Heat shock transcription factors and the hsp70 induction response in neural and non-neural tissues of the hyperthermic rat during postnatal development

by

Andrew John Morrison

A thesis in conformity with the requirements

For the degree of Master of Science

Graduate Department of Zoology

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ABSTRACT

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Heat shock transcription factor 1 (HSF1) levels increase in brain regions during postnatal rat development, but decrease in kidney and heart. HSF1 protein levels in brain and kidney correlate with levels of HSF DNA-binding activity and the magnitude of hsp70 protein induction after thermal stress in both neonatal and adult rats. There appears to be more HSF1 protein in adult brain than is needed for induction of hsp70 after thermal stress, suggesting that HSF1 may have other roles in addition to its role as a stress-inducible activator of heat shock genes. HSF2 protein levels decline during postnatal rat development in brain regions, kidney and heart. Gel mobility shift analysis showed that HSF2 is not in a DNA-binding form in the neonatal rat, suggesting that HSF2 may not be involved in the constitutive expression of heat shock proteins in early postnatal development. There is no apparent correlation between levels of HSF2 protein and basal levels of hsp90, hsp70, hsc70 and hsp60.

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
BS	brain stem
BSA	bovine serum albumin
C	degrees Celsius
Cb	cerebellum
CH	cerebral hemisphere
cm	centimetre
CNS	central nervous system
cpm	counts per minute
ΔT	change in temperature
d	day
DNA	deoxyribonucleic acid
E	embryonic day
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
Fb	forebrain (brain stem plus cerebral hemispheres)
g	times the force of gravity
H	heart
HEPES	N-(2-hydroxyethy!)piperazine-N'-(2-ethanesulfonic acid)
hr	hour
HS	heat shock
HSBP1	HSF binding protein 1
hsc	heat shock cognate protein
HSE	heat shock element
HSF	heat shock transcription factor
hsp	heat shock protein
IAA	isoamyl alcohol
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
K	kidney
kDa	kilodalton
L	liver
µg	microgram
µl	microlitre
M	molar
mA	milliampere
MAPK	mitogen-activated protein kinase
mg	milligram

min	minute
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
PP2A	protein phosphatase 2A
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	standard saline citrate
STAT	signal transducer and activator of transcription
TBST	tris-buffered saline Tween 20
TE	tris-EDTA
TNF-α	tumor necrosis factor alpha
Unstr.	unstressed
UTR	untranslated region
wk	week

Some of the data presented in Section 3 of this thesis (Figures 1A, B; 2A, B, 3A, 5-8) have been submitted for publication. This is to certify that I, Andrew J. Morrison, carried out the research documented in the following publication.

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1. INTRODUCTION

1.1 The heat shock response

The heat shock response is a cellular reaction to stressful stimuli that occurs in organisms ranging from bacteria to humans (Lindquist and Craig, 1988; Nover, 1991; Morimoto et al., 1994). This response involves the repression of ongoing gene expression and the induction of a set of genes encoding heat shock proteins (hsps) (Lindquist and Craig, 1988; Morimoto et al., 1994). The induction of hsps also occurs during cell cycle progression, differentiation and development (Morimoto et al., 1994). Hsps are involved in the repair of cellular damage and in the protection of cells from subsequent stress (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993; Hightower and Li, 1994; Morimoto et al., 1994). Many hsps are also expressed constitutively in the unstressed cell, where they cooperatively act as molecular chaperones to facilitate many cellular processes such as protein synthesis, folding and assembly, translocation across membranes, degradation, and the regulation of kinases and transcription factors (Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Rutherford and Zuker, 1994; Hartl, 1996; Gottesman et al., 1997; Bukau and Horwich, 1998).

The heat shock response can be triggered by a plethora of stress signals including heat shock, amino acid analogues, transition heavy metals, ischemia or tissue injury (Lis and Wu, 1993; Morimoto, 1993; Wu, 1995). The accumulation of denatured or abnormal proteins is thought to be the trigger for the heat shock response (Goff and Goldberg,

1985; Ananthan et al., 1986; Mifflin and Cohen, 1994). Stress causes proteins to unfold, and they are then prone to misfolding and aggregation. Heat shock proteins capture these non-native intermediates and maintain them in their intermediate folded states in order to avoid aggregation. Then they either refold the non-native intermediates or help target them for degradation (Chiang et al., 1989).

Overexpression of one or more hsps is often sufficient to protect cells and tissues against otherwise lethal exposures to diverse environmental stresses, including toxic chemicals and extreme temperatures (Parsell and Lindquist, 1994). Cells that have been given a sublethal heat shock can then survive a subsequent severe, otherwise lethal heat shock (Li and Werb, 1982). This process is called thermotolerance and is caused partly by the accumulation of hsps after the initial heat shock. Heat acclimation leads to an increased basal level of hsp70 (Maloyan et al., 1999). After heat stress, peak hsp70 mRNA levels are attained faster in heat acclimated rats than in controls (Maloyan et al., 1999). Most of the work on the heat shock response has been carried out on tissue culture systems rather than *in vivo*. This thesis addresses the heat shock response in an intact animal. Heat shock proteins are classified according to molecular size into the following families: hsp100, hsp90, hsp70, hsp60, hsp40 and small hsps.

1.2 The mammalian hsp70 family

Hsp70 proteins are the most conserved proteins found in all species, and have therefore been used to establish phylogenies between even the most distantly related organisms (Gupta and Golding, 1993; Gupta and Singh, 1994; Gupta et al., 1994). Hsp70

proteins are encoded by multigene families, consisting of both constitutively expressed and stress-inducible members (Welch, 1992). In mammalian cells, hsc70 and hsp70 are found in the cytosol and nucleus, Bip is found in the endoplasmic reticulum, and mhsp70 is found in the mitochondria (Morimoto and Santoro, 1998). All of these proteins are members of the hsp70 family. Hsc70 is constitutively expressed at a high level in mammalian cells, but is usually not induced after stress; hsp70 is expressed at a very low level under non-stress conditions but is highly induced by stress. In the rat, there are two coding genes for hsp70: the hsp70-1 gene and the hsp70-2 gene (Akcetin et al., 1999).

Hsp70 proteins have a multidomain organization consisting of a 45 kDa amino-terminal ATPase, an 18 kDa carboxyl-terminal peptide binding domain, and a 7 kDa intra- and interdomain EEVD regulatory motif (McKay et al., 1994; Freeman et al., 1995; Zhu et al., 1996). The peptide binding domain interacts with unfolded proteins through recognition of a cluster of hydrophobic residues (Blond-Elguindi et al., 1993; Rudiger et al., 1997). These hydrophobic residues would be buried in the interior of a native protein, but might be exposed in a non-native protein. When hsp70 is bound to ADP it has a high affinity for denatured proteins, but when it is bound to ATP it has a low affinity for denatured proteins (Frydman and Hartl, 1994). By hydrolyzing ATP hsp70 goes from a low affinity state to a high affinity state; this process is reversed when hsp70 releases ADP and binds to ATP again (Frydman and Hartl, 1994). This process is aided by cochaperones of the DnaJ family and other accessory proteins (Hohfeld et al., 1995; Takeyama et al., 1997), and results in cycles of binding and release of non-native proteins (Frydman and Hartl, 1994). The consequence of hsp70 interactions with the non-native proteins is to shift the equilibrium of protein folding and refolding reactions toward

productive on-pathway events by functioning as kinetic traps to prevent off-pathway intermediates that may lead to the formation of aggregates. By binding to hydrophobic regions of the protein that are likely to be misfolded, hsp70 changes their structure in a way that gives the protein another chance to fold.

Hsp/hsc70 binds to nascent polypeptides as they emerge from the ribosome to prevent premature folding before the protein is completely synthesized (Beckmann et al., 1990; Nelson et al., 1992). The formation of the correct tertiary structure of the protein requires the presence of the complete protein or at least a complete folding domain (usually 100-200 amino acids) (Frydman and Hartl, 1994). Along with other chaperones, hsp70 holds non-native polypeptides in a soluble, refolding competent state, thus preventing aggregation. Hsp70 helps to maintain newly translated proteins which are targeted to a cellular compartment or organelle in an unfolded state so that they can be translocated through the membrane, and also helps with the translocation process itself (Chirico et al., 1988; Deshaies et al., 1988). Mitochondrial hsp70 helps to transport preproteins into the mitochondrion via an ATP-dependent interaction with the membrane anchor Tim44 which functions to generate a pulling force and unfold preproteins (Voisine et al., 1999).

After stress, hsp70 migrates into the nucleus (Welch and Feramisco, 1984) and nucleolus (Pelham, 1984), where it may help repair damaged preribosomes and restore nuclear function. Hsp/hsc70 and hsp110, a diverged hsp70 family member, preferentially bind AU-rich RNA *in vitro* (Henics et al., 1999). These hsps may act as RNA-binding entities *in vivo* to guide the appropriate folding of RNA substrates for subsequent regulatory processes like mRNA degradation and/or translation (Henics et al., 1999).

Hsp70 proteins also have roles in protein degradation, reorganization of tubulin and intermediate filaments, translation initiation, nuclear protein import and export, ribosome assembly, interaction with DNA-topoisomerase I and DNA synthesis (Nover, 1991, 1994; Ciavarra et al., 1994). The hsp70 gene is potently induced in mammalian cells at the G1/S phase transition of the cell cycle, suggesting that hsp70 plays an important role in cell cycle progression (Kao et al., 1985; Milarski and Morimoto, 1986). In non-transformed, unstressed cells hsp70 is expressed at very low levels; however, hsp70 is expressed at elevated levels in most tumors and in many transformed cell lines (Volloch and Sherman, 1999). A recent study showed that when hsp70 is overexpressed in Rat-1 fibroblasts, the cells become oncogenically transformed (Volloch and Sherman, 1999). Thus hsp70 may play an active role in oncogenic transformation (Volloch and Sherman, 1999). Hsc70 is a clathrin-uncoating ATPase that helps remove clathrin coats from endocytic vesicles (Chappell et al., 1986).

The human hsp70 promoter contains consensus-binding sites for the factors CTF, CBF, Sp1, and TFIID, as well as two heat shock elements (HSEs) (Morgan et al., 1987; B. Wu et al., 1987; Morgan, 1989; Williams et al., 1989; Lum et al., 1990). The proximal HSE is sufficient for heat shock induction (Wu et al., 1986; Williams and Morimoto, 1990), and consists of five contiguous alternating NGAAN units, three perfect and two imperfect matches to the consensus sequence (Amin et al., 1988; Xiao and Lis, 1988). The CCAAT and TATA elements are essential for basal expression of the human hsp70 gene in nonheat-shocked cells, and the Sp1 site also contributes to basal transcription (Wu et al., 1986; Williams et al., 1989).

1.3 Hsps in the mammalian brain and kidney

During postnatal development of the rat brain, hsp90 protein levels decrease slightly in the cerebellum and basal hsp70 protein levels increase in the cerebral hemispheres (D'Souza and Brown, 1998). During the postnatal development of the rat kidney, hsp90 protein levels decrease dramatically and hsc70 protein levels also decrease (D'Souza and Brown, 1998). High levels of hsc70 mRNA exist in neuronal cell populations in control animals, but expression was not detected in glial cell populations (Sprang and Brown, 1987; Brown, 1990; Manzerra and Brown, 1990, 1992a, b; Pardue et al., 1992). Hsp90 is preferentially expressed in neuronal cell populations in the unstressed mammalian brain (Quraishi et al., 1996). Hsp90, hsc70 and hsp60 proteins in the unstressed rat kidney are localized to the convoluted tubules of the renal cortex (D'Souza and Brown, 1998). Hsp70 is induced in the cerebellums of rats trained for an active avoidance task, but is not induced in other brain regions (Ambrosini et al., 1999). Induction of hsp70 may therefore be attributed to learning in the cerebellum (Ambrosini et al., 1999). In the golden-mantled ground squirrel hsp70 mRNA levels in both brain and peripheral tissues appear to vary with the animal's arousal level (Bitting et al., 1999). In this diurnal animal, hsp70 expression is increased during the day (Bitting et al., 1999).

Hyperthermia is particularly damaging to the embryonic central nervous system, and can cause severe malformations in exposed embryos (Edwards, 1986; Edwards et al., 1997). Proliferating cells are particularly sensitive to temperature elevations, with immediate death of cells in mitosis with temperature elevations of 1.5 – 2.5°C (Edwards, 1986). Previous studies in our laboratory have shown a differential induction of hsp70 in

various cell types in the mammalian brain in response to a physiologically relevant increase in body temperature (Brown, 1990, 1994; Brown and Sharp, 1999). Oligodendrocytes and some microglia induce hsp70 mRNA after heat shock, while glial fibrillary acidic protein-positive astrocytes and neurons of the forebrain do not (Sprang and Brown, 1987; Foster and Brown, 1996, 1997). However, abundant levels of HSF1 exist in forebrain neurons, and this HSF1 is present in the nuclei of these neurons in both control and hyperthermic animals (Brown and Rush, 1999).

In response to ischemia and kainic acid, hsp70 is induced primarily in neurons with a pattern of induction correlating with the known histopathology of the insult (Vass et al., 1988, 1989; Gonzales et al., 1989). After localized tissue injury, namely a small surgical cut in the rat cerebral cortex, hsp70 mRNA is expressed by both neurons and glia at the injury site (Brown et al., 1989). It is possible that the high levels of hsc70 and hsp90 proteins in neurons prevents physiological heat shock conditions from damaging these cells, and that this is why there is no heat shock response in most neurons in response to those conditions. Cerebellar granule cells are the only type of neuron which induces hsp70 mRNA in the 1-hr hyperthermic rabbit (Sprang and Brown, 1987; Brown, 1990; Manzerra and Brown, 1992a). However, there is a delayed accumulation of hsp70 mRNA (Manzerra et al., 1993) and hsp90 β mRNA (Quraishi et al., 1996) in Purkinje neurons after heat shock. This delayed response may be due to after effects of the heat shock rather than to direct heat effects (Brown, 1994). In contrast to hsp70, no change in hsc70 mRNA levels occurs in the 1-hr heat shock rabbit brain (Brown and Rush, 1990). Synaptic transmission at the *Drosophila* larval neuromuscular junction is protected by

prior exposure to heat shock that strongly induces expression of hsps, particularly hsp70 (Karunanithi et al., 1999).

Currie et al. (1983) were unable to detect any induction of hsp70 protein in rat brain due to hyperthermia until the rats were more than 2 wk old, but they found high levels of induction in the older rat. However, Brown (1983) observed a robust induction of translatable hsp70 mRNA in heat shocked neonatal rat brain. Furthermore there was a lower threshold temperature required for hsp70 mRNA induction in the neonatal rat brain compared to the adult (Brown, 1983). Currie et al. (1983) found high levels of posthyperthermic hsp70 induction in liver, kidney and heart at all ages. Recently it was discovered that renal tubules from 8 – 10 day old rats have a greater HSF activation and hsp70 induction after stress than do renal tubules from mature rats (Gaudio et al., 1998).

1.4 Induction of hsps in stressed cells

In heat-stressed *Drosophila*, transcription of nonheat-shock mRNAs is repressed while heat shock genes are activated (Vazquez et al., 1993). Strikingly different responses occur with different genes (Vazquez et al., 1993). For example, transcription of histone H1 genes are severely inhibited even under mild heat shock conditions, transcription of the actin 5C gene is inhibited to an average extent, and the core histone genes and heat shock cognate genes are more resistant to inhibition (Vazquez et al., 1993). In the oomycete *Achlya ambisexualis* transcription of non-heat shock genes is repressed after heat shock (S. Brunt, personal communication). In particular, transcription of the actin gene is reduced several-fold (S. Brunt, personal

communication). However, in mammalian cells transcription of most nonheat shock mRNAs are only slightly affected by heat shock (Sadis et al., 1988; Ghosal and Jacob, 1996; D'Souza et al., 1998), although transcription of rRNA is inhibited significantly (Sadis et al., 1988; Ghosal and Jacob, 1996). Transcription of the β-actin gene is decreased during heat shock in HeLa cells (Abravaya et al., 1991b).

After heat shock, hsp mRNAs are selectively transported to the cytoplasm, as observed for hsp70 mRNA in heat-treated yeast (Moore et al., 1987; Liu et al., 1996). The transport of other poly(A) mRNAs to the cytoplasm is inhibited during stress (Liu et al., 1996; Tani et al., 1996). Severe heat shock blocks the splicing of intervening sequences from mRNA precursors (Yost and Lindquist, 1988). Certain hsp genes such as hsp70 lack introns, and are thus able to escape splicing arrest (Jolly and Morimoto, 1999). However, other hsp genes such as hsp90 do contain introns, which suggests that some intron-containing hsp transcripts can be correctly spliced during stress (Muhich and Boothroyd, 1989; Minchiotti et al., 1991; Osteryoung et al., 1993). Some of the unspliced mRNA transcripts that accumulate after a severe heat shock leave the nucleus and enter the pool of cytoplasmic mRNA (Yost and Lindquist, 1988). Translation of these mRNAs proceeds into the introns, resulting in the production of abnormal proteins (Yost and Lindquist, 1988). Thus the repression of normal transcription, which usually accompanies the heat shock response, may protect the cell from the large-scale synthesis of these aberrant proteins (Yost and Lindquist, 1988). Adenine-rich sequences in the 5' untranslated region (UTR) of heat shock gene mRNAs are recognized by ribosomes, which preferentially translate them at high temperatures (Klemenz et al., 1985; McGarry and Lindquist, 1985). Meanwhile the translation of non-hsp mRNAs is repressed (Jolly

and Morimoto, 1999). However, the expression of heat shock genes is regulated primarily at the level of transcription.

1.4.1 The heat shock element

The heat shock response is tightly controlled at the level of transcription, and in larger eukaryotes it is mediated by a family of heat shock transcription factors (HSFs) (Morimoto et al., 1994, 1996; Wu, 1995) which recognize and bind to heat shock elements (HSEs) present in the promoters of all heat shock genes (Fernandes et al., 1994; Wu, 1995). HSEs are made up of multiple adjacent and inverse iterations of the pentanucleotide motif 5'-nGAAn-3' (Fernandes et al., 1994). There are usually 3 – 6 of these 5-bp units in a functional HSE (Fernandes et al., 1994). The number of HSEs varies between different heat shock genes, as does the distance between the HSEs (Fernandes et al., 1994). Studies indicate that, while a single HSE is sufficient for inducibility, multiple HSEs can act in a cooperative way (Tanguay, 1988). It seems that the efficiency of transcriptional activation is related, within limits, to the number of HSEs (Tanguay, 1988). However, the presence of HSEs does not necessarily confer heat inducibility, as shown by their presence in the constitutively expressed non-heat-inducible heat shock cognate genes (Tanguay, 1988). HSEs can be positioned at different distances from the TATA box in either orientation, similar to enhancer elements (Tanguay, 1988). It is likely that each 5-bp unit is a binding site for one of the DNA-binding domains of the HSF trimer (Perisic et al., 1989). A complete, minimal binding site for trimeric HSF is provided by three 5-bp units (Fernandes et al., 1994). The

binding affinity of *Drosophila* HSF increases as the number of 5-bp units within an HSE increases, showing that HSF binds cooperatively (Topol et al., 1985; Xiao et al., 1991). Particularly strong binding occurs when there are 6 or more 5-bp units in an HSE (Fernandes et al., 1994).

HSF (at least human HSF) is unable to bind to HSEs which are packaged in nucleosomes (Taylor et al., 1991). Therefore (at least in *Drosophila*) the chromatin structures of uninduced heat shock gene promoters are in an open configuration (Wu, 1980; Costlow and Lis, 1984). On the uninduced hsp70 promoter, in both humans and *Drosophila*, there is a paused, transcriptionally engaged RNA polymerase ternary complex present over a narrow region centered at +21 to +35 in *Drosophila* (Rougvie and Lis, 1988; Giardina et al., 1992; Rasmussen and Lis, 1993) and at +45 in humans (Brown et al., 1996). In response to heat shock, not only does the initiation rate increase but also the transit time of the polymerase through the pause is drastically reduced. This is one of the best-characterized examples of regulated elongation in eukaryotes (Brown et al., 1998). The presence of a paused polymerase at the 5' end of uninduced heat shock genes may help these genes to respond rapidly to a heat shock (Fernandes et al., 1994).

1.4.2 Heat shock factors

In yeast and *Drosophila*, only one HSF has been cloned (Wiederrecht et al., 1988; Clos et al., 1990; Gallo et al., 1991; Morimoto, 1998). However, plants and larger animals have multiple HSFs (Scharf et al., 1990, 1993; Sarge et al., 1991; Schuetz et al., 1991; Nakai and Morimoto, 1993; Treuter et al., 1993; Czarnecka-Verner et al., 1995;

Nover et al., 1996; Nakai et al, 1997). Among vertebrates HSFs 1, 2 and 4 are ubiquitous, whereas HSF3 has been characterized only in avian species (Morimoto, 1998). The diversity of HSFs may provide redundancy and specialization of stress signals, permit differential control of the transcription rate of heat shock genes, and allow for interactions with other regulatory factors from other genetic networks (Morimoto, 1998).

Yeast HSF is essential for cell growth or viability in the absence of stress (Wiederrecht et al., 1988; Sorger and Pelham, 1988; Gallo et al., 1993). The HSF of the yeast *Saccharomyces cerevisiae* is required for the basal expression of heat shock genes as well as the induction of heat shock genes in response to heat stress (Sorger, 1990; Smith and Yaffe, 1991). *Drosophila* HSF is also essential for the heat shock response but, unlike yeast HSF, it is dispensable for general cell growth or viability under normal conditions (Jedlicka et al., 1997). *Drosophila* HSF is required for oogenesis and larval development (Jedlicka et al., 1997). After heat shock, HSF is localized to 164 sites on *Drosophila* polytene chromosomes (Westwood et al., 1991). Some of these sites appear to be developmental loci where HSF binds in order to repress transcription (Westwood et al., 1991). In vertebrates HSF1 is functionally analogous to yeast and *Drosophila* HSF as the principal stress-induced transcription factor (Rabindran et al., 1991; Sarge et al., 1991; Nakai and Morimoto, 1993). Mice lacking HSF1 can reach adulthood (McMillan et al., 1998). Constitutive expression of multiple hsps in cultured $hsf1^{-/-}$ embryonic cells was unaffected, but stress-induced transcription of hsps does not occur (McMillan et al., 1998). However, over- or underexpression of HSF1 in a cell does not necessarily result in change in stress-induced expression of heat shock genes. For example $hsf1^{+/-}$

heterozygous primary embryonic fibroblasts have ~50% as much HSF1 protein as wild type cells, but induce equivalent amounts of hsp70 mRNA after heat shock (McMillan et al., 1998). Overexpression of human HSF1 in murine cells does not increase the levels of heat shock gene expression after heat stress (Mivechi et al., 1995). This may be because the cells already contain enough HSF1 for a maximal heat shock response (Mivechi et al., 1995). In the embryonic chicken, the magnitude of the heat shock response as determined by Northern blotting does not always correlate with the level of HSF1 expression (Kawazoe et al., 1999). However, this may suggest that HSF3 is sufficient for induction of the heat shock response in chicken (Kawazoe et al., 1999).

HSF1 binds to an HSE in the prointerleukin 1β gene and represses its transcription, apparently by modulating the activity of adjacent factors such as NF-IL6 and Spi-1/PU.1 (Cahill et al., 1996). Since IL-1β expression in blood monocytes and tissue macrophages contributes to potentially damaging responses such as toxic shock, fever and inflammation (Dinarello et al., 1986; Dinarello, 1987; Schmidt and Abdulla, 1988; Velasco et al., 1991; Kappel et al., 1991), the repression of the prointerleukin 1β gene may be useful in limiting those responses (Cahill et al., 1996). In the $hsf1^{-/-}$ knockout mouse, there is exaggerated tumor necrosis factor alpha (TNF-α) production after endotoxin challenge, suggesting that HSF1 normally suppresses this pro-inflammatory cytokine (Xiao et al., 1999). Fever-like temperatures inhibit cytokine expression (Dinarello et al., 1986; Schmidt and Abdulla, 1988; Velasco et al., 1991; Kappel et al., 1991), and HSF1 may mediate this inhibition (Cahill et al., 1996). It would be interesting to investigate whether cytokine transcription is repressed in $hsf1^{-/-}$ monocytes after heat shock, as it is in normal monocytes. It would also be interesting to

see whether the transcription of the β-actin gene is decreased in hyperthermic $hsf1^{-/-}$ mice, as it is in normal mammalian cells (Abravaya et al., 1991b). HSF1 has also been shown to repress the c-fos gene (Chen et al., 1997). Two isoforms of HSF1 protein exist in mammalian cells, which arise from alternative splicing of HSF1 pre-mRNA (Goodson and Sarge, 1995; Fiorenza et al., 1995).

HSF2 is not activated by heat stress, but it is in a DNA-binding form during early mouse embryonic development, spermatogenesis, and in human erythroleukemia K562 cells exposed to hemin (Theodorakis et al., 1989; Sistonen et al., 1992, 1994; Mezger et al., 1994; Sarge et al., 1994; Rallu et al., 1997). These results suggest that HSF2 activity is associated with development and differentiation (Morimoto, 1998). K562 erythroleukemia cells are capable of differentiating along either erythroid or megakaryocytic lineages. When K562 cells undergo hemin-mediated erythroid differentiation, HSF2 protein levels rise (Pirkkala et al., 1999). During megakaryocytic differentiation, expression of HSF2 is rapidly down-regulated, leading to a complete loss of HSF2 protein (Pirkkala et al., 1999). Therefore, HSF2 could function as a lineage-restricted transcription factor during differentiation of K562 cells along either the erythroid or the megakaryocytic pathway (Pirkkala et al., 1999). HSF2 might regulate the testis-specific hsp70 gene (hsp70.2) (Rallu et al., 1997). Mouse HSF2 is in a DNA-binding form in all embryonal carcinoma cell lines tested and in embryonal stem cells, under normal growth temperature (Mezger et al., 1994; Murphy et al., 1994). HSF2 is activated during preimplantation stages (Mezger et al., 1994).

HSF2 is activated by downregulation of the ubiquitin-dependent protein degradation machinery (Mathew et al., 1998). Thus HSF2 has a role in the heat shock

response as a molecular response to the flux of non-native proteins targeted for protein degradation, as a complement to HSF1, which is principally activated by the flux of newly synthesized non-native proteins (Morimoto, 1998). Consequently, the kinetics of HSF1 activation typically is very rapid compared to the delayed activation profile of HSF2 (Sistonen et al., 1994; Morimoto, 1998). After activation by proteasome inhibitors, HSF2 induces the same set of heat shock genes that are induced during heat shock by HSF1 (Mathew et al., 1998). However, HSF1 and HSF2 may differ in their preferences for the consensus HSE and for numbers of HSE pentamer binding sites required for optimal binding (Kroeger and Morimoto, 1994). HSF2 may therefore have distinct target genes from those of HSF1, as well as different specificities for common target genes (Leppa et al., 1997a; Liu et al., 1997). For example, human HSF2 (hHSF2) and a constitutively active mutant of human HSF1 (hHSF1lz4m) both activate target gene transcription in yeast cells in response to thermal stress (Liu et al., 1997). However, hHSF1lz4m more strongly activates transcription of the yeast SSA3 gene, containing a consensus HSE with five consecutive pentameric nGAAn repeats, while hHSF2 selectively activates transcription of the yeast CUP1 gene, which has three nGAAn repeats with a gap between the second and third pentamers (Tanabe et al., 1999). The SSA3 gene is not significantly activated by hHSF2, and the CUP1 gene is only weakly activated by hHSF1lz4m (Tanabe et al., 1999).

In addition to its role as a transcription factor, HSF2 may be a regulator of protein phosphatase 2A (PP2A) (Hong and Sarge, 1999). PP2A is involved in the regulation of a number of important cellular processes, including intermediary metabolism, signal transduction, and cell cycle progression by dephosphorylating and thereby modulating

the activity of proteins that control these processes (Wera and Hemmings, 1995; Mumby, 1995; Barford, 1996; Faux and Scott, 1996; Cohen, 1997). PP2A is composed of a core heterodimer containing a protein called PR65 (A subunit) and a catalytic subunit. HSF2 interacts specifically with PR65 and blocks the binding of the catalytic subunit to PR65, probably by directly competing with the catalytic subunit for binding to PR65 (Hong and Sarge, 1999). Because PR65 association modulates the activity of the catalytic subunit (Chen et al., 1989; Kamibayashi et al., 1992; Agostinis et al., 1992; Wera et al., 1995; Turowski et al., 1997), this could lead to alterations in cellular PP2A activity (Hong and Sarge, 1999). The dual function of HSF2, as a transcriptional regulator of heat shock gene expression and a regulator of PP2A activity, may provide a mechanism for cross-talk between regulation of hsp expression and PP2A-regulated pathways, particularly those pathways involved in control of cell division (Hong and Sarge, 1999). Alternatively the two functions of HSF2 may be independent of one another (Hong and Sarge, 1999). Two isoforms of HSF2 exist in mammalian cells, arising from alternative splicing of HSF2 pre-mRNA (Goodson et al., 1995; Fiorenza et al., 1995).

In avian cells HSF3 and HSF1 are both expressed, and both are activated by stress (Nakai et al., 1995; Tanabe et al., 1997). Cells that lack HSF3 are severely compromised for induction of the heat shock response, even though HSF1 is expressed (Tanabe et al., 1998). In HSF3$^{+/-}$ cells, which have ~50% of the level of HSF3 protein as wild-type cells, the levels of heat shock mRNAs after extreme heat shock are only ~50% of that in wild-type cells (Tanabe et al., 1998). At intermediate heat shock temperatures, where HSF1 oligomerizes to an active trimer in wild-type cells, HSF1 remained as an inert monomer in the HSF3 null cell line (Tanabe et al., 1998). In HSF3 null cells, the basal

expression of the hsp90α, hsp90β and hsp110 genes is reduced, revealing that HSF3 also plays a significant role in the constitutive expression of heat shock genes (Tanabe et al., 1998). There are two isoforms of HSF4, HSF4a and HSF4b, derived from alternative RNA splicing events (Tanabe et al., 1999). HSF4a is functionally distinct from other vertebrate HSFs in that it lacks any activity as a positive transactivator (Nakai et al., 1997). Overexpression of HSF4a leads to a reduction in the basal expression of heat shock genes, presumably through constitutive DNA binding at the HSEs in their promoters (Nakai et al., 1997). However, overexpression of HSF4a does not interfere with HSF1-mediated heat shock gene transcription after heat shock (Nakai et al., 1997). The HSF4b isoform acts as a transcriptional activator (Tanabe et al., 1999). HSF4a is preferentially expressed in the heart, brain, skeletal muscle, and pancreas (Nakai et al., 1997). HSF4b mRNA is more abundant than HSF4a in mouse tissues, and HSF4b protein is present in brain and lung (Tanabe et al., 1999). Human HSF4b, like human HSF2 and a constitutively active mutant of human HSF1 (hHSF1lz4m) can complement the viability defect of an *S. cerevisiae* hsfΔ strain (Liu et al., 1997; Tanabe et al., 1999). Interestingly, wild type human HSF1 cannot complement the hsfΔ strain, because it does not trimerize in yeast (Liu et al., 1997). Human HSF4b activates target gene transcription with HSF1-like specificity in yeast (Tanabe et al., 1999).

1.4.3 Domains of HSF

Among the various cloned HSF genes there is an overall sequence identity of 40% at the amino acid level (Sarge et al., 1991; Morimoto et al., 1996). HSF homologues

from different species (eg. human HSF1 and mouse HSF1) show 80 - 90% conservation of amino acid sequence (Rabindran et al., 1991; Sarge et al., 1991; Morimoto et al., 1996). There are two domains in HSF which are highly conserved at the structural and amino acid sequence levels. These sequences are the winged helix-turn-helix DNA binding domain (Harrison et al., 1994; Vuister et al., 1994; Schultheiss et al., 1996) and an adjacent 80 residue hydrophobic repeat (HR-A/B) essential for trimer formation (Sorger and Nelson, 1989; Clos et al., 1990; Peteranderl and Nelson, 1992). The third helix of the DNA-binding domain is the recognition helix (Wu, 1995). Ten out of the fifteen amino acids which comprise helix 3 and the preceding turn are invariant in all cloned HSFs, making this the most highly conserved sequence in HSF (Wu, 1995). The trimerization domain (HR-A/B) has three arrays of hydrophobic heptad repeats and seems to constitute a leucine zipper (Landschulz et al., 1988; Cohen and Parry, 1990, 1994). Most leucine zipper proteins associate as homo- or heterodimers, so the trimeric assembly of HSF is very unusual (Wu, 1995).

There is also a structurally conserved carboxy-terminal transactivation domain in HSFs (Chen et al., 1993; Green et al., 1995; Shi et al., 1995; Zuo et al., 1995; Wisniewski et al., 1996), but the amino acid sequence of this domain is not widely conserved in evolution (Green et al., 1995). Activation of HSF1 leads to exposure of this transcriptional activation domain, leading to increased transcription of hsp genes (Fritsch and Wu, 1999). With the exception of the HSF of budding yeast and human HSF4, HSFs also contain another hydrophobic repeat (HR-C) located adjacent to the transactivation domain; this domain may suppress trimerization by interacting with HR-A/B (Nakai and Morimoto, 1993; Rabindran et al., 1993). Other sequences exist between HR-A/B and

HR-C which negatively regulate DNA binding and transcriptional activation (Nieto-Sotelo et al., 1990; Hoj and Jakobsen, 1994; Green et al., 1995; Shi et al., 1995; Zuo et al., 1995). In *Saccharomyces cerevisiae* HSF there is an amino-terminal transactivation domain which is not present in other HSFs (Sorger, 1990).

1.4.4 HSF1 regulation

In the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF is trimeric and largely bound to DNA *in vivo*, even in the absence of stress (Jakobsen and Pelham, 1988, 1991; Gross et al., 1990). *S. cerevisiae* HSF apparently acquires enhanced ability to stimulate transcription upon heat stress (Sorger et al., 1987; Jakobsen and Pelham, 1988). However, *Drosophila*, human and mouse HSFs are maintained in a latent, monomeric state until the onset of heat stress, when the monomers are converted quantitatively into trimers (Westwood et al., 1991; Westwood and Wu, 1993; Baler et al., 1993; Sarge et al., 1993; Sistonen et al., 1994). Trimerization of HSF is central to the acquisition of high-affinity binding to the HSE for higher eukaryotic HSFs (Wu, 1995).

In mammalian cells, HSF1 mediates the induction of heat shock genes in response to temperature elevation and other stresses, while HSF2 is not activated in response to heat shock and most other forms of cellular stress (Morimoto et al., 1994, 1996; Wu, 1995). In the control state, HSF1 exists as a non-DNA-binding monomer (Sarge et al., 1993; Westwood and Wu, 1993). The DNA-binding and transactivation domains of HSF1 are negatively regulated by intramolecular interactions with the central region of

HSF1 and by constitutive phosphorylation at critical serine residues (Knauf et al., 1996; Kline and Morimoto, 1997).

Mitogen-activated protein kinases (MAPKs) of the ERK-1 family phosphorylate HSF1 on two serine residues and repress transcriptional function at control temperature (Chu et al., 1996). Glycogen synthase kinase 3 and protein kinase C also phosphorylate HSF1 on two distinct serine residues and repress its transcriptional activity at 37°C (Chu et al., 1998). Coupling HSF1 repression to protein kinase activities associated with normal anabolic function may help ensure suppression of HSF1 at 37°C during growth and recovery from stress (Chu et al., 1998; He et al., 1998). However, this constitutive phosphorylation does not prevent HSF1 transcriptional activation after heat shock (Chu et al., 1996).

After heat shock HSF1 trimerizes and thus acquires DNA-binding activity. The trimeric form of HSF has a greater than 1000-fold increase in affinity for the HSE than the monomeric form (Wu, 1984, 1985; Kingston et al., 1987; Sorger et al., 1987; C. Wu et al., 1987; Zimarino and Wu, 1987; Taylor et al., 1991; Kim et al., 1995b). Furthermore, HSF1 becomes inducibly phosphorylated on serine and threonine residues after heat shock (Hoj and Jakobsen, 1994; Cotto et al., 1996; Knauf et al., 1996; Kline and Morimoto, 1997; Xia and Voellmy, 1997; Xia et al., 1998), and this phosphorylation can be detected as a decrease in the mobility of the factor on a SDS-polyacrylamide gel (Sarge et al., 1993; Jurivich et al., 1994; Mathur et al., 1994). Stress-induced alterations in the phosphorylation level of *Drosophila*, yeast or human HSF1 probably play no role in regulating trimerization or DNA binding (Sorger and Pelham, 1988; Nieto-Sotelo et al., 1990; Cotto et al., 1996; Knauf et al., 1996; Kline and Morimoto, 1997; Xia and

Voellmy, 1997; Fritsch and Wu, 1999). The acquisition of DNA-binding activity by HSF1 is independent of and precedes the event of inducible phosphorylation, and the acquisition of transcriptional activity is linked to the inducible phosphorylation (Cotto et al., 1996; Xia and Voellmy, 1997). In heat-shocked *S. cerevisiae*, transcription of hsp70 is correlated with increased phosphorylation of HSF at serine and threonine residues (Sorger and Pelham, 1988; Sorger, 1990). However, studies on *S. cerevisiae* and mammalian HSF suggest that inducible phosphorylation is not essential for transcription (Sarge et al., 1993; Hoj and Jakobsen, 1994). For example, when 3T3 cells are treated with the proline analog azetidine, HSF1 hyperphosphorylation does not occur, despite induction of HSF1 DNA-binding activity and transcription of hsp70 and hsp90 (Sarge et al., 1993). When mice are subjected to whole body hypothermia (cold treatment) HSF1 DNA-binding activity is induced and increased expression of hsp70 mRNA occurs in various tissues, but HSF1 does not appear to undergo inducible phosphorylation (Cullen and Sarge, 1997). Phosphorylation of HSF may act in the deactivation of transcriptional competence (Hoj and Jakobsen, 1994; Mivechi et al., 1994; Mivechi and Giaccia, 1995; Chu et al., 1996; Knauf et al., 1996; Kim et al., 1997; Kline and Morimoto, 1997; Chu et al., 1998; Xia et al., 1998; Kim and Li, 1999). The area of regulation seems to be serines 303 and 307 for human HSF1, and MAP kinases appear to be the regulatory enzymes (Mivechi et al., 1994; Mivechi and Giaccia, 1995; Chu et al., 1996; Knauf et al., 1996; Kline and Morimoto, 1997; Kim et al., 1997; Chu et al., 1998).

Although heat shock and most other stresses convert HSF1 to the fully active trimer, sodium salicylate treatment activates HSF1 to an intermediate trimeric state, which is bound *in vivo* to the hsp70 gene promoter, yet is transcriptionally inert (Jurivich

et al., 1992). This intermediate, transcriptionally inert trimeric form of HSF1 is analogous to the control form of *S. cerevisiae* HSF, while the fully active trimeric form of HSF1 is analogous to the activated form of *S. cerevisiae* HSF (Sorger et al., 1987). Thus the regulation of HSF has acquired additional levels of control during evolution (Morimoto, 1998).

When HeLa cells are exposed to a prolonged 42°C heat stress, transcriptional induction of the hsp70 gene and activation of HSF occur only during the initial phase of the heat shock and then attenuate, despite the continued exposure to the elevated temperature (Mosser et al., 1988). However, attenuation of the heat shock response occurs more rapidly if the cells are returned to 37°C after a 40 min, 42°C heat shock (Abravaya et al., 1991). Levels of activated HSF are higher when cells are heat shocked at 43°C rather than 42°C (Abravaya et al., 1991). There is a tight correlation between levels of activated HSF and the fold of transcriptional induction of hsp70 (Abravaya et al., 1991; D'Souza et al., 1998). After thermal stress there is a very rapid induction of hsp70 transcription, with activation apparent even at 1 min following temperature elevation (Abravaya et al., 1991). Genomic footprinting analysis shows that attenuation of hsp70 transcription is mediated by the release of bound HSF1 from the HSE of the promoter (Abravaya et al., 1991). The release of bound HSF from the hsp70 promoter *in vivo* occurs faster than would be predicted from *in vitro* measures of dissociation, suggesting that *in vivo* release of bound HSF may be a facilitated event (Abravaya et al., 1991). After thermal stress the transcriptional activation, maintenance, and attenuation of hsp70, hsp90 and hsp60 follow a similar time course (Abravaya et al., 1991; D'Souza et al., 1998). This may suggest that within any one species, HSEs in the various heat shock

promoters have similar affinities for HSF, allowing a coordinate pattern of response to heat shock (Abravaya et al., 1991).

Molecular chaperones such as hsp90 have been shown to help in maintaining HSF1 in an inert, non-DNA-binding state under nonstress conditions (Ali et al., 1998; Zou et al., 1998). Hsp70 and the cochaperone hdj1 interact directly with the transactivation domain of HSF1 and repress heat shock gene transcription (Shi et al., 1998). In the same study Hsc70 was also shown to bind to the activation domain of HSF1 (Shi et al., 1998). It is thus possible that hsc70, like hsp70, can repress the transactivation ability of HSF1. Hsp70 chaperones and cochaperones alone do not prevent the formation of HSF1 trimers, showing that the acquisition of transcriptional activity is a distinct process from regulation of trimer formation (Morimoto, 1998). Hsp70 does not affect activation of HSF1 DNA-binding nor inducible phosphorylation of HSF1 (Shi et al., 1998). However, overexpression of hsp70 accelerates HSF trimer dissociation, although HSF and hsp70 associate under both non-stressed and heat-stressed conditions (Price and Calderwood, 1992; Mosser et al., 1993; Rabindran et al., 1994).

HSF that was translated *in vitro* in reticulocyte lysates, purified *Drosophila* HSF and recombinant mouse and human HSF1 can acquire DNA binding after an *in vitro* heat shock (Mosser et al., 1990; Goodson and Sarge, 1995b; Larson et al., 1995; Zhong et al., 1998). *In vitro* translated HSF1 or purified HSF can also be activated by low pH (pH 6.5) or salicylate (Mosser et al., 1990; Zhong et al., 1998). These experiments show that HSF can directly respond to certain stresses, but they do not exclude a role for other negative regulators that may keep HSF in a repressed state (Morimoto, 1998). The cellular environment determines the temperature for activation of HSF. For example,

growing HeLa cells at temperatures less than 37°C reduces the temperature required to completely activate HSF1 (Abravaya et al., 1991). Similarly, human HSF1 expressed in *Drosophila* cells becomes activated at the temperature of the *Drosophila* heat shock response (37°C) (Clos et al., 1993).

HSF1 is activated by multiple stress conditions, including heat shock, oxidative stress, and amino acid analogues that lead to the synthesis of non-native proteins (Morimoto et al., 1990, 1994b, 1996; Wu, 1995). Experimental evidence links the activation of the heat shock response to increased levels of denatured and misfolded proteins (Baler et al., 1992). Injection of denatured protein into unstressed *Xenopus* oocytes results in the induction of HSF activity (Ananthan et al., 1986; Mifflin and Cohen, 1994). Pretreatment with protein synthesis inhibitors blocks the induction of HSF and heat shock gene transcription, suggesting that nascent protein chains are a critical target for protein damage (Mosser et al., 1988; Amici et al., 1992).

The latest theory of how HSF1 is activated by stress to a DNA-binding form is the following (Zou et al., 1998). In the unstressed cell, most HSF1 is bound to either hsp90 or an hsp90-containing multichaperone complex. This complex is unstable and continuously dissociates and reassembles. After stress, nonnative proteins accumulate and compete with HSF1 for binding to hsp90. As a result, unbound HSF1 protein accumulates. HSF1 trimerization is strongly favoured both because of the increased concentration of unbound HSF1 and because the competing pathway (reassembly of HSF1-hsp90 complexes) is blocked. The HSF1 trimerization reaction is strongly dependent on concentration (Larson et al., 1988). Hsp70 may also be involved in

regulating the trimerization of HSF1 to a lesser extent (Mosser et al., 1993; Rabindran et al., 1994).

After stress human HSF1 relocalizes within the nucleus to form large, irregularly shaped granules distinct from other nuclear bodies (Sarge et al., 1993; Cotto et al., 1997; Jolly et al., 1997). However, in mouse 3T3 cells HSF1 does not form these stress granules (Sarge et al., 1993). Instead there is only a diffuse HSF1 staining throughout the nucleus in those cells after heat shock (Sarge et al., 1993). Since the number of HSF1 foci correlates with the ploidy of human cells, the HSF1 granules may have a chromosomal target (Morimoto, 1998). The kinetics of the appearance and disappearance of HSF1 granules parallels the activation and attenuation of HSF1 and the transcription of heat shock genes (Cotto et al., 1997). Furthermore the appearance of HSF1 granules is a reliable visual indicator of the transcriptional activity of HSF1 (Cotto et al., 1997). Sodium salicylate, which induces HSF1 trimers that are transcriptionally inert (Jurivich et al., 1992; Cotto et al., 1996), does not cause HSF1 granules to form, while other stresses that induce transcriptionally active HSF1 trimers to form do cause the formation of HSF1 granules (Cotto et al., 1997). However, the granules do not seem to colocalize with sites of hsp90α, hsp90β or hsp70 gene transcription (Jolly et al., 1997). Furthermore, stress granules are present in heat-shocked mitotic cells that are devoid of transcription (Jolly et al., 1999). This shows that the stress granules are involved in a function distinct from transcription (Jolly and Morimoto, 1999). The HSF1 stress granules may represent a novel nuclear compartment where the multistep activation of HSF1, including trimerization, phosphorylation, and acquisition of DNA binding activity

takes place, and from which transcriptionally competent HSF1 trimers are dispensed to hsp genes (Jolly and Morimoto, 1999).

Although the heat-shocked form of HSF1, or its homologue the *Drosophila* HSF, is known to be localized to the nucleus, there is some controversy regarding the location of the unshocked form (Wu, 1995). In one study, it was seen in both nucleus and cytoplasm (Sarge et al., 1993), while in other studies it was seen predominantly in the nucleus (Westwood et al., 1991; Wu et al., 1994; Mercier et al., 1999). In the unstressed rat brain, HSF1 is localized to the nucleus in both neurons and glia (Brown and Rush, 1999). In early *Drosophila* embryos HSF is restricted to the cytoplasm even after heat shock, and hsp70 cannot be induced during this period (Wang and Lindquist, 1998). HSF moves from the cytoplasm to the nucleus in the absence of stress precisely when the capacity to induce hsp70 is acquired (Wang and Lindquist, 1998). Thus the nuclear transport of HSF controls the inducibility of hsp70 in early *Drosophila* embryos (Wang and Lindquist, 1998). Apparently, in early embryos the high rate of nuclear division cause the detrimental effects of hsp70 on cell division to outweigh its beneficial effects on survival, resulting in the evolution of a mechanism to restrict its expression (Wang and Lindquist, 1998).

Complexes of hsp70 with HSF trimers have been detected during attenuation of the heat shock transcriptional response (Abravaya et al., 1992; Baler et al., 1992; Shi et al., 1998). Overexpression of hsp70 or hdj-1/hsp40 in the absence of stress prevents the inducible transcription of heat shock genes (Mosser et al., 1993; Rabindran et al., 1994; Shi et al., 1998). However, hsp70 chaperones and cochaperones alone are insufficient to prevent HSF1 from trimerizing, thus showing that acquisition of transcriptional activity is

a distinct process from the regulation of trimerization (Morimoto, 1998). Hsp70 and hdj-1 repress the transcriptional activity of HSF1 during the attenuation of the heat shock response (Morimoto, 1998). There is also evidence for the association of HSF1 with hsc70 *in vivo* (Nunes and Calderwood, 1995). Hsc70 also binds to the HSF1 activation domain (Shi et al., 1998). However, because hsc70 is normally engaged in a variety of cellular activities, including protein synthesis, protein assembly and translocation, and protein degradation, it may not be available to negatively regulate HSF1 (Shi et al., 1998). During attenuation of the heat shock response, HSF1 trimers are negatively regulated by HSF binding protein 1 (HSBP1), which binds both to the hydrophobic heptad repeat of HSF1 and to hsp70, thus leading to the dissociation of HSF1 trimers and the appearance of HSF1 inert monomers (Satyal et al., 1998; Morimoto, 1998). HSBP1 is conserved from *Caenorhabditis elegans* to humans (Morimoto, 1998).

The hsp90β promoter is activated by both the NF-IL6 (C/EBPβ) and the signal transducer and activator of transcription-3 (STAT-3) transcription factors, which have binding sites that are located close to the HSE (Stephanou et al., 1998). These two transcription factors are activated by the binding of interleukin-6 (IL-6) to its receptor (Akira et al., 1994). HSF1 interacts with NF-IL6 on the promoter of IL1B during repression of that gene (Cahill et al., 1996; Housby et al., 1999). HSF1 can bind directly to NF-IL6 (Housby et al., 1999). The hsp70 and hsp90β promoters are activated by the STAT-1 transcription factor (Stephanou et al., 1999). The STAT-1 signaling pathway is activated by interferon-γ (IFN-γ) (Stephanou et al., 1999). A short sequence in both the hsp70 and hsp90β promoter is important for mediating IL-6 and IFN-γ signaling as well as the heat shock response (Stephanou et al., 1999). This sequence contains the HSE and

a STAT-like binding site (Stephanou et al., 1999). STAT-1 and HSF1 interact with one another via a protein-protein interaction and produce a strong activation of transcription (Stephanou et al., 1999). Interestingly, HSF1 is able to bind to the promoter and modulate hsp promoter activity under non-heat stress conditions when STAT-1 is activated (Stephanou et al., 1999).

Thermal induction of hsp70 is repressed by overexpression of the Ku-70 subunit of the Ku protein (Li et al., 1995; Yang et al., 1996a, 1996b). Thermal induction of other heat shock proteins is not affected in Ku-overexpressing cell lines, nor is the phosphorylation state or DNA-binding ability of HSF1 affected (Li et al., 1995; Yang et al., 1996a, 1996b). This suggests that Ku protein plays a role in the modulation of the heat shock response *in vivo* (Li et al., 1995). Ku protein is one component of a DNA-dependent protein kinase, which phosphorylates many different transcription factors such as Sp1 (Gottlieb and Jackson, 1993), c-Jun (Bannister et al., 1993), p53 (Lees-Miller et al., 1990) and RNA Pol II (Dvir et al., 1993) *in vitro*. Furthermore Ku protein may have a role as a transcription factor (Kim et al., 1995). Ku autoantigen directly modulates RNA Pol I-mediated transcription (Knuth et al., 1990; Kuhn et al., 1993; Hoff and Jacob, 1993). After heat shock the amount of Ku-70 subunit decreases in a time-dependent manner, and disappears after 3 hr of heat shock (Ghosal and Jacob, 1996). This correlates well with the inhibition of RNA Pol I-directed transcription of the rRNA gene after heat shock in mouse lymphosarcoma cells (Ghosal and Jacob, 1996).

Certain small molecules modulate HSF1 activity. Non-steroidal anti-inflammatory drugs (NSAIDS), prostaglandins and arachidonic acid lower the temperature at which the heat shock response is observed (Morimoto, 1993; Lee et al.,

1995). All of these substances are involved in the inflammatory response (Jurivich et al., 1992). Exposure to aspirin or indomethacin at concentrations comparable to clinical levels results in the priming of human cells for subsequent exposure to heat shock and other stresses, the enhanced transcription of heat shock genes, and protection from thermal injury (Amici et al., 1995). Non-steroidal anti-inflammatory drugs also inhibit cytokine expression, possibly due to their ability to activate HSF1 to a DNA-binding form (Housby et al., 1999). Development of new drugs that activate a heat shock response in human cells may aid in the treatment of diseases that cause tissue damage, including stroke, neurodegenerative disease, myocardial insufficiency and trauma (Morimoto and Santoro, 1998).

1.4.5 Regulation of HSF2 and other HSFs

The lowest molecular weight isoform of HSF2, HSF2-β, is a less potent transcriptional activator than the larger HSF2-α isoform (Goodson et al., 1995). HSF2-β seems to act as a negative regulator of HSF2 DNA-binding activity and transcriptional induction of heat shock genes during hemin-mediated erythroid differentiation of K562 cells (Leppä et al., 1997b). As mentioned previously, HSF2 is activated by a downregulation of the ubiquitin-dependent protein degradation machinery (Mathew et al., 1998). HSF2 DNA-binding activity is induced in mammalian cells by exposure to the proteasome inhibitors hemin, MG132, and lactacystin (Mathew et al., 1998). HSF2 is a short-lived protein (Mathew et al., 1998). There is an increased synthesis and decreased degradation of HSF2 protein after proteasome inhibition, resulting in the accumulation of

HSF2 (Mathew et al., 1998). However, levels of HSF2 mRNA do not increase, showing that HSF2 levels are posttranscriptionally regulated (Mathew et al., 1998). There is a correlation of HSF2 activity with HSF2 protein levels (Mathew et al., 1998). This correlation between HSF2 protein accumulation and activation is also observed when HSF2 is overexpressed by transient transfection (Finley et al., 1987). The regulation of HSF2 may be similar to that of the *Escherichia coli* heat shock promoter-specific σ^{32} subunit of RNA polymerase (Mathew et al., 1998). Under normal conditions σ^{32} is a short-lived protein (Tilly et al., 1989). Upon heat shock σ^{32} levels increase, chiefly due to decreased degradation by the FtsH protease, and this ensures that the heat shock genes are induced (Tilly et al., 1989; Herman et al., 1995; Tomoyasu et al., 1995).

Ubiquitination and proteasome activities are modulated during stress (Finley et al., 1988; Hayes and Dice, 1996; Hilt and Wolf, 1996; Sherman and Goldberg, 1996; Varshavsky, 1997) and during development and differentiation (Shimbara et al., 1992, 1993; Hochstrasser, 1995; Varshavsky, 1997). Proteasome inhibition results in expression of heat shock proteins (Zhou et al., 1996; Bush et al., 1997; Lee and Goldberg, 1998), and these heat shock proteins seem to be involved in ubiquitin-proteasome-mediated protein degradation (Mathew et al., 1998). Inhibition of proteasome activity results in the accumulation of misfolded proteins destined for proteasomal degradation (Mathew et al., 1998). Therefore HSF1 can be activated by proteasome inhibition in some cell lines (Kawazoe et al., 1998; Kim et al., 1999). In avian cells, HSF3 is also activated by proteasome inhibitors (Kawazoe et al., 1998). Induction of DNA-binding activity in HSF2 is accompanied by a transition from an inert, dimeric state to an activated trimer (Morimoto, 1998).

The avian heat shock factor HSF3 seems to be involved in the persistent activation of heat shock genes following severe stress (Tanabe et al., 1997). HSF3 is not activated by mild stress that will activate HSF1; it is only activated by severe stress (Tanabe et al., 1997). HSF3 activation after stress occurs more slowly than HSF1 activation, but HSF3 is activated for a longer period than HSF1 (Tanabe et al., 1997). After severe heat shock, HSF1 aggregates and cannot bind to DNA any longer, but HSF3 remains activated (Tanabe et al., 1997). Denaturation of nascent peptides may be the trigger for the activation of both HSF3 and HSF1 (Tanabe et al., 1997). Activation of HSF3 after heat shock is accompanied by nuclear translocation, acquisition of DNA-binding activity, and conversion from an inert dimeric form to a DNA-binding trimeric form (Tanabe et al., 1997). However, cells that lack HSF3 are severely compromised for induction of the heat shock response, even though HSF1 is expressed (Tanabe et al., 1998). HSF3 interacts with other transcription factors, and can be activated by the c-Myb proto-oncogene, independent of stress, via direct protein-protein interaction between the HSF3 and Myb DNA binding domains (Kanei-Ishii et al., 1997). Because c-Myb participates in cell proliferation, this regulatory pathway may provide a link between cellular proliferation and the stress response (Kanei-Ishii et al., 1997). c-Myb protein is highly expressed during the G_1 to S transition of the cell cycle and is required for the G_1 to S transition and maintenance of the proliferative state (Graf, 1992). The expression of the hsp70 gene is also induced at the G_1 to S transition in non-heat-shocked cells (Milarski and Morimoto, 1986). Therefore c-Myb-induced activation of HSF3 may contribute to the cell cycle-dependent expression of stress-responsive genes (Kanei-Ishii et al., 1997).

Another heat shock factor, HSF4, has been found in mammalian cells (Nakai et al., 1997; Tanabe et al., 1999). HSF4 lacks the carboxyl-terminal hydrophobic repeat that is shared among all other vertebrate HSFs, and it exists in a trimeric, DNA-binding form in unstressed cells (Nakai et al., 1997; Tanabe et al., 1999). There are two isoforms of HSF4, derived from alternative RNA splicing events (Tanabe et al., 1999). HSF4a acts as an inhibitor of the constitutive expression of heat shock genes, but its DNA-binding activity is lost *in vitro* upon heat shock (Morimoto, 1998). Like HSF1, HSF4b shows retarded mobility on an SDS-PAGE gel after heat shock, suggesting that HSF4b may likewise be phosphorylated in response to heat shock (Tanabe et al., 1999). The HSF4b isoform elevates the expression of heat shock genes in the absence of stress (Tanabe et al., 1999). Heat shock and other stresses stimulate transcription of target genes by HSF4b in human cells, although the ability of HSF4b to activate transcription is much lower than that of HSF1 (Tanabe et al., 1999). Therefore HSF4b may regulate the constitutive expression of heat shock genes, and may also induce the expression of these genes in response to stress in a manner analogous to yeast HSF (Tanabe et al., 1999). HSF4 is constitutively localized to the nucleus (Nakai et al., 1997; Tanabe et al., 1999).

1.5 HSF in the mammalian brain

HSF2 may be involved in mouse embryonic development (Rallu et al., 1997). HSF2 exists in a DNA-binding form in mouse embryos during post-implantation development (Rallu et al., 1997). This DNA-binding activity disappears between E13.5 (embryonic day 13.5) and E15.5 in the limbs and tailbud, but persists after E15.5 in the

CNS (Rallu et al., 1997). *In situ* hybridization shows that at E12.5 HSF2 mRNA is preferentially localized to the ventricular or ependymal layers of the neural tube, corresponding to the proliferative cell-rich zones, in contrast to postmitotic cells (Rallu et al., 1997). This suggests that HSF2 may have a role in neural proliferation (Rallu et al., 1997). However, immunocytochemistry revealed no obvious correlation between the levels of HSF2 and the levels of a number of developmentally regulated heat shock proteins in the CNS (Rallu et al., 1997). This may indicate that the function of HSF2 during development extends beyond induction of classical hsps (Morano and Thiele, 1999). The developmental regulation of many of the inducible hsps may occur through other, as yet unidentified transcription factors (Morano and Thiele, 1999). It is noteworthy that during mouse embryogenesis the smallest, inhibitory, isoform of HSF2 is quantitatively the major one (Rallu et al., 1997). Immunocytochemistry of the postnatal day 2 rat brain shows that HSF2 is localized to the nuclei of cortical and brainstem neurons (Brown and Rush, 1999). In the postnatal day 30 rat, HSF2 protein is localized in the cytoplasm of Purkinje neurons in the cerebellum and neurons of the brainstem and cerebral cortex (Brown and Rush, 1999). In the rat spinal cord, HSF2 is scattered in both nucleus and cytoplasm of the motoneurons of the ventral horns, while glial cells show a nuclear staining (Stacchiotti et al., 1999). There is no activated HSF2 in adult mouse tissues (Fiorenza et al., 1995), except testis (Sarge et al., 1994).

HSF1 does not seem to show any constitutive DNA-binding activity in postimplantation mouse embryos (Rallu et al., 1997). However, $hsf1^{-/-}$ knockout mice have an increased chance of prenatal death, and $hsf1^{-/-}$ female mice are infertile (McMillan et al., 1998). Furthermore, $hsf1^{-/-}$ mice have defects of the chorioallantoic

placenta, growth retardation, and exaggerated tumor necrosis factor alpha production resulting in increased mortality after endotoxin challenge (Xiao et al., 1999). However, the constitutive hsp expression is not altered appreciably by the disruption of the HSF1 gene (McMillan et al., 1998; Xiao et al., 1999), suggesting that HSF1, like *Drosophila* HSF, might be involved in regulating other important genes or signaling pathways (Xiao et al., 1999). In the rat brain, immunocytochemistry shows that HSF1 protein is prepositioned in the nucleus in a range of neuronal and glial cell types throughout postnatal development (Brown and Rush, 1999). In the unstressed rat spinal cord HSF1 is distributed in the perinuclear compartment of selected neurons of the gray matter, but localized predominantly to the nuclei of astrocytes, oligodendrocytes and ependymal cells (Stacchiotti et al., 1999). In this tissue the different ability of neurons vs. glial cells to respond to stress may be correlated with different constitutive localization of HSF1 (Stacchiotti et al., 1999).

As previously mentioned, most neurons do not induce hsp70 after heat shock. Marcuccilli et al. (1996) found that the inability of primary cultured rat hippocampal neurons to induce a heat shock response after hyperthermia was caused by a lack of HSF1 protein in those cells. However, this HSF1 deficit appears to be a feature of this *in vitro* system, since hippocampal neurons can induce hsp70 *in vivo* in response to severe stress such as ischemia (Nowak et al., 1994). A retinoblastoma cell line (Y79), which has some neuronal-like features, has attenuated hsp70 induction but normal hsp90α induction after heat stress (Mathur et al., 1994). HSF1 is activated normally in these cells, but does not bind to the hsp70 promoter (Mathur et al., 1994). Furthermore adjacent hsp70 promoter elements, previously characterized as sites of constitutive interaction with basal

transcription factors (Abravaya et al., 1991b) are unoccupied in Y79 cells (Mathur et al., 1994). Since a transfected hsp70 promoter was heat-inducible in Y79 cells, the deficiency of induction of the endogenous hsp70 gene may be a consequence of regulation of the chromatin structure (Mathur et al., 1994). This suggests that inducible transcription of hsp70 requires other factors in addition to HSF1, perhaps involved in regulating access to the hsp70 locus (Mathur et al., 1994). It is not known whether or not HSF1 is activated after heat stress in neurons *in vivo*. It is possible that neurons are not stressed sufficiently by a fever-like hyperthermic episode to induce a heat shock response. A decline in the stress-induced expression of hsp70 with age occurs in a variety of tissues from rodents (Gutsmann-Conrad et al., 1999). For example, the induction of hsp70 mRNA and protein after heat shock in hepatocytes and splenocytes of old (20 – 22 months) female Fisher 344 rats is markedly less than in young adult (4 – 8 months) female rats (Gutsmann-Conrad et al., 1999). Although levels of HSF1 protein are similar in splenocytes and hepatocytes from old female rats compared to young adult female rats, the levels of HSE-binding are lower in splenocytes from old rats (Gutsmann-Conrad et al., 1999).

1.6 Thesis objectives

The magnitude of induction of hsp70 after hyperthermic stress in brain, kidney and other tissues changes as postnatal development proceeds (Currie et al., 1983; Gaudio et al., 1998; Xia et al., 1999). Since HSF1 is responsible for the stress response in mammals (Morimoto et al., 1994, 1996; Wu, 1995), it was of interest to determine

whether HSF1 protein levels differed during development in various tissues and whether levels of HSF1 protein correlated with the extent of the hsp70 stress response. The constitutive levels of hsp70, hsc70, hsp90 and hsp60 in the rat brain and kidney change during postnatal development (D'Souza and Brown, 1998). Since HSF2 may serve as a developmental regulator of heat shock gene expression during embryogenesis (Loones et al., 1997), HSF2 protein levels in the developing rat brain and kidney were investigated in order to see if there was a correlation between postnatal HSF2 levels and constitutive levels of hsps. Gel mobility shift analysis was used to determine if HSF was in a DNA-binding form in these tissues during development. In addition, heat shock experiments were carried out on 2-day neonatal and 40-day adult rats to measure DNA-binding activation and hyperphosphorylation of HSF1, and also subsequent induction of hsp70 protein in the brain, kidney and liver.

2. MATERIALS AND METHODS

2.1 Animals

Wistar rats were purchased from Charles River, kept on a 12 hr light/dark cycle at 23°C, and fed ad libitum.

2.2 Induction of Hyperthermia

Body temperature of 2-day old Wistar rat pups was elevated from 30°C to 40°C by placing the animals 12 cm below an infrared lamp. In a second experiment, body temperature of 2-day old Wistar rat pups was elevated from 35°C to 40°C by placing the animals 23 cm below an infrared lamp. The initial temperature of the rat pups was lower in the first experiment because they had spent some time away from the mother. The capacity for thermoregulation is not yet fully developed in 2-day rats. Body temperature was monitored with a needle thermistor probe placed under the animals' armpits. Following maintenance of body temperature at the elevated level for 5 min, the animals were left at room temperature for various lengths of time before sacrifice by decapitation. This method of heat-shocking 2-day rats has been previously described (Brown, 1983; Brown and Rush, 1990). Littermates were used in this study.

Body temperature of 40-day old Wistar rat adults was elevated by 3.3±0.8°C above normal body temperature (38.3±0.7°C) by placement of animals in a dry incubator preheated to 42°C. Body temperature was monitored using a rectal thermistor probe.

Maximum rectal temperature was reached 40 min after placement in the incubator. Rats were maintained at the increased body temperature of plus 3.3±0.8°C for various lengths of time before sacrifice by decapitation, or else maintained at the increased body temperature for 1 hr and then left at room temperature for various lengths of time before sacrifice. Brown and Rush (1999) successfully used this method to heat-shock adult rats.

2.3 Gel Mobility Shift Assays

2.3.1 Tissue preparation

Neural regions, kidney and liver were quickly removed from sacrificed Wistar rats (Charles River), and subjected to fast freezing on tin foil placed over powdered dry ice. Tissue extracts were prepared by homogenization, in a glass/teflon homogenizer, of previously frozen tissue samples in 5 volumes of homogenization buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin) as described previously (Brown and Rush, 1996). This homogenization buffer ("buffer C+") was a modification of "buffer C", previously described by Sarge et al. (1994). Homogenization was immediately followed by centrifugation of 1 ml aliquots in a microfuge at 12,000*g* for 2 min at 4°C. The supernatant was quickly drawn off without disturbing the pellet and frozen in aliquots of 50 µl in powdered dry ice, followed by storage at -70°C. Protein concentrations were determined using the Bio-Rad protein assay.

2.3.2 Labeling of HSE oligonucleotide

A synthetic, self-complementary HSE oligonucleotide (5'-CTAGAAGCTTCTAGAAGCTTCTAG-3') was obtained from Vetrogen Corp. The self-annealed HSE oligonucleotide was end labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Mandel Scientific). The mixture was extracted with phenol:chloroform:IAA and then spun through a 3 ml column of G25-50 sephadex in TE buffer in a swinging bucket centrifuge.

2.3.3 Non-denaturing gel electrophoresis

Native gel mobility shift assays were carried out in duplicate, as described by Sarge et al. (1991), with a self-complementary consensus HSE oligonucleotide (5'-CTAGAAGCTTCTAGAAGCTTCTAG-3') which contained four perfect inverted 5-NGAAN-3' repeats after annealing. This oligonucleotide has been previously used in gel mobility shift assays to detect HSF1 and HSF2 DNA-binding activity in rat (Higashi et al., 1995). Tissue extract containing 30 µg of protein was pipetted into an eppendorf tube, and the volume was adjusted to 5 µl with buffer C+. Then 20 µl of "Reaction Mix" (10 mM Tris pH 7.5, 1 mM EDTA, 5% glycerol) containing 0.5 µg poly(dI-dC) and 50,000 cpm of HSE probe (~0.5 ng) were added and the mixture was incubated at room temperature for 20 min before loading on the gel. This method was used for the kidney data in Fig. 3A and for all of the data in Figs. 3B, 5A, 6A and 6B. For the other gel mobility shift assays, HSE probe was not added to the Reaction mix. Instead, all the

other ingredients for the gel shift reaction were added first and incubated for 20 min at room temperature. Then 50,000 cpm of HSE probe was added, followed by a further 20 min incubation at room temperature, before loading on the gel. This reduced nonspecific signal. The gel shift reactions were run on a 4% nondenaturing polyacrylamide gel for 2.5 hr at 15 mA/gel, dried on a gel dryer and exposed to Kodak X-OMAT film with an intensifying screen at -70°C. Data shown are representative of independent experiments carried out on two sets of animals.

2.3.4 Gel supershift assays

For experiments involving the preincubation of tissue extracts with HSF1 or HSF2 polyclonal antibodies prior to gel mobility shift analysis, the procedure described by Sarge et al. (1993, 1994) was followed, using antibodies donated by Kevin Sarge (University of Kentucky) and Richard Morimoto (Northwestern University). Briefly, tissue extracts were pre-incubated with HSF1 or HSF2 polyclonal antibodies diluted in TSG buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol) at room temperature for 20 min. Then Reaction Mix, containing 0.5 µg of poly(dI-dC) and 50,000 cpm of HSE probe, was added and the mixture was incubated at room temperature for a further 20 min before loading on the gel. Data shown are representative of independent experiments carried out on two sets of animals, except where otherwise noted.

2.4 Western Blot Analysis

2.4.1 Tissue preparation

Homogenates previously used for the gel shift assays were used for the Western blot analysis. Whole cell homogenates were prepared from control and 6 hr post-hyperthermic 2-day rat tissues (Fig. 5C). These tissues were homogenized in 5 volumes of 0.32 M sucrose. It was noted that HSF1 protein could not be detected in these samples, and hence was likely degraded because of the absence of protease inhibitors in the homogenization solution. Heart tissue (Figs. 1C and 2C) was prepared as a whole cell homogenate in buffer C+. Protein levels were determined using the Bio-Rad protein assay. Protein samples (100 µg of protein) were boiled for 5 min in solubilizing solution (4 M urea, 1% SDS, 1% β-mercaptoethanol, 10% glycerol) to solubilize the proteins. Bromophenol blue dye was added to the samples prior to loading on the gel.

2.4.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out on 10% gels plus a 5% stacking gel, using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie blue stain and used to adjust sample volumes for equal loading. Then a second set of gels was run for the Western blots. Protein was transferred electrophoretically for 17 – 18 hr to a nitrocellulose membrane in transfer buffer (50 mM boric acid, 4 mM β-mercaptoethanol, 2 mM EDTA).

2.4.3 Processing of Western blots

Blots were stained with Ponceau S to verify equal loading. The blots were then washed 4 x 5 min in TBST (10 mM Tris, 0.25 M NaCl, 0.05% Tween 20, pH 7.5) and then blocked. Blots that would later be probed with HSF1 or HSF2 primary antibodies were blocked overnight in 5% Carnation milk powder, 5% goat serum, 0.02% sodium azide in TBST. Blots that would later be probed with C92 or 1477 primary antibodies were blocked for 2 hr in 5% Carnation milk powder in TBST. After blocking, the blots were rinsed briefly with TBST and then incubated with primary antibody solution overnight. The primary antibody solution contained the appropriate primary antibody diluted in 1% purified BSA (fatty acid free), 0.02% sodium azide in TBST. Then the blots were washed 4 x 10 min in 1% BSA (98% pure) in TBST, and incubated for 2 hr at room temperature with horseradish peroxidase-labeled secondary antibody (Sigma) in 1% BSA (98% pure) in TBST. Blots which were probed with HSF1, HSF2 or 1477 primary antibodies were then processed with a goat anti-rabbit IgG (A6154; diluted 1:10,000) secondary antibody. Blots which were probed with C92 primary antibody were subsequently processed with a goat anti-mouse IgG (A4416; diluted 1:5000) secondary antibody. All blots were then washed 6 x 5 min in TBST. Protein bands were then visualized using ECL Western blotting detection reagents (Amersham RPN 2106). Some of the Western blots that were originally incubated with HSF1 or C92 primary antibody were stripped with 2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris pH 6.7 for 30 min at 50°C, and then probed again with another antibody. This solution completely

removes these antibodies from the blot, as was verified by doing ECL on stripped blots. Data shown for the 40-day rat heat shock experiment are representative of independent experiments carried out on three sets of animals. Other data shown are representative of independent experiments carried out on two sets of animals.

2.4.4 Antibodies used for Western blot analysis

The following primary antibodies were used for Western blot analysis:

1) C92 mouse monoclonal anti-human hsp70 (StressGen; SPA810; Welch and Suhan, 1986), diluted 1:5000.
2) 1477 rabbit polyclonal anti-human hsc70 (gift from R. Tanguay), diluted 1:50,000.
3) HSF1 rabbit polyclonal anti-mouse antibody (gift from R. Morimoto and K. Sarge), diluted 1:25,000.
4) HSF2 rabbit polyclonal anti-mouse antibody (gift from R. Morimoto and K. Sarge), diluted 1:33,000.

2.5 DNA Quantification

Cerebellum and kidney were quickly removed from sacrificed Wistar rats (Charles River), fast frozen and homogenized in buffer C+ as described above. The whole cell homogenate was stored at -20°C. DNA concentration was established by the "Dische" or "boiling" diphenylamine reaction. Briefly, the DNA concentration of each sample was first estimated by lysing the tissue in a 0.2 M NaOH, 1% SDS solution and

then measuring the OD_{260}. Then duplicate aliquots of approximately 100 µg of DNA from each sample were pipetted into glass test tubes, and to each was added enough 1 x SSC solution (0.15 M NaCl, 0.015 M sodium citrate) to bring the total volume to 1.0 ml. Then 2.0 ml of diphenylamine reagent (1% (w/v) diphenylamine, 2.75% (v/v) concentrated sulfuric acid in glacial acetic acid) was added to each tube, and the tubes were heated in a boiling water bath for 10 min. The OD_{600} was measured on a Phillips Pye Unicam PU8610 UV/VIS kinetics spectrophotometer and compared to a standard curve prepared using known concentrations of stock DNA (from calf thymus). Independent experiments were performed on three sets of Wistar rats (Charles River).

3. RESULTS

3.1 Changes in levels of HSF1 and HSF2 during postnatal development

A previous study done in our laboratory showed that HSF1 protein levels increase in the rat cerebellum during postnatal development up to day 30, while HSF2 levels decline (Brown and Rush, 1999). This study has now been extended in the present work by examining developmental changes in HSF1 and HSF2 protein levels in additional neural regions and in kidney, and by extending the analysis to postnatal day 98. Fig. 1A shows changes in HSF1 protein levels in three brain regions and kidney of the rat from postnatal day 2 to 98. HSF1 levels increase in the brain regions from day 2 to 20/30 and then decline. In the kidney, HSF1 protein levels are high at day 2, but decline during subsequent development (Fig. 1A). Therefore, HSF1 protein levels seem to be regulated in a tissue-specific manner.

In order to compare HSF1 levels in the different tissues, samples from 2- and 30-day rats were run side by side (Fig. 1B). At postnatal day 2, HSF1 levels are highest in the kidney, followed by cerebellum, cerebral hemisphere and brain stem (Fig. 1B). However, by day 30, HSF1 levels are highest in cerebellum, followed by brain stem and cerebral hemisphere. HSF1 levels in kidney at day 30 were too low to be detected by the Western analysis. Isoforms of HSF1 protein were detected, which likely result from alternative splicing of HSF1 pre-mRNA (Fiorenza et al., 1995; Goodson and Sarge, 1995). Fig. 1C shows HSF1 protein levels in the heart from 2- to 32-days. HSF1 levels decline over this period.

Fig. 1. HSF1 protein levels in rat tissues from postnatal day 2 to 98

Aliquots of whole tissue extracts (100 μg protein) from three different brain regions and kidney were obtained from animals at the indicated postnatal ages (2 to 98 days) and subjected to Western blot analysis with a 1:25,000 dilution of HSF1 antibody. Proteins of approximately 70 kDa molecular weight were detected.

A) Developmental changes in HSF1 protein levels. Numerals indicate postnatal age in days.

B) Levels of HSF1 protein in the different tissues at 2- and 30-days. Samples from the various tissues at each of the two different ages were run side by side to obtain a better comparison between the different tissues. 2d, 2-day rat; 30d, 30-day rat.

C) Aliquots of whole tissue extracts (100 μg protein) from heart were obtained from animals at the indicated postnatal ages (2 to 32 days) and subjected to Western blot analysis with a 1:25,000 dilution of HSF1 antibody. H, heart.

Cb, cerebellum; BS, brain stem; CH, cerebral hemispheres; K, kidney.

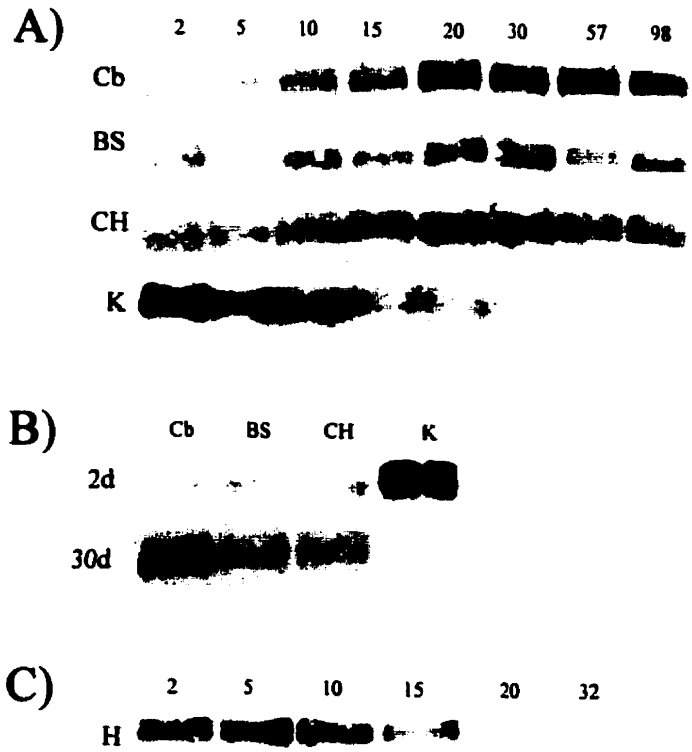

A)
2
5
10
15
20
30
57
98
Cb
BS
CH
K
B)
Cb
BS
CH
K
2d
30d
C)
2
5
10
15
20
32
H

Fig. 2A shows changes in HSF2 protein levels in various tissues of the rat from postnatal day 2 to 98. In the three brain regions and kidney, HSF2 levels decline during postnatal development. This suggests that HSF2 may have a role in early rat development. At both day 2 and day 30, HSF2 levels are higher in the three brain regions than in kidney (Fig. 2B). This may indicate that HSF2 plays a greater role in the postnatal brain than in the kidney. Isoforms of HSF2, which are generated by alternative splicing of HSF2 pre-mRNA, have been reported in mouse (Fiorenza et al., 1995; Goodson et al., 1995). Thus the multiple bands that appear in brain and kidney in Fig. 2 likely correspond to different isoforms of HSF2. Both the HSF1 and the HSF2 antibodies have previously been shown to be highly specific in discriminating mammalian HSF1 and HSF2 (Sarge et al., 1993, 1994). Fig. 2C shows HSF2 protein levels in the heart from 2- to 32-days. HSF2 levels decline over this period.

In the Western blots shown in Figs. 1 and 2, 100 μg of a whole cell extract was loaded in each lane. However, the number of cells contained in that 100 μg of protein may vary during development or between different tissues, and this could influence the observed differences in HSF1 and HSF2 levels between the tissues. Since HSF1 and HSF2 are transcription factors, their concentrations in a tissue should be linked more closely to DNA content than to protein levels. In order to investigate this possibility, the amount of DNA per 100 μg of protein was determined in rats ranging in age from 2 to 32 days. DNA levels in the cerebellum increased from 8.3±0.4 μg DNA/100 μg protein at postnatal day 2 to 9.8±0.6 μg DNA/100 μg protein at day 32. Over the same time period, DNA levels in kidney increased from 6.6±3.1 μg DNA/100 μg protein to 8.6±0.9 μg DNA/100 μg protein, and DNA levels in heart decreased from 8.6±2.4 μg DNA/100 μg

Fig. 2. HSF2 protein levels in rat tissues during postnatal development

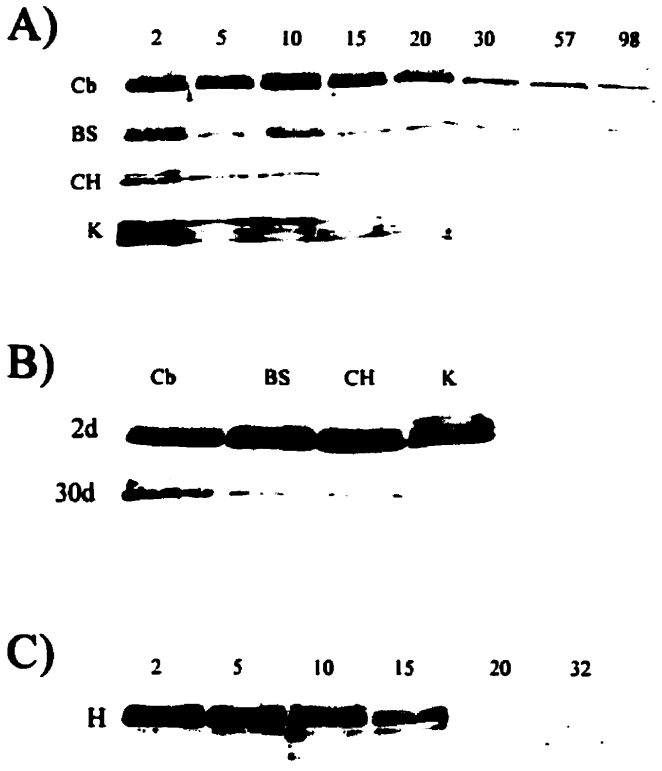
Aliquots of whole tissue extracts (100 μg protein) from three different brain regions and kidney were obtained from animals at the indicated postnatal ages (2 to 98 days) and subjected to Western blot analysis with a 1:50,000 dilution of HSF2 antibody. Proteins of approximately 70 kDa molecular weight were detected.

A) Developmental changes in HSF2 protein levels. Numerals indicate postnatal age in days.

B) Levels of HSF2 protein in the different tissues at 2- and 30-days. Samples from the various tissues at each of the two different ages were run side by side to obtain a better comparison between the different tissues. 2d, 2-day rat; 30d, 30-day rat.

C) Aliquots of whole tissue extracts (100 μg protein) from heart were obtained from animals at the indicated postnatal ages (2 to 32 days) and subjected to Western blot analysis with a 1:50,000 dilution of HSF2 antibody. H, heart.

Cb, cerebellum; BS, brain stem; CH, cerebral hemispheres; K, kidney.


A)
2
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20
30
57
98
Cb
BS
CH
K
B)
Cb
BS
CH
K
2d
30d
C)
2
5
10
15
20
32
H

protein to 3.1±1.5 μg DNA/100 μg protein. These DNA changes, which are reflective of cell numbers per 100 μg protein, are not of sufficient magnitude to explain the large developmental variations in HSF1 and HSF2 levels in the cerebellum and kidney shown in Figs. 1 and 2. However, the decrease in cell numbers per 100 μg protein in heart may partially explain the developmental decrease in HSF1 and HSF2 in that tissue.

3.2 Constitutive HSF activation during development

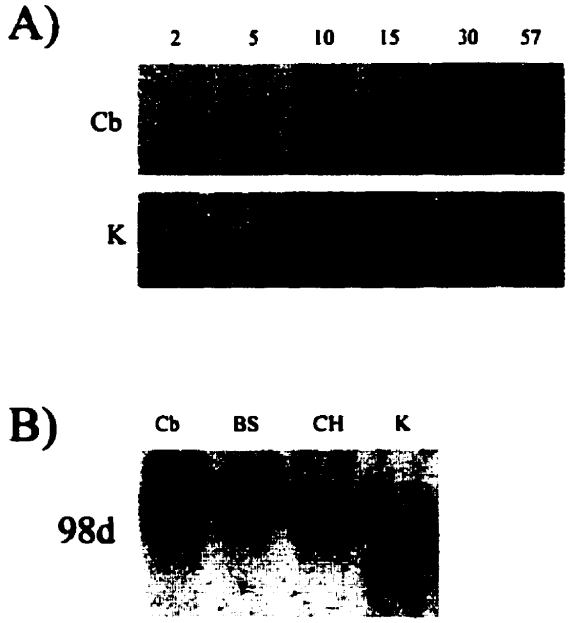
HSF2 has been proposed to regulate heat shock gene expression during developmental events (Sistonen et al., 1992, 1994; Loones et al., 1997; Sarge and Cullen, 1997). In order to investigate this possibility, gel mobility shifts were performed to establish the levels of HSF-HSE binding activity under nonstress conditions in different rat tissues during postnatal development (Fig. 3A). If HSF2 is in a DNA-binding form, it could be involved in regulation of constitutive expression of heat shock genes. In the cerebellum and kidney, constitutive HSF-HSE binding activity increased during postnatal development (Fig. 3A), however, binding activity was not observed in either tissue in the unstressed 2-day rat. This suggests that HSF2 is not activated in the neonatal rat, despite being present at high levels (Fig. 2). Since HSF2 is not in a DNA-binding form, it is probably not involved in constitutive heat shock gene transcription in the neonatal rat. However, it may be influencing development by means of its effect on PP2A activity. HSF-HSE binding activity at later postnatal ages shows that a portion of the transcription factor in cerebellum and kidney is in a DNA-binding form, and thus it may play a role in constitutive expression of heat shock genes at later ages (Fig. 3A). In the 98-day rat there

Fig. 3. Constitutive HSF activation in various tissues

A) Gel mobility shift analysis of HSE-binding activity in cerebellum and kidney during postnatal development. Extracts of the two tissues were obtained from animals at the indicated postnatal ages (2 to 57 days) and subjected to gel shift analysis using a labeled oligonucleotide probe which contained four inverted repeats of the HSE consensus sequence 5'-NGAAN-3'.

B) Gel mobility shift analysis of HSE-binding activity in three brain regions and kidney of the 98-day rat. 98d, 98-day rat.

Cb, cerebellum; K, kidney; BS, brains stem; CH, cerebral hemispheres.


A)
2
5
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30
57
Cb
K
B)
Cb
BS
CH
K
98d

is fairly high HSF DNA-binding activity in cerebellum, followed by cerebral hemispheres, brain stem and kidney (Fig. 3B).

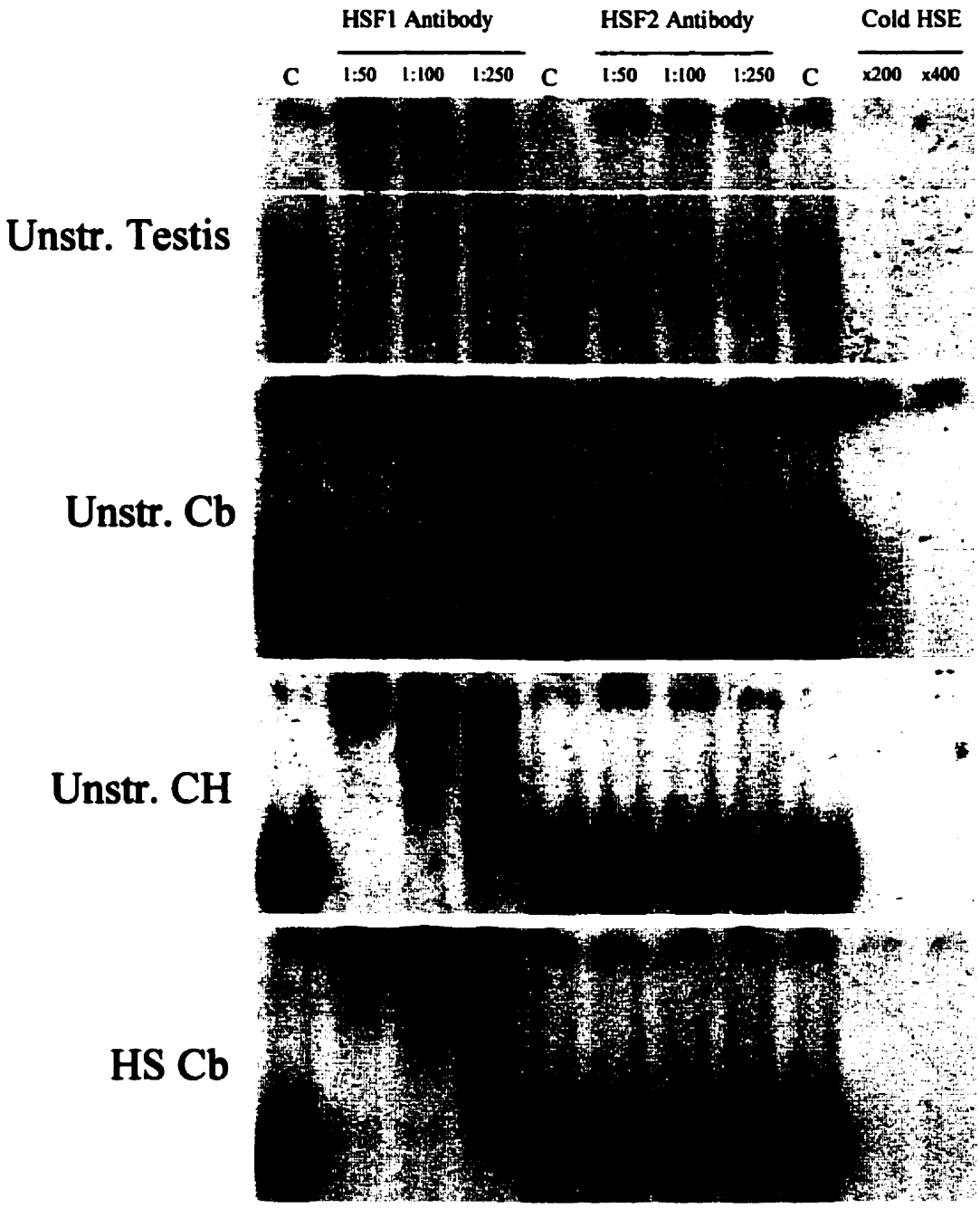
Rat testis has previously been shown to have constitutive HSF2 DNA-binding activity (Higashi et al., 1995). In order to confirm that the oligonucleotide used in the gel mobility shifts was a good target for activated HSF2, a supershift gel mobility shift assay was performed on 98-day rat testis (Fig. 4). The amount of HSF-HSE complex in testis is diminished by the addition of either HSF1 or HSF2 antibody, suggesting that both HSF1 and HSF2 are in a DNA-binding form and that the oligonucleotide probe is bound by activated HSF2. The oligonucleotide probe was identical to that used by Sarge et al. (1994), who successfully used it to detect HSF2 DNA-binding activity in mouse testis. The HSF DNA-binding activity in unstressed cerebellum and cerebral hemispheres was investigated with a supershift assay. The DNA-binding activity was found to be due to activated HSF1 and not to HSF2 in both cases (Fig. 4). As expected, the HSE-binding activation in heat shocked cerebellum was also due to HSF1 and not to HSF2 (Fig. 4). The DNA-binding activity in these tissues was shown to be specific for the HSE probe by adding an excess of nonradioactive probe. In all cases the signal disappeared, showing that it is specific for HSE sequences (Fig. 4).

3.3 The Heat Shock Response in the 2-day Rat

It was of interest to establish whether the different HSF1 levels in 2-day brain regions and kidney (Fig. 1B) correlated with the magnitude of the heat shock response in these tissues. Body temperatures of 2-day rats were elevated from 30°C to 40°C for 5

Fig. 4. Supershifts on HSF-HSE complexes in various adult rat tissues.

Gel shift reactions were preincubated with either HSF1 or HSF2 primary antibodies prior to the addition of the oligonucleotide probe. The antibodies were added at either a 1:50, 1:100 or 1:250 concentration. In order to test the specificity of the complex, an excess of cold oligonucleotide probe was added to the gel shift reactions prior to the addition of the oligonucleotide probe. Either a 200-fold or a 400-fold excess of this probe was added. Unstr., unstressed; HS, heat shocked; Cb, cerebellum; CH, cerebral hemispheres; C, control (no antibody or cold HSE probe added).


HSF1 Antibody
HSF2 Antibody
Cold HSE
C
1:50
1:100
1:250
C
1:50
1:100
1:250
C
x200
x400
Unstr. Testis
Unstr. Cb
Unstr. CH
HS Cb

min, and the animals were then allowed to recover at room temperature for various time periods before sacrifice. Fig. 5A shows the magnitude of HSF activation in brain, kidney and liver at time points after heat shock. HSF DNA-binding activation was observed at 25 min after the end of the heat shock, but had disappeared by 55 min. The highest HSF activation occurred in the kidney, followed by liver and brain.

When HSF1 becomes hyperphosphorylated after heat shock, phosphorylation of the transcription factor can be detected as a decrease in the mobility of the factor on a SDS-polyacrylamide gel (Sarge et al., 1993; Jurivich et al., 1994; Mathur et al., 1994). Western analysis of the 2-day rat kidney showed a slight decrease in the mobility of HSF1 on a SDS-polyacrylamide gel at 25 min posthyperthermia (Fig. 5B). This is consistent with stress-induced phosphorylation of the transcription factor. The change in mobility of HSF1 was not observed at 55 min, concurrent with the disappearance of HSF-HSE binding activity.

There are only basal levels of hsp70 protein in kidney at 55 min after heat shock (Fig. 5B). However, 6 hr after hyperthermia a robust induction of hsp70 protein was apparent in kidney and liver, with a lower induction in brain (Fig. 5C). Therefore, the higher level of HSF1 protein and the higher level of HSF-HSE binding activity in 2-day kidney compared to brain (Fig. 1B, 5A) correlated with the greater induction of hsp70 protein in kidney compared to brain after thermal stress. High basal amounts of hsp70 were detected in the unstressed liver, with smaller amounts in kidney (Fig. 5C). Constitutive hsc70 levels are slightly higher in brain, followed by kidney and liver (Fig. 5C). No induction of hsc70 protein occurred after heat shock in any of the three tissues (Fig. 5C).

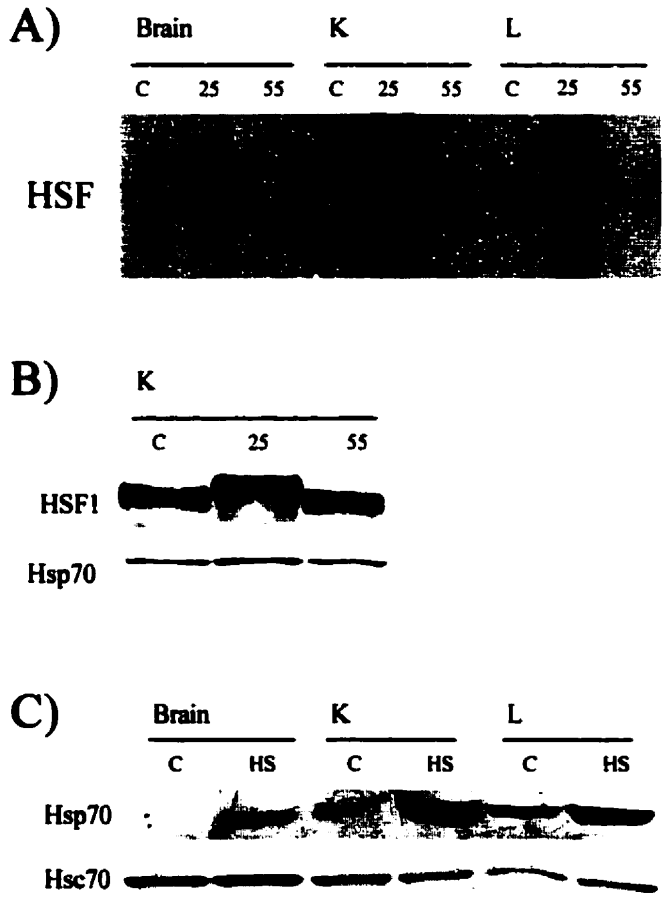
Fig. 5. HSF1 activation and hsp70 and hsc70 levels after high change in temperature heat shock in tissues from 2-day rats

A) Gel mobility shift analysis of HSE-binding activity in extracts of brain, kidney and liver following hyperthermia (change in temperature = 10°C). Extracts were prepared from control rats (C) and from rats 25 or 55 min following heat stress.

B) Extracts of kidney were prepared from control rats (C) and from rats 25 or 55 min following hyperthermia. Aliquots (100 μg protein) were subjected to Western blot analysis with either a 1:25,000 dilution of HSF1 antibody or a 1:5,000 dilution of C92 (anti-hsp70) antibody.

C) Aliquots (100 μg protein) of brain, kidney and liver were obtained from control 2-day rats (C) and from 2-day rats 6 hr post-hyperthermia (HS), and subjected to Western blot analysis with either a 1:5,000 dilution of C92 (anti-hsp70) antibody or with a 1:50,000 dilution of 1477 (anti-hsc70) antibody.

K, kidney; L, liver.


A)
Brain
K
L
C 25 55 C 25 55 C 25 55
HSF
B)
K
C 25 55
HSF1
Hsp70
C)
Brain
K
L
C HS C HS C HS
Hsp70
Hsc70

In order to see if the magnitude of the change in body temperature influenced the heat shock response in the 2-day rat, body temperature was elevated from 35°C to 40°C for 5 min. These rats had an initial body temperature of 35°C, because they were taken away from their mother only a short time before the heat stress. This heat stress resulted in a change in temperature of 5°C compared to the previous experiment, where the change in temperature was 10°C (Fig. 5). HSF activation was highest immediately after heat shock and had diminished greatly by 10 min after heat shock in the three tissues studied (Fig. 6A). In order to compare the magnitude of HSF DNA-binding activation after heat shock in the different tissues, samples of the three different tissues from unstressed and stressed animals were run side by side (Fig. 6B). The highest HSF activation occurred in the kidney, followed by liver and brain (Fig. 6B), as was observed in Fig. 5A, however, there was no detectable decrease in the mobility of HSF1 in kidney on a SDS polyacrylamide gel, suggesting that HSF1 may not be hyperphosphorylated after a change in temperature of 5°C (Fig. 6C). This suggests that HSF1 hyperphosphorylation does not always accompany HSF1 DNA-binding activity following thermal stress. Because no rats that were given the low change in temperature heat shock were then allowed to recover at room temperature for 5 – 10 hr, it is not known whether hsp70 is induced after this heat shock. Perhaps further work will address this question. It would be interesting to know whether HSF1 that is activated by heat shock to a DNA-binding, but nonhyperphosphorylated form is transcriptionally competent. The duration of HSF DNA-binding activation was longer when the change in temperature was higher (compare Fig. 5A and Fig. 6A). Additionally, HSF1 appeared to be hyperphosphorylated after the high change in temperature (+10°C), but not detectably hyperphosphorylated

Fig. 6. HSF activation in 2-day old rat tissues following low change in temperature heat shock

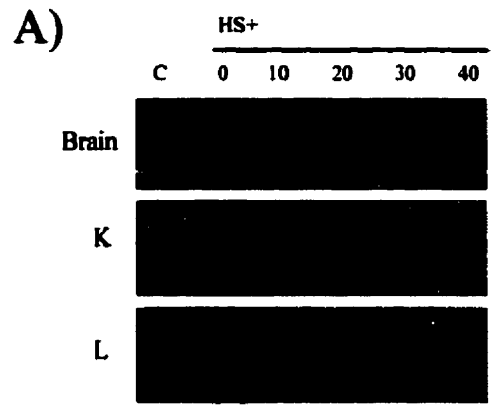
Gel mobility shift analysis of HSE-binding activity in extracts of brain, kidney and liver following hyperthermia (change in temperature = 5°C).

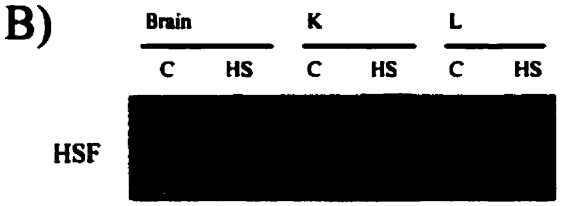
A) Extracts of the tissues were prepared from control 2-day rats (C) and from 2-day rats immediately following hyperthermia (0) or after 10, 20, 30 or 40 min of recovery at room temperature following hyperthermia.

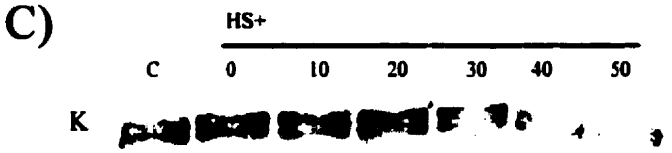
B) In order to more accurately compare the amount of HSE-binding activity in the three tissues after heat shock, samples of each tissue from control animals (C) or from animals immediately after heat shock (HS) were run side by side.

C) Extract of kidney was prepared from control 2-day rats (C) and from 2-day rats immediately following hyperthermia (0) or after 10, 20, 30, 40 or 50 min of recovery at room temperature following hyperthermia. Aliquots (100 μg protein) were subjected to Western analysis with a 1:50,000 dilution of HSF1 antibody.

K, kidney; L, liver.


A)
HS+
C
0
10
20
30
40
Brain
K
L


B)
Brain
K
L
C
HS
C
HS
C
HS
HSF


C)
HS+
C
0
10
20
30
40
50
K

after the low change in temperature (+5°C). Therefore the magnitude of HSF1 activation appeared to be influenced by the magnitude of the change in temperature.

3.4 Heat Shock of the 40-day Rat

In order to see whether the differences in HSF1 protein levels previously observed in adult brain regions and kidney (Fig. 1) correlated with the magnitude of the stress response in these organs, a heat shock experiment was performed on 40-day rats. Since HSF1 levels in brain stem and cerebral hemispheres are similar thoughout development (Fig. 1A), these regions were combined into a single fraction (forebrain) in this experiment. To establish the kinetics of HSF1 activation, the body temperatures of 40-day old rats were raised by a physiologically relevant increase of 3.3±0.8°C. After 30 min of heat shock, when HSF1 activation was maximal, the amount of activated HSF1 was greatest in the cerebellum, followed by forebrain, liver and kidney (Fig. 7B). Western blotting demonstrated that the level of HSF1 was higher in the adult cerebellum than the forebrain, while HSF1 levels in the kidney were too low to be detected by the Western analysis (Fig. 7C).

After 30 min of heat shock there was only slightly more activated HSF1 in the forebrain than in kidney (Fig. 7B). This contrasts with the observation that high levels of HSF1 protein exist in the 40-day rat forebrain, while levels of HSF1 protein in kidney are undetectably low. This suggests that not all of the HSF1 protein in forebrain was activated. The inactivated HSF1 was likely present in the cell populations in the forebrain that do not induce hsp70 mRNA after a fever-like heat shock.

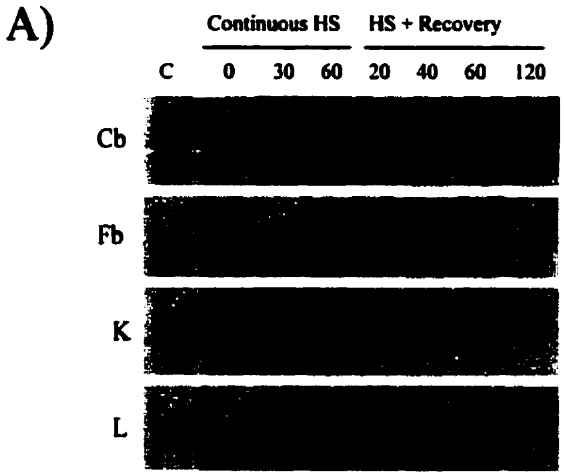
Fig. 7. HSF-HSE binding activity and hyperphosphorylation in 40-day old rat tissues during heat shock recovery

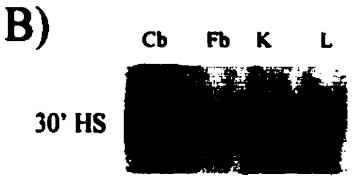
A) Gel mobility shift analysis of HSE-binding activity in extracts of cerebellum, forebrain (brain stem and cerebral hemispheres), kidney and liver of 40-day rats following hyperthermia. Extracts of the tissues were prepared from control rats (C) and from rats that were maintained for 0, 30, or 60 min at the elevated temperature (continuous heat shock), and from rats that were maintained at the elevated temperature for 1 hr followed by 20, 40, 60, or 120 min of recovery at room temperature (heat shock + recovery).

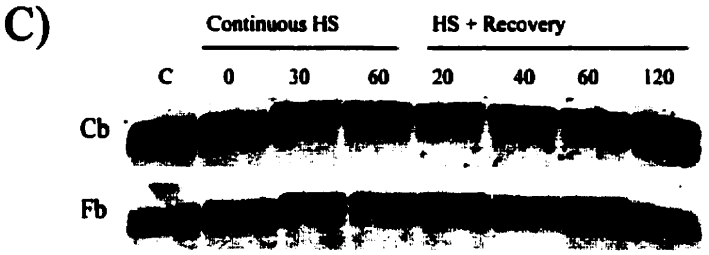
B) HSF-HSE binding activity in the different tissues after the rats were maintained for 30 min at the elevated temperature. Samples from the various tissues were run side by side to obtain a better comparison between the different tissues. 30' HS, 30 min heat shock.

C) Western blot of HSF1 protein in two brain regions during heat shock recovery experiment on 40-day rats. Aliquots (100 μg protein) of cerebellum and forebrain were obtained from control rats (C) and from rats after they were heat shocked as in *A*. The samples were subjected to Western blot analysis with a 1:50,000 dilution of HSF1 antibody. There was insufficient HSF1 protein in kidney to be detected by the Western analysis.

Cb, cerebellum; Fb, forebrain (brain stem + cerebral hemispheres); K, kidney; L, liver; HS, heat shock.


A)
Continuous HS
HS + Recovery
C
0
30
60
20
40
60
120
Cb
Fb
K
L


B)
Cb
Fb
K
L
30' HS


C)
Continuous HS
HS + Recovery
C
0
30
60
20
40
60
120
Cb
Fb

To establish the kinetics of HSF1 deactivation during recovery from heat shock, 40-day old adult rats were maintained at the elevated temperature for 1 hr and then allowed to recover at room temperature for either 20, 40, 60, or 120 min before sacrifice. HSF1 still remained in a DNA-binding form at 120 min after the end of the heat shock in the adult cerebellum, but not in forebrain, kidney or liver (Fig. 7A).

Hsp70 protein was induced in brain regions, kidney and liver after 60 min of heat shock (Fig. 8A). The highest hsp70 levels were observed at the longest timepoint, 2 hr after the end of the heat shock, at which time the level of hsp70 protein was greatest in kidney and liver, followed by cerebellum and then forebrain. However, there were very low basal levels of hsp70 in brain regions, but a relatively high basal level of hsp70 in kidney (Fig. 8A). Therefore, the hyperthermic brain had the greater magnitude of induction of hsp70 compared to kidney, in agreement with the higher levels of HSF1 protein and its DNA binding activity in brain versus kidney. The observation that hsp70 protein induction is greater in cerebellum than in forebrain correlates with the relative HSF1 levels in the two brain regions (Fig. 7C).

Constitutive hsc70 is not induced by hyperthermia in brain, kidney or liver (Fig. 8B). Hsc70 protein levels are higher in brain regions than in kidney or liver. The high levels of constitutive hsc70 in brain may protect it from the effects of stress, preventing denaturation of proteins and thus dampening the stress response.

Western analysis of the two adult brain regions showed a slight decrease in the mobility of HSF1 on a SDS-polyacrylamide gel after 30 min of continuous heat shock (Fig. 7C), suggesting that HSF1 was hyperphosphorylated. In forebrain the inducible phosphorylation of HSF1 was reduced after 20 min of recovery, but in cerebellum it

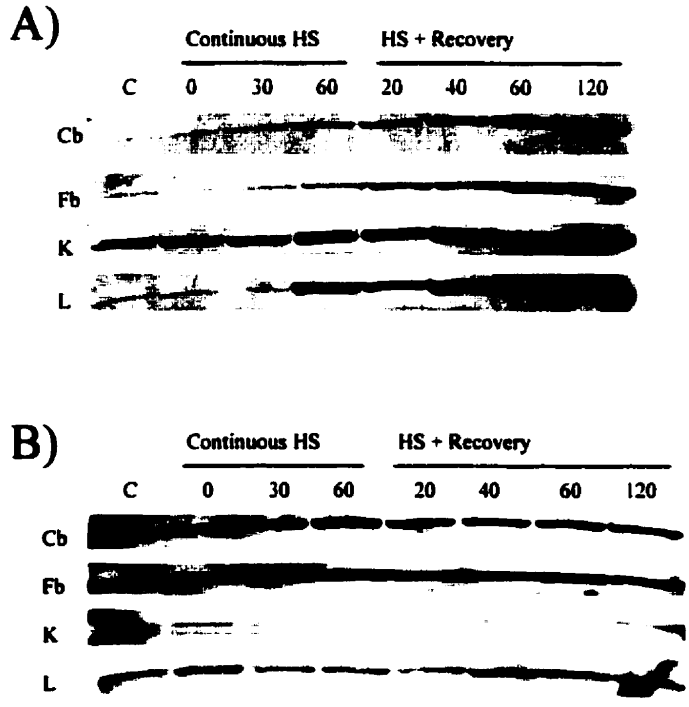
Fig. 8. Western blot analysis of hsp70 and hsc70 protein levels in 40-day old rat tissues following heat shock

Aliquots (100 µg protein) of cerebellum, forebrain, kidney and liver were obtained from control rats (C) and from rats that were maintained for 0, 30, or 60 min at the elevated temperature (continuous heat shock), and from rats that were maintained at the elevated temperature for 1 hr followed by 20, 40, 60, or 120 min of recovery at room temperature (heat shock + recovery).

A) Analysis of hsp70 levels (Western blot analysis with a 1:5000 dilution of C92 antibody).

B) Hsc70 levels (Western blot analysis with a 1:50,000 dilution of 1477 antibody).

Cb, cerebellum; Fb, forebrain (brain stem + cerebral hemispheres); K, kidney; L, liver; HS, heat shock.


A)
Continuous HS
HS + Recovery
C
0
30
60
20
40
60
120
Cb
Fb
K
L
B)
Continuous HS
HS + Recovery
C
0
30
60
20
40
60
120
Cb
Fb
K
L

remained maximal until 60 min of recovery (Fig. 7C). This longer period of hyperphosphorylation may contribute to the higher induction of hsp70 in cerebellum than in forebrain (Fig. 8A). There was some variability in how high the body temperature of the individual rats rose during the heat shock procedure. It was observed that HSF1 hyperphosphorylation persisted longest in those rats that experienced the highest temperature rise during the heat stress (data not shown). In forebrain HSF1 hyperphosphorylation was reduced before HSF1 returned to a non-DNA-binding form (Fig. 7A, C). HSF1 was not inducibly phosphorylated in the unstressed cerebellum, despite the presence of some HSF1 in the DNA-binding form (Fig. 7A, C). Since the acquisition of transcriptional activity is linked to the hyperphosphorylation of HSF1 (Cotto et al., 1996), this suggests that the HSF1 in unstressed cerebellum may not be transcriptionally competent.

In the unstressed animal, neural HSF1 appears as a doublet; however, in the stressed animal the hyperphosphorylated HSF1 appears as a single band (Fig. 7C). This is somewhat puzzling because one would expect to see a doublet in both cases, corresponding to the two protein isoforms of HSF1 (Goodson and Sarge, 1995). Almost all of the HSF1 in the cerebellum seems to be hyperphosphorylated and hence presumably activated. Most, but not all, of the HSF1 in the forebrain is hyperphosphorylated. This is the expected result in the cerebellum, where the large majority of cells induce hsp70 mRNA after heat shock. However, it is surprising that the majority of HSF1 is hyperphosphorylated in the forebrain, where the neurons, astrocytes and some microglia do not induce hsp70 mRNA after heat shock at fever-like temperatures (Foster and Brown, 1997). In conjunction with the gel shift results (Fig.

7B), this indicates that some HSF1 in heat shocked forebrain neurons and astrocytes may exist in a hyperphosphorylated form that does not bind DNA.

4. DISCUSSION

Since HSF1 is responsible for the stress response in mammals (Morimoto et al., 1994, 1996; Wu, 1995), levels of this transcription factor may influence the magnitude of hsp70 induction following thermal stress. The increase in HSF1 protein levels in brain with development up to postnatal day 20/30 parallels an increase in the heat shock response, as measured by hsp70 induction after heat shock in rat (Currie et al., 1983). It has been shown that the stress response of immature rat renal tubules is greater than that of mature rats (Gaudio et al., 1998), paralleling the developmental decrease of HSF1 protein in kidney. Therefore it seems that developmental changes in HSF1 protein levels have an effect on the magnitude of the heat shock response in brain and kidney.

In the 2-day rat, higher levels of HSF1 protein and HSF-HSE binding activity are present in hyperthermic kidney compared to brain regions, and this is correlated with the level of induction of hsp70 protein after thermal stress. In the adult rat, higher levels of HSF1 protein and HSF-HSE binding activity are present in hyperthermic brain compared to kidney, and this correlates with the magnitude of induction of hsp70 protein in these two organs after thermal stress. There are high basal levels of hsp70 protein in kidney and therefore, presumably, high basal levels of hsp70 mRNA. Since heat shock markedly increases the stability of hsp70 mRNA (Theodorakis and Morimoto, 1987), stabilization of the relatively large amount of hsp70 mRNA in the hyperthermic kidney may contribute to the high expression of hsp70 protein in that tissue. In brain, not all cell types induce hsp70 after a physiologically relevant increase in body temperature (Brown, 1990, 1994; Brown and Sharp, 1999). For example, oligodendrocytes and some

microglia induce hsp70 mRNA after heat shock, whereas large neurons and GFAP-positive astrocytes in the forebrain do not (Sprang and Brown, 1987; Foster and Brown, 1996, 1997). This is despite the fact that abundant levels of HSF1 are present in forebrain neurons, and this HSF1 is localized to the nuclei of these neurons in both control and hyperthermic animals (Brown and Rush, 1999). However, in the cerebellum, most cells do induce hsp70 after heat stress.

Despite the very low levels of HSF1 in adult kidney, the organ can nevertheless induce a large amount of hsp70 after heat shock. Therefore, why is it necessary for adult brain regions to have such high levels of HSF1? Naively, we might question whether HSF1 is entirely responsible for the induction of hsp70 after heat shock. However, Xiao et al. (1999) have shown that hsf1(-/-) mice cannot induce hsp70 after hyperthermia. Hsp90 and hsc70 levels are much higher in adult rat brain than in kidney (Fig. 7B; D'Souza and Brown, 1998), and those proteins may dampen the heat shock response in brain at low stress levels. A high level of HSF1 in the brain may be necessary to lower the threshold for the heat shock response in the presence of large amounts of hsc70 and hsp90. Another possibility is that high levels of neural HSF1 are necessary to repress non-hsp genes. There is evidence that *Drosophila* HSF binds to dozens of non-hsp genes, perhaps repressing their transcription after heat shock (Westwood et al., 1991). In heat shocked human monocytes, activated HSF1 binds to an HSE in the prointerleukin 1β gene and represses transcription of that gene (Cahill et al., 1996). HSF1 may also inhibit expression of a wide spectrum of other cytokines (Housby et al., 1999; Xiao et al., 1999). It may be that HSF1 is involved in the repression of a range of other non-heat shock genes. At all developmental stages, a higher percentage of non-repeated DNA is

transcribed in mammalian brain than in kidney (Brown and Church, 1972). The complexity of transcription increases in brain from the newborn to the 2-wk-old stage before leveling off at the 6-wk-old stage (Brown and Church, 1972). However, the complexity of RNA transcription in kidney appears to decrease with the development of the animal (Brown and Church, 1972). Interestingly, this pattern matches the developmental expression of HSF1 protein in those tissues. Therefore, an additional function of HSF1 may be to shut off genes following heat shock.

HSF1 may be performing other functions as well. For example, HSF1 has been shown to repress the transcription of the c-fos gene, an immediate early gene that controls responses to extracellular stimuli for growth and differentiation (Chen et al., 1997). This repression did not require binding to the c-fos promoter (Chen et al., 1997). There may be a regulatory interaction between the DNA-dependent protein kinase (DNA-PK) and HSF1 (Nueda et al., 1999). Recently it was shown that HSF1 is activated to a DNA-binding form in the G(1) phase of the cell cycle in HeLa cells in the absence of stress (Bruce et al. 1999). However, entry into G(1) does not lead to HSF1 hyperphosphorylation or induction of hsp70 (Bruce et al., 1999). HSF1 may play a role in cell cycle regulation (Bruce et al., 1999). HSF1 may influence postnatal rat development in ways that do not involve the induction of heat shock genes. The developmental functions of *Drosophila* HSF are not mediated through the induction of heat shock genes (Jedlicka et al., 1997). As mentioned previously, hsf1(-/-) knockout mice have multiple phenotypic abnormalities, despite the fact that basal hsp expression is not altered appreciably (McMillan et al., 1998; Xiao et al., 1999). This suggests that

HSF1, like *Drosophila* HSF, might be involved in regulating other important genes or signaling pathways (Xiao et al., 1999).

The initial temperature of heat shocked 2-day rats affected the duration of HSF-HSE binding activity and the hyperphosphorylation of HSF1. Rats which were at 30°C before the 40°C heat shock experienced a longer duration of HSF-HSE binding activity than those which were initially at 35°C. This result agrees with Abravaya et al. (1991), who found that HeLa cells grown at 35°C experienced a greater magnitude and duration of HSF-HSE binding after heat shock than cells grown at 37°C. Also heat-stressed 2-day rats which were initially at 30°C showed apparent stress-induced phosphorylation of HSF1, while rats which were initially at 35°C did not.

HSF1 is hyperphosphorylated in the hyperthermic 40-day rat brain and the duration of this hyperphosphorylation is proportional to the severity of the heat shock. The majority of the HSF1 is hyperphosphorylated in the hyperthermic 40-day rat forebrain, implying that it must be hyperphosphorylated even in forebrain neurons, which are known not to induce hsp70 after heat shock, despite having ample amounts of HSF1 protein (Brown and Rush, 1999). This conclusion is bolstered by a previous investigation done in our laboratory on the hyperthermic rabbit (Brown and Rush, 1996). That study showed that in three neuronal-enriched regions (cerebral cortex, hippocampus and thalamus) the large majority of HSF1 protein was apparently hyperphosphorylated after a fever-like heat shock (Brown and Rush, 1996). This is despite the fact that forebrain neurons do not induce hsp70 mRNA after a fever-like heat shock (Brown, 1994). In conjunction with the observation that the majority of HSF1 in forebrain apparently is not in a DNA-binding form, this suggests that some HSF1 molecules exist in a

hyperphosphorylated but non-DNA-binding form in the hyperthermic 40-day rat forebrain.

HSF1 is not hyperphosphorylated in unstressed cerebellum, despite the presence of some HSF1 in the DNA-binding form. Since the acquisition of transcriptional activity is linked to the hyperphosphorylation of HSF1 (Cotto et al., 1996), this suggests that the HSF1 in the unstressed cerebellum may not be transcriptionally competent. However, the constitutively activated HSF1 may be performing some other function. The activation of HSF1 is probably at least a two-part process, and an intermediate form exists that binds HSE but does not activate heat shock gene transcription (Sorger and Pelham, 1987; Price and Calderwood, 1991; Bruce et al., 1993). Preliminary results indicate that the intermediate form of HSF1 is able to repress transcription (Cahill et al., 1996). Therefore the HSF1 in the unstressed cerebellum may be involved in the repression of transcription.

A significant amount of HSF-HSE binding activity is seen in cerebellum at day 30. The constitutive HSF DNA-binding activity may be caused by the large amount of HSF1 protein in cerebellum after day 30. When HSF1 is overexpressed in murine cells, it shows constitutive DNA-binding activity (Mivechi et al., 1995). A similar phenomenon may occur in cells where HSF1 is normally expressed at a high level. However, there are high levels of HSF1 protein in 2-day kidney, yet no constitutive HSF activation. There is higher HSF activation later on in kidney development, despite falling levels of HSF1 protein. This shows that there are other factors involved in constitutive HSF1 activation besides the concentration of HSF1 protein in the cell.

It has been suggested that constitutive HSF DNA-binding activity may allow the cerebellum to mount a quicker heat shock response under stressful conditions (Cullen and

Sarge, 1997). If it is necessary for there to be some constitutively active HSF1 in the rat cerebellum, then that might explain why HSF1 protein levels are high in that region. A high level of HSF1 protein is a simple way to ensure that some HSF1 in cerebellum will be in a DNA-binding form. However, in the 40-day rat, the induction of hsp70 protein did not seem to occur any faster in cerebellum than it did in kidney or liver (Fig. 8A).

HSF2 protein levels decline in brain and kidney during postnatal rat development. The fact that HSF2 levels are higher in the immature rat may suggest a developmental role of HSF2. However, there is some HSF2 in adult tissues, which indicates that HSF2 has a function in the mature animal. HSF2 levels are higher in brain than in kidney. Even though HSF2 levels are high in the 2-day rat, there is no constitutive HSF-HSE binding activity in 2-day cerebellum or kidney, suggesting that HSF2 is not involved in gene transcription at this time. During postnatal development of the rat brain, hsp90 protein levels decrease slightly in the cerebellum but not in other brain regions, basal hsp70 protein levels increase in the cerebral hemispheres but not in cerebellum, hsp60 protein levels increase in all brain regions, and hsc70 levels do not change significantly (D'Souza and Brown, 1998). During the postnatal development of the rat kidney, hsp90 and hsc70 protein levels decrease dramatically, but not hsp60 and hsp70 levels (D'Souza and Brown, 1998). Therefore the large developmental decrease in HSF2 levels in brain does not correlate with the basal levels of these hsps. The large decrease in HSF2 levels in kidney does correlate with the pronounced decrease in hsp90 and hsc70 in that tissue, but does not correlate with hsp60 or hsp70 expression. Overall, the postnatal developmental changes in HSF2 levels do not correlate with constitutive hsp levels. Furthermore, the onset of constitutive HSF DNA-binding activity in adult brain does not

seem to be correlated with constitutive hsp expression. Therefore it would seem that HSF2 is not involved in the expression of heat shock genes in brain or kidney under nonstress conditions. Similarly, Rallu et al. (1997) found no obvious correlation between the expression patterns of the major heat shock proteins and that of HSF2 during mouse embryonic development. However, another heat shock factor, HSF4b, which could act as an activator of heat shock genes under normal conditions, has recently been found in mammals (Tanabe et al., 1999).

As mentioned before, the smaller isoform of HSF2, HSF2-β, may act as a negative regulator of HSF2 activity during hemin-mediated erythroid differentiation of K562 cells (Leppä et al., 1997). The HSF2-β isoform is present in higher amounts than the HSF2-α isoform in the postnatal brain and kidney. This may explain the lack of any HSF2 activation in either of these tissues, despite the high levels of HSF2 protein in the 2-day brain and kidney. Immunocytochemistry of the rat brain shows that HSF2 is localized to the nuclei of neurons at day 2, and in the cytoplasm at day 30 (Brown and Rush, 1999). The localization of HSF2 in the nuclei of neurons in the 2-day rat brain suggests that the transcription factor may be involved in transcribing heat shock genes at this time, however, the observation that there is no HSF-HSE binding activity in the 2-day rat brain (Fig. 3A) argues against this conclusion.

Recently, it was shown that HSF2 is activated when the ubiquitin-proteasome pathway is inhibited (Mathew et al., 1998). It is known that many neurodegenerative diseases, as well as normal aging, are characterized by an accumulation of ubiquitinated proteins in neurons and some glia cells (Alves-Rodrigues et al., 1998). Neurons may be especially sensitive to malfunction of the ubiquitin/ATP-dependent pathway (Alves-

Rodrigues et al., 1998). The cytoplasmic localization of HSF2 in adult brain neurons (Brown and Rush, 1999) indicates that HSF2 is not involved in gene transcription in these cells under normal conditions, however, it is possible that the relatively high amounts of HSF2 protein in brain are there to protect against the accumulation of ubiquitinated proteins. The transcription factor may be quiescent, but ready to be activated by a malfunction of the ubiquitin-proteasome pathway. Additionally, HSF2 may influence rat development via its previously discussed influence on PP2A activity. Creation of an HSF2(-/-) deficient mouse would clarify the function of HSF2.

In summary, HSF1 levels rise in brain regions and decline in kidney and heart during postnatal rat development. In both neonatal and adult rats, HSF1 protein levels in brain and kidney correlate with levels of HSF-HSE binding activity and the magnitude of hsp70 protein induction after thermal stress. There appears to be more HSF1 protein in adult brain than is needed for stress-induced expression of hsp70, suggesting that HSF1 may have other roles in addition to its role as a stress-inducible activator of heat shock genes. In the hyperthermic adult rat forebrain, most HSF1 is not in a DNA-binding form, but most HSF1 is hyperphosphorylated. This indicates that some HSF1 in that tissue is hyperphosphorylated, but not in a DNA-binding form. Thus, hyperphosphorylation of HSF1 can occur without acquisition of DNA-binding activity. HSF2 protein levels decline during postnatal rat development in brain regions and kidney, suggesting a role for HSF2 in development. However, gel mobility shift analysis showed that HSF2 is not in a DNA-binding form in the neonatal rat, suggesting that HSF2 may not be involved in the constitutive expression of hsps at that time. There is no apparent correlation between levels of HSF2 protein with basal levels of hsp90, hsp70, hsc70 and hsp60.

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