nuclear events. This could also explain the difference in the accumulation pattern of hsp27 and hsp70 upon heat shock and cadmium treatment in my experiments.

4.4. Relationship between Hyperthermia and the Heat Shock Response

Exposure of postimplantation Sprague-Dawley rat embryos on days 9, 10, 11 and 12 of gestation to an *in vitro* heat shock of 43°C for 15, 30 or 60 minutes resulted in the accumulation of hsp70 mRNA (Mirkes, Grace and Little, 1991). Embryos on day 9 of gestation exhibited the most pronounced accumulation of hsp70 RNA, while embryos on days 10 to 12 of gestation exhibited an increasingly attenuated accumulation of this heat shock protein, particularly after the more acute exposures to heat, such as 43°C for 30 or 60 minutes. The different durations of heat used in my experiments (5, 10, 15 or 20 minutes) lead to a dose-dependent increase in the accumulation of hsp70 mRNA concentrations as the dose of heat shock was increased from 5 to 20 minutes. In Mirkes, Grace and Little's work, as the durations of heat shock increased from 15 to 30 to 60 minutes, a different response was observed. Hsp70 mRNA was elevated with a 30 minutes heat treatment when compared to that at 15 minutes, however when the embryos were exposed for 60 minutes to 43°C, the accumulation of hsp70 mRNA decreased. A possible reason for this decrease in hsp70 mRNA is that the heat shock used in the experiment of Mirkes, Grace and Little is too embryolethal. That is, exposure of the embryos to 60 minutes of heat treatment causes damage so severe to the embryos that they are unable to mount a heat shock response.

In the developing mammalian embryo, the induction of members of the small heat shock protein family has been far less documented. Mirkes (1987) analyzed the synthesis of any new proteins by examining the amount of [^{3}H] leucine incorporation on the fluorographs of two dimensional polyacrylamide gels. Following a 30 minute exposure at 43°C, the synthesis of a set of eight proteins with molecular masses ranging between 28 and 82 kilodaltons was apparent. In order to determine the kinetics of the induction and decay of heat shock proteins synthesis, embryos were exposed to 43°C for 30 minutes and then returned to 37°C. The synthesis of all eight heat shock proteins was detected in extracts from embryos heat shocked for 30 minutes at 43°C and immediately cultured for 1 hour in the presence of [^{3}H] leucine; this is denoted the 0 hour time point. The synthesis of the 28 kDa heat shock protein was turned off between 1 and 3 hours post heat shock. The synthesis of the 31.5, 39, 69, 78 and 82 kDa heat shock proteins was turned off between 3 and 9 hours after heat shock. On the other hand, the synthesis of 33.5 and 34 kDa heat shock protein decayed to the non-heat shock level between 3 and 9 hours post heat shock.

4.5. Relationship between Cadmium and the Heat Shock Response

Utilizing *Drosophila* embryonic culture, Bournias-Vardiabasis and Buzin (1986) developed an *in vitro* assay for detecting teratogenic agents. They discovered that drugs which inhibit the differentiation of embryonic cells also induced the synthesis of three small proteins, identified as the low molecular weight *Drosophila* heat shock proteins, hsp22a, hsp22b and hsp23. Conversely, drugs that do not inhibit differentiation to a significant extent also do not induce these heat shock proteins. When the effects of teratogens, such as cadmium, on protein synthesis was examined by two-dimensional gel electrophoresis, two of the small heat shock proteins (hsp22 and hsp23) were induced. The addition of cadmium to such a system was also found to reduce the number of myotubes and ganglia when compared to control cells.

Heat-induced cross-tolerance to cadmium was investigated in two inbred strains of mice, BALB/c and SWV (Kapron-Bras and Hales, 1992). Embryos were pretreated 5 minutes at 43°C to induce thermotolerance, and subsequently treated with an embryotoxic concentration of cadmium, 1.75 μM. Each strain responded differently to the heat pretreatment; cross-tolerance was induced in SWV but not in BALB/c mice. The variation in the development of cross tolerance in both strains did not correlate with the increased synthesis of a 68 kDa heat shock protein. That is, there was an increase in the immunoreactive hsp68 in embryo and yolk sac tissues following the mild heat treatment as detected by Western blotting, but there was not an excess production of this protein in the strain that developed cross-tolerance, SWV. In BALB/c and SWV mice, the cadmium treatment did not result in the induction of hsp68 synthesis. Similarly, embryo tissues in my studies did not mount a heat shock response. In addition, the development of malformations did not correlate with the accumulation of hsp27 and hsp70 mRNAs.

An understanding of the biochemical pathways by which the heat shock factor is activated in response to metal ions and heat shock will reveal whether multiple pathways for stress gene activation are available. The observation that the metal-responsive element in several heat shock gene promoters corresponds to the heat shock element has implications for heavy metal transcriptional regulation (Stuart et al., 1984). Either the heat shock element-binding protein factor is a heavy-metal responsive protein distinct from metallothionein regulatory proteins that act on the human metallothionein gene or, perhaps, heat shock factor activity is indirectly regulated by a metal-sensing factor (Morimoto, Tissieres and Georgopoulos, 1990). Comparison of the upstream promoter regions of the hsp70 and the metallothionein I and II genes, which are transcriptionally induced following incubation with the metals cadmium, copper and zinc (Levinson, Oppermann and Jackson, 1980; Karin et al., 1984; Watowich and Morimoto, 1988), does not suggest a common mechanism of shared transcription factors. Yet, because transcription of all three genes responds to the same metal ions, it is likely that common metal ion sensitive intermediates in the pathway of transcription activation transmit this information to transcription factors such as heat shock factors. The presence of different transcriptional regulatory elements for heat-induced and heavy-metal ion-induced stress response can explain the differential pattern of hsp27 and hsp70 mRNA accumulation in response to heat or cadmium treatment in my experiments. There might be different regulatory elements acting at different levels depending on the nature of the stress.

4.6. Relationship between Teratogenicity and the Heat Shock Response

In 1984, German formulated the hypothesis that exposure of the embryo to stress, such as a heat shock, during its development could account for many intrauterine developmental anomalies. Moreover, the precise period during gestation when the stress is administered would determine the nature of the malformation. The magnitude and duration of the heat shock response depends, in some cases, on the magnitude and duration of the stimulus. Thus, the induction of the heat shock response in the mammalian embryo during the critical period of organogenesis can alter the established program of activation and inactivation of genetic material essential for normal intrauterine development. This in turn may lead to the development of anatomical malformations.

Recently, Mirkes and Doggett (1992) provided evidence that the 72 kilodalton heat shock protein is a biomarker of heat-induced embryotoxicity. By exposing rat embryos on day 10 of gestation to elevated temperatures (40°C to 43°C) for various lengths of time (2.5

minutes to 18 hours), hsp72 was observed to be induced by all hyperthermic exposures that induced alterations in the normal pattern of growth and development in rat embryos. Previously, Mirkes (1985) showed that rat embryos exposed *in vitro* to temperatures ranging from 41°C to 43°C resulted in malformed embryos. Moreover, as the temperature increased, the length of exposure required to produce abnormal embryos was decreased. Recent studies indicated that exposure of embryos to hyperthermia leads to the rapid accumulation of hsp70 mRNA (Walsh et al., 1989; Harris, Juchau and Mirkes, 1991; Mirkes, Grace and Little, 1991) and the synthesis of a set of heat shock proteins visualized by two dimensional gel electrophoresis (Mirkes, 1987; Walsh et al., 1987). Therefore, the hyperthermia-induced stress response is related to hyperthermia induced teratogenicity. My heat shock experiments support this hypothesis. However, the use of a stress other than heat shock, such as cadmium, did not support this hypothesis. In my experiments, even though the dose of cadmium used was teratogenic, the stress response was not induced until 6 hours after the addition of this heavy metal.

Since the initial discovery of the induction of heat shock protein synthesis by heat shock and other forms of stress, it has become apparent that heat shock proteins play a role in the normal life-span of the cell as well as in situations where the cell is under stress. The fact that proteins as highly conserved as the heat shock proteins are used by many organisms for normal growth and development processes, as well as for survival from environmental insults, suggests at least two different explanations for the function of these proteins. The obvious one is that the organism needs to protect itself at specific stages of its life from potential damage by stressful agents and from all the literature cited in this thesis, heat shock proteins fit this role. The alternative hypothesis is that the heat shock proteins are used for normal growth and metabolic activities and their role in stress is to enable the cell to return to a normal pattern of growth that has been temporarily blocked as the result of stress. That is they are essential elements for the homeostasis of the cell. Many of the biological properties that are now known for heat shock proteins and the variety of biological activities associated with heat shock protein induction strongly support this hypothesis. But the ultimate resolution of the role of heat shock proteins in biology will come when we understand the true function of these proteins.

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