# Regulation of Heat Shock Factor 1 (HSF1) DNA-binding and transcription

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy in the
Department of Anatomy and Cell Biology
University of Saskatchewan
Saskatoon

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Summer 2003

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# Abstract

Cellular stress invokes a protective response in which heat shock factor 1 (HSF1) is activated to increase heat shock protein (Hsp) expression. HSF1 exists as a latent monomer in unstressed cells. Upon stress HSF1 forms homotrimers, increasing its affinity for the heat shock DNA element upstream of all Hsp genes. A second conformational change is required for HSF1 to gain transcriptional competence. During prolonged heat shock or following the resumption of normal conditions HSF1 DNA-binding and transcriptional activities are reduced and HSF1 returns to the monomeric state in a process called attenuation. During the activation/deactivation cycle HSF1 is modified by small ubiquitin-related modifier (SUMO-1) conjugation and undergoes several phosphorylation and dephosphorylation events that modulate HSF1 activity. Hyperphosphorylation of HSF1 is hypothesized to trigger HSF1 transcriptional activity. HSF1 also interacts with a dynamic series of Hsp90/Hsp70-based chaperone heterocomplexes that negatively regulate DNA-binding, and transcriptional activity, and promote attenuation. This thesis was aimed at characterizing the mechanisms regulating HSF1 DNA-binding, and transcriptional activity. Expression of human HSF1 in *Xenopus* oocytes altered the set-point of DNA-binding in response to heat indicating that both the cellular environment and innate properties of the molecule allow HSF1 to set its activation/deactivation set-point in response to stress *in vivo*. HSF1 DNA-binding but not transcription was activated in oocytes treated with a high temperature heat shock. Further characterization of this observation determined that HSF1 activated by a brief

high temperature heat shock inhibited transcriptionally competent HSF1 from activating transcription. It was hypothesized that this phenomenon exists to ensure the eventual death of the cell due to the accumulation of excessive damage and potential mutation caused by severe stress. The most significant observation made in this thesis is that Hsp expression was detected in oocytes injected with reporter plasmid only during recovery from a high temperature heat shock. These results led to the proposal of a model in which HSF1 trimers are either assembled in a transcriptionally incompetent form or one that has the potential to become transcriptionally competent during stress, prior to DNA-binding. The identity of HSF1-binding proteins that interact with HSF1 at different stages of activation/deactivation was characterized in an effort to assign regulatory roles to these proteins. HSF1 was detected in a high molecular weight complex (350-600 kDa) during all phases of the activation/deactivation cycle. HSF1 at different stages of activation was tested for interaction with specific molecular chaperones by electrophoretic mobility supershift analysis. Hsp90, p23, FKBP52, Hip and Hop are all associated with transcriptionally active and inactive HSF1 suggesting that interaction of HSF1 with any of these molecules does not activate HSF1 transcriptional activity. These results do not exclude the possibility that the function of these molecular chaperones may change during activation of HSF1 transcription or that post-translational modifications may be the primary mechanism that drives HSF1 from a transcriptionally inactive to active form.

# Acknowledgments

This work was supported by salary awards from the National Sciences and Engineering Council (NSERC) and the Canadian Institutes of Health Research (CIHR) and by CIHR operating grants.

I would like to sincerely thank Dr. Nick Ovsenek for mentoring me throughout my graduate education. Intellectually, I have grown by leaps and bounds since we have met and I owe that in large part to you. My colleagues throughout the years have also played a significant role in my scientific development, especially Andrew Ficzycz, Steven Bharadwaj, Johnny Xavier, Adnan Ali, Renaud Conde, and Christine Mcloughlin. I would like to thank the support staff of the Department, especially Tonya McGowan and Gregg Parchomchuk. I would like to thank the present and past members of my thesis committee Dr's Pat Krone, Bill Roesler, Helen Nichol, and Colin Rasmussen for helpful comments and discussions. I would also like to thank Dr. Dick Mosser for his vigorous revision of my manuscript.

Most importantly I would like to thank my wife whose tireless encouragement, support and belief that I could succeed inspired me pursue my goals. I would like to dedicate this thesis to my children Samual, Jacob and our new baby boy expected soon.

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## List of Abbreviations

ATP – adenosine triphosphate
BSA – bovine serum albumin
CAT – chloramphenicol acetyl transferase
CIP – calf intestinal phosphatase
CMV – cytomegalovirus
CTD – C-terminal domain
CyP40 – cyclophilin 40
dATP – deoxyadenosine triphosphate
dCTP – deoxycytidine triphosphate
dGTP – deoxyguanosine triphosphate
DHSF – *Drosophila* HSF
DNA – deoxyribonucleic acid
dTTP - deoxythymidine triphosphate
EDTA – ethylene-diaminetetra-acetic acid
EGTA – Ethylene glycol-bis(2-aminoethylether)-N,N,$N^1$,$N^1$-tetraacetic acid
FKBP – FK506 binding protein
GAF – GAGA associated factor
EMSA – electrophoretic mobility shift assay
Hip – HSP70 interacting protein
HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2 Hydroxyethyl)piperazine-$N^1$-(2-ethanesulfonic acid)
HHSF1 – human HSF1
Hop – Hsp90-Hsp70 organizing protein
HSE – heat shock element
HSF – heat shock factor
IgG – immunoglobulin G
KLH – keyhole limpet hemocyanin
LB – Luria broth
OR2 – Operation repair media 2
PCNA – proliferating cell nuclear antigen
PMA – phorbol-12-myristate-13-acetate
PP5 – protein phosphatase 5
RPM – revolutions per minute
SEC – size exclusion chromatography
SDS – sodium dodecylsulfate
SDS-PAGE - sodium dodecylsulfate polyacrylamide gel electrophoresis
SL2 – Schneider line 2
TAE – Tris-Cl acetate EDTA buffer
TBST – Tris buffered saline with Tween-20
TCM – Tris-Cl, calcium, magnesium buffer
TEMED - N,N,$N^1$,$N^1$-Tetramethylethylenediamine
TFIID – Transcription factor II D
TLC – thin layer chromatography

TPA - 12-O-tetradecanoylphorbol 13-acetate
Tris-Cl – Tris (hydroxymethyl) – aminomethane chloride
UV - ultraviolet
YY1 – Ying Yang 1
YY1BE – YY1 binding element

# 1 Introduction

## *1.1 The Cellular Response to Stress*

Common to all cells is the ability to respond to acute or toxic conditions in the environment and repair damage caused by stressful conditions. Cellular stress can be defined as any situation that results in large scale protein unfolding, misfolding or aggregation. Exposure of cells to stress results in the inducible expression of a family of genes encoding heat shock or stress proteins (Hsp) that function to restore homeostasis and help the cell survive (Lindquist and Craig, 1988; Morimoto et al., 1997; Morimoto, 1994). Hsps are a diverse group of proteins that are divided into families based on molecular weight. Major families of Hsps include Hsp100, Hsp90, Hsp70, Hsp60, the small chaperones, and ubiquitin. In addition to the role that Hsps play during stress, members of each family of chaperones are constitutively expressed and function in normal protein folding, transport, regulation, and degradation in unstressed cells (reviewed in (Frydman, 2001; Glover and Tkach, 2001; Haslbeck, 2002; Naylor and Hartl, 2001; Picard, 2002)). The upregulation of Hsp synthesis is not only beneficial to the cell at the time of the initial stress but cells previously exposed to sub-lethal heat stress are known to become tolerant when re-exposed to a normally lethal challenge (Lindquist and Craig, 1988). Thus, Hsps can serve a protective role in the cell if over-expressed prior to stress. The complex nature of the stress response is highlighted by the diversity and number of conditions leading to the production of stress proteins. Generally, inducers of Hsps can be divided into three categories: (i) environmental

stresses such as heat shock, and exposure to amino acid analogues, drugs, toxins, and heavy metals, (ii) non-stressful conditions such as during development and differentiation, exposure to growth factors, during stages in the cell cycle, and (iii) pathophysiological stresses such as viral and bacterial infection, fever and inflammation, neuronal injury, oxidant injury, ischaemia, aging, and cancer (Morimoto, 1996; Morimoto et al., 1997). Exposure of cells to elevated temperatures has been the most widely used experimental inducer of stress.

Early studies of the stress response indicated that Hsps participate in the regulation of their own expression (DiDomenico et al., 1982). This conclusion is based on several different observations. Experiments designed to monitor the production of Hsps in response to stress revealed that the level of Hsp induction achieved is related to the degree of stress, i.e. a higher heat shock temperature elicited a greater increase in Hsp levels. Hsp synthesis continues following the resumption of normal conditions, and the amount of time that is required for the cell to resume basal levels of Hsp synthesis varies with the severity of the heat shock. This data can be explained by a model in which Hsps are required to repair damage caused by the stress and are unavailable to prevent their own synthesis until that damage is repaired. In addition, blocking the normal production of Hsps during heat shock and during recovery leads to the continued accumulation of Hsps suggesting that newly synthesized Hsps are required to repress their own synthesis.

Other general phenomena, characteristic of the stress response, support a model in which Hsps participate in their own regulation. During prolonged stress, such as 2-3 hour heat shock, transcription of Hsps diminishes, reaching pre-stress levels in a process called attenuation (Abravaya et al., 1991a). Attenuation of the heat shock response is

coincident with the accumulation of Hsps (Abravaya et al., 1991a). These results were the first to indicate that regulation of Hsps involved a feedback loop in which newly expressed Hsps were involved in reducing their own synthesis. The role of Hsps in their own regulation is discussed in detail below.

## 1.2 Transcriptional regulation of heat shock proteins

Basal expression of Hsps depends primarily on a number of *cis*-acting DNA elements in the promoters of heat shock genes. *Cis*-acting promoter elements of the human Hsp70 gene have been mapped by deletion and mutagenesis and include the CCAAT box (centered at –65 and –147), Sp1 binding sites (centered at –46, -167, and -241 ), and an ATF/AP1 binding site (centered at –34) (Abravaya et al., 1991b; Greene and Kingston, 1990; Greene et al., 1987; Morgan, 1989; Morgan et al., 1987; Williams et al., 1989; Williams and Morimoto, 1990; Wu et al., 1986b). The CCAAT box interacts with two transcriptional activators, CBF (CCAAT binding factor) and CBTF (CCAAT box transcription factor) (Lum et al., 1990; Morgan et al., 1987). AP1 and ATF (activation transcription factor) have both been shown to bind the ATF/AP1 site (Ovsenek et al., 1990; Taylor and Kingston, 1990). Sp1, ATF, and AP1 are all positive regulators of basal Hsp70 transcription. *In vivo* footprinting studies reveal that these basal promoter elements are occupied at 37°C and factor binding is not altered during stress (Pelham, 1982; Wu, 1984a). These transcription factors are, therefore, largely responsible for the basal level of Hsp70 transcription (Abravaya et al., 1991b). Heat shock promoters also contain a functional TATA box which is occupied by TBP/TFIID

now known as members of the preinitiation complex in unshocked and heat shocked cells (Abravaya et al., 1991b). Other *cis*-elements within the Hsp70 promoter elements have been identified including serum response elements (Wu and Morimoto, 1985; Wu et al., 1987a). The existence of these and other *cis*-acting promoter elements are thought to be the vehicle through which basal Hsp70 transcription is regulated in response to oncogenes such as the adenovirus E1A gene product (Kao et al., 1985; Taylor and Kingston, 1990; Williams et al., 1989; Wu et al., 1986a) and to environmental (Wu and Morimoto, 1985; Wu et al., 1987a) and developmental cues (Milarski and Morimoto, 1986; Ovsenek et al., 1990).

Upregulation of Hsps in response to stress is primarily controlled at the transcriptional level. Heat and stress induction of Hsps requires a *cis*-acting element upstream of all heat shock genes termed the heat shock element (HSE). The HSE was originally identified as a DNA element that, when linked to a heterologous reporter gene, could render expression of that reporter heat inducible (Amin et al., 1988; Corces et al., 1981; Mirault et al., 1982; Pelham and Bienz, 1982; Xiao and Lis, 1988). A consensus HSE has been defined as three contiguous inverted repeats of the 5 base pair sequence whose consensus is AGAAn (Cunniff and Morgan, 1993; Fernandes et al., 1994; Kroeger and Morimoto, 1994). The human Hsp70 promoter (that controls the most dramatically upregulated Hsp in cells following stress) contains two HSEs, only one, centered at –98, is required for heat shock induction (Williams and Morimoto, 1990). HSEs are not bound by any protein in unstressed cells. During stress heat shock transcription factor (HSF) binds the HSE (Wu, 1980; Wu, 1984a; Wu, 1984b). Soon after identification of the HSE, the first HSFs were purified, cloned, and characterized (Sorger et al., 1987; Wu et al., 1987b).

Genetic deletion of yeast HSF has revealed that HSF is essential for cell viability during unstressed conditions, and is required for the upregulation of Hsps during stress, survival of the organism in response to extreme stress, and the establishment of thermotolerance (Gallo et al., 1993; Smith and Yaffe, 1991; Sorger and Pelham, 1988; Wiederrecht et al., 1988). The requirement of yeast HSF for cell viability implies that it may have some role in the regulation of basal Hsp expression. In higher eukaryotes, however, HSF1 (the HSF family member present in all cells that is primarily responsible for upregulating Hsps in response to stress as discussed above) is required for stress induced expression of Hsps and the establishment of thermotolerance, but is not required for cell viability or the basal expression of Hsps (Jedlicka et al., 1997; McMillan et al., 1998). Thus it appears that HSF1 is not involved in the basal expression of Hsps in higher eukaryotes.

A number of HSF family members have been identified in different eukaryotes such as frog, chicken, mouse, rat, and human cells, each sharing similarities in their DNA-binding specificity and homologies in their DNA-binding and oligomerization domains (reviewed in (Pirkkala et al., 2001)). HSF1 is present in all eukaryotic cells and is the main HSF that responds to pathophysiological stresses, environmental stresses, and other non-stressful conditions discussed above (Morimoto et al., 1997). Other HSFs (HSF2,3,4) are either specific to some organisms, restricted to specific tissues, or are activated by different conditions. The existence of multiple HSFs is thought to allow cells to respond to stimuli that cannot activate HSF1, and may also serve to modify the level and duration of Hsp expression in response to stresses that also activate HSF1.

## 1.3 Heat Shock Factor 1

Stress induced HSF1 activity is regulated through changes in its activity rather than changes in its steady state levels, synthesis, or stability. HSF1 exists in unstressed cells as a latent monomer and is transiently activated upon stress (Baler et al., 1993; Sarge et al., 1993; Westwood et al., 1991; Zimarino and Wu, 1987). There are a number of discrete steps involved in full activation of HSF1 including: formation of homotrimers associated with high affinity binding for the HSE, and activation of the transcriptional activation domain (see references below). Activation of HSF1 correlates with its concentration into several bright staining foci called granules in mammalian cells (Jolly et al., 1997). The biological purpose of these granules is unclear, as one of the dominant granules localizes at a heterochromatic site on chromosome 9 containing a specific subfamily of satellite III repeats in human cells, and the others do not localize to any particular nuclear structure, sites of DNA replication or RNA synthesis or loci of the major heat shock inducible genes (Cotto et al., 1997; Jolly et al., 2002; Jolly et al., 1997). Deactivation/attenuation of HSF1 is characterized by the loss of high affinity binding to the HSE and separation of HSF1 trimers to monomers (Abravaya et al., 1992; Abravaya et al., 1991a; Sarge et al., 1993). HSF1 can be deactivated after a brief stress followed by resumption of normal conditions or following long term continuous heat shock (Abravaya et al., 1991a; DiDomenico et al., 1982). Concomitant with activation of HSF1 is the post-translation SUMO-1 modification (small ubiquitin-related modifier) and phosphorylation of HSF1 (see below). Although some phosphorylation and SUMO-1 modification events have been shown to modulate HSF1 activity, the precise role of

post-translation modification of HSF1 remains unclear. HSF1 has also been shown to interact with a number of chaperones similar to the Hsp90 complex that regulates steroid receptors discussed above (see below). The full nature of the Hsp90 chaperone heterocomplex that interacts with HSF1 at each step in the activation/deactivation pathway has not been determined nor has the role for the chaperone heterocomplex in the regulation of HSF1 activity. HSF1 also interacts with a number of other chaperones, and binding proteins whose role in HSF1 regulation is being determined.

Regardless of the nature of the stress, HSF1 must become activated in order to elevate Hsp synthesis. One of the central questions concerning the cellular response to stress is the identity of the stress sensor and if a universal stress sensor exists that mediates HSF1 activity in response to the myriad of stresses that result in Hsp synthesis. It is still unknown whether HSF1 regulation is imparted by the intrinsic nature of the molecule i.e. if HSF1 can directly detect and respond to stress, or whether sensation of stress is dependent on cellular factors. Purified HSF1 can be reversibly activated to trimerize and bind DNA in response to heat (Farkas et al., 1998; Goodson and Sarge, 1995; Larson et al., 1995; Zhong et al., 1998), hydrogen peroxide (Zhong et al., 1998), and low pH (Farkas et al., 1998; Zhong et al., 1999) indicating that under certain conditions HSF1 can directly sense stress. However, under the same experimental conditions HSF1 is not affected by salicylate, dinitrophenol, ethanol, and arsenite, all conditions that activate HSF1 *in vivo* (Zhong et al., 1998). Other experiments suggesting that the temperature at which HSF1 is activated is not an absolute condition, argue against HSF1 as a direct sensor of heat stress. For example, HSF1 is activated at a lower temperature (40°C-41°C) in HeLa cells grown at a temperature of 35°C (as

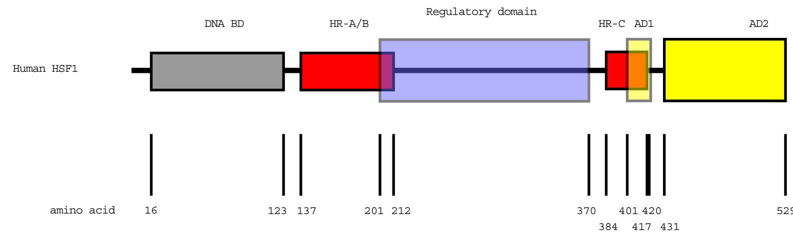
opposed to the optimal 37°C) (Abravaya et al., 1991a). Human HSF1 exhibits a lower temperature threshold for activation of DNA-binding in male germ cells that are physiologically maintained at a lower ambient temperature (Sarge, 1995; Sarge et al., 1995). In addition, human HSF1 expressed in *Drosophila* cells whose normal physiological temperature is 25°C is activated at 32°C as opposed to 42°C in human cells (Clos et al., 1993). These results indicate that although HSF1 can directly respond to stress the exact set-point of activation is dependent on interaction with the cellular environment.

Thus far Hsp70 has been championed as the universal stress sensor (Craig and Gross, 1991). However, the contribution of other chaperones such as the Hsp90 chaperone complex that has recently been shown to interact with and modulate HSF1, and the plethora of signal transduction pathways activated by stress, cannot be discounted as contributing to the regulation of HSF1 activation. The details of HSF1 activation/deactivation and regulatory models including the potential that various chaperones and signal transduction pathways act as the stress sensor will be discussed in the following sections.

### 1.4 HSF1 functional domains

HSF1s from different species share common features/domains depicted in Figure 1A. The DNA-binding domain, hydrophobic heptad repeat domains, regulatory domain, and activation domains have been identified through numerous mutational analyses (reviewed in (Wu, 1995)). HSF1 contains three arrays of hydrophobic heptad repeats

A




B

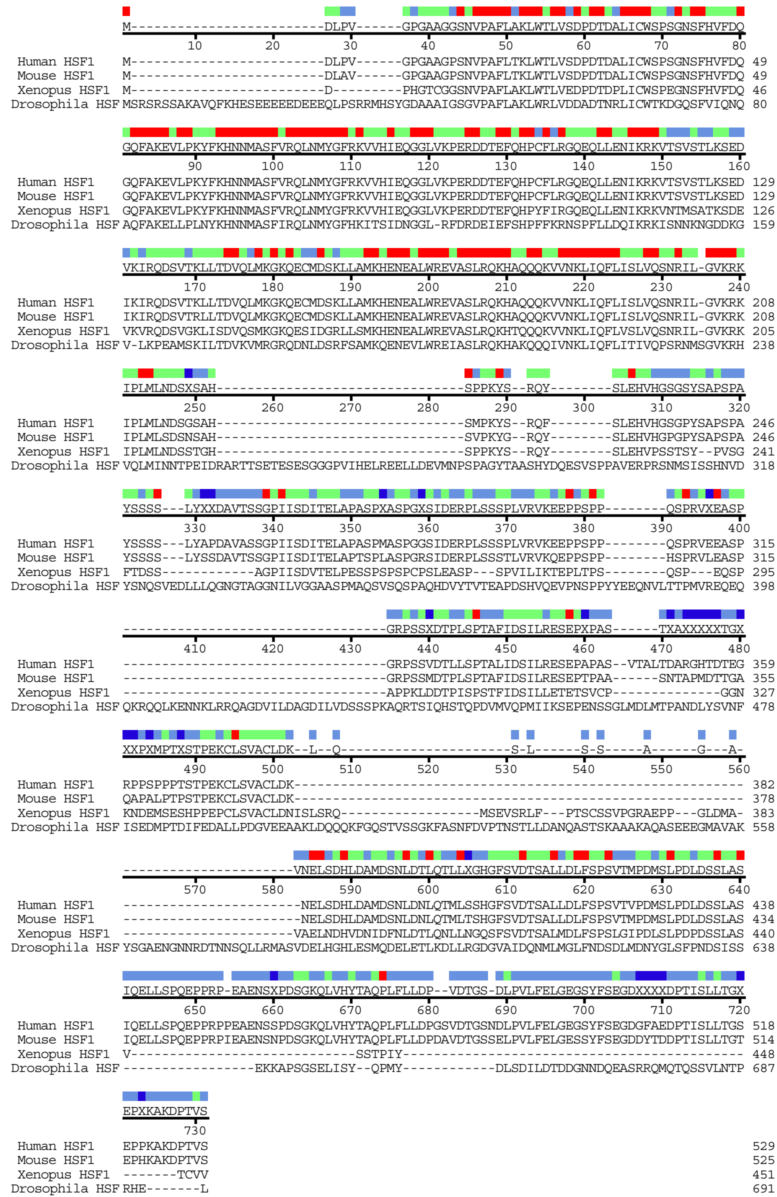



**Figure 1. Organization of HSF1 protein. (A)** Schematic representation of human HSF1. DNA binding domain (DNA BD), hydrophobic heptad repeats (HR-A/B), the regulatory domain, hydrophobic repeat (HR-C), and the AD1 and AD2 activation domains are depicted. Also indicated are the boundaries of these functional domains. This schematic has been adapted from (Wu, 1995; Green et al., 1995; Newton et al., 1996; Pirkkala et al., 2001). **(B)** An alignment of the predicted amino acid sequences of human HSF1, mouse HSF1, *Xenopus* HSF1, and *Drosophila* HSF. The common sequence is indicated and the sequence similarity between these HSF1s is indicated (red-100%, green-75%, light blue-50%, dark-blue represents regions of dissimilarity within the consensus). The alignment was carried out using Megalign (Lasergene 99).

(HR-A/B) at the N-terminus of the protein. The fourth heptad repeat (HR-C) is found at the C-terminus of HSF1 and associates with the heptad repeats A/B in unshocked cells to form an intramolecular coiled coil, a structure that represses trimer formation (Rabindran et al., 1993; Sarge et al., 1993; Zuo et al., 1994; Zuo et al., 1995). Activation of HSF1 involves the disruption of these intramolecular interactions and the formation, through the amino-terminal heptad repeats (HR-A/B), of intermolecular α-helical coiled coils to form an HSF1 trimer (Peteranderl and Nelson, 1992; Sorger and Nelson, 1989). Concomitant with HSF1 trimer formation is the increased affinity of HSF1 for the HSE. The DNA-binding domain (found near the N-terminus) is the most well conserved of all HSF1 functional domains (Figure 1B) (Wu, 1995). The DNA-binding motif is a helix-turn-helix similar to that found in catabolite activator protein (Damberger et al., 1994; Vuister et al., 1994a; Vuister et al., 1994b; Weber et al., 1997; Wu, 1995). An unconserved transcriptional activation domain has been mapped to the C-terminus of HSF1 (Bonner et al., 1992; Nieto-Sotelo et al., 1990; Shi et al., 1995; Sorger, 1990; Wisniewski et al., 1996). The activation domain of human HSF1 can be further dissected into two distinct activation domains AD1 and AD2 (Green et al., 1995). A negative regulatory region lies between the N-terminal and C-terminal heptad repeats (Green et al., 1995; Newton et al., 1996). This domain represses the transcriptional activation domain of unshocked HSF1 and can render heat shock inducibility to a chimeric protein containing an activation domain (Green et al., 1995; Newton et al., 1996).

Major functional domains are highly conserved as evidenced by the findings that *Drosophila* HSF or human HSF1 can functionally substitute for yeast HSF (Gallo et al.,

1993; Liu et al., 1997). Despite conserved functional domains, there is widespread variation in the size of HSF1 genes. HSF1 proteins range in size from 301 amino acids (*Lycopersicon peruvianum* HSF24) to 833 amino acids (*Saccharomyces cerevisiae* HSF1). Unconserved regions may be responsible for the slight differences in the activities of HSF1s from different species.

## *1.5 Subcellular Localization*

The subcellular localization of HSF1 has been a subject of controversy. Whereas some studies report that latent HSF1 is a cytoplasmic protein that translocates to the nucleus upon heat shock (Baler et al., 1993; Morimoto, 1998; Sarge et al., 1993; Sistonen et al., 1994; Zandi et al., 1997), other studies have indicated that HSF1 remains a nuclear protein prior to and during stress (Mercier et al., 1997; Mercier et al., 1999; Orosz et al., 1996; Westwood et al., 1991; Wisniewski et al., 1996; Wu et al., 1994). The discrepancy seems to lie in part to the techniques and tools used to determine the subcellular localization of HSF1 in different studies. Biochemical fractionation of tissue culture cells followed by western blot analysis consistently finds HSF1 fractionating with cytoplasmic proteins in unstressed cells and nuclear proteins in heat shocked cells (Baler et al., 1993; Mercier et al., 1999; Sistonen et al., 1994). Use of this technique to determine the subcellular localization of proteins is not always consistent with findings using immunofluorescence microscopy. For example, Hsp27 and most steroid receptors excluding the glucocorticoid receptor fractionate with cytoplasmic proteins using different biochemical extraction methods (Beaulieu et al., 1989; Perrot-Applanat et al.,

1992). In contrast, immunofluorescence microscopy using specific antibodies have determined that these proteins are exclusively nuclear at all times (King and Greene, 1984; Perrot-Applanat et al., 1992; Welshons et al., 1984). Immunofluorescence microscopy of HSF1 in unshocked human, mouse, and *Drosophila* cells has suggested that HSF1 is a cytoplasmic protein (Baler et al., 1993; Zandi et al., 1997), however similar studies using the same cell types but using different HSF1 antibodies have suggested that HSF1 is a nuclear protein in unstressed cells (Mercier et al., 1999; Orosz et al., 1996; Westwood et al., 1991). The critical difference in these studies is that different antibodies were used to localize HSF1. Baler and Zandi (Baler et al., 1993; Zandi et al., 1997) have suggested that the antibodies used by Westwood, Orosz, and Mercier (Mercier et al., 1999; Orosz et al., 1996; Westwood et al., 1991) only recognized the trimeric active form of HSF1 and therefore could not detect cytoplasmic HSF1 in unshocked cells. To address this point Mercier (Mercier et al., 1999) carried out a careful study using several human HSF1 antibodies on different cell types and quantified the cytoplasmic and nuclear fluorescence in unshocked and heat shocked cells. The quantity and proportion of HSF1 in the nucleus was essentially the same in unshocked and heat shocked cells indicating that most of the HSF1 (80%) is in the nucleus before and after heat shock. These results imply that antibodies that detected HSF1 in the cytoplasm in other studies may have cross-reacted with cytoplasmic proteins. Recently, a GFP-human HSF1 chimera has been visualized in the nucleus of unshocked and heat shocked HeLa cells, however, it was not known whether the chimeric protein was not inadvertently activated in control cells due to excessive expression (Cotto et al., 1997; Dai et al., 2000). Although the subcellular localization of HSF1 remains a controversial issue, there is substantial evidence indicating that

translocation of HSF1 from the cytoplasm to the nucleus is probably not a part of the multi-step process in HSF1 activation and that stress must eventually be detected in the nucleus.

## *1.6 Evidence for a 2-step HSF1 activation mechanism*

The study of yeast HSF has been useful in the development of a 2-step mechanism of activation of HSF1 from higher eukaryotes. Unlike HSF1 molecules in metazoans and more advanced yeasts such as *S. pombe*, HSF in *S. cerevisiae* and *K. lactis* exists as constitutively HSE-bound homotrimers in the absence of stress (Jakobsen and Pelham, 1988; Sorger et al., 1987). In the absence of stress, DNA-bound HSFs are transcriptionally inactive (Jakobsen and Pelham, 1988; Sorger et al., 1987). It has been suggested that the structure of the transcriptionally inactive HSF might involve interaction of the transcriptional activation domains with the C-terminal end of the DNA-binding domain (DBD), since mutation of a conserved amino acid in the DBD (R274) reduced interaction of the DBD with the activation domain and rendered HSF constitutively active (Chen and Parker, 2002). Upon stress, induction of transcriptionally active HSF requires a conformational change inhibiting the interaction between DNA-binding and transcriptional activation domains thus releasing or exposing the activation domain.

The DNA-binding activity of metazoan HSF1 can be activated with the absence of transcriptional activity by treatment of cells with non-steroidal anti-inflamatory agents such as salicylate or indomethicin (Bharadwaj et al., 1998; Cotto et al., 1996; Giardina

and Lis, 1995; Jurivich et al., 1995; Jurivich et al., 1992; Lee et al., 1995; Locke and Atance, 2000; Winegarden et al., 1996). In human cells, heat can activate the transcriptional activity of HSF1 in cells pretreated with salicylate or indomethicin (Cotto et al., 1996; Lee et al., 1995). This indicates that the DNA-bound transcriptionally inactive HSF1 may represent a true intermediate in the activation of HSF1. In contrast, heat cannot induce HSF1 transcriptional activity in rat, *Drosophila*, or yeast cells pretreated with salicylate (Giardina and Lis, 1995; Locke and Atance, 2000; Winegarden et al., 1996). The ability of heat to render DNA-bound HSF1 transcriptionally competent in human cells but not in yeast, *Drosophila,* and rat may reflect a difference in how heat or salicylate affects these cell types. These studies have led to the hypothesis that activation of HSF1 in metazoans occurs in a stepwise manner during which HSF1 homotrimers bind HSEs and only following another conformational change does HSF1 become transcriptionally active. Activation of the DNA-bound but transcriptionally incompetent HSF1 intermediate has been linked to stress induced phosphorylation which will be discussed below.

## *1.7 Post-translational modification*

### 1.7.1 Phosphorylation

HSF1 has been proposed to be regulated at least in part by post-translational modifications including phosphorylation, and SUMO-1 modification. Generally, HSF1 is phosphorylated to some extent in unshocked cells and becomes hyperphosphorylated

upon heat shock (Cotto et al., 1996; Rabindran et al., 1994; Sorger and Pelham, 1988; Winegarden et al., 1996). There is also evidence that several sites on HSF1 are dephosphorylated in response to stress (Fritsch and Wu, 1999). The extent to which HSF1 is phosphorylated in both inactive and active states, coupled with the large number of dynamic phosphorylation events occurring between these states, has made it difficult to determine the role of phosphorylation in the regulation of HSF1 activity. Although a number of studies have addressed this phenomenon, the precise phosphorylation/dephosphorylation events critical in modulating HSF1 under a variety of stress conditions and the identity of the kinases and/or phosphatases involved have not been clearly established. While there is sufficient evidence to suggest that changes in phosphorylation serve to modulate HSF1 activity, it appears that no single phosphorylation event has been identified that is absolutely required to regulate HSF1 activity. A review of the literature in this area reveals that there is little consensus as to the role of phosphorylation events or the kinases and phosphatases involved in HSF1 regulation.

Discrepancies concerning the complexity of HSF1 phosphorylation has made the analysis of HSF1 phosphorylation difficult. Tryptic digest patterns of $^{32}P$ labeled HSF1 observed in studies by (Kline and Morimoto, 1997; Knauf et al., 1996) and Xia (Xia et al., 1998) vary widely. Xia (Xia et al., 1998) observed only enhanced phosphorylation of 4 different peptides of human HSF1 between unshocked and heat shocked cells whereas Kline (Kline and Morimoto, 1997) detected 5 different constitutively phosphorylated peptides and 14 inducibly phosphorylated peptides of mouse HSF1. In a study with *Drosophila* HSF1, Fritsch (Fritsch and Wu, 1999) detected 17 different constitutively phosphorylated peptides, 5 with increased intensity upon heat shock, 8

newly phosphorylated peptides upon heat shock, and at least 3 different dephosphorylated peptides upon heat shock. Discrepancies in the number of phosphorylated peptides between these studies cannot be due to the difference in the number of available serines as discussed by Fritsch (Fritsch and Wu, 1999) but probably reflect differences in labeling conditions, extract preparation, and peptide mapping and detection procedures. It is clear that identification, characterization, and determination of the role of phosphorylation of HSF1 is made difficult by the lack of precise knowledge of the scale of the changes in phosphorylation.

Several lines of evidence suggest that alterations in the phosphorylation state of HSF1 are not absolutely required for regulation of trimerization and DNA-binding during activation. Bacterially produced HSF1 is not phosphorylated yet is trimeric and can bind DNA (Clos et al., 1990; Rabindran et al., 1991). In more advanced yeasts, *Drosophila*, and human cells, salicylate treatment rapidly activates DNA-binding (and in *Drosophila* chromosomal puffing is activated as well), however, salicylate does not induce hyperphosphorylation or transcriptional activation (Jurivich et al., 1995; Jurivich et al., 1992; Lee et al., 1995; Winegarden et al., 1996). Furthermore, the tryptic digest patterns of $^{32}P$ labeled HSF1 are similar between unshocked and salicylate treated cells (Cotto et al., 1996).

Treatments of cells with agents that inhibit or activate cellular kinases or phosphatases have also been used to assess the role of phosphorylation in the regulation of HSF1 DNA-binding. Some reports indicate that pharmacological agents have little or no effect on HSF1 DNA-binding. For example, treatment of cells with pervanadate (a tyrosine kinase inhibitor) can induce HSF1 hyperphosphorylation but has no effect on the DNA-binding ability of HSF1 (Park and Liu, 2000). Treatment of *Drosophila* cells

with the phosphatase inhibitors okadaic acid, calyculin A, and orthovanadate, the kinase inhibitors staurosporine K252b, chelerythrine, H-7, KT5720, KT5823, and KT5926 did not on their own affect the DNA-binding activity of HSF1 in unshocked or heat shocked cells (Fritsch and Wu, 1999). Similarly, treatment of mammalian cells with the phosphatase inhibitor calyculin A (Xia and Voellmy, 1997) and kinase inhibitors staurosporine (Erdos and Lee, 1994), H-7, and GF-X (Xia and Voellmy, 1997) did not affect HSF1 DNA-binding in unshocked or heat shocked cells. In contrast, others have shown that the kinase inhibitors sodium orthovanadate (Mivechi and Giaccia, 1995) and H-7 (Ohnishi et al., 1999) reduce the strength of HSF1 DNA-binding activity, and the kinase activator TPA (12-O-tetradecanoylphorbol 13-acetate) (Chu et al., 1998; Holmberg et al., 1997; Holmberg et al., 1998) increases the strength of HSF1 DNA-binding activity during heat shock. It is possible that discrepancies in the reported effects of some of these agents in the modulation of HSF1 DNA-binding activity are due to differences in treatment conditions and cells types. In addition, specific inhibition of GSK3β increases heat induced HSF1 DNA-binding activity and over-expression of GSK3β decreases heat induced HSF1 DNA-binding activity (Xavier et al., 2000). There is only one report where the use of a general phosphatase inhibitor NaF was shown to activate HSF1 DNA-binding in the absence of stress (Cheng et al., 1998). Overall, these studies indicate that changes in HSF1 phosphorylation are not essential for the acquisition of DNA-binding during stress, but that phosphorylation and dephosphorylation of HSF1 appear to modulate HSF1 DNA-binding ability.

Several studies have suggested that alterations in HSF1 phosphorylation play a role in increasing the transcriptional activity and the eventual deactivation of HSF1.

Because hyperphosphorylation of yeast HSF is associated with transcriptional activation of heat shock genes, it has been suggested that phosphorylation might activate HSF1 transcriptional activity in metazoans (Sorger, 1990; Sorger and Pelham, 1988). This hypothesis was tested by cell treatments with kinase/phosphatase inhibitors/activators in which HSF1 had either been partially activated by pretreatment with salicylate or over-expression of HSF1 *in vivo* (both treatments activate DNA-binding but not transcription in the absence of stress) (Clos et al., 1990; Rabindran et al., 1991). Phosphatase inhibition by calyculin A treatment in mammalian cells resulted in hyperphosphorylation of salicylate-activated HSF1 and transcription of a reporter gene under control of a heat shock promoter (Xia and Voellmy, 1997). Similarly, over-expressed trimeric HSF1 in human cells was rendered transcriptionally competent by treatment with PMA (a protein kinase C stimulator) in the absence of stress, an effect that was reversed with the co-treatment of staurosporine (a PKC inhibitor) (Ding et al., 1997). As stated previously, heat shock activates transcription of Hsps in a cell that has been pretreated with salicylate in human cells (Cotto et al., 1996). The only detectable difference between salicylate activated HSF1 and heat activated HSF1 either in the presence or absence of salicylate is the increase in serine phosphorylation (Cotto et al., 1996).

Results from experiments with inhibitors and activators of kinases and phosphatases also support a role for HSF1 hyperphosphorylation in the acquisition of transcriptional competence. For example, treatment of cells with serine/threonine protein kinase inhibitors H7, staurosporine, genistein, 2-aminopurine, and calphostin C inhibit Hsp expression whereas serine/threonine phosphatase inhibitor okadaic acid potentiates Hsp gene expression in cells exposed to a mild heat shock (Chang et al.,

1993; Erdos and Lee, 1994; Ohnishi et al., 1999; Xia and Voellmy, 1997; Yamamoto et al., 1994).

In addition to some suggestions that phosphorylation of HSF1 by GSK3 and/or JNK may play a role in deactivation (discussed below), other studies have suggested that hyperphosphorylation acts to deactivate HSF1. A conserved heptapeptide domain of *K.lactis* HSF designated as CE2 has been shown to restrain the C-terminal activator (CTA) (Chen et al., 1993). The CE2 domain is hyperphosphorylated upon heat shock and when a stretch of serine rich residues adjacent to CE2 is mutated to alanines, HSF is not deactivated (Hoj and Jakobsen, 1994). Mutation of these residues to aspartic acid however did not have any detectable effect on the activity of HSF (Jakobsen and Pelham, 1991). Conversely, treatment of heat-treated human cells with calyculin A (a protein phosphatase inhibitor) has been shown to prolong the existence of HSF1 trimers after heat shock (Xia and Voellmy, 1997). These experiments suggest that phosphorylation and/or dephosphorylation events may play a role in the deactivation and disassembly of HSF1.

In addition to the non-specific approaches used to assess the role of phosphorylation in the modulation of HSF1 activity discussed above, the roles of specific phosphorylation events, and various kinases and their associated signal transduction pathways has been examined. Several key phosphorylation events that influence HSF1 activity, all in the regulatory domain of human HSF1, have been characterized. To summarize, human HSF1 is phosphorylated at S230 intermittently in unshocked cells. During heat shock S230 becomes more stably phosphorylated and this is associated with increased transcriptional activity. S303, S307, and S363 of human HSF1 are all constitutively phosphorylated and function to repress the transactivation

domain in the unshocked state. Signal transduction pathways that have been implicated in the regulation of HSF1, possibly by phosphorylation of these sites, include calcium/calmodulin (CaM) dependent kinases, protein kinase C (PKC), the mitogen activated kinase pathways (MAPK) ERK (extracellular signal regulated kinase), JNK (c-Jun N-terminal kinase), and the p38 kinase. A discussion of each phosphorylation event and its associated signal transduction cascade is presented below.

Phosphorylation of serine 230 (S230) in the regulatory domain has been established as an event that greatly increases the transcriptional activity of HSF1 during heat shock (Holmberg et al., 2001). Phosphopeptide mapping of human HSF1 has determined that HSF1 is phosphorylated at S230 under normal growth conditions *in vivo* and its phosphorylation is increased upon heat shock (Holmberg et al., 2001). Introduction of human HSF1 S230A mutants into HSF1 -/- cells reduced transcription from the endogenous Hsp70 gene after a heat shock by more than 50% indicating that phosphorylation of S230 is a potent stimulator but not on its own essential for HSF1 transcriptional activity (Holmberg et al., 2001). Mutation of S230 to alanine had no effect on the DNA-binding ability of HSF1 suggesting that phosphorylation of S230 modulates only the transcriptional activity of HSF1 (Holmberg et al., 2001). In this study, calmodulin/calcium dependent kinase II (CaMKII) was shown to phosphorylate S230 *in vitro* and overexpression of CaMKII in cells increased the transcriptional activity of HSF1 in heat shocked cells, but did not activate transcription in unshocked cells (Holmberg et al., 2001). In a different study examining the role of CaMKII in HSF1 regulation activation of HSF1 was enhanced by treatment of cells with KC62, a specific CaMKII inhibitor (Soncin et al., 2000). Although there are some discrepancies amongst different studies as to the role of CaMKII in the regulation of HSF1 during

stress it is clear that phosphorylation of S230 during heat shock increases the transactivation potential of HSF1.

From early observations of the increase in intracellular calcium levels during stress, the involvement of a calcium dependent process that modulates HSF1 activity has been proposed. Cytoplasmic calcium concentration increases following heat shock and does not return to pre-heat shock levels until 45 minutes after resumption of normal conditions in *Drosophila*, mouse and human cells (Drummond et al., 1986; Kiang et al., 1992; Price and Calderwood, 1991). Several studies using pharmacological agents that increase internal calcium concentrations report transcriptional upregulation of Hsp genes in the absence of heat (Resendez et al., 1985; Welch et al., 1983; Wu et al., 1981; Yamamoto et al., 1994). This indicates that calcium modulators may induce DNA-binding as well as transcription. Despite this evidence of a role for $Ca^{2+}$ in regulating Hsp synthesis, blocking the $Ca^{2+}$ rise during heat shock using pharmacological agents revealed that the rise of $Ca^{2+}$ is not required for Hsp synthesis in *Drosophila* cells (Drummond et al., 1986). Both positive and negative regulatory effects on HSF1 DNA-binding activity have been observed in different experiments analyzing the effect of $Ca^{2+}$ on HSF1 activity *in vitro*, and *in vivo* (Elia et al., 1996; Mosser et al., 1990; Soncin et al., 2000). Elevating $Ca^{2+}$ levels in a HeLa cell free system activated DNA-binding (Mosser et al., 1990), and elevation of $Ca^{2+}$ levels in cells by addition of the calcium ionophore A23187 prolonged HSF1 DNA-binding during long term heat shock in human K562 cells (Elia et al., 1996). In contrast the calcium ionophore A23187 has been reported to decrease HSF1 DNA-binding activity during heat shock in human THP1 cells (Soncin et al., 2000). Taken together these results indicate that $Ca^{2+}$

dependent processes may have multiple roles in HSF1 activation and deactivation, and there may be differences in these roles depending on the cell type.

The PKC group of kinases has been implicated in both positively and negatively regulating HSF1. There are 11 members of the PKC family of kinases that can be further divided into three subgroups: conventional PKCs include α, β and γ which are regulated by phosphatidylserine, calcium and diacylglycerol; novel protein kinase Cs such as δ, ε, η, θ which are regulated by diacylglycerol and phosphatidylserine; and atypical protein kinases ζ, ι, and λ which only respond to phosphatidylserine and in contrast to conventional PKCs do not respond to phorbol esters (reviewed in (Newton, 1997)). Interestingly, despite minor increases in cytosolic calcium observed during heat shock as mentioned above, none of these PKC isozymes were reported to be upregulated during heat shock in human cells (Holmberg et al., 1998). Nevertheless, upregulation of PKC activity by treating cells with the phorbol ester 12-*O*-tetradecanoylphorbol 12-acetate (TPA) accelerates the acquisition of DNA-binding activity by HSF1, and enhances Hsp expression (Chu et al., 1998; Holmberg et al., 1997; Holmberg et al., 1998). A key point is that TPA treatment does not activate HSF1 in unstressed cells and so heat shock is still required for activation (Chu et al., 1998; Holmberg et al., 1997; Holmberg et al., 1998). Phorbol-12-myristate-13-acetate (PMA) treatment of cells in which HSF1 is bound to the HSE in the absence of stress through HSF1 over-expression also stimulated transcription of the Hsp70 gene (Ding et al., 1997). Activation with TPA and PMA were reversed by also treating cells with PKC inhibitors bisindolylmaleimide I, (Holmberg et al., 1998) and staurosporine (Ding et al., 1997). Interestingly, in these experiments upregulation of HSF1-dependent transcription was restricted, in that Hsp70

but not Hsp90 was upregulated (Holmberg et al., 1997). These results suggest that although PKC isozymes are not activated during heat stress, activation of different PKC isozymes during normal cellular activities might serve to prime HSF1 and potentiate the response particularly by upregulating the Hsp70 class of chaperones (Holmberg et al., 1997; Holmberg et al., 1998). Over-expression of Hsp70 in unstressed cells down-regulates PKC activity (Ding et al., 1998) suggesting that a feedback loop allows the return of HSF1 to a pre-primed state.

Alternatively, a negative regulatory role for PKC activity in the regulation of HSF1 has also been described. Chu (Chu et al., 1996 1998) identified S363 in the regulatory domain of human HSF1 as a constitutively phosphorylated site in unstressed and heat stressed cells that is phosphorylated by PKCα *in vivo* (Chu et al., 1998). Over-expression of PKCα in cells containing HSF1 bound to the HSE in the absence of stress via HSF1 over-expression led to down-regulation of HSF1 dependent transcription without altering DNA-binding activity (Chu et al., 1998). These results suggest that phosphorylation of S363 functions to repress the transcriptional activity of HSF1 in unstressed cells. However, because S363 is constitutively phosphorylated and heat shock can activate HSF1 transcriptional activity, S363 phosphorylation is not sufficient to prevent activation of HSF1 by heat (Chu et al., 1998). These results do not exclude the possibility that PKC activation can potentiate HSF1-dependent transcription by phosphorylation of HSF1 at a different site as suggested by (Holmberg et al., 1997; Holmberg et al., 1998).

The role of two constitutively phosphorylated residues in the regulatory regions of human, and murine HSF1s and their effect on the transcriptional ability of HSF1 have

been addressed. Mutation of serines 303 and 307 to alanine render HSF1-chimeras bound to the HSE by overexpression transcriptionally active in the absence of heat (Knauf et al., 1996). Conversely, if these serines are mutated to glutamic acid, to mimic phosphorylated residues, the resultant molecules are transcriptionally inactive in the absence of stress but remain heat-inducible (Knauf et al., 1996). Although human and murine HSF1 are both constitutively phosphorylated at serine residues at positions 303 and 307 it appears that mutation of S303 to alanine is sufficient to deregulate HSF1 transcriptional activity in murine cells (Kline and Morimoto, 1997) and mutation of S307 to alanine is sufficient to deregulate HSF1 transcriptional activity in human cells (Xia et al., 1998). Since S303 is conserved in human, mouse, chicken, fly, and frog (Xavier et al., 2000) it is likely that constitutive phosphorylation of this residue underlies a conserved mechanism to repress the transcriptional activity of HSF1 in unstressed cells.

An alternative and more dynamic role of phosphorylation of the regulatory domain has been proposed by Xia (Xia et al., 1998). Phosphorylation of the regulatory domain was either weak or absent in cells over-expressing HSF1 chimeras that were constitutively active, suggesting that phosphorylation of S307 only occurred on inactive HSF1 (Xia et al., 1998). Presumably S307 is dephosphorylated during the course of HSF1 activation (Xia et al., 1998). What has not been demonstrated by any group is the significance of dephosphorylation of either S303 and/or S307 in the context of a monomeric inactive HSF1 molecule. Although mutation of HSF1 at S303 and/or S307 to alanine (Knauf et al., 1996) can derepress the transcriptional activity of HSF1 that has been partially active and is bound to DNA, a dephosphorylation event such as this may not have any effect on the conversion of HSF1 from a monomer to trimer. Thus,

transcriptional activation of HSF1 dephosphorylated at S303 and/or S307 may still require the prior activation of HSF1 at the level of DNA-binding.

The ERK1/ERK2 pathway has also been linked to HSF1 phosphorylation. ERK1/ERK2 are members of the MAPK superfamily and are activated by growth factors, cytokines, viral infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents and carcinogens, resulting in the activation of kinases which phosphorylate serine/threnine-proline motifs in a Ras-Raf-dependent manner (for review see (Johnson and Lapadat, 2002; Widmann et al., 1999)). The ERK1/2 pathway is also mildly activated by heat shock (Kyriakis et al., 1994). Several observations support the involvement of the ERK kinase pathway in the regulation of HSF1. ERK1/2 has been shown to phosphorylate serine 307 of human HSF1 *in vitro* (Chu et al., 1996; Chu et al., 1998; Kim et al., 1997; Knauf et al., 1996) and in a Ras-Raf-dependent manner *in vivo* (Kim et al., 1997; Knauf et al., 1996) resulting in the negative regulation of HSF1 transcriptional activity. Over-expression of a dominant inhibitory mutant of ERK1 increases the expression of an HSF1-dependent promoter (Mivechi and Giaccia, 1995). ERK/MAPK pathways have also been proposed to negatively regulate HSF1 in an indirect manner by activation of the RSK2 serine-threonine kinase. RSK2 is primarily activated by the ERK pathway and has been shown to regulate gene expression by phosphorylation of serum response factor, c-Fos and c-Jun (Blenis, 1993). RSK2 has been shown to phosphorylate HSF1 *in vitro* (Stevenson et al., 1999). Cells derived from patients from Coffin-Lowry Syndrome, which are functionally deficient in RSK2 activity, were used to determine that RSK2 plays a minor role in negatively regulating HSF1 activation at 37°C and a more significant role in repressing HSF1 DNA-binding

ability during heat shock (Wang et al., 2000). It remains to be seen if RSK2 negatively regulates HSF1 activity by a GSK3 dependent mechanism (see below) or by directly phosphorylating HSF1.

It has been proposed that phosphorylation of S307 by ERKs primes the phosphorylation of S303 by GSK3 (α or β) (Chu et al., 1996; Chu et al., 1998; He et al., 1998). Recombinant human HSF1 can be phosphorylated weakly on S303 by GSK3 *in vitro* but ERK pretreatment greatly increases the level of HSF1 phosphorylation by GSK3 (Chu et al., 1996; Chu et al., 1998). Over-expression of GSK3β in *Xenopus* oocytes and human cells has been shown to inhibit HSF1 DNA-binding and transcriptional activity while specific inhibition of GSK3β increases both DNA-binding and transcriptional activity of HSF1 in heat shocked cells (Bijur and Jope, 2000; Xavier et al., 2000). Taken together these observations support a role of GSK3β in the down-regulation of HSF1 activity.

Several studies conflict as to when GSK3β exerts its effect to down-regulate HSF1 activity. As discussed above some studies suggest that GSK3β functions to phosphorylate HSF1 at S303 and negatively regulate HSF1 transcriptional activity in unstressed cells (Chu et al., 1996; Chu et al., 1998; Kline and Morimoto, 1997; Knauf et al., 1996). This is consistent with the finding that GSK3 is constitutively active in cells (reviewed in (Plyte et al., 1992)). Other experiments including the kinetics of GSK3 activity during heat shock have suggested that the primary role of GSK3β is to phosphorylate HSF1 during recovery and promote deactivation of HSF1. GSK3 activity is mildly stimulated during the first hour of heat shock (He et al., 1998; Xavier et al., 2000). Nuclear GSK3β levels increase following 1 hour of heat shock (Xavier et

al., 2000). More importantly GSK3β activity increases 3 fold during the first two hours of recovery (He et al., 1998). Additionally, inhibition of GSK3β activity delays HSF1 attenuation during recovery (Bijur and Jope, 2000; Xavier et al., 2000). Over-expression of GSK3β also facilitates the disappearance of HSF1 granules that develop in the nucleus during a heat shock (He et al., 1998).

It is possible that GSK3 could participate in the down-regulation of HSF1 transcriptional activity by increased phosphorylation of S303 if HSF1 is incompletely phosphorylated on S303 in unshocked cells. The regulation of GSK3 activity during normal cellular events is consistent with the hypothesis that only a fraction of HSF1 molecules may be phosphorylated on S303 in unstressed cells. Although GSK3 activity is not acutely regulated, several signaling pathways can serve to moderately down-regulate GSK3 activity. GSK3 can be inhibited by activation of the Wnt pathway (Cook et al., 1996), agents that stimulate the phosphatidylinositol 3-kinase (PI-3K) (Coffer et al., 1998), certain PKC isotypes (Goode et al., 1992), p90rsk which is activated by the ERK1/2 MAPK pathway (Eldar-Finkelman et al., 1995), and agonists that increase intracellular cAMP concentrations in a PKA-dependent manner (Fang et al., 2000; Li et al., 2000). Any event that could activate signaling pathways upstream of GSK3 could result in the inhibition of GSK3 and the eventual dephosphorylation of HSF1 on S303 by non-specific phosphatases. A clear role for HSF1 phosphorylation by ERK and GSK3 would require precise knowledge of the dynamics of HSF1 phosphorylation at ERK and GSK3 target sites.

The c-Jun N-terminal kinase (JNK) MAPK phospho-relay module has also been implicated in the regulation of HSF1 activity. JNKs (1,2, and 3) are all activated in

response to environmental stress, radiation and growth factors (Johnson and Lapadat, 2002; Kyriakis et al., 1994). JNK activity increases dramatically during high temperature heat shock (45°C for human cells) and decreases following the resumption of normal conditions (Dai et al., 2000). Endogenous JNK activity is not as strongly activated in response to moderate heat shock such as 42°C in human cells (Dai et al., 2000; Kyriakis et al., 1994; Park and Liu, 2001). The literature contains contradictory evidence that JNK acts as both a positive or negative regulator of HSF1 activity. Experiments by Dai (Dai et al., 2000) suggest that JNK negatively regulates HSF1 DNA-binding and transcriptional activities and that the primary role of JNK in the regulation of HSF1 may be to promote the recovery of HSF1 following heat shock. JNK associates with HSF1 in unshocked and heat shocked cells. Anisomycin-directed elevation of JNK activity decreased HSF1 DNA-binding during the initial stages of heat shock, accelerated loss of HSF1 DNA-binding during recovery from heat shock, and repressed Hsp70 transcription in heat stressed cells. Over-expression of JNK in cells reduced transcription of Hsp70 and increased the rate of disappearance of HSF1 containing granules formed during high temperature heat shock. In contrast, other studies suggest that JNKs act as a positive regulators of HSF1. Treatment of cells with SB203580 (a JNK inhibitor) decreased the phosphorylation of HSF1 in response to stress and SB203580 treatment or transfection of cells with a dominant negative form of JNK suppressed heat induced transcription of Hsp70 (Park and Liu, 2001). Similar results were observed upon glucose starvation of human cells (Lee and Corry, 1998). Expression of a dominant negative version of Rac (a small GTPase) upstream of JNK in

the MAPK phospho-relay module decreased HSF1 DNA-binding activity and transcriptional activity (Han et al., 2001).

The potential phosphorylation sites on HSF1 targeted by JNKs have been investigated. JNK has been shown to phosphorylate HSF1 within the nuclear localization sequence but the role of this phosphorylation event has not been examined (Dai et al., 2000). JNKs have been shown to phosphorylate human HSF1 in the regulatory domain between amino acid residues 280-370 *in vitro* (Kim et al., 1997). More recently JNK has been shown to specifically phosphorylate S363 on human HSF1 *in vitro* (Dai et al., 2000), a residue that has also been shown to be a target of ERKs and certain PKC isozymes (Chu et al., 1998). As stated previously, JNK over-expression reduced Hsp70 transcription (Dai et al., 2000). However, HSF1-dependent transcription was not repressed in cells over-expressing both JNK and the S363A mutant of human HSF1 suggesting that JNK phosphorylation of S363 negatively regulates HSF1 transcriptional activity during heat shock (Dai et al., 2000). As discussed above the role of HSF phosphorylation on S363 during heat shock and recovery is unknown. Chu has noted that S363 is constitutively phosphorylated in unshocked and heat shocked cells and mutation of S363 to alanine mimicking an unphosphorylated residue did not prevent HSF1 activation in response to heat (Chu et al., 1996; Chu et al., 1998). It is also important to note that phosphorylation of HSF1 on S363 by PKCα was shown to reduce HSF1 transcriptional activity as has phosphorylation of S363 by JNK (Chu et al., 1998). Thus it is not clear whether JNKs are positive or negative regulators of HSF1, and the exact targets of JNK on HSF *in vivo* have not been determined.

The p38 MAPK phospho-relay module has also been implicated in the regulation of HSF1. The p38 MAPK module is regulated by many of the conditions that activate the JNK/MAPK modules such as pro-inflammatory cytokines and environmental stress, but the time course and extent of activation differs between the two MAPK pathways (Raingeaud et al., 1995). Support for a role for p38 in the regulation of HSF1 comes from the observation that p38 phosphorylates human HSF1 in the regulatory domain (Knauf et al., 1996) between amino acids 280 and 370 *in vitro* (Kim et al., 1997). An inhibitor of p38 (SB203580) eliminated the phosphorylation of HSF1 and the upregulation of Hsp70 in response to 100 μM cadmium (Hung et al., 1998). These results suggest that phosphorylation of HSF1 in the regulatory domain by p38 during stress positively regulates HSF1.

When considering the potential regulating interactions of members of the MAPKs with HSF1 it is important to consider mechanisms that exist to prevent cross talk between MAPK pathways (discussed in (Weston et al., 2002; Widmann et al., 1999)). Thus, even if a stimulus activated each MAPK to varying degrees and with different kinetics, each MAPK pathway may serve a distinct role in regulating HSF1 activity throughout the activation/deactivation pathway in response to certain stresses. This point is best illustrated by a study that implicates ERK1/2 as a positive regulator of HSF1 in response to low concentrations of cadmium and p38 as a positive regulator of HSF1 in response to high concentrations of cadmium (Hung et al., 1998). An inhibitor of p38 (SB203580) eliminated the phosphorylation of HSF1 and the upregulation of Hsp70 in response to 100 μM cadmium but not 60 μM cadmium. A specific inhibitor of ERKs (PD98059) eliminated the phosphorylation of HSF1 and the upregulation of Hsp70 in response to 60 μM cadmium and not 100 μM cadmium. Although other

groups have provided evidence that SB203580 is a specific inhibitor of p38 (Cuenda et al., 1995; Lee et al., 1993; Young et al., 1993; Young et al., 1997) other studies have shown SB203580 inhibits both p38 and JNK at concentrations lower that those used in the study by Hung (Hung et al., 1998). As JNK activity was not measured in this study it is difficult to attribute the specific phosphorylation of HSF1 in cell treated with high cadmium concentrations specifically to p38. Nevertheless, this study illustrates the possibility that each MAPK pathway may regulate HSF1 dependent on the stress or the severity of the stress.

### 1.7.2 SUMO-1 modification

In addition to numerous phosphorylation/dephosphorylation events, HSF1 is also modified by SUMO-1 addition during stress (Hong et al., 2001). SUMO-1 is an 11kDa protein with homology to ubiquitin that can become conjugated to proteins and modulate their function (Hay, 2001; Kretz-Remy and Tanguay, 1999; Melchior, 2000; Muller et al., 2001; Yeh et al., 2000). Experiments have characterized the role of SUMO-1 modification of human HSF1 (Hong et al., 2001). HSF1 was found to be modified by SUMO-1 conjugation on lysine 298 within the regulatory domain during heat shock. Modification of HSF1 *in vitro* in a reconstituted SUMO-1 reaction system activated DNA-binding in the absence of stress. A K298R mutant of HSF1 could not bind DNA in the same system. The K298R HSF1 mutant also displayed decreased transcriptional activation of a reporter gene, compared to wild type HSF1, and the mutant did not localize to nuclear punctate granules following heat shock. These nuclear localization

characteristics were likely to be secondary to decreased DNA-binding activity of the mutant. Hong (Hong et al., 2001) suggests that SUMO-1 modification during heat shock disrupts the intramolecular interaction between the trimerization domain and C-terminal leucine zippers and promotes formation of trimers and the associated acquisition of DNA-binding. Recently, a link between phosphorylation of HSF1 at S303 and S307 in the regulatory domain and SUMO-1 modification has been made (Hietakangas et al., 2003). S303A/S307A HSF1 mutants are not SUMO-1 modified *in vitro* or *in vivo*. Conversely, S303D/S307D HSF1 mutants are efficiently SUMO-1 modified both *in vitro* and *in vivo* indicating that HSF1 must be phosphorylated at S303 and S307 prior to SUMO-1 modification. Although SUMO-1 modification is involved in the activation of HSF1 it is probably not essential as purified HSF1 can rapidly trimerize in the absence of SUMO-1 modification (Clos et al., 1990; Rabindran et al., 1991).

Although it is clear that HSF1 is SUMO-1 modified and extensively phosphorylated during the activation/deactivation pathway, it remains to be seen whether any HSF1 phosphorylation events are absolutely necessary for HSF1 activity? If post-translational modifications are indeed necessary for HSF1 activation *in vivo*, then at least one or more signal transduction pathways must also be essential for HSF1 activity. A review of the current state of knowledge suggests that many of the identified post-translational modifications serve to modulate the stress response by increasing or decreasing the ability of HSF1 to respond to the stressful situation either at the level of DNA-binding during activation and deactivation or transcriptional activity. The preponderance of evidence suggest that HSF1 hyperphosphorylation is essential for HSF1 to assume the transcriptionally active conformation once bound to DNA. A

specific phosphorylation event that drives conformational changes from transcriptionally inactive to active states has not been identified, and it is possible that multiple phosphorylation events including phosphorylation at S230, are responsible for this conformational change.

### *1.8 HSF1 interacting proteins*

Several models have been advanced to predict how cells regulate HSF1 in response to seemingly unrelated stress stimuli. Many if not all of the stress conditions resulting in activation of the stress response exert a common physiological effect which is the increase in the proportion of misfolded and damaged proteins to correctly folded proteins (reviewed in (Voellmy, 1996)). Early experiments by DiDomenico (DiDomenico et al., 1982) suggested that Hsps act to negatively regulate their own expression. A model was subsequently developed suggesting that the activity of HSF1 is autoregulated by the intracellular level of free Hsp70 family proteins (reviewed in (Craig and Gross, 1991)). In this model Hsp70 was hypothesized to maintain HSF1 in monomeric form in unstressed cells, and stress would increase the pool of misfolded proteins that compete with HSF1 for Hsp70, thereby freeing HSF1 to trimerize and bind DNA. In the case of a transient heat shock, the model proposed that HSF1 is deactivated once cellular protein damage is repaired, increasing the intracellular pool of free Hsp70 that can interact with HSF1. During sustained heat shock Hsp70, expression is continually increased to a point where the level of Hsp70 exceeds that required for repair of damaged cellular proteins. Excess Hsp70 is then free to interact with HSF1 and attenuate the stress response. In this model therefore Hsp70 represented the universal

stress sensor. Recent data suggests that HSF1 is also regulated at least in part by an Hsp90-containing chaperone heterocomplex perhaps in conjunction with Hsp70. Discussed below is the experimental evidence supporting the role of molecular chaperones in the regulation of HSF1, including the conversion of HSF1 from a monomer to a homotrimer, DNA-binding, transcriptional activation, and the reconversion of HSF1 from trimers to monomers upon termination of the stressful state.

A general effect of Hsp/c70 on the activation of heat shock proteins has been observed in a number of different experimental models. Over-expression of Hsp70 in mammalian cells prior to heat shock reduced heat shock induced expression from a heat shock promoter (Baler et al., 1992). Treatment of cells with cycloheximide, a protein synthesis inhibitor, decreased the heat-induced expression of Hsp70, an effect which was overcome by a severe heat shock (Baler et al., 1992). It was suggested that reducing protein synthesis levels and protein concentration in cells increased the effective levels of Hsp70 that could then act to negatively regulate its own synthesis in response to a moderate heat shock (Baler et al., 1992). A severe heat stress however could sequester free Hsp70 and thus allow for activation of the stress response (Baler et al., 1992). The *Xenopus* oocyte has also been used as a model system to study the regulation of HSF1. The heat shock response can be activated with proteins are injected into the nucleus of *Xenopus* oocytes, only if they are denatured prior to injection (Ananthan et al., 1986). A similar but more detailed examination of this phenomenon revealed that the ability of exogenous proteins to activate the stress shock response when injected into oocytes depended on its propensity to form aggregates and not necessarily on the degree of modification (Mifflin and Cohen, 1994a). Experiments by Mifflin (Mifflin and Cohen, 1994a) also revealed that the dependence of the stress response on the amount of protein

injected was non-linear, indicating that a negative regulatory factor had been titrated by injection of the denatured protein. Injection of Hsc70 protein directly into the nucleus of *Xenopus* oocytes reduced the stress response induced by denatured proteins, and the observed level of reduction correlated with the ability of Hsc70 to bind the denatured proteins (Mifflin and Cohen, 1994b).

Earlier models suggested that Hsp70 could repress HSF1 oligomerization and therefore maintain HSF1 in an inactive monomeric state in unstressed cells. Consistent with this, constitutive over-expression of Hsp70 in human tissue culture cells has been shown to reduce heat shock induced DNA-binding by HSF1 in a dose dependent manner (Mosser et al., 1993). A decrease in Hsp/c70 by RNAi in *Drosophila* cells elevated Hsp synthesis in the absence of heat and increased the DNA-binding activity of HSF1 (Marchler and Wu, 2001).

Many other studies also support a role for Hsp/c70 in the disassembly HSF1 trimers following heat shock. For example, during long-term stress of mammalian cells at 42°C, HSF1 DNA-binding and transcription attenuate and this is associated with a large increase in the pool of available Hsps (Abravaya et al., 1991a). In heat shocked mammalian cells experiencing a general block in protein synthesis by long term heat shock at 43°C (Welch and Suhan, 1986), HSF1 did not attenuate suggesting that free Hsps are required to attenuate the response (Abravaya et al., 1991a). In addition, Hsp levels did not increase and HSF1 DNA-binding does not attenuate in *Xenopus* oocytes during long-term stress (Gordon et al., 1997). Because Hsp70 is the most upregulated Hsp during heat shock, it was proposed to be the chaperone primarily responsible for the disassembly of HSF1 trimers following heat shock. Experiments in which Hsp70

protein levels were manipulated *in vivo* support a role for Hsp70 in the disassembly of HSF1 trimers. HSF1 DNA-binding is attenuated more quickly in cell lines constitutively over-expressing Hsp70 (Mosser et al., 1993) or in cells directly injected with Hsp/c70 protein (Bharadwaj et al., 1999). Constitutive over-expression of Hsp70 in *Drosophila* and rat cells did not affect the level of HSF1 DNA binding during activation but prolonged DNA-binding following heat shock, indicating that the primary role of Hsp70 is to mediate the disassembly of HSF1 during recovery (Kim et al., 1995; Rabindran et al., 1994). Consistent with this, a decrease in Hsp/c70 by RNAi in *Drosophila* cells delayed recovery of HSF1 DNA-binding (Marchler and Wu, 2001) and inhibition of Hsp/c70 by injection of antibodies directly into *Xenopus* oocytes delayed recovery of HSF1 DNA-binding (Bharadwaj et al., 1999). Thus, there is ample experimental evidence to suggest that Hsp/c 70 regulates HSF1 homotrimerization during activation and deactivation.

Direct interactions between Hsp/c70 and HSF1 have been assessed by a variety of techniques. Hsp/c70-HSF1 interactions have been observed by co-immunoprecipitation assays (Rabindran et al., 1994; Shi et al., 1998) and using electrophoretic mobility supershift analysis (Abravaya et al., 1992; Baler et al., 1992). Hsp/c70-substrate interaction and chaperone activity is linked to the binding of Hsp70 to ADP or ATP (Hartl, 1996). In the ATP-bound state Hsp70 binds and releases substrates quickly whereas the ADP bound state of Hsp70 binds and releases substrates slowly (Hartl, 1996). ATP can disrupt Hsp/c70 substrate interactions *in vitro* (Hartl, 1996). This test is used to determine the specificity of Hsp/c70-substrate interactions. Differences in the ATP-dependence of HSF1-Hsp/c70 interactions have been observed. Rabindran and Baler determined that Hsp70-HSF1 interactions were not disrupted in the presence of

ATP in rat cells (Rabindran et al., 1994) and human cells (Baler et al., 1992), whereas Shi, and Abravaya observed that ATP disrupted Hsp/c70 interactions in human cells (Abravaya et al., 1992; Shi et al., 1998). Abravaya, and Baler also observed that addition of ATP to human cell extracts *in vitro* altered the mobility of HSF1-HSE complexes (Abravaya et al., 1992; Baler et al., 1992). The inability of Hsp70-HSF1 interactions to be consistently altered by increasing or decreasing ATP levels may indicate that differences in extraction and detection protocols could result in loss of components of a perhaps larger chaperone complex which could affect the ability of Hsp70 to hydrolyze ATP (Hartl et al., 1994). Regardless, different experimental procedures resulting in differences in detection of Hsp/c70-HSF1 complexes has highlighted the weak or transient nature of Hsp70-HSF1 interactions (Baler et al., 1996).

There is evidence that Hsp70 in co-operation with accessory proteins also modulates HSF1 transcriptional activity. Mutagenic analysis of HSF1 has revealed that Hsp70 and the co-chaperone Hdj1 (Hsp40) bind the activation domain of HSF1 (Shi et al., 1998). Over-expression of either Hsp70 can negatively regulate the transcriptional activity of HSF1 without affecting DNA-binding or phosphorylation (Shi et al., 1998). As both Hsp70 and Hsp40 are significantly increased upon heat shock, it has been proposed that Hsp70, in conjunction with Hsp40, acts as a repressor of HSF1 transcriptional activity primarily during the deactivation of HSF1 and prior to the dissociation of HSF1 from the HSE (Shi et al., 1998). The existence of a transcriptionally inactive DNA-bound trimer following heat shock has been observed during kinetic analysis of the heat shock response (Abravaya et al., 1991a). Such a model of HSF1 deactivation implies that HSF1 deactivation is a two step process, first involving a decrease in transcriptional activity followed by disassembly of the HSF1

trimer and a release of the HSF1 trimer from DNA. The *Drosophila* homologue of Hsp40 (DROJ1) was discovered to interact with *Drosophila* HSF in an unbiased screen for proteins that could interact with HSF (Marchler and Wu, 2001). Fine mapping of this interaction revealed that DROJ1 interacted with the region between the N and C-terminal leucine zippers, a region shown to modulate HSF trimerization (Marchler and Wu, 2001). Constitutive over-expression of DROJ1 slightly delayed the onset of the heat shock response (Marchler and Wu, 2001). Depletion of DROJ1 de-repressed Hsp synthesis in the absence of heat, and enhanced HSF DNA-binding in response to heat (Marchler and Wu, 2001). This study demonstrated a role for Hsp40 in the negative regulation of HSF1 DNA-binding and possibly transcriptional activity through direct interaction of Hsp40 with HSF between the N and C-terminal leucine zippers (Marchler and Wu, 2001). This study did not exclude the possibility that Hsp40 can interact with the activation domain of HSF because DROJ1-HSF interactions were demonstrated using truncated molecules lacking the activation domain (Marchler and Wu, 2001).

Experiments in the yeast system have also supported a model in which Hsp70 serves to negatively regulate HSF1 transcriptional activity. In yeast the critical HSEs required for stress-induced transcription of Hsps are constitutively occupied by HSF trimers, thus negative regulation of Hsp genes by Hsp/c70 in yeast occur at the level of transcriptional activation. Transcription of a reporter gene under control of a heat shock promoter was decreased by artificially raising the levels of Hsc70 in yeast by constitutive over-expression, and alternately increased by lowering the levels of normally constitutively expressed Hsc70 family members in yeast by genetic deletion (Boorstein and Craig, 1990; Stone and Craig, 1990). Analysis of the role of Hsp/c70 (SSA) and a related but functionally distinct chaperones SSB has led to the hypothesis

that Hsp70 protein family members can modulate transcriptional activation in two distinct ways (Bonner et al., 2000). In this model, SSA proteins act to regulate the HSF conformation change that is eventually necessary for HSF to revert to its low activity conformation (Bonner et al., 2000). Consistent with this hypothesis, deletion of SSA proteins renders HSF constitutively active (mentioned above (Boorstein and Craig, 1990)). In addition, the interaction of SSA with HSF is transient as suggested by the difficulty in co-immunoprecipitating HSF with SSA (Bonner et al., 2000). SSB forms large ATP-sensitive complexes with HSF, and in contrast to the interaction with SSA, the interaction with SSB is much more stable and can be detected throughout the activation/deactivation process (Bonner et al., 2000). This has led to the hypothesis that SSB acts to directly down-regulate the transcriptional activity of HSF by steric interference with the transcriptional activation domain (Bonner et al., 2000). As deletion of SSB proteins in yeast only leads to a minor upregulation of HSF activity, SSB probably plays a minor role in the regulation of HSF (Bonner et al., 2000). Since preferred substrates of SSB are upregulated during vigorous cell growth, SSB has also been postulated to link the regulation of HSF and transcription of stress proteins with the growth rate (Maicas et al., 1988; Ohba, 1997; Warner, 1989; Warner et al., 1985). In the context of a feedback loop model, SSB substrates would be high during periods of cell growth, limiting the amount of SSB that could interact with HSF resulting in upregulation of HSF activity. During periods of decreased cell growth, the pool SSB would be free to interact with HSF and down-regulate its activity.

A novel protein has recently been described called heat shock binding protein (HSBP1) that can negatively regulate HSF1, but the mechanism of regulation is not yet clear (Satyal et al., 1998). HSBP1 was discovered in an unbiased screen for HSF1

binding proteins that could bind the hydrophobic repeats of the trimerization domain (Satyal et al., 1998). HSBP1 is an evolutionary conserved constitutively expressed protein that is localized to the nucleus and its expression is not regulated by heat shock (Satyal et al., 1998). It is a small 76 amino acid protein comprised of putative coiled coil oligomerization domains without other obvious structural or functional domains (Tai et al., 2002). HSBP1 only interacts with trimeric phosphorylated HSF1 (Satyal et al., 1998). During recovery, or during prolonged heat stress HSBP1 loses affinity for HSF1 and transiently binds Hsp70 in the time that HSF1 transcriptional and DNA-binding activity is down-regulated (Satyal et al., 1998). Over-expression of HSBP1 repressed both HSF1 DNA-binding and transcription (Satyal et al., 1998).

It has been shown in a variety of cell types that artificial elevation of cellular levels of Hsps increases the rate of transition of HSF1 trimers to monomers during recovery, and during prolonged heat shock (Abravaya et al., 1991a; DiDomenico et al., 1982; Kim et al., 1995; Mosser et al., 1993; Rabindran et al., 1994). These observations coupled with the detection of Hsp/c70-HSF1 trimer interactions suggests that members of the 70 kDa family of heat shock proteins are involved in the regulation of HSF1, but does not exclude the possibility that other heat shock proteins are also involved. Using a cross-linking agent, mouse HSF1 has been found in a 400-500kDa complex in unstressed cells and in a smaller complex in stressed cells (Nunes and Calderwood, 1995) indicating that HSF1 can potentially interact with other proteins in addition to Hsp/c70.

Hsp90 and a number of co-chaperones present in the Hsp90 chaperone machine known to regulate steroid receptors have been identified as a functionally relevant components of the large HSF1 complex. Hsp90 has been shown to interact with both monomeric and trimeric HSF1 *in vitro*, and *in vivo* in *Xenopus* oocytes and human tissue

culture cells (Ali et al., 1998; Guo et al., 2001; Nadeau et al., 1993; Nair et al., 1996; Xia et al., 1998). Studies in several cell types and model systems suggest that Hsp90 is a negative regulator of HSF1 DNA-binding and a modulator of HSF1 transcriptional ability. Increasing Hsp90 levels in a rabbit reticulate lysate decreases the heat inducibility of HSF1 DNA-binding activity, and immuno-depletion of Hsp90 activates HSF1 DNA-binding activity (Xia et al., 1998). Disassembly of HSF1 trimers was delayed during recovery from heat shock by treatment of *Xenopus* oocytes with geldanamycin, an agent that disrupts Hsp90-target interactions (Grenert et al., 1997; Roe et al., 1999; Schulte et al., 1998; Stebbins et al., 1997), or immuno-sequestering Hsp90 by antibody microinjection (Ali et al., 1998).

Hsp90 has also been shown to modulate HSF1 transcriptional activity. In some cell types treatment with geldanamycin or other benzoquinoid ansamycin antibiotics like herbimycin A (reviewed in (Rinehart and Shield, 1976)) can activate both HSF1 DNA-binding and transcription of Hsps in the absence of any other stress (Bagatell et al., 2000; Kim et al., 1999; Knowlton and Sun, 2001; Lu et al., 2002). Herbimycin A can increase the levels of Hsps in cells treated in the apparent absence of HSF1 activation in other cell types (Hegde et al., 1995). Thus depending on the cell type, the benzoquinoid ansamycin, and the quantity of drug, differential effects of the inhibition of Hsp90 on HSF1 activity can be observed.

The role of Hsp90 as a negative regulator of HSF1 transcriptional activity has also been observed using an alternative technique. Steroid receptors such as the androgen, glucocorticoid, mineralcorticoid, and progesterone receptors are regulated by a well-characterized Hsp90-containing molecular chaperone complex. Briefly Hsp70 and Hsp90 chaperones and co-chaperones are involved in the assembly and maturation of

unliganded steroid receptors (reviewed in (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997)). Ligand binding is accompanied by dissociation of the receptor and chaperone heterocomplex (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997). At this point the steroid receptor can bind steroid responsive elements and activate transcription (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997). Thus, steroid receptors sequester Hsp90-containing chaperone heterocomplexes and can effectively decrease the intracellular pool of Hsp90. This effect can be reversed by the addition of hormone ligand. Addition of dexamethasone, a glucocorticoid receptor agonist, to cells decreased stress induced HSF1 transcriptional activity without affecting DNA-binding activity (Wadekar et al., 2001). Dexamethasone treatment also inhibited HSF1 dependent transcription of a constitutively active HSF1 mutant in unstressed cells indicating that members of the chaperone heterocomplex that interact with steroid receptors act to negatively regulate the HSF1 transcriptional activity (Guo et al., 2001; Wadekar et al., 2001). Over-expression of any of a number of different steroid receptors in cells has been shown to activate transcription of an Hsp promoter driven reporter gene and this affect can be nullified by addition of hormone ligands (Xiao and DeFranco, 1997). Over-expression of the glucocorticoid receptor, or introduction of denatured proteins in cells expressing a HSF1 chimera that has constitutive DNA-binding activity but no transcriptional activity decreases the association of HSF1 with Hsp90, FKBP52, and p23 (other members of the Hsp90 chaperone machine that interacts with steroid receptors) and activates transcription (Guo et al., 2001). Hsp90 has also been shown to be a negative regulatory of HSF1 transcriptional activity in yeast in unstressed and stressed cells (Duina et al., 1998). Taken together these results indicate that Hsp90 or other members of the chaperone

heterocomplex that regulate steroid receptors act to negatively regulate HSF1 DNA-binding and transcriptional activity.

The identity and functional role of members of the Hsp90 chaperone heterocomplex known to regulate steroid receptors and interact with HSF1 varies between different experimental model systems and experimental design. Examination of the composition of molecular chaperone complexes using a tagged HSF1 fusion protein in an unstressed *in vitro* rabbit reticulate lysate revealed that HSF1 interacts with a chaperone complex consisting of Hsp90, Hsp70, p60 (Hop), FKBP51, FKBP52, CyP40, and p23 (Nair et al., 1996). The contribution of additional co-chaperones in this study such as Hip was not examined and cannot be dismissed. Immunoprecipitation of Hsp90, FKBP52, and p23 containing complexes from cells indicate that the association of HSF1 with these proteins occurs in a stepwise manner (Guo et al., 2001). In particular, Hsp90 but not FKBP52 appears to be associated with HSF1 monomers (Guo et al., 2001). DNA-bound HSF1 is found associated with Hsp90, FKBP52, and p23 via the HSF1 regulatory domain (Guo et al., 2001). In the model presented by Guo, (Guo et al., 2001) Hsp90, FKBP52 and p23 associate with DNA-bound HSF1 and this association must be disrupted for transcriptional activation to occur. Using extracts from heat treated *Xenopus* oocytes, antibodies to Hsp90, FKBP52, and p23 were shown to supershift HSF1:HSE complexes *in vitro* indicating that these chaperones were bound to the trimeric DNA-bound form of HSF1 (Bharadwaj et al., 1999). Immuno-targeting of Hsp90, and p23 by injection of antibodies into *Xenopus* oocytes activated HSF1 DNA-binding in the absence of stress (Bharadwaj et al., 1999). Immuno-targeting of Hsp90, p23, FKBP52, FKBP52, Hip, Hop, or Hsp/c70 can all delay recovery of HSF1 DNA-binding following heat shock (Bharadwaj et al., 1999). Conversely, over-expression of

Hsp/c70, Hsp90, Hip, and Hop in *Xenopus* oocytes accelerated the recovery of HSF1 DNA-binding following heat shock (Bharadwaj et al., 1999). Over-expression of Cyp40, FKBP51, and FKBP52 delayed the recovery of HSF1 DNA-binding following heat shock and over-expression of p23 had no affect on the recovery of HSF1 DNA-binding (Bharadwaj et al., 1999). Addition of purified Hsp90 decreased HSF1 DNA-binding in response to stress, and immuno-depletion of Hsp90 activated DNA-binding in the absence of stress in a rabbit reticulate lysate (Zou et al., 1998). In this system immuno-depleting Hop, Hip, Hsp40, p23, or Cyp40 had no affect on HSF1 DNA-binding (Zou et al., 1998). These latter results are curious given the significant effects observed after over-expressing and depleting these co-chaperones *in vivo* and the apparent association of HSF1 with HOP, CyP40, p23, FKBP51, and FKBP52 in an ATP-dependent manner in reticulate lysates (Guo et al., 2001). These results indicate that HSF1 DNA-binding, both in unshocked cells and during recovery, and transcriptional activity appear to be negatively regulated by a dynamic Hsp90 containing chaperone heterocomplex including proteins important in the regulation of steroid receptors.

## 1.9 Regulation of steroid receptors

How similar are the mechanisms by which HSF1 and steroid receptors are regulated by Hsp90-containing chaperone heterocomplexes, and can models of the regulation of steroid receptor by chaperones be applied to the regulation of HSF1? As mentioned above, the maturation and activity of steroid receptors is largely controlled by

interaction and co-operation of heat shock protein chaperones (reviewed in (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997)). Regulation of steroid receptors by molecular chaperones can be divided into three separate stages including assembly, maturation (during which the receptor is competent to respond to ligand) and disassembly and recycling or degradation (Morimoto, 2002) (Figure 2).

Assembly and maturation of steroid receptors involves a dynamic remodeling of a chaperone complex that serves to prime the steroid receptor to bind to ligand (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997). The following is a summary of the current model of steroid hormone receptor maturation including the glucocorticoid and progesterone receptors. This model has been refined by a number of studies on different steroid receptors using several *in vivo* and *in vitro* systems and biochemical systems using purified components. The use of the rabbit reticulocyte lysate system has been particularly useful in the dissection of the steps required for steroid receptors to mature and become competent to bind ligand. The initial step in the maturation of a steroid receptor is the binding of Hsp40 to the aporeceptor with a stoichiometry of 1:1 (Hernandez et al., 2002a; Morishima et al., 2000a). Hsp40 then recruits Hsp70 by direct interaction with the Hsp70 TPR (tetratricopeptide) domain (Gebauer et al., 1997). Typically Hsp70 chaperones unfolded substrates or folding intermediates by binding to and stabilizing exposed hydrophobic sequences (reviewed in (Hartl, 1996)). Hsp70-substrate interaction and chaperone activity is linked to the binding of Hsp70 to ADP or ATP (Hartl, 1996). In the ATP-bound state Hsp70 binds and releases substrates quickly whereas the ADP-bound state of Hsp70 binds and releases substrates slowly (Hartl, 1996). The intrinsic ATPase properties of Hsp70 contribute to cycling between ATP and ADP-bound states (Hartl, 1996). In addition, co-chaperones including Hsp40, and

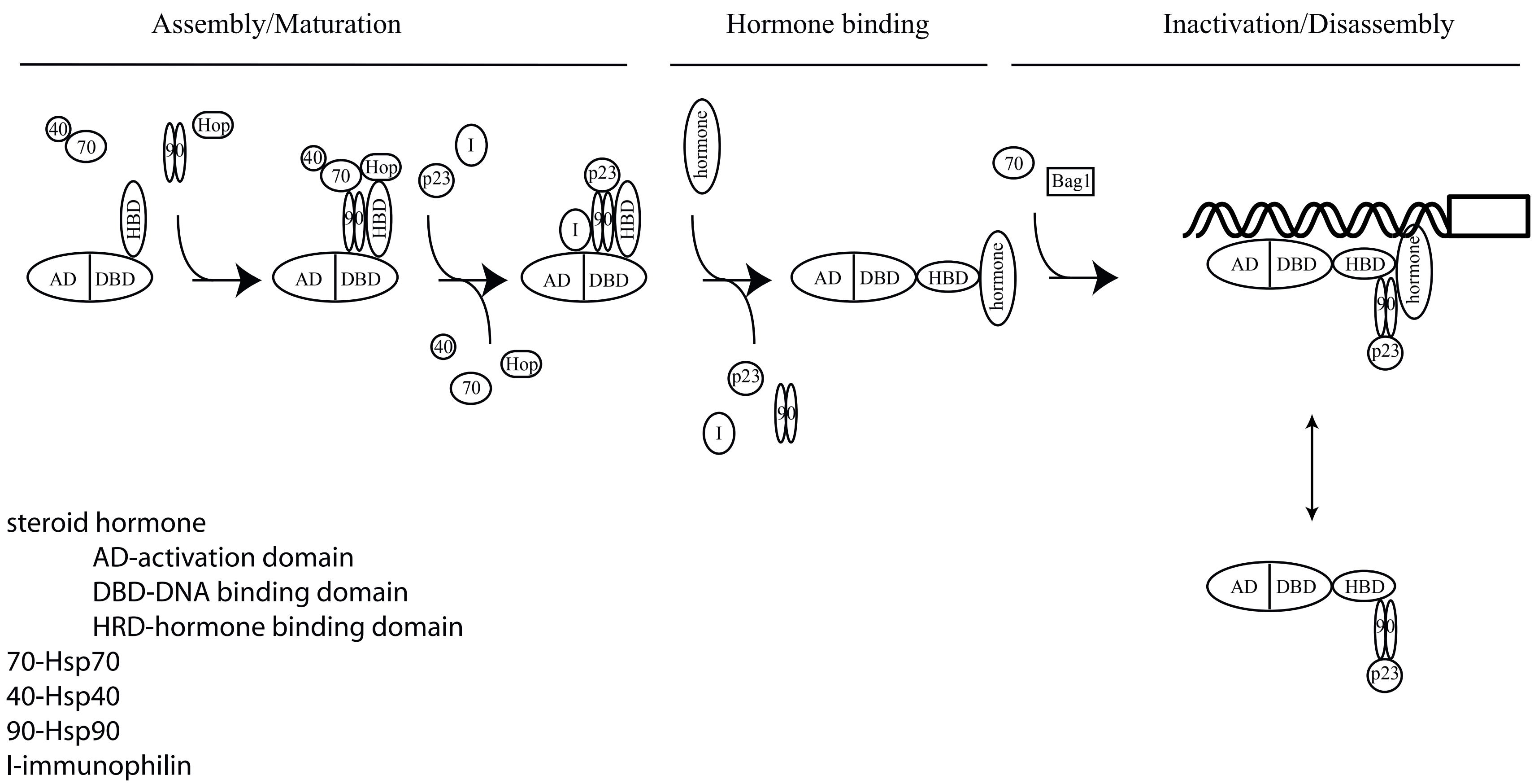



**Figure 2. Assembly and disassembly of intracellular hormone receptors by molecular chaperones.** For a description refer to the Introduction, section 1.9. Adapted from Morimoto, 2002.

Hip serve to regulate either the ATPase activity of Hsp70 or the exchange of ADP for ATP (Hartl, 1996). Hsp70 is recruited to the steroid receptor in the ATP-bound state (Morishima et al., 2000b), and Hsp40 stimulates the ATPase activity of Hsp70 (Freeman et al., 1995; Minami et al., 1996; Szabo et al., 1994) such that Hsp70 is quickly converted to the ADP-bound state that more stably binds the receptor (Morishima et al., 2001). Hip can bind the ATPase domain of Hsp70 and increase its chaperone activity by stabilizing the ADP-bound state (Hohfeld et al., 1995). ADP-bound Hsp70 is the preferred binding partner of Hop which associates with Hsp70 via its N-terminal TPR domain (Chen et al., 1996; Lassle et al., 1997). Hop recruited to the Hsp70 aporceptor complex is prebound to Hsp90 via C-terminal TPR domains on the respective proteins (Chen et al., 1996; Lassle et al., 1997). As both Hop and Hsp40 interact with the same TPR domain on Hsp70, Hsp40 is thought to be excluded from the chaperone-aporeceptor heterocomplex at this stage (Chen et al., 1996; Lassle et al., 1997). The role of Hop is to transfer the aporeceptor from Hsp70 to an Hsp90 dimer (Chen and Smith, 1998; Hernandez et al., 2002b; Kosano et al., 1998; Murphy et al., 2001). Hsp90 chaperone activity is dependent on intrinsic ATPase activity (Obermann et al., 1998; Panaretou et al., 1998), however, binding of Hsp90 to substrates is ATP-independent, thus Hsp90 dimers recruited by Hop are not bound to nucleotide (Chadli et al., 2000; Prodromou et al., 2000; Young and Hartl, 2000). Hop can inhibit the ATPase activity of Hsp90 (Prodromou et al., 1999). Dissociation of Hop-Hsp70 from the Hsp90-substrate complex is hypothesized to occur simultaneously with binding of ATP to the ATPase domain of Hsp90 (Prodromou et al., 1999). This also induces a dimerization of the N-terminal domains of Hsp90 forming a molecular clamp enclosing the substrate (Chadli et al., 2000; Prodromou et al., 2000). p23 is then recruited to the N-terminal ATPase

domain of Hsp90 which is proposed to stabilize the aporeceptor complex (Chadli et al., 2000; Chen et al., 1998; Dittmar et al., 1997). A mature aporeceptor complex contains the aporeceptor, a dimer of Hsp90 bound to ATP, p23 and an immunophilin (discussed below). Thus a minimal system containing Hsp90, Hsp70, Hsp40, Hop and p23 can properly assemble a stable steroid receptor capable of binding hormone with high affinity *in vitro* in reticulocyte lysates (Dittmar et al., 1998; Kosano et al., 1998). Hormone binding initiates the release of the Hsp90-containing chaperone heterocomplex which is followed by receptor dimerization, DNA-binding (Tsai et al., 1988) and transcriptional activation of hormone responsive genes (Pratt and Toft, 1997).

During the ATPase cycle discussed above, Hsp90 associates with one of several competing immunophilins, FK506 binding protein 51 (FKBP51), FK506 binding protein 52 (FPBK52), cyclophilin 40 (Cyp40) or protein phosphatases 5 (PP5) by interaction of immunophilin TPR domains with the Hsp90 TPR domain (Owens-Grillo et al., 1995; Owens-Grillo et al., 1996; Ratajczak and Carrello, 1996; Silverstein et al., 1997; Young et al., 1998). Immunophilins are characterized by their ability to bind to the immunosuppressant drugs FK506 (FKBP51, FKBP52, PP5) or cyclosporin A (CyP40) (reviewed in (Pratt and Toft, 1997)). All immunophilins contain a TPR domain linked to one functional domain such as a peptidylprolyl isomerase domain (FKBP51, FKBP52, CyP40) or a protein phosphatase domain (PP5) (Pratt and Toft, 1997). Cyclosporin A, and FK506 inhibit the peptidylprolyl isomerase activity of immunophilins (Pratt and Toft, 1997). FKBP52, and CyP40 also contain calmodulin binding domains that are not well characterized (Pratt and Toft, 1997) and FKBP51 contains a calcium/calmodulin independent calcineurin binding site (Li et al., 2002). Although each of the immunophilins have been detected in heterocomplexes with Hsp90, the role of these

proteins in the regulation of steroid receptors is not well known (Nair et al., 1997; Silverstein et al., 1997). Since treatment of cells with either FK506 or cyclosporin A does not affect glucocorticoid receptor or progesterone receptor-Hsp90 heterocomplex formation or proper folding of the hormone binding domain, it has been suggested that peptidylprolyl activity of these immunophilins are not required in steroid receptor chaperone heterocomplexes (Hutchison et al., 1993; Owens-Grillo et al., 1995; Renoir et al., 1994). Other studies report that immunosuppressants can affect the activity of steroid receptors, however, it is unclear that these effects are directly attributable to the action on immunophilins in the receptor complexes (discussed in (Pratt and Toft, 1997)).

The most well characterized role of immunophilins in the regulation of steroid receptors has come from studies of the glucocorticoid receptor. The glucocorticoid receptor is found in the cytoplasm in most mammalian cell types in the absence of hormone and must translocate into the nucleus once bound to hormone (Picard and Yamamoto, 1987). Evidence suggests that nuclear localization of glucocorticoid receptors requires the exchange of FKBP51 for FKBP52 upon binding of hormone (Davies et al., 2002). The immunophilin switch is associated with increased subsequent association of the receptor complex with dynein, a microtubule motor protein, and nuclear translocation (Davies et al., 2002; Galigniana et al., 2002; Galigniana et al., 2001). The association of FKBP52 with dynein requires the peptidylprolyl isomerase domain of FKBP52 (Davies et al., 2002; Galigniana et al., 2002; Galigniana et al., 2001). Interestingly, PP5 has also been shown to participate in nucleoplasmic shuttling of the glucocorticoid receptor (Dean et al., 2001) and both PP5 and CyP40 peptidylprolyl isomerase domains have also been shown to interact with dynein (Galigniana et al., 2002). Although it appears that hormone binding is concomitant with

Hsp90 release, recent evidence (Bagchi et al., 1990; Denis et al., 1988; Mendel et al., 1986; Sanchez et al., 1987) from the role of FKBP52 and Hsp90 (Davies et al., 2002; Galigniana et al., 2002; Galigniana et al., 2001) in the intracellular trafficking of the glucocorticoid receptor suggest that the chaperone heterocomplex remains temporarily associated with activated receptors prior to DNA-binding. The peptidylprolyl isomerase domain of FKBP52 has also been shown to potentiate hormone-dependent transcription of glucocorticoid receptors, an effect associated with increased hormone binding (Riggs et al., 2003). FKBP51, CyP40 or PP5 could not potentiate hormone dependent transcription in a similar manner (Riggs et al., 2003). Additional roles for FKBP52 or the other immunophilins in hormone receptor regulation have yet to be defined (Chinkers, 2001; Pratt and Toft, 1997).

The Hsp70 co-chaperone BAG-1 interacts with many nuclear hormone receptors and negatively regulates their activity following hormone binding (Cato and Mink, 2001; Liu et al., 1998; Zeiner and Gehring, 1995). Cytoplasmic steroid receptors such as the glucocorticoid receptor recruit BAG-1 and Hsp70 following hormone binding, and these proteins accompany the steroid receptor to the nucleus (Schneikert et al., 2000; Schneikert et al., 1999). BAG-1 binds the ATPase domain of Hsp70 and promotes both ATPase activity and the release of ADP that inhibits the chaperone activity of Hsp70 both *in vitro* and *in vivo* (Bimston et al., 1998; Hohfeld and Jentsch, 1997; Nollen et al., 2000; Takayama et al., 1997). Binding of BAG-1 has been shown to inhibit steroid binding, and negatively regulate receptor DNA-binding and transcription in an Hsp70-dependent manner with glucocorticoid receptor, and in an Hsp70-independent manner with retinoic acid receptor (Connell et al., 2001; Kanelakis et al., 1999; Kullmann et al., 1998; Liu et al., 1998; Schneikert et al., 2000; Schneikert et al., 1999). The negative

regulatory role of BAG-1 has been proposed to temper the response of steroid receptors to ligand binding or function to limit the duration of the response (Cato and Mink, 2001).

Following activation, steroid receptor-hormone complexes must be deactivated. For the glucocorticoid receptor, both initiation and duration of activation occurs with a half-life of approximately 5-10 minutes (Freeman and Yamamoto, 2001). The half-life of the receptor itself is approximately 11-25 hours, thus, deactivation of the receptor must be regulated by means other than receptor degradation (Freeman and Yamamoto, 2001). Current models suggest that receptor-hormone complexes are continuously disassembled into components that can be recycled into functional receptors, enhancing the speed of the response to hormone (Freeman and Yamamoto, 2001). Deactivation of the receptor and disassembly of the complex could potentially involve disrupting interaction of receptors with hormone, DNA, and with co-activators (Freeman and Yamamoto, 2001). As discussed above, Hsp70 and the co-chaperone BAG-1 act as negative regulators of liganded steroid receptor activities that may act to temper the response or deactivate the receptor (Cato and Mink, 2001). In addition, Hsp90 has also been implicated in the deactivation of steroid receptors. Hsp90 can de detected at hormone response elements in a hormone dependent manner (Freeman and Yamamoto, 2002). Inhibition of Hsp90 abrogates the release of glucocorticoid receptor from chromatin following hormone withdrawal (Liu and DeFranco, 1999) and increasing Hsp90 levels attenuates the response of cells to glucocorticoid possibly by active dissociation of the receptor from DNA (Kang et al., 1999; Sabbah et al., 1996). p23 has also been detected at hormone response elements in a hormone dependent manner and has been shown to play an active role in decreasing the interaction of receptor with

DNA, and disrupting transcriptional activation (Freeman et al., 2000; Freeman and Yamamoto, 2002). Recycling of receptors would include resetting the receptor to respond to new or continuous signals as described above for nascent receptors, a process that probably requires the same chaperones involved in the deactivation and disassembly of the receptor complex (Freeman and Yamamoto, 2002).

Current models of HSF1 regulation by the Hsp90-heterocomplex are discussed below (section 1.11).

## *1.10 Mechanism of transcriptional activation of Hsps*

It is clear that transcription of heat shock genes in response to stress in higher organisms is dependent on HSF1. Stress induced formation of homotrimers is the most critical mechanism by which HSF1 becomes competent to bind the HSE, but other factors at the promoter can contribute to the affinity of HSF for the promoter. For example, HSF1 has been shown to bind cooperatively to the promoters of heat shock genes that contain multiple heat shock elements. The existence of a particular chromatin architecture established by TFIID (transcription factor II D, GAF (GAGA associated factor), and a paused RNA polymerase II can increase HSF1 binding to the promoter (discussed below). Elongation of the polymerase is also stimulated by HSF1 in co-operation with the GAF, NURF, and SWI/SNF chromatin remodeling factors.

In unshocked cells the RNA polymerase II holoenzyme at the $5^1$ end of the Hsp70 gene is transcriptionally engaged and forms a 25 nucleotide transcript, the elongation of which is arrested until heat induction (Rougvie and Lis, 1988). Paused RNA polymerase

II has been detected at the human Hsp70 promoter, and *Drosophila* Hsp27, and Hsp27 promoters (Brown et al., 1996; Rasmussen and Lis, 1993; Rougvie and Lis, 1988; Rougvie and Lis, 1990) and is a common feature of other non-heat shock genes (Law et al., 1998). Overcoming this pause in elongation is hypothesized to be the rate-limiting step in the expression of Hsps as the initiation of transcription by RNA polymerase II remains more rapid than the resumption of elongation by a paused polymerase molecule, particularly when cells are heat shocked at intermediate temperatures (O'Brien and Lis, 1991).

The dominant factor that contributes to paused RNA polymerase II on Hsp genes in unshocked cells is the presence of nucleosomes (Benjamin and Gilmour, 1998; Brown et al., 1996). Transcription driven by the Hsp70 promoter proceeds in unstressed cells under conditions that do not allow for efficient chromatin assembly, but is repressed under conditions of efficient chromatin assembly (Landsberger and Wolffe, 1995a; Landsberger and Wolffe, 1995b). Increasing nucleosome assembly at a heat shock promoter beyond physiological levels gradually decreases transcription despite the presence of active HSF1 (Becker et al., 1991).

There are several proteins and protein complexes involved in chromatin remodeling at heat shock loci that are required for efficient transcription. HSF1 is a type IIB transcription factor (Blau et al., 1996) that activates transcription by stimulating both elongation and initiation (Brown et al., 1998; Sandaltzopoulos and Becker, 1998). Stimulation of elongation can occur via the HSF1-dependent disruption of chromatin structure (Brown and Kingston, 1997). HSF1 alone has been shown to be capable of restructuring nucleosomes on the Hsp26 promoter *in vitro* (Wall et al., 1995). The GAGA factor (GAF) is also responsible for chromatin remodeling both prior to and

during heat shock. Unlike mammalian Hsp70 promoters, the *Drosophila* Hsp70 promoter is not constitutively occupied by transcription factors such as CTF/CBF, Sp1, ATF/AP1 but it is occupied by GAF, and TFIID (Wu, 1984a). GAF is a constitutively expressed transcription factor that binds with high affinity to GAF binding elements at several sites in the promoter of the *Drosophila* Hsp70 gene (Lee et al., 1992; O'Brien et al., 1995). In unshocked cells GAF is important in establishing an open promoter required for basal Hsp expression (Elgin et al., 1993; Glaser and Lis, 1990; Lu et al., 1992). The binding of GAF is a critical step in regulating Hsp expression before and after heat shock since binding of TFIID, establishment of a paused polymerase, and heat induced binding of HSF1 are reduced in the absence of GAF (Shopland et al., 1995; Shopland and Lis, 1996; Weber et al., 1997). In addition to its role in the promoter region, GAF is also found associated along the heat shock gene in a position coincident with the position of the elongating RNA polymerase II in a heat inducible manner (O'Brien et al., 1994). Thus GAF may play an additional role in Hsp transcription by modifying chromatin all along the gene (O'Brien et al., 1994). GAF-directed nucleosome disruption is accomplished in conjunction with an ATP-dependent nucleosome remodeling complex (NURF) and only occurs during heat shock (Tsukiyama et al., 1994; Tsukiyama and Wu, 1995). NURF is an ISWI (imitation switch) containing class of chromatin remodeling protein complex that remodels chromatin by facilitating bidirectional displacement of nucleosomes in *cis* without disrupting the integrity of the nucleosomes themselves (Georgel et al., 1997; Guschin and Wolffe, 1999; Hamiche et al., 1999; Langst and Becker, 2001; Langst et al., 1999; Muchardt and Yaniv, 1999; Tsukiyama et al., 1994). Binding of HSF1, GAF, and

NURF to the promoters of heat shock genes all participate in increased transcription upon stress.

Chromatin remodeling of the human Hsp70 gene involves the SWI/SNF chromatin remodeling machine. Although GAF type activity is important in the regulation of the *Drosophila* Hsp70 promoter, it does not appear to be the dominant mechanism by which mammalian Hsp70 gene is regulated in adult tissues (Bevilacqua et al., 2000). SWI/SNF is an ATP-dependent chromatin remodeling complex that has been reported to positively or negatively remodel chromatin by three distinct biochemical mechanisms (reviewed in (Sudarsanam and Winston, 2000; Vignali et al., 2000)). SWI/SNF can either remodel existing nucleosomes, making the surrounding DNA more accessible (Schnitzler et al., 1998), facilitate the bidirectional displacement of nucleosomes in *cis* (Lorch et al., 1999; Whitehouse et al., 1999), or facilitate the transfer of nucleosomes to other DNA molecules in *trans* (Owen-Hughes et al., 1996). In order to stimulate elongation, human HSF1 selectively recruits SWI/SNF to the Hsp70 promoter by directly associating with BRG1 (the ATPase subunit of human SWI/SNF) (Sullivan et al., 2001). This association is dependent on phenylalanine residues in the AD1 and AD2 activation domains of human HSF1 that have been shown to selectively stimulate elongation (Brown et al., 1998). Recruitment of SWI/SNF has been shown to stimulate release of polymerase II from the paused state (Brown et al., 1996), perhaps by remodeling nucleosomes within the Hsp70 coding region. Interestingly, by introducing a dominant negative form of the ATPase required for SWI/SNF activity, de La Serna was able to show that SWI/SNF activity was required for the transcriptional upregulation of Hsp70 in response to arsenite and cadmium treatment but not heat shock (de La Serna et al., 2000). Thus heat treatment may either activate another mechanism

that remodels chromatin or other positive regulatory factors activated by heat may overcome the negative regulatory effects of chromatin condensation (de La Serna et al., 2000).

Despite the requirement of activated HSF1 for chromatin remodeling at heat shock loci, the presence of HSF1 is not sufficient to decondense chromatin. Salicylate treatment, which can activate HSF trimerization and DNA-binding activity but not transcription, has been shown to induce chromosomal puffing at heat shock loci in *Drosophila* polytene chromosomes at low concentrations (Winegarden et al., 1996). This suggests that HSF1 DNA-binding and chromosomal puffing (a reflection of chromatin decondensation) are not necessarily linked. This also suggests that HSF1 binding to the promoters of heat shock genes and chromatin decondensation are not the only events required for transcriptional upregulation.

Stimulation of RNA polymerase II elongation at heat shock loci has also been proposed to be regulated by phosphorylation of the CTD (C-terminal domain) since the paused polymerase is hypophosphorylated at the CTD and escape from the pause is associated with a hyperphosphorylation (O'Brien et al., 1994). The P-TEFb kinase has been implicated as the major kinase responsible for the phosphorylation of the CTD in *Drosophila* since it is recruited to heat shock loci specifically during heat shock (Lis et al., 2000). P-TEFb is a heterodimer consisting of Cdk9 (Zhu et al., 1997) and cyclin T (Peng et al., 1998) that has been shown to be critical in allowing polymerase II to become competent for elongation (Marshall et al., 1996; Marshall and Price, 1995). P-TEFb recruitment is dependent on the presence of HSF1, but the presence of HSF1 alone is not sufficient to recruit P-TEFb indicating that other factors are required (Lis et al., 2000). Artificial recruitment of P-TEFb to heat shock loci can activate transcription

without heat shock, highlighting the potential importance of P-TEFb to heat shock transcription (Lis et al., 2000). It is not known whether P-TEFb can stimulate transcriptional elongation by a mechanism other than phosphorylation of the CTD.

HSF1 has been hypothesized to promote transcription initiation by binding to several general transcription factors. Stimulation of transcriptional initiation has been shown to be dependent on acidic residues in both activation domains of human HSF1 (Brown et al., 1998). Current models propose that transcription factors stimulate transcriptional initiation by recruiting general factors to form a pre-initiation complex (reviewed in (Ptashne and Gann, 1990; Tjian and Maniatis, 1994)). Both the AD1 and AD2 domains of human HSF1 have been shown to physically interact with TATA-binding protein (TBP) and TFIIB (Mason and Lis, 1997; Yuan and Gurley, 2000), and full-length HSF1 interacts with a subunit of TFIIA (Yuan and Gurley, 2000). In addition, the negative regulatory domain of human HSF1 has been shown to interact with TFIID in stressed and unstressed cells, probably through contact with one or more of the TBP associated factors (TAFs) (Yuan and Gurley, 2000).

Several models describing how the interaction of HSF1 with general transcription factors regulate HSF1 transcriptional activity under different conditions have been developed (Yuan and Gurley, 2000). The most advanced model suggests that interaction of the negative regulatory domain with TAFs prevents productive interaction of the activation domain with TBP and TFIIB when HSF1 is bound to the promoter but not transcriptionally active (i.e. when activated by salicylate) (Yuan and Gurley, 2000). Once HSF1 has made the conformational change resulting in transcriptional activity, the activation domain is free to interact with TBP and TFIIB to stimulate reinitiation (Brown et al., 1998; Sandaltzopoulos and Becker, 1998). HSF1 may stimulate reinitation by

recruitment of the holoenzyme through contacts of the activation domain and TFIIB, and interaction of HSF1 with TBP and TFIIA may function to stabilize the interaction of these general transcription factors with the start site (Sandaltzopoulos and Becker, 1998).

Interaction of the HSF1 activation domain with TBP has also been proposed to stimulate release of the stalled polymerase (Mason and Lis, 1997). It has been proposed that interaction of the conserved acidic H-domain of RNA polymerase II with TBP is a contributing factor that prevents RNA polymerase from elongating past +45 (Rasmussen and Lis, 1993). Interaction of active HSF1 with TBP has been shown to displace RNA polymerase II from TBP (Mason and Lis, 1997) and this has been proposed as a trigger that releases RNA polymerase from a stalled state. Competition of HSF1 with RNA polymerase II for binding to TBP would then facilitate release of the RNA polymerase from the paused state during subsequent rounds of reintiation (Mason and Lis, 1997).

## *1.11 Model of HSF1 activation/deactivation*

Contemporary models of HSF1 regulation hypothesize that HSF1 DNA-binding, transcriptional activities and deactivation and disassembly of HSF1 trimers during recovery are regulated by a Hsp70/Hsp90 based chaperone system. This model was proposed from the studies discussed above. Most notably, the following studies were instrumental in the construction of this model ((Ali et al., 1998; Bharadwaj et al., 1999; Guo et al., 2001; Marchler and Wu, 2001; Shi et al., 1998; Zou et al., 1998) and reviewed in (Morimoto, 2002)). In this model, HSF1 exists in a monomeric form in

unstressed cells. Monomeric HSF1 interacts with Hsp70, Hsp90, and Hsp40 that serve to repress trimerization and DNA-binding in the absence of stress. During stress, HSF1 forms homotrimers and gains affinity for the HSE upstream of heat shock genes. Trimeric, DNA-bound HSF1 interacts with Hsp70, Hsp40, Hsp90, FKBP52, and p23 which all serve to negatively regulate HSF1 transcriptional activity. The acquisition of HSF1 transcriptional activity is thought to require the release of Hsp90, and the hyperphosphorylation of HSF1. While activating transcription, HSF1 stimulates elongation by recruiting a number of chromatin remodeling factors, and by directly stimulating chromatin remodeling. HSF1 also directly increases transcriptional initiation through direct interaction with the general transcriptional machinery including TBP, TAFs, TFIIA, and TFIIB. Following recovery from heat shock or during long term heat shock, HSF1 trimers are disassembled and DNA-binding is reduced. Hsp70, Hsp40, Hsp90, and p23 have all been proposed to regulate the deactivation and disassembly of HSF1 trimers. Thus, during the activation/deactivation cycle, HSF1 interacts with a dynamic series of Hsp90 containing chaperone heterocomplexes in a manner that is remarkably similar to but probably not identical to those involved in the regulation of steroid receptors. HSF1 activity can also be modulated by a number of Hsp70 and Hsp90 co-chaperones, and by post-translational modification, but the precise role of these events is unclear.

## *1.12 Xenopus oocyte model system*

There are many properties of the *Xenopus* oocyte that make it amenable to studying gene expression and the heat shock response. Large numbers of oocytes can be

obtained quickly and easily at any time during the frog's reproductive cycle. The oocyte has a tremendous capacity for mRNA and protein production. It has been estimated that one oocyte can synthesize 20 ng of total RNA and 400 ng of total protein per day (Gurdon and Wickens, 1983). Perhaps the most useful feature of the *Xenopus* oocyte is its large size which facilitates relatively simple microinjection techniques. The stage VI oocyte (used in my thesis) is a single cell with a diameter of approximately 1.3 mm. The size of the oocyte makes it possible to introduce exogenous materials into the oocyte easily by microinjection. Oocytes will transcribe mRNA from injected DNA constructs and translate exogenous mRNA efficiently and accurately (Gurdon et al., 1971). The oocyte can therefore act as an *in vivo* transcription/translation system. Consistent levels of expression of a particular gene from cell to cell is easily achieved by microinjection of expression constructs in oocytes compared to the cell to cell variability realized during transfection of constructs into tissue culture cells. Microinjection can also be used to introduce materials that do not easily cross the cell membrane including certain pharmacological agents, antibodies, antisense oligonucleotides etc. The introduction of antibodies can sometimes be used to sequester a particular molecules, effectively decreasing the level or activity of that protein. This technique has been successfully used to study the role of chaperones in modulating the activity of HSF1 (Ali et al., 1998; Bharadwaj et al., 1999).

Although the oocyte has an active heat shock response, there are several features inherent in the oocyte model system that must be taken into consideration when studying HSF1 activity. After reaching a certain size the chromosomes of stage VI oocytes (used in this study) condense and transcription halts (Davidson, 1986). At that point gene expression in the oocyte is dependent on the translation of a vast store of mRNA

produced prior to stage VI (Davidson, 1986). Transcription of the endogenous genome does not resume after fertilization until the mid-blastula transition (Davidson, 1986). Upregulation of Hsps from genomic DNA is not readily measurable in the single nucleus of an oocyte (Horrell et al., 1987). Transcriptional upregulation of HSF1-dependent genes, however, is measurable when reporter plasmids are injected into the oocyte (Landsberger and Wolffe, 1995b). Reporter constructs containing the Hsp70 promoter driving expression of chloramphenicol acetyl-transferase have been used to measure HSF1-mediated gene expression in oocytes (Landsberger and Wolffe, 1995b).

The oocyte is also particularly useful in studying HSF1 binding proteins since it can be easily extracted from cells in low salt conditions (Karn et al., 1992) due to the unusually high ratio of HSF1 to chromatin that does not exist in other cell types. In tissue culture cells there is a lower ratio of HSF1 to genetic material. Thus when HSF1 is activated in tissue culture cells, a large proportion of activated HSF1 is associated with chromatin. High salt is required to extract activated HSF1 in tissue culture cells to break the bond between HSF1 and the HSE. The ability to extract HSF1 in a low salt buffer from oocytes provides the opportunity to observe protein-protein interactions which are destroyed in high ionic conditions (Bharadwaj et al., 1999).

# 2 Experimental Objectives

Studies in our lab and in others have demonstrated that HSF1 can be regulated both by chaperone heterocomplexes and post-translational modification. Exactly how HSF1 is regulated by HSF1 binding proteins and post-translational modification in unstressed cells and during the activation/deactivation pathways has not been established. My thesis was aimed at studying the mechanism by which HSF1 is activated in response to stress and deactivated upon resumption of normal conditions, and examining the contribution of HSF1 binding proteins and post-translational modification during these stages. Specific aims were to:

1. Qualitatively determine the contribution of the cellular environment to the modulation of the HSF1 activation set-point. The *Xenopus* environment was tested for its ability to modify the set-point of HSF1 DNA-binding activity for HSFs from different species that have different activation set-points than *Xenopus* HSF1 (XHSF1).

2. Develop a set of antibodies specific to XHSF1 that could be used in this and subsequent studies. Prior to this study the only antibody that could bind XHSF1 specifically was an anti-mouse HSF1 polyclonal antibody (Sarge et al., 1993). The antibody was difficult to obtain in large quantities. A set of polyclonal antibodies to 5 different regions of XHSF1 was produced and characterized.

3. Characterize DNA-binding and transcriptional activities of HSF1 when activated by high temperature heat stress. It was observed in experiments under objective 1 that HSF1 recovery from high temperature heat shock was significantly delayed. I characterized the temperature and duration of heat stress required to elicit a failure in the HSF1 recovery mechanism. I also tested the hypothesis that the failure in attenuation was due to the lack of an appropriate signal delivered during a lower temperature heat shock. The transcriptional activity of XHSF1 activated by high temperature heat shock was also characterized both during the stress and during the recovery period.

4. Establish several conditions in the *Xenopus* oocyte model system in which extracts could be prepared that contained HSF1 locked into each stage of the activation/deactivation cycle. A summary of the treatments and at what stage of the activation/deactivation cycle are shown in the following table:

| *Inactive* | *DNA-bound but not transcriptionally active* | *Fully activated* | *Recovered* |
|---|---|---|---|
| 18°C | 80mM salicylate 60min,<br>39°C 20 min | 33°C 30min,<br>39°C 20min-24hr 18°C | 33°C 30min-15min 18°C |

These treatments were then used as a basis to identify changes in HSF1-interacting proteins and define their roles in the modulation of HSF1. XHSF1 from each stage of activation and deactivation was tested for interaction with each member of the Hsp90 chaperone hetercomplex known to interact with fully active HSF1. Several

experimental lines were also pursued to identify post-translation modifications unique to each stage of activation and deactivation without success.

# 3 Methods and Materials

## *3.1 Tissue culture*

### 3.1.1 Oocyte isolation

*Xenopus laevis* frogs were obtained from Xenopus 1 (Ann Arbour, MI). To extract ovary lobules, female frogs were anesthetized in 0.15% Tricaine (w/v), 0.1% Tris-Cl (Tris (hydroxymethyl) – aminomethane chloride) (w/v) for 15 minutes. *Xenopus* ovaries were surgically extracted by making an incision in the right or left ventral abdomen and through the muscle wall. Ovary lobules were removed through the incision and cut away from the abdomen. The body and muscle wall were each sutured with 4-6 sutures using surgical thread (Ethicon silk 4.0). Ovaries were placed in a 0.2% Collagenase (type IV) (w/v) solution diluted in calcium-free OR2 (Operation repair media 2) buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 10 mg/ml streptomycin sulphate, 10 mg/ml benzyl penicillin, 5 mM HEPES pH 7.8 (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid N-(2 Hydroxyethyl) piperazine-$N^1$-(2-ethanesulfonic acid))) (Wallace et al., 1973) to remove follicle cells. Following 4 hours of constant agitation (swirling at 150 rpm (revolutions per minute)) free oocytes were rinsed and placed in petri dishes containing OR2 as above supplemented with 1 mM $CaCl_2$ (Wallace et al., 1973). Healthy oocytes were selected under a dissecting microscope for use in experiments based on morphological characteristics such as homogeneous color in

the animal and vegetal poles and shape. For all experiments outlined in this thesis, stage IV oocytes (staged according to Dumont (Dumont, 1972)) were used. Oocyte extraction and initial manipulation were carried out at room temperature.

### 3.1.2 Drosophila SL2 tissue culture

*Drosophila* Schneider line 2 (SL2) suspension cells were grown in Shields and Sang M3 insect medium (Quality Biological) as per manufacturer's instructions supplemented with 10% heat-inactivated fetal bovine serum (Hy-Clone), 10 mg/ml streptomycin sulphate, 10 mg/ml benzyl penicillin. Cells were grown at 18°C in T-75 tissue culture flasks for suspension cells (Starstedt). Cells at a concentration of 1–1.4 X $10^7$ cells/ml as determined by a hemocytometer were transferred to 50-ml polypropylene tubes at a volume of 2.5–6.5 ml. Cells were aerated in these tubes by shaking at 175 rpm, 21°C, for 4 hours prior to the experiment.

## *3.2 Stress treatments*

Healthy oocytes were incubated at different temperatures indicated in the figures by placing them in petri dishes containing preheated OR2 and immersion of sealed dishes in a temperature controlled water bath or exposed to sodium salicylate dissolved in OR2 at the desired concentration. *Drosophila* SL2 cells were placed in a temperature controlled water bath at 37°C for heat treatment.

## 3.3 Protein Extracts

### 3.3.1 Drosophila tissue culture cells

Extracts of *Drosophila* tissue culture cells were prepared by the method described in (Winegarden et al., 1996). Following treatment, 1 ml aliquots of cells were transferred to 1.5 ml microcentrifuge tubes, and pelleted at 400Xg, at 4 °C, for 2 min. Supernatants were aspirated off, and cell pellets were immediately frozen under liquid nitrogen. Pellets were thawed by resuspension in five pellet volumes of lysis buffer (10 mM HEPES pH 7.9, 0.4 M NaCl, 0.1 mM EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,$N^1$,$N^1$-tetraacetic acid), 5.0% glycerol (w/v), 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and then centrifuged at 17,500Xg for 10 min at 4 °C. Supernatants were stored at -80 °C.

### 3.3.2 Oocyte extracts

Extracts for electrophoretic mobility shift assay and chromatography were prepared by the method described in (Karn et al., 1992). Following treatment healthy oocytes were placed in a 1.5 ml centrifuge tubes and the excess OR2 was removed. Oocytes were then lysed by a micropipette in 10 µl of 50 mM Tris-Cl pH 8.0, 20% glycerol (w/v), 50 mM KCl, 0.1 mM EDTA (ethylene-diaminetetra-acetic acid), 10

μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were centrifuged at 17500Xg for 5 minutes at 4°C, and the supernatants were stored at -80°C. Extracts for chloramphenicol acetyl transferase assays were lysed by a micropipette in 10 μl of 10 mM Tris-Cl pH 7.4 as in (Landsberger and Wolffe, 1995b). The lysates were centrifuged at 17500Xg for 5 minutes at 4°C and the supernatants were transferred to a fresh microtubes and stored at -80°C.

### *3.4 Bacterial transformation*

DH5α cells (BRL) were routinely used for cloning and plasmid amplification. Competent cells were prepared using a protocol described in (Ausubel et al., 1987). Briefly DH5α cells were prepared using a culture of DH5α in the logarithmic phase of cell growth grown in LB (Luria broth) media (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl in double distilled $H_2O$ (dd $H_2O$)) at 37°C shaking at 200 rpm. Cells were pelleted by centrifugation at 1600Xg, resuspended in 2.5 ml 50 mM $CaCl_2$ and incubated on ice for 1 hour. Cells were pelleted by centrifugation at 1600Xg and resuspended in 0.5 ml cold 50 mM $CaCl_2$.

Cell transformation was performed using the $CaCl_2$ protocol described in (Ausubel et al., 1987). To transform these cells 50 ng of plasmid or 10 μl ligation mixture were diluted 1:3 with TCM buffer (10 mM Tris-Cl pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$), added to 100 μl competent cells and incubated on ice for 1hour. The mixtures were then heated at 42°C for 2 minutes and then allowed to recover for 15 minutes at room

temperature. Prewarmed LB media (300 µl) was added and the cells were shaken at 37°C at 200 rpm for 15 minutes before being spread on LB (15 g/l agar) plates supplemented to 0.1 µg/ml ampicillin.

### 3.5 Plasmid preparation

Plasmid preparations were carried out by the method of alkaline lysis (Ausubel et al., 1987). Single colonies growing on LB plates supplemented to 0.1 µg/ml ampicillin were picked using a sterile toothpick, transferred to liquid LB media, and grown overnight at 37°C shaking at 200 rpm. Overnight cultures were separated into 1.5 ml aliquots in microtubes and centrifuged at 10,000Xg for 1 minute. The pellets were thoroughly resuspended in 100 µl of cold 50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0. Cells were lysed by the addition of 200 µl of fresh 0.2 N NaOH, 1% SDS (sodium dodecylsulfate) (w/v). The solutions were mixed 10 times by inversion and incubated on ice for 10 minutes. Next, 150 µl of cold 3 M potassium acetate was added, mixed by vortex, and the solutions incubated on ice for 5 minutes. The solutions were then centrifuged at 17,500Xg for 10 minutes at 4°C. To eliminate contamination by RNA, 2.5 µl RNase A (10 mg/ml) was added to the supernatants followed by incubation at 37°C for 30 minutes. Plasmid DNA was extracted by adding 500 µl phenol:chloroform (1:1), vortexing, and centrifugation at 17,500Xg for 5 minutes at room temperature. The upper phase was removed, placed in a fresh microtube, and plasmid DNA was precipitated by adding 2 volumes ice cold absolute ethanol to the upper phase. Following 10 minutes of incubation on ice, plasmid DNA was pelleted by

centrifugation at 17,500Xg for 10 minutes at 4°C. The pellets were resuspended in 20 µl $ddH_2O$. Plasmid concentrations were determined by visual inspection of aliquots of the samples on ethidium bromide stained 1% agarose gels (Ausubel et al., 1987) and comparison with DNA of known concentration. Briefly, DNA was mixed with loading dye (5% glycerol, 0.025% bromophenol blue (w/v), and 0.025% xylene cyanol (w/v) final concentration) and run on a 1.0% agarose gel in 1XTAE. The agarose gel was made by dissolving agarose in 1XTAE, 0.04 M Tris-acetate, 0.002 M EDTA (ethylene-diaminetetra-acetic acid) in a microwave. Before pouring into the casting tray, the warm gel solution was supplemented with 0.5 µg/ml ethidium bromide. DNA was visualized on a UV transilluminator.

## *3.6 Description of Drosophila and Human HSF1 clones*

The pCMV:hHSF1 (human HSF1) construct was a generous gift from A. Wolffe (NIH). The construct contains the gene encoding hHSF1 downstream of the cytomegalovirus promoter, a constitutive promoter. This construct was used to express hHSF1 in *Xenopus* oocytes.

In order to express DHSF (*Drosophila* HSF) in oocytes, the gene encoding DHSF was subcloned from the pHSFpoly(A) construct (Clos et al., 1990) into the multiple cloning site of pcDNA3.1 (Invitrogen) which contains the CMV promoter and allows constitutive expression of the inserted gene. This construct was named pCMV:DHSF. Briefly 2 µg of pHSFpoly(A) was digested with EcoR1 (Pharmacia) for 4 hours at 37°C. For isolation of the HSF coding region, components of the reaction were separated by

size by agarose gel electrophoresis as described above. The size of the DNA fragments was estimated by comparison to DNA of known sizes separated on the same gel. The band whose size corresponded to DHSF was excised and extracted using a Qiagen gel purification kit according to manufacturers instructions. To prepare the vector 2 μg of pcDNA3.1 was digested with EcoR1 for 4 hours at 37°C, then treated with calf intestinal phosphatase (NEB) for 15 minutes at 37°C. The enzyme was then inactivated by incubation at 85°C for 15 minutes. Digested pcDNA3.1 and DHSF were combined in a 1:1 molar ratio and incubated with T4 DNA ligase (Pharmacia) overnight at 16°C. The mixture was then transformed into DH5α as described above and ampicillin resistant colonies were screened for correct orientation by Stu1 digestion (Pharmacia).

## *3.7 Electrophoretic mobility shift assay (EMSA)*

### 3.7.1 Annealing oligonucleotides

Oligonucleotides (Life Technologies) were resuspended at 1nmol/μl in $ddH_2O$. To anneal the oligonucleotides they were combined at 100 nmol/ml each in annealing buffer (10 mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8.0) and heated at 65°C for 10 minutes in a thermocycler. The reaction was allowed to cool gradually over two hours to room temperature.

### 3.7.2 $^{32}$P labeling of oligonucleotides

Double stranded oligonucleotides were end filled with $\alpha$–$^{32}$P dCTP. The heat shock element and YY1 binding elements are described below (the nucleotides indicated in lower case represent the nucleotides that were incorporated in the reaction):

Heat Shock Element

$5^1$ - GGGCGTCATAGAATATTCTCGAATTCTAAATCAGG - $3^1$

$3^1$ – cccgcagtatcttataaGAGCTTAAGATTTAGTCC - $5^1$

This is an artificial heat shock element containing the consensus HSF DNA binding site defined as three contiguous inverted repeats of AGAAn (Cunniff and Morgan, 1993; Fernandes et al., 1994; Kroeger and Morimoto, 1994; Rabindran et al., 1991).

YY1 Binding Element

$5^1$ – GGATCCTCGGCCGTCATGGCGCTGCAGGAGGCggatc - $3^1$

$3^1$ – cctagGAGCCGGCAGTACCGCGACGTCCTCCGCCTAG - $5^1$

This represents the YY1 binding element from the internal coding sequence of the mouse histone 3.2 gene (Ficzycz et al., 2001).

Oligonucleotides were labeled using a modification of a protocol described in (Ausubel et al., 1987). For labeling reactions, 100 ng of YY1BE or 12 pmol HSE were added to microtubes containing 2.5 mM each of dATP, dTTP, and dGTP, 50U DNA polymerase I Large Fragment (Klenow) (New England Biolabs), 10 mM Tris-Cl (pH 7.5), 0.5 mM MgCl2, 0.75 mM dithiothreitol, 4.0 µl $^{32}$P-αdCTP (3000Ci/mmol) (Perkin Elmer) to a volume of 12 µl. The reactions were then incubated at 37°C for 20 minutes. To ensure that the oligonucleotides were filled in completely dCTP was then added to a final concentration of 80 µM and reactions were further incubated at 37°C for 5 minutes. To stop the reactions an equal volume of stop buffer containing 1.0% SDS (w/v), 20 mM EDTA (pH 8.0) was added. Unincorporated $^{32}$P-αdCTP was removed by centrifugation of the reactions in a swinging bucket rotor at 1600Xg, through 1.0 ml of size exclusion chromatography resin (G25 Sephadex hydrated in 10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0). $^{32}$P-labelled double stranded oligonucleotides were stored and -20°C.

### 3.7.3 DNA binding reaction and non-denaturing electrophoresis

To assay for DNA binding activity, 10 µl of protein extracts (20 µg protein) were incubated with 50 cps (counts per second) of labeled YY1BE or HSE diluted in a solution containing 10 mM Tris-Cl (pH 8.0), 50 mM NaCl, 1 mM EDTA (pH 8.0), 1 µg poly (dI-dC), and 5% glycerol (w/v) (final concentration) to a volume of 20 µl. Reactions were incubated on ice for 20 minutes. The same binding reactions were used

for supershift analysis except that 1-2 μl of antibody was added to the protein extracts and incubated for 20 minutes on ice prior to addition of the labeled oligonucleotides. In some cases the protein extracts were treated with 1 μl calf intestinal phosphatase (CIP) and incubated for 20 minutes on ice prior to addition of the labeled oligonucleotides. In some cases 10 μg BSA was added to equalize the protein content, and ATP (adenosine triphosphate) was added to a final concentration of 0.1 mM before addition of labeled oligonucleotides.

For electrophoresis, loading dye, 5% glycerol (w/v), 0.025% bromophenol blue (w/v), and 0.025% xylene cyanol (w/v) (final concentration) was added and the samples were then loaded onto 5% non-denaturing polyacrylamide gels as described in (Karn et al., 1992). Gels were prepared by preparing a solution containing 5% polyacrylamide (w/v) (29:1 acrylamide:bis-acrylamide) 6.7 mM Tris-Cl (pH 7.5), 1 mM EDTA (pH 8.0), and 3.3 mM sodium acetate. The solutions were polymerized with 0.1% ammonium persulfate (v/v) (final concentration), 0.1% (v/v) TEMED (N,N,$N^1$,$N^1$-Tetramethylethylenediamine) (final concentration) and poured immediately 1.5 mm vertical slab gels as described in (Ausubel et al., 1987). Gels were electrophoresed for 2.5 hours at 150V in a buffer containing 6.7 mM Tris-Cl (pH 7.5), 1 mM EDTA (pH 8.0), and 3.3 mM sodium acetate, dried on Whatmann 3MM paper, and exposed to autoradiographic film overnight (Kodak X-OMAT 5) with an intensifying screen at -80°C. 4-8% gradient non-denaturing gels were used for EMSA depicted in figures 5A,B, 6A,B,C, 7B, 8A,B,C, and 26 in order to enhance the separation of different HSF1 complexes. Gradient gels were prepared using a BIORAD gradient former as per manufacturers instructions.

## 3.8 Discontinuous denaturing protein electrophoresis (SDS-PAGE)

For protein analysis SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was carried out using a protocol from (Ausubel et al., 1987). Samples to be run on denaturing gels were first diluted to 1X sample buffer (50 mM Tris-Cl pH 6.8, 16% (v/v) glycerol, 1.6% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue) and incubated at 100°C for 4 minutes. Samples were briefly centrifuged at 17,500Xg , cooled on ice and loaded onto 1.5 mm thick vertical slab gels consisting of a lower separating gel (10% acrylamide (w/v) (29:1 acrylamide:bis-acrylamide), in 375 mM Tris-Cl (pH 8.8), 0.1% (w/v) SDS polymerized using 0.1% (v/v) ammonium persulfate, 0.1% (v/v) TEMED), and an upper stacking gel (4% acrylamide (29:1 acrylamide:bis-acrylamide), in 125 mM Tris-Cl (pH 6.8), 0.1% (w/v) SDS polymerized using 1% (v/v) of 10% ammonium persulfate, 0.1% (v/v) TEMED). Gels were electrophoresed for 45 minutes at 200V in a buffer containing 25 mM Tris-Cl (pH 8.3), 0.2M glycine, 0.1% (w/v) SDS or until the bromophenol blue reached the end of the gel.

### 3.8.1 Protein staining

Proteins in gels were routinely stained using Coomassie brilliant blue by incubating the gel in a tray containing 0.05% Coomassie Brilliant Blue R, 40%

methanol, 10% acetic acid for 20 minutes as described in (Ausubel et al., 1987). The gel was then placed in a tray containing destaining solution (40% methanol, 10% acetic acid, 50% water) until the Coomassie stained bands of proteins were clearly visible over background. Proteins were stained by incubating gels in Sypro Ruby Red stain (BIORAD) overnight as per manufacturer's instructions where indicated. Proteins in Sypro stained gels were visualized using a UV transilluminator.

## *3.9 Immunoblotting*

Following electrophoresis, proteins were electroblotted onto nitrocellulose using a semi-dry transfer unit (BIORAD) using manufacturers instructions. To prevent non-specific association of antibodies with the membrane, the blots were blocked with 5% milk powder (w/v) in TBST (Tris buffered saline with Tween-20) (20 mM Tris-Cl pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20) for 30 minutes at 4°C while shaking as described in (Ausubel et al., 1987). Blots were then incubated overnight with primary antibodies diluted in TBST supplemented with 5% milk powder (w/v) (most primary antibodies were diluted at 1:5000) at 4°C while shaking. Blots were then washed 3 times for 4 minutes each in TBST at room temperature. Blots were then incubated with the appropriate secondary antibodies diluted in TBST (1:10,000) supplemented with 5% milk powder (w/v) for 1-2 hours at room temperature while shaking. Blots were then washed 3 times for 4 minutes each in TBST. Horseradish peroxidase conjugated secondary antibodies were visualized by a chemiluminescence detection system (Dupont

NEN) as per manufacturer's instructions, followed by autoradiography using X-ray film (Kodak X-OMAT 5).

### *3.10 Plasmid Microinjection*

Microinjections were performed using a Narishige microinjector (model IM 300) under a dissecting microscope. To express DHSF or hHSF1 in oocytes, 20 nl of a solution containing 300 pg of pCMV:DHSF or pCMV:hHSF1 plasmids were injected into the nuclei of healthy oocytes. The concentration of plasmid was varied where indicated in order to control the amount of HSF1 produced. The oocytes were incubated at 18°C overnight to allow for transcription and translation of the exogenous proteins.

To measure the amount of heat inducible transcription in oocytes, 20 nl of a solution containing 30 pg of the reporter gene pHsp70:CAT plasmid which contains 251 base pairs of the *Xenopus* Hsp70 promoter driving expression of the chloramphenicol acetyl transferase gene (CAT) (described in (Landsberger and Wolffe, 1995b)) was injected into the nuclei of healthy oocytes. To control for global alterations in transcription, oocytes treated in parallel to those injected with pHsp70:CAT were injected with 20 nl of a solution containing 60 pg of the reporter gene pCMV:CAT plasmid which contains the cytomegalovirus promoter driving expression of the CAT gene into the nuclei of healthy oocytes (described in (Landsberger and Wolffe, 1995b)). This cytomegalovirus promoter is a strong promoter. Following injection, oocytes were incubated for 4 hours at 18°C to allow for nuclear localization and DNA packaging

(Landsberger and Wolffe, 1995b). Oocytes were then subject to stress or control treatments after which they were allowed to recover at 18°C in fresh OR2 for 12 hours to allow for transcription and translation of the reporter genes. In some experiments, stress treatments were performed prior to injection of pHsp70:CAT or pCMV:CAT reporter plasmids as indicated in the figure legend.

### *3.11 Chloramphenicol acetyl transferase assay*

Chloramphenicol acetyl transferase assays were performed as described by (Gorman et al., 1982). Briefly, 10 µl of protein extract was incubated in 670 µM acetyl CoA (final concentration), 12.5 µCi $^{14}$C-chloramphenicol in 30 µl reactions. Reactions were incubated for 40 minutes at 37°C. Chloramphenicol was extracted by adding 250 µl ethyl acetate followed by centrifugation of the samples at 17500Xg for 5 minutes. The upper phase was lyophilized and the dried pellets were resuspended in 10 µl ethyl acetate. Samples were spotted onto a 7.5 cm silica TLC (thin layer chromatography) plate 1.5 cm from the base. Ascending chromatography was performed in a chamber containing 95% chloroform (v/v), 5% methanol (v/v) as the solvent. After the solvent front migrated 6.5 cm, the plates were removed, dried and exposed to autoradiographic film (Kodak X-OMAT 5) overnight at room temperature.

### 3.12 Antibody production

#### 3.12.1 Antigens

Antigens corresponding to five regions of *Xenopus* HSF1 (XHSF1) were ordered from the Alberta Peptide institute as both N-terminal Keyhole Limpet Hemocyanin (KLH) or Bovine Serum Albumin (BSA) conjugates. Conjugates were synthesized using an Applied Biosystems Model 430A Peptide synthesizer with t-Boc and/or Fmoc Na-protection and HBTU chemistry. Peptides were further purified by reversed phase HPLC. The amino acid sequences of the peptides are as follows:

CS3171 – MDPHGTCGGSNVPAF

CS3172 – PDSSLASVSSTPIYT

CS3173 – VKPERDDTEFQHPYF

CS3174 – GNKNDEMSESHPPEP

CS3175 – PSLEASPSPVILIKT

The rational for selecting these peptides was as follows. Peptides CS3171 and CS3172 correspond to N-terminal and C-terminal sequences of XHSF1 (Figure 3). The termini of the protein have the greatest chance of being exposed on the surface of the intact protein, thus antibodies to these regions would likely recognize the native form of the protein. Peptide CS3173 corresponds to the DNA binding region of XHSF1 as determined by sequence comparison with other HSF1s where the DNA binding region has been mapped (Wu, 1995) (Figure 1). This peptide was predicted to bind all isoforms and family members of HSF as well as HSFs from all species (Wu, 1995). Peptide CS3174 and CS3175 correspond to stretches of amino acids with distinct sequences

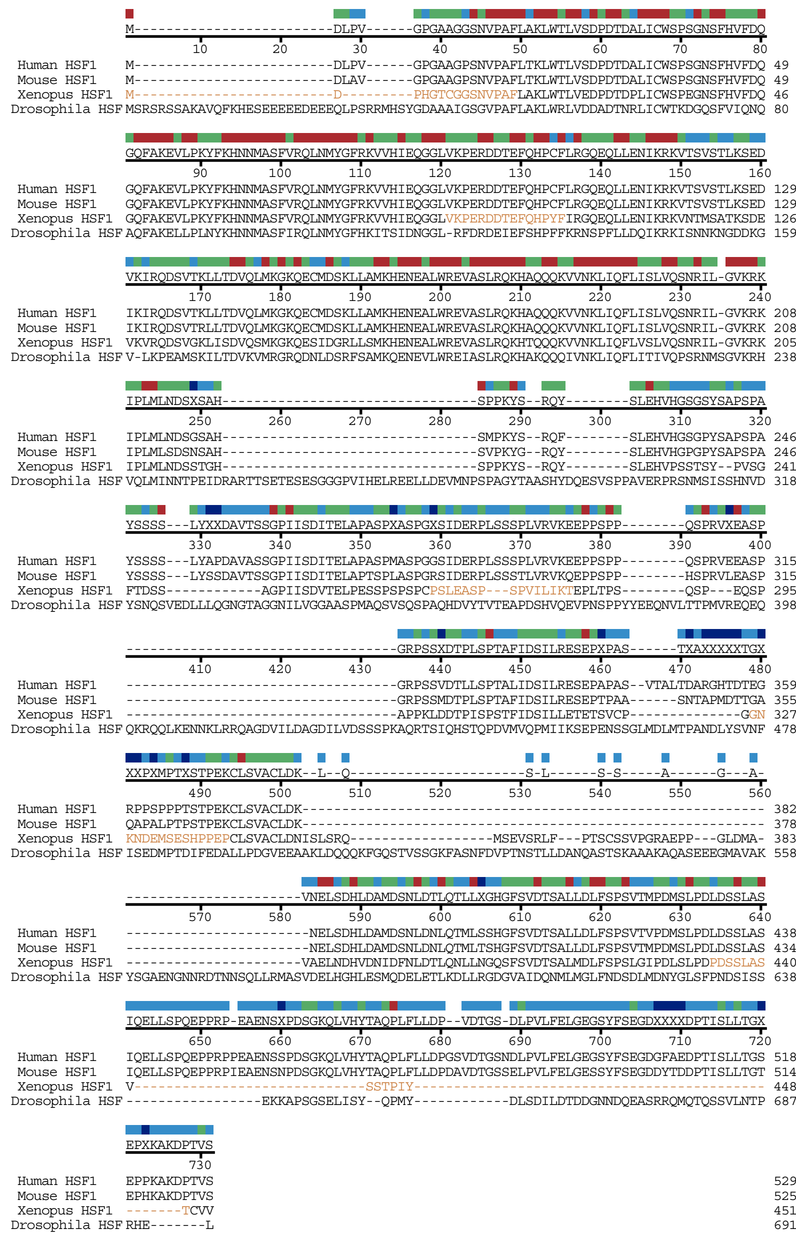


**Figure 3. Peptides from which antibodies to XHSF1 were produced.** The identity of KLH and BSA conjugated peptides that were produced at the Alberta Peptide Institute are indicated. An alignment of the predicted amino acid sequences of human HSF1, mouse HSF1, *Xenopus* HSF1, and *Drosophila* HSF is depicted. The common sequence is indicated and the sequence similarity between these HSF1s is indicated (Red-100%, green-75%, light blue-50%, dark-blue represents regions of dissimilarity within the consensus). The alignment was carried out using Megalign (Lasergene 99). Peptide sequences (CS3171 - MDPHGTCGGSNVPAF, CS3172 - PDSSLASVSSTPIYT, CS3173 - VKPERDDTEFQHPYF, CS3174 - GNKNDEMSESHPPEP, CS3175 - PSLEASPSPVILIKT) are highlighted in orange. Peptides were used as immunogen in rabbits for antibody production.

exclusive to XHSF1. Antibodies to these peptides would be predicted to bind to XHSF1 and not HSFs from other species. Peptides were dissolved in $ddH_2O$. CS3175 was particularly insoluble in $ddH_2O$ and so was dissolved in glacial acetic acid. Before injection into animals, the solution containing peptide was exchanged with 0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic pH 7.5 by dialysis.

### 3.12.2 Antibody production

Production of antibodies was carried out at the University of Saskatchewan Animal Facility using a protocol described in (Harlow and Lane, 1988). Two New Zealand white rabbits were used for each of the peptide antigens described above. Briefly, rabbits were initially pre-bled (5 ml) to obtain preimmune sera. Rabbits were exposed to antigen by initially injecting 500 µg of KLH conjugated peptide antigen mixed 1:1 with Freunds complete adjuvant subcutaneously. Only 250 µl of volume was injected at each site. Fourteen days later 500 µg of KLH conjugated peptide antigen mixed 1:1 with Freunds incomplete adjuvant was injected subcutaneously. Injections of antigen in Freunds incomplete adjuvant as described above were continued every 14 days for 6 cycles. Rabbits were sacrificed and the blood collected by heart puncture by a veterinarian. Blood collected from the animals was allowed to clot overnight at 4°C. Serum was obtained by centrifugation of the clotted blood for 10 minutes at 1600Xg at 4°C. Serum was stored at -4°C for the short term (up to 12 months) and at -80°C for the long term.

### 3.12.3 Enzyme linked immuno-sorbant assay

The method for enzyme linked immuno-sorbant assay (ELISA) was adapted from Harlow and Lane (1988) (Harlow and Lane, 1988). ELISAs were carried out in 96 well polystyrene plates. In order to coat the wells of the plate with antigen, 50 μl of antigen (5 μg/ml) was diluted in carbonate-bicarbonate buffer (pH 9.6) (15 mM anhydrous sodium carbonate, 35 mM sodium bicarbonate) and placed in the wells. Plates were incubated overnight at room temperature in a humid chamber. Wells were washed 3 times with phosphate buffered saline (pH 7.5) supplemented with 0.5 g/l bovine serum albumin (fraction V). To prevent proteins from binding to the polystyrene non-specifically, the wells were blocked by filling with blocking buffer (phosphate buffered saline (pH 7.5) supplemented with 10 g/l bovine serum albumin (fraction V) in the wells to completely fill them. Plates were incubated overnight at room temperature in a humid chamber. Wells were washed 2 times with wash buffer (phosphate buffered saline (pH 7.5) supplemented with 0.5 g/l bovine serum albumin (fraction V)). Primary antibodies, diluted in wash buffer, were added to the wells and incubated for 1-2 hours at room temperature. Five fold serial dilutions (1:100, 1:500, 1:2500, 1:12500, 1:62500, 1:312500, 1:1562500) were distributed across the wells of a plate to ensure that a level of primary antibody would be tested that would be within the linear range of the assay. Wells were washed 3 times with wash buffer. Secondary antibody (goat anti-rabbit IgG-HRP conjugate; BIORAD), diluted 1:2500 in wash buffer, was added to the wells and incubated for 1-2 hours at room temperature. Wells were washed 5 times with wash buffer and wells were emptied thoroughly. To assay for HRP activity, 125 μl substrate

(2,2$^1$-Azino-Bis(3-Ethylbenzthiazoline-6-sulfonic acid)) (Sigma A3219) was added to each well. Plates were developed at room temperature on a rotating platform for 20 minutes. Reactions were quantified by a plate reader (SLT Spectra) set at 405 nm. The efficacy of the protocol was confirmed by using the rabbit Anti-PKB (protein kinase B) polyclonal antibody (Stressgen #KAP-PK004C) and the peptide from which it was derived Akt (PKB) peptide FHVETPEEREEWTC (Stressgen #KPT-PK004E) as control antibody and antigen respectively.

## *3.13 anti-HSF chromatography*

### 3.13.1 Conjugation of antibodies to protein-A sepharose beads

This method was adapted from Harlow and Lane (1988) (Harlow and Lane, 1988) to use the MAPSII buffer system (BIORAD) that has been designed to maximize binding and recovery of antibodies. Sera was diluted 1:3 in MAPSII binding buffer (BIORAD) and filtered through a 0.2 µm filter. The sera was then added to washed affi-prep protein A resin (BIORAD) to a ratio of 1:1. The mixture was incubated for 1 hour at room temperature on a rotary shaker set to 50 rpm. Material that did not bind the resin was isolated by centrifugation of the resin at 1600Xg for 3 minutes at room temperature. The resin was washed 2 times with 0.2 M sodium borate (pH 9.0). Antibodies were covalently linked to the resin by resuspending the resin in 0.2 M sodium borate (pH 9.0) supplemented with 20 mM dimethylpimelimidate and incubated

for 30 minutes at room temperature on a rotary shaker. Reactions were stopped by washing the resin in 0.2 M ethanolamine (pH 8.0) and further incubated with a fresh 0.2 M ethanolamine (pH 8.0) overnight on a rotary shaker. Antibody-conjugated resins were either packed in a high pressure column (BIORAD) or used in batch.

### 3.13.2 anti-XHSF1 chromatography

The anti-HSF chromatography protocol was designed to use the MAPSII buffer system (BIORAD) using the manufacturers instructions. The composition of the MAPSII buffers are not disclosed by the manufacturer but by testing the buffers it appears that the binding buffer is a high salt buffer at (pH 9.0). The MAPSII elution buffer is a low salt buffer (pH 3.0). Protein extracts containing the total protein from 30 oocytes (300 µl) were loaded onto an anti-HSF column using a Bio-Logic HR high performance liquid chromatography machine (BIORAD) at a flow rate of 1 ml/min in MAPSII binding buffer. The column was washed extensively with MAPSII binding buffer until all non-specific binding proteins had been removed as judged by OD 280 nm reading from the flow through. XHSF1 was eluted from the column using MAPSII elution buffer (BIORAD) at a flow rate of 1 ml/min at which time 500 µl fractions were collected. To neutralize the eluate, 100 µl of Tris-Cl (pH 9.0) was added to the fractions. Proteins were precipitated by adding 2 volumes of -20°C acetone and incubation of the samples at -20°C overnight. Samples were centrifuged at 17500Xg, the supernatant aspirated, and the pellets resuspended in sample buffer containing 50 mM Tris-Cl (pH

6.8), 1.6% (w/v) sodium dodecyl sulfate, 4% (v/v) β-mercaptoethanol, 16% (v/v) glycerol, 0.02% (w/v) bromophenol blue.

### 3.13.3 Size exclusion chromatography

The protocol for size exclusion chromatography was modified from protocols supplied with the column by the manufacturer. Protein extracts representing 30 oocytes (300 μl) were loaded onto a Superdex 200HR 30 size exclusion column (separation range 10-700 kDa) using a Bio-Logic HR high performance liquid chromatography machine at a flow rate of 0.5 ml/min using a buffer containing 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA (pH 8.0). Fractions (500 μl) were collected and precipitated with 2 volumes of acetone at -20°C overnight. Samples were centrifuged at 17500Xg, the supernatant aspirated and the pellets resuspended in sample buffer containing 50 mM Tris-Cl (pH 6.8), 1.6% (w/v) sodium dodecyl sulfate, 4% (v/v) β-mercaptoethanol, 16% (v/v) glycerol, 0.02% (w/v) bromophenol blue. The column was calibrated using gel filtration standards (BIORAD 151-1901) containing the following size standards: thyroglobulin (670 kDa), globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), vitamin B-12 (1.35 kDa).

### *3.14 Antibodies*

In addition to the XHSF1 polyclonal antibodies described above, the following antibodies were used in various experiments as indicated in the figure legends: goat anti-rabbit IgG-HRP conjugate (BIORAD), goat anti-mouse IgG-HRP conjugate (BIORAD), anti-PCNA (sc-53 Santa Cruz), anti-DHSF (Clos et al., 1990), anti-hHSF1 (Mercier et al., 1999; Rabindran et al., 1991), anti-MmHSF1 (Sarge et al., 1993), anti-XHSF1 (Mercier et al., 1997), anti-Hsp90 (OSU B3 kind gift of B. Matts), anti-p23 (clone JJ3, gift from D. Toft) (Johnson et al., 1994), anti-FKBP52 (clone Hi52c, gift from D. Smith) (Nair et al., 1997), anti-Hip (clone 2G6, gift from D. Smith) (Prapapanich et al., 1996), anti-Hop (clone f5, gift from D. Smith) (Smith et al., 1993), anti-Cyp40 (PA3-023, Affinity Bioreagents), anti-SUMO-1 (OPA1-07200, Affinity Bioreagents), anti-Ubiquitin (SPA-210, Stressgen).

# 4 Results

## *4.1 Determinants of the set point of HSF1 activation and deactivation*

### 4.1.1 Activation of HSF1 DNA-binding

HSF1 activation first involves the oligomerization of HSF1 from a monomer to a homotrimer at which point HSF1 acquires the ability to bind the HSE with high affinity (Baler et al., 1993; Westwood et al., 1991). For that reason, when HSF1 is said to have acquired DNA-binding activity, it may be assumed that HSF1 has also formed homotrimers. The oligomeric change can be triggered *in vivo* by elevation of the ambient temperature, however, there is variation amongst organisms in the temperature at which HSF1 binding is induced. For example, *Xenopus* HSF1 (XHSF1) is optimally activated at 33°C (Gordon et al., 1997), *Drosophila HSF* (DHSF) is optimally activated at 36°C (Clos et al., 1990), and human HSF1 (hHSF1) is optimally activated at 42°C (Abravaya et al., 1991a). It is not known whether HSF1 can act as the cellular thermometer and trimerize by directly sensing the change in temperature or whether molecular chaperones act as the stress sensor and regulate HSF1 oligomerization and

DNA-binding. In order to investigate the relative contribution of the HSF1 molecule and the cellular environment to the activation of HSF1 DNA-binding, characterization of the activities of *Drosophila* and human HSF1 expressed in *Xenopus* oocytes was initiated. If DHSF or hHSF1 maintained their activation set-points in the oocyte, it would indicate that innate properties of the HSF were most critical in determining the set-point of activation. If the set-point of activation was reprogrammed in the new environment, it would indicate that cellular factors were most critical in determining the set-point of activation.

Assessment of DHSF and hHSF1 DNA-binding in oocytes was dependent on the ability to differentiate exogenous HSFs from XHSF1 in a DNA-binding assay. HSF DNA-binding activity was measured using an electrophoretic mobility shift assay (EMSA) with radiolabeled HSE. A common domain of all HSFs is the highly conserved DNA-binding domain that enables all HSFs to bind the HSE with high affinity. Thus the DNA-binding element used in the EMSA could not differentiate between HSFs from different species. Beyond the highly conserved DNA-binding domain and other highly conserved functional domains discussed in the introduction, HSFs from different species also contain many unconserved regions and there is a wide range of sizes of HSFs between different species. For example, DHSF is approximately 110 kDa (Clos et al., 1990), hHSF1 is approximately 82 kDa (Rabindran et al., 1991), and XHSF1 is approximately 67 kDa (Stump et al., 1995). Thus, homotrimers of DHSF, hHSF1 and XHSF1 were expected to be separable in the electrophoretic mobility shift assay. In order to test this hypothesis, the position of the XHSF:HSE complex was compared to the DHSF:HSE complex in the same gel by comparing the gel shift from extracts from

**Figure 4. DNA binding activity of different quantities of DHSF expressed in *Xenopus* oocytes.** *Xenopus* oocytes injected with indicated quantities of pCMV:DHSF were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C) or heat shocked (H) at 36°C for 20 minutes. Following treatment, protein extracts were prepared and subjected to EMSA using radiolabeled HSE. *Drosophila* SL2 cells were treated as unshocked (U, 18°C) or heat shocked (H) at 36°C for 20 minutes. Protein extracts were prepared and subjected to EMSA using radiolabeled HSE. The positions of DHSF:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

heat shocked *Xenopus* oocytes and *Drosophila* SL2 cells (Figure 4). As expected, DHSF had less mobility in the gel compared to XHSF1. Therefore, it was surmised that the DNA-binding activities of DHSF and XHSF1 could be identified in the same sample due to the difference in the molecular weight of these proteins.

Overexpression of HSFs *in vivo* leads to inappropriate trimerization and activation of DNA-binding activity (Sarge et al., 1993; Stump et al., 1995). Thus, it was important to determine what level of protein expression of exogenous HSF would produce detectable amounts of HSF that would remain inactive in unshocked oocytes and retain the potential for DNA-binding in stressed cells. DHSF was expressed in oocytes by injection of a plasmid bearing the DHSF gene under control of the constitutive CMV (cytomegalovirus) promoter (pCMV:DHSF). In order to express a range of DHSF levels, oocytes were injected with various quantities (30-750pg) of pCMV:DHSF (Figure 4). Oocytes were then incubated overnight at 18°C to allow transcription and translation of DHSF. Extracts of unshocked and heat shocked oocytes (36°C for 20 minutes) were then prepared and subject to EMSA to test for HSF DNA-binding ability. Low concentrations (30 pg) of plasmid did not produce detectable levels of DHSF. Injection of 150-300 pg of plasmid into oocytes resulted in the formation of HSF1-HSE complexes in cells treated at 36°C not present in uninjected heat shocked samples. As this complex was present only in plasmid injected oocytes, was heat inducible, and had an electrophoretic mobility similar to DHSF from SL2 cells, it was presumed to be comprised of DHSF. At higher plasmid concentrations (750 pg) DHSF bound the HSE in unshocked oocytes and did not bind the HSE at 36°C, indicating that at this level of expression, the regulatory mechanisms that maintain HSF in a monomeric state in

unshocked cells and trimerized in heat shocked cells had failed. These results indicated that under carefully controlled conditions of expression, DHSF could be properly regulated in the *Xenopus* environment. Based on these results, subsequent experiments in which exogenous HSFs were expressed in oocytes were performed by injecting 300 pg of expression plasmid and the oocytes were incubated overnight before treatment.

The set-point of exogenous HSF activation in oocytes was determined by treating pCMV:DmHSF and pCMV:HuHSF1-injected oocytes at a range of temperatures and assaying for HSE binding by EMSA. The set-point of DHSF and hHSF1 activation was then compared to the temperature activation profile of XHSF1. Oocytes were treated at the indicated temperatures for 20 minutes which is sufficient to observe strong HSF1 DNA-binding activity. Oocytes were only treated at 42°C for 10 minutes because oocytes died after longer durations at this temperature, as determined by morphological characteristics including cell mottling, nuclear envelope breakdown and loss of plasma membrane integrity. Little or no XHSF1 DNA-binding activity was observed in oocytes treated at 18°C, 27°C, and 30°C (Figure 5A,B). XHSF1 DNA-binding was activated optimally in oocytes treated at temperatures between 33 and 36°C (Figure 5A,B). XHSF1 DNA-binding was also activated at 39°C but to a lesser extent. XHSF1 DNA-binding is absent in oocytes treated at 42°C. Interestingly, the non-specific band typically observed in electrophoretic mobility shift analysis under these conditions (Gordon et al., 1997) was less prominent in 36°C treated oocytes and absent in 39°C and 42°C treated oocytes. It is possible that at higher temperatures the protein that binds the HSE non-specifically is damaged or becomes aggregated, thus inhibiting its ability to bind DNA. Western blot analysis of these extracts with a PCNA antibody indicated that

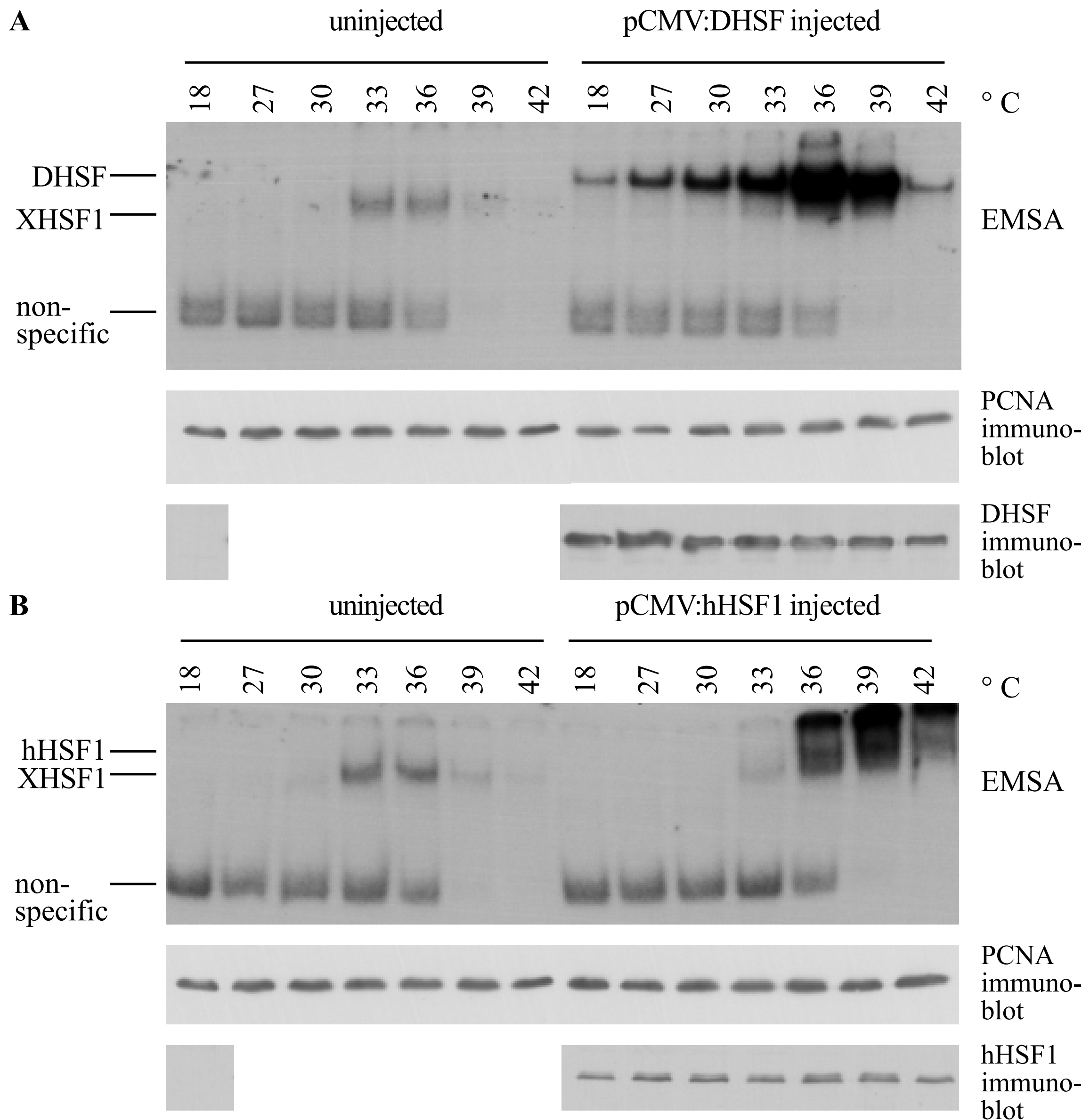



**Figure 5. Temperature activation profile of DHSF and hHSF1 DNA binding activity expressed in *Xenopus* oocytes. (A)** *Xenopus* oocytes injected with pCMV:DHSF were incubated at 18°C for 24 hours and subsequently treated at indicated temperatures for 20 minutes except for oocytes treated at 42°C which were heat shocked for 10 minutes. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of DHSF:HSE, XHSF1:HSE and the non-specific DNA binding protein:HSE complexes are indicated on the left. The same extracts were also run on SDS-PAGE and immunoblotted for PCNA or DHSF where indicated. **(B)** Oocytes injected with pCMV:hHSF1 were incubated at 18°C for 24 hours and subsequently treated at indicated temperatures for 20 minutes except for oocytes treated at 42°C which were heat shocked for 10 minutes. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of hHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. The same extracts were also run on SDS-PAGE and immunoblotted for PCNA or hHSF1 where indicated.

changes in the level of active XHSF1 was not due to differences in protein concentration in each extract (Figure 5A,B).

DHSF DNA-binding activity was minimal at 18°C when expressed in oocytes (Figure 5A). DHSF DNA-binding activity increased when oocytes were treated at increasing temperatures reaching a maximum at 36°C. DHSF DNA-binding activity was reduced in oocytes treated at 39°C compared to those treated at 36°C and was minimal in 42°C treated oocytes. Western blot analysis of pCMV:DmHSF injected samples with a PCNA antibody indicated that each extract contained equal protein levels (Figure 5A). Western blot analyses of these extracts with an antibody to DHSF indicated that DHSF was expressed equally in all samples (Figure 5A). Thus the differences in DHSF DNA-binding activity observed between these treatments was due to the differential activation of DHSF and not due to variation in total protein levels or DHSF levels. The activation profile of DHSF in response to this range of temperatures was similar in oocytes as had previously been observed in *Drosophila* cells (Clos et al., 1990). These results suggest that the mechanism that controls the set-point of DHSF activation functions similarly whether DHSF is in *Xenopus* oocytes or in its endogenous environment.

DNA-binding activity of hHSF1 was undetectable in oocytes treated at 18°C, 27°C, or 33°C (Figure 5B). hHSF1 DNA-binding activity was detected in oocytes treated at 36°C reaching a maximum in oocytes treated 39°C. Minimal hHSF1 DNA-binding activity was detected in 42°C treated oocytes. Western blot analyses of these samples confirm that all samples contained the same amount of total protein, and contained the same amount of hHSF1 (Figure 5B). In human cells, hHSF1 is inactive at

37°C, the normal physiological temperature, and hHSF1-HSE complexes are not detected in cells exposed to mild heat shock up to 40°C (Mosser et al., 1990). hHSF1 DNA-binding activity is low but detectable in human cells treated at 41°C and is maximal in cells treated at temperatures between 42°C and 45°C (Mosser et al., 1990). Interestingly, in the oocyte environment hHSF1 DNA-binding activity was not activated at the same temperatures that activate XHSF1 or DHSF. However, in the oocyte environment hHSF1 DNA-binding activity was also activated at temperatures (36°-39°C) that fail to activate hHSF1 in human cells. This has led me to hypothesize that neither the inherent properties of HSF1 nor the cellular environment are solely responsible for controlling the activation set-point of HSF1. If hHSF1 DNA-binding is activated by directly responding to heat one would expect that the temperature activation profile of hHSF1 in oocytes would have been the same as that in human cells. Conversely, if hHSF1 DNA-binding is activated by interaction with regulatory factors in the cellular environment then one would expect that the temperature activation profile of hHSF1 in oocytes would have been the same as that of XHSF1. The results obtained in these experiments suggest that a combination of these two factors must be responsible for controlling the monomer to trimer transition of HSF1 in response to heat stress. It is important to note that these conclusions are only valid if hHSF1 and XHSF1 did not form heterotrimers in the experiments discussed above. This possibility was examined further and described in section 4.1.3.

It is known that stresses other than heat can activate HSF1. Therefore it was of interest to determine if DHSF and hHSF1 was activated to the same degree by a stress unrelated to heat shock. The set-point of exogenous HSF activation in response to stress

other than heat was examined by treating oocytes expressing DHSF and hHSF1 to salicylate (Figure 6). As reported previously (Bharadwaj et al., 1998), treatment of oocytes with 50-90 mM salicylate activated XHSF1 DNA-binding but levels of XHSF1-HSE complex formation were not as high as that induced by heat shock at 33°C (Figure 6A). DHSF has been shown to be much more sensitive to exposure to salicylate since DNA-binding can be activated by as little as 10 mM in *Drosophila* SL2 cells as measured by EMSA (Winegarden et al., 1996). hHSF1 is also more sensitive than XHSF1 to exposure to salicylate in human cells. hHSF1 DNA-binding can be activated by treatment with 30 mM salicylate in human cells (Jurivich et al., 1992). The experiment shown in Figure 6B shows that DHSF DNA-binding in oocytes was activated by treatment of cells with 10-90 mM salicylate. Also, hHSF1 DNA-binding in oocytes was activated by treatment of cells with 30-90 mM salicylate (Figure 6C). Therefore it appears that exogenous HSF1s expressed in oocytes retain their activation set-points in response to salicylate. These results indicate that identity of the HSF1 determines the activation set-point in response to salicylate. HSF1, however, does not directly respond to salicylate. Zhong et al have shown that DNA-binding activity of purified DHSF can be activated by heat but not by salicylate, indicating that cellular factors are required to activate DHSF DNA-binding in response to heat (Zhong et al., 1998). Observations in the present experiments (Figure 6) probably reflect a difference in how the cellular factors that regulate HSF1 DNA-binding interact with different species of HSF1.

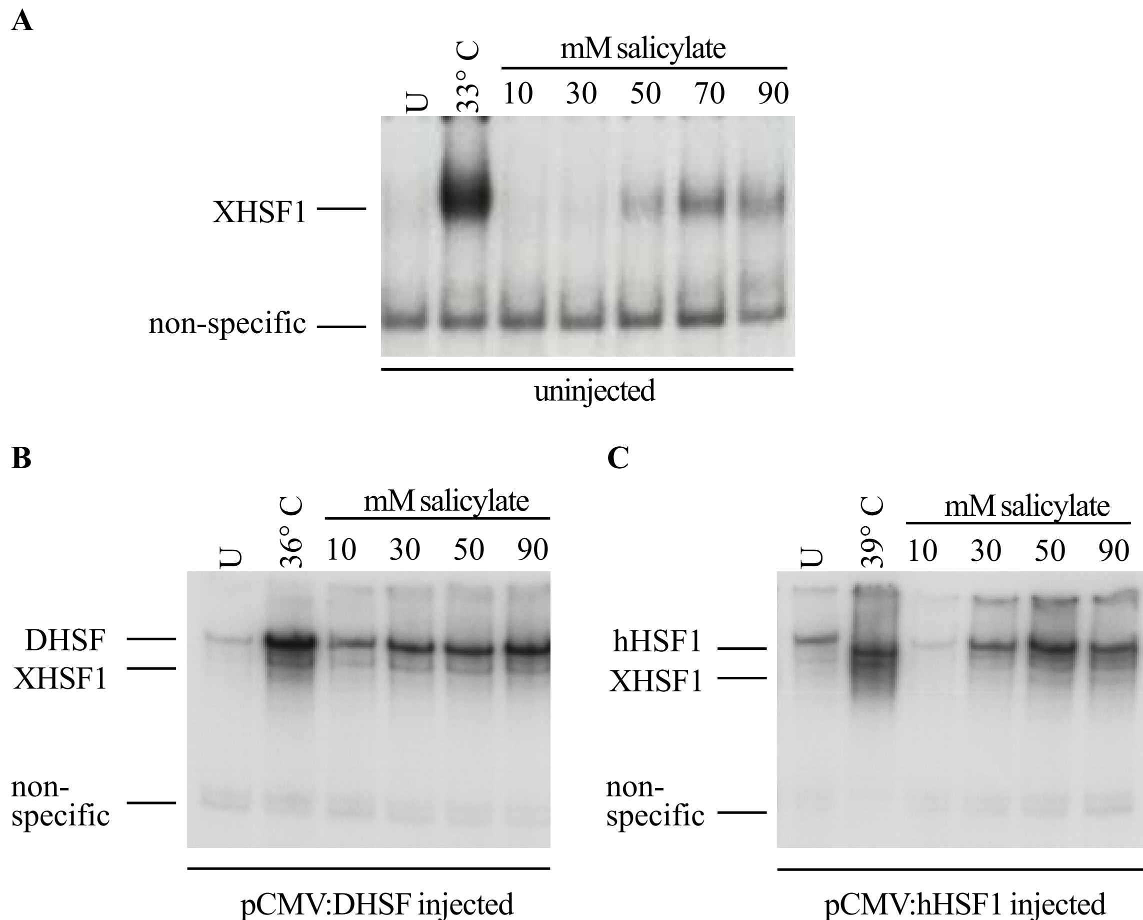



**Figure 6. Induction of DHSF and hHSF1 DNA binding activity in *Xenopus* oocytes exposed to salicylate. (A)** Uninjected *Xenopus* oocytes were treated as unshocked (U, 18°C) or heat shocked at 33°C for 20 minutes, or incubated in media containing the indicated concentration of salicylate for 1 hour. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** Oocytes injected with pCMV:DHSF were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C) or heat shocked at 36°C for 20 minutes, or incubated in media containing the indicated concentration of salicylate for 1 hour. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of DHSF:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(C)** Oocytes injected with pCMV:hHSF1 were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C) temperature, heat shocked at 39°C for 20 minutes, or incubated in media containing the indicated concentration of salicylate for 1 hour. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of hHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

### 4.1.2 Recovery of HSF1 DNA-binding

Many studies have established that HSF1 DNA-binding activity recovers following the removal of cells from the stressful condition (Abravaya et al., 1991a; DiDomenico et al., 1982; Gordon et al., 1997; Mosser et al., 1988). To determine the relative contributions of cellular environment and properties inherent to the HSF1 molecule on the recovery of DNA-binding, DHSF and hHSF1 were expressed in oocytes as above, then the oocytes were heat treated for 20 minutes at the indicated temperatures and the attenuation of HSF1-DNA-binding activity associated with the disassembly of trimers during recovery, following a return to 18°C, were analyzed by EMSA (Figure 7). Oocytes injected with DHSF expression plasmids were heat shocked at 36°C and oocytes injected with hHSF1 expression plasmids were heat shocked at 39°C in order to optimally activate each HSF1. The data presented in Figure 7A,B shows that endogenous XHSF1 DNA-binding recovered within 5 to 10 minutes following a 20 minute heat shock 33°C or 36°C heat shock. This is similar to the pattern that was previously observed in our laboratory (Gordon et al., 1997). DHSF DNA-binding in oocytes also recovered within 5 to 10 minutes following a 20 minute heat shock at 36°C (Figure 7A). The time frame of recovery was similar to that reported for DHSF in *Drosophila* cells (Winegarden et al., 1996). XHSF1 DNA-binding did not recover following a 39°C heat shock within 20 minutes (Figure 7). Additional experiments

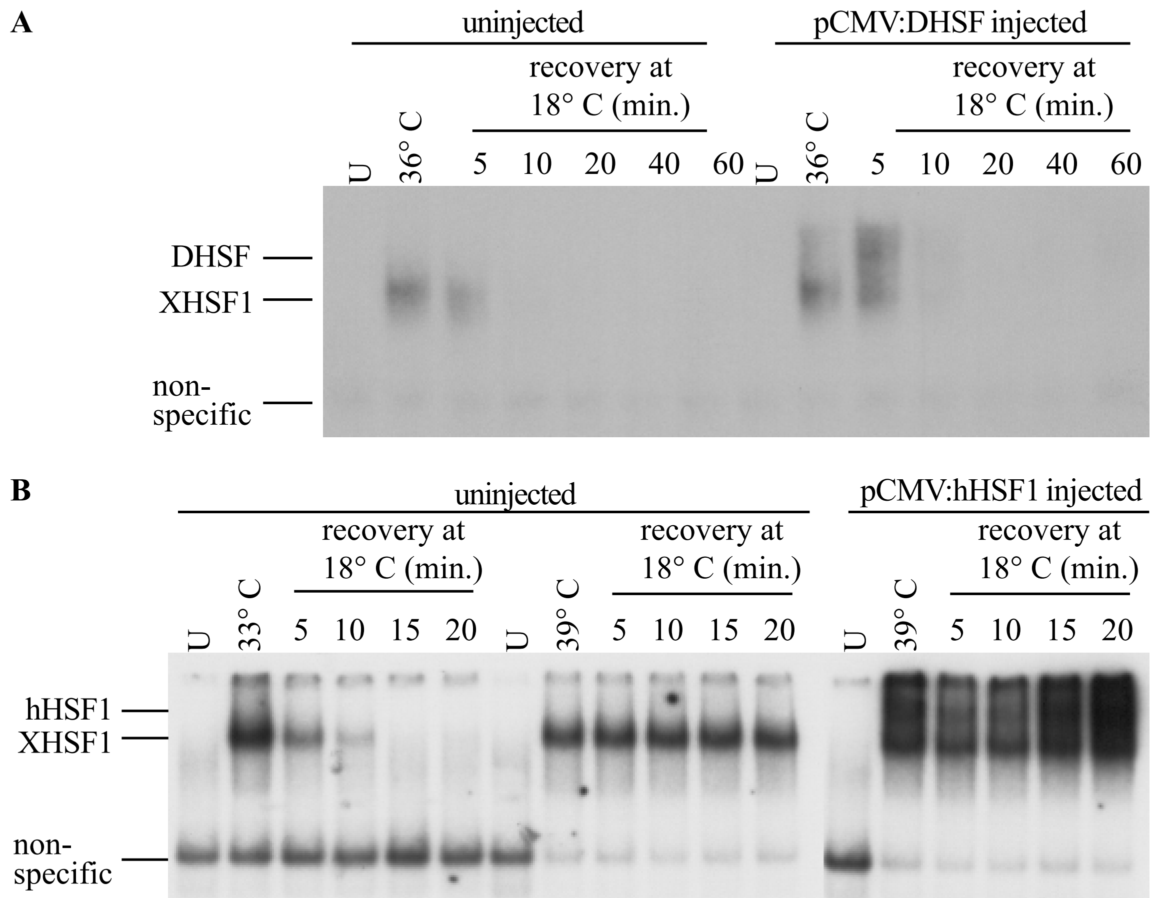



**Figure 7. Recovery of DHSF and hHSF1 DNA binding activity in *Xenopus* oocytes following the removal from heat.** **(A)** Uninjected or pCMV:DHSF injected *Xenopus* oocytes were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C) or heat shocked at 36°C for 20 minutes. Following heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of DHSF:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** Uninjected or pCMV:hHSF1 injected oocytes were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C), or heat shocked at 33°C for 20 minutes, or heat shocked at 39°C for 20 minutes where indicated. Following heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of hHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

show that XHSF1 DNA-binding does not recover following a 20 minute heat shock at 39°C even after 24 hours at 18°C (Figure 14). hHSF1 expressed in oocytes does not recover from a 20 minute heat shock at 39°C even after 20 minutes of recovery (Figure 7B). The inability of XHSF or hHSF1 DNA-binding to recover following a heat shock at high temperature was examined in more detail in section 4.3 of the results. Direct comparison of the recovery kinetics of hHSF1 in oocytes compared to hHSF1 in human cells is not possible because hHSF1 is not activated in human cells following a 39°C heat shock. The recovery profiles of DHSF and hHSF1 expressed in oocytes mirror the kinetics of recovery observed for the endogenous factor from the same degree of heat stress. Particularly for hHSF1 these results indicate that the cellular environment rather than the inherent properties of the HSF molecule controls recovery of HSF1 DNA-binding following heat shock in contrast to what is hypothesized to occur during activation.

### 4.1.3 Hetero-oligomers of exogenous and endogenous HSFs in heat shocked oocytes

A previous study in which DHSF and hHSF1 were expressed in the same cell type showed that these HSFs did not form heterotrimers, and the respective homotrimers were clearly resolved by EMSA (Clos et al., 1993). Using polyclonal antibodies specific to DHSF and hHSF1, Clos (Clos et al., 1993) was able to show that each band in the EMSA represented pure hHSF1 or DHSF homotrimers. Due to the difficulties in clearly separating trimers presumably containing DHSF, hHSF1 and XHSF1 in EMSAs

(Figures 4,5,6,7), the possibility remained that DNA-bound HSF1 observed in these assays might be composed of hetero-oligomers containing both exogenous and endogenous HSFs. To address the possibility that hetero-oligomers of DHSF, hHSF1 and XHSF1 could have formed in these experiments, antibodies designed to the different species of HSF were used in supershift EMSAs. The expectation was that specific antibodies would bind to their target and reduce the electrophoretic mobility of XHSF1, DHSF, and hHSF1-containing HSE bound complexes (supershift). If, for example, an antibody to XHSF1 supershifted the faster migrating XHSF1:HSE complexes and did not supershift the slower migrating DHSF:HSE complexes and conversely an antibody to DHSF supershifted DHSF:HSE complexes and not XHSF1:HSE complexes it could be concluded that XHSF1 and DHSF did not form heterocomplexes when expressed in the same cell. If however, antibodies to XHSF1 supershifted both HSF:HSE complexes then it would indicate that XHSF1 and DHSF formed hetero-oligomers. Similarly, antibodies to hHSF1 could be used to differentiate between the more slowly migrating hHSF1 homotrimers, and the faster migrating XHSF1 homotrimers in EMSA.

Antibodies used in supershift assays included an antibody produced against mouse HSF1 (MHSF1) (Sarge et al., 1993) that had previously been shown to bind XHSF1 (Ali et al., 1998), an antibody produced against a MBP-tagged N-terminal deleted XHSF1 (Mercier et al., 1997), an antibody produced against a MBP-tagged hHSF1 (hHSF1a) (Mercier et al., 1999), an antibody produced against purified hHSF1 (hHSF1b) (Rabindran et al., 1991), and an antibody produced against purified DHSF (Clos et al., 1990). In order to ensure that these antibodies were specific for their targets and did not cross react with XHSF1, each antibody was added to extracts of uninjected heat shocked (33°C for 20 minutes) *Xenopus* oocytes prior to EMSA (Figure 8A). MHSF1, XHSF1,

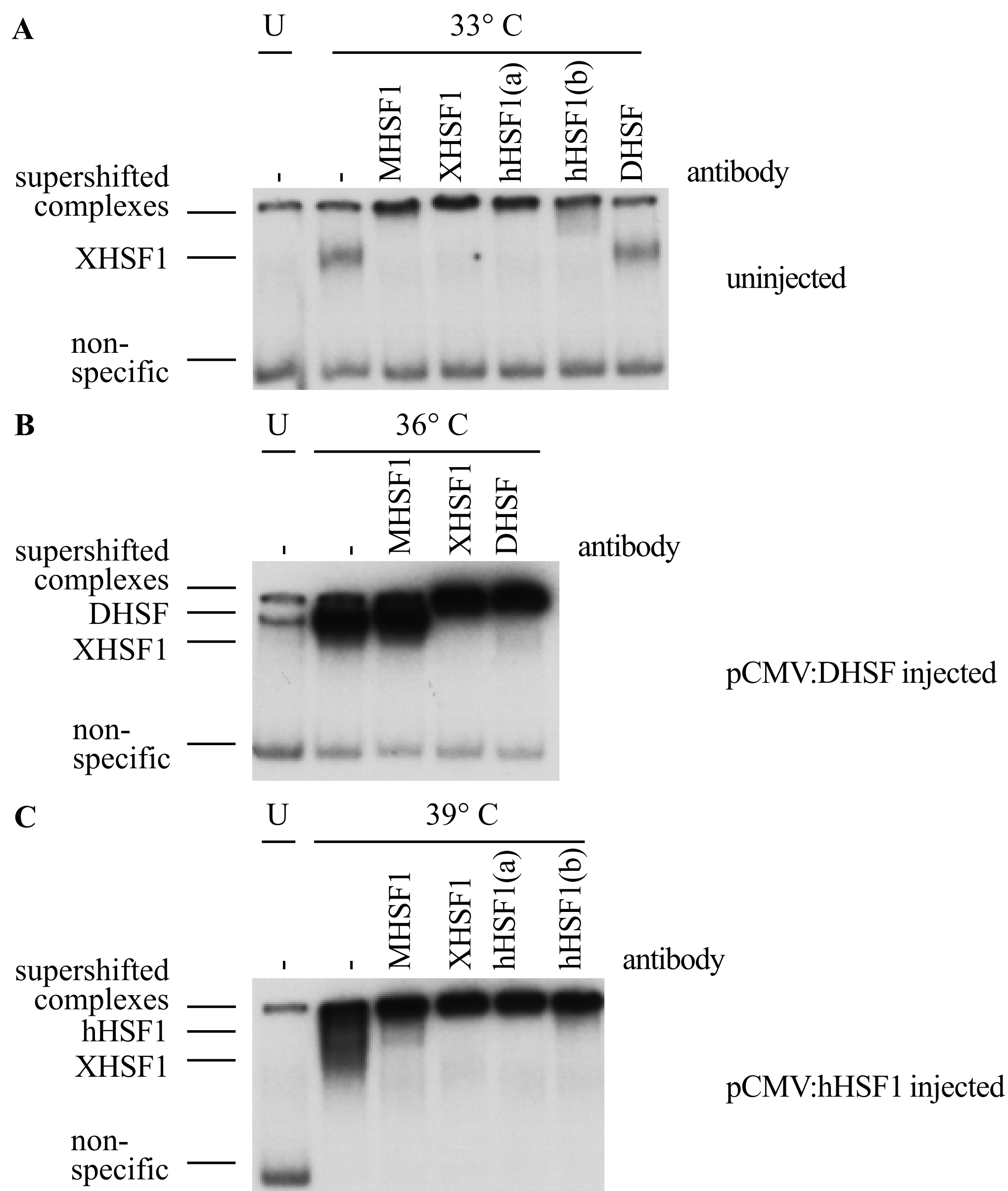



**Figure 8. Hetero-oligomerization of XHSF1 with DHSF and hHSF1 expressed in *Xenopus* oocytes. (A)** *Xenopus* oocytes were treated as unshocked (U, 18°C) temperature or heat shocked at 33°C for 20 minutes. Following treatment, protein extracts were prepared and subjected to EMSA using radiolabeled HSE. Where indicated antibody was added to the heat shocked protein extract prior to addition of HSE. The positions of supershifted XHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** Oocytes injected with pCMV:DHSF were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C), or heat shocked at 36°C for 20 minutes. Following treatment, protein extracts were prepared and subjected to EMSA using radiolabeled HSE. Where indicated antibody was added to the heat shocked protein extract prior to addition of HSE. The positions of supershifted DHSF:HSE and XHSF1:HSE, DHSF:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(C)** Oocytes injected with pCMV:hHSF1 were incubated at 18°C for 24 hours and subsequently treated as unshocked (U, 18°C), or heat shocked at 39°C for 20 minutes. Following treatment, protein extracts were prepared and subjected to EMSA using radiolabeled HSE. Where indicated antibody was added to the heat shocked protein extract prior to addition of HSE. The positions of supershifted hHSF1:HSE and XHSF1:HSE, hHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

hHSF1a and hHSF1b antibodies also supershifted the endogenous XHSF1-HSE complexes indicating that these antibodies cross reacted with XHSF1 (Figure 8). DHSF antibody did not supershift XHSF1 trimers in uninjected oocytes (Figure 8).

To examine the possibility that DHSF and XHSF1 formed hetero-oligomers, supershift analysis using MHSF1, XHSF1, and DHSF antibodies was performed on extracts of *Xenopus* oocytes expressing DHSF. DHSF was expressed in oocytes as in Figure 5 and heat shocked at 36°C for 20 minutes in order to activate DHSF. MHSF1, XHSF1, and DHSF antibodies were added to heat shocked extracts of oocytes expressing DHSF and subject to EMSA (Figure 8B). In this experiment, it was particularly difficult to differentiate two HSF:HSE complexes compared to experiments in Figure 4,5,6,7 due to the intensity of the more slowly migrating DHSF:HSE complex compared to the faster migrating endogenous XHSF1:HSE complex. MHSF1 antibodies did not alter the gel shift pattern compared to extracts in which no antibody had been added. XHSF1 antibodies supershifted both HSF:HSE complexes. That XHSF1 antibodies supershifted both fast and slowly migrating HSF:HSE bands either indicates that DHSF and XHSF1 formed hetero-oligomers or that XHSF1 antibodies cross-react with DHSF, however, the different effects of MHSF1 and XHSF1 antibodies make it difficult to differentiate between these two possibilities. DHSF antibodies supershifted the more slowly migrating HSF:HSE complex presumed to be DHSF homotrimers. Addition of DHSF antibodies did not alter the mobility of the HSF:HSE complex that has the same electrophoretic mobility as XHSF1 homotrimers from uninjected cells. Because anti-DHSF antibodies were shown to not cross react with XHSF1 homotrimers

in EMSAs (Figure 8A) these results using DHSF antibodies indicate that DHSF and XHSF1 did not form heterotrimers.

To examine the possibility that hHSF1 and XHSF1 formed hetero-oligomers, supershift analysis using MHSF1, XHSF1, hHSF1a and hHSF1b antibodies was performed on extracts of *Xenopus* oocytes expressing hHSF1. hHSF1 was expressed in oocytes as in Figure 5 and heat shocked at 39°C for 20 minutes in order to activate hHSF1. MHSF1, XHSF1, hHSF1a and hHSF1b all supershifted both slowly and faster migrating HSF:HSE complexes (Figure 8C). If these MHSF1 and XHSF1 antibodies are specific to XHSF1 and do not cross react with hHSF1, these results would indicate that hHSF1 and XHSF1 could form hetero-oligomers, however, results described in Figure 8A indicate that hHSF1 and XHSF1 contain similar epitopes that result in the cross reactivity of antibodies produced against hHSF1 with XHSF1. Because of the apparent cross reactivity of available hHSF1 antibodies and the strong possibility that antibodies to XHSF1 could cross react with hHSF1, it cannot be determined whether XHSF1 and hHSF1 form hetero-oligomers when expressed in the same cell.

## *4.2 Production of antibodies to XHSF1*

### 4.2.1 Titre of peptide antibodies

As mentioned above, we lacked a large supply of antibody specific to XHSF1. The production of antibodies to XHSF1 was undertaken in order to facilitate the use of

the *Xenopus* oocyte model system to study heat shock and XHSF1 regulation. An antibody was needed that could be used to quantify XHSF1 from samples in a western blot and that could recognize the native form of the protein *in vivo*, or in EMSA. In order to produce a large quantity of antigen with which to immunize animals, the gene for XHSF1 was subcloned into a bacterial expression vector that would produce a chimera of XHSF1 with a nickel binding protein domain consisting of 6 histidine residues at the N-terminus of the protein. Expression and purification of this protein yielded a truncated version of XHSF1 that proved unsuitable for antibody production (data not shown). Subsequently, 5 peptides corresponding to several regions of XHSF1 were manufactured and used as antigens for injection of rabbits. The identity of the peptides (produced at the Alberta peptide institute) and their position in the molecule are outlined in Figure 3. These peptides were chosen with specific reasoning and criteria. Peptides to both the N and C termini (CS-3171, CS3172) were chosen in order to obtain an antibody which would most likely bind the native protein because either the N or C terminus of a globular protein are usually exposed to the cytoplasm. Peptide CS-3173 was chosen in order to obtain an antibody that would bind the conserved DNA-binding region of XHSF1. An antibody to this region could potentially inhibit DNA-binding of HSF trimers, and could potentially bind HSF1s from multiple species because it bound the DNA-binding domain, a region that is conserved across species. CS-3174 and CS-3175 were chosen because of their uniqueness to XHSF1 that would specifically recognize XHSF1 and not HSF1s from other species. In addition, the relative hydrophobicity of these peptides potentially makes them more immunogenic which is an asset when producing antibodies (Harlow and Lane, 1988). All peptides were screened using the program BLAST for homology to known proteins in order to reduce potential

cross-reactivity. Peptides were ordered as BSA and KLH conjugates. Following a prebleed to obtain preimmune sera, KLH conjugates were used to immunize rabbits (2 rabbits per conjugate) at the University of Saskatchewan animal facility as outlined in the Materials and Methods.

The titre of each antibody was measured to qualitatively determine the concentration of antibody the rabbits had produced specific to the antigens. To measure the titre of all the antisera collected (2 rabbits for each of 5 antigens) an enzyme linked immunosorbant assay (ELISA) protocol was developed. The ELISA protocol was developed in order to optimize experimental parameters for the detection of peptide antibodies. To test the protocol, commercially available antibodies to PKB and the peptide from which it was derived were used in the ELISA protocol outlined in the Materials and Methods (Figure 9). Background was measured by performing the ELISA in the absence of primary antibody (indicated as 0 for primary antibody dilution). Positive reactions above background were measured in wells in which antibody had been added at 1:100, 1:500, and 1:2500. The intensity of the reactions in these wells decreased with an increase in the dilution of primary antibody. Increasing dilutions of primary antibody beyond 1:2500 did not produce a measurable reaction above background. The successful detection of PKB antibodies to its peptide antigen validates this method for the detection of antibodies to KLH-CS3171, 3172, 3173, 3174, and 3175 peptide antigens.

The titres of sera obtained from KLH-conjugated peptide-injected rabbits were tested by ELISA against corresponding BSA conjugated peptides (Figure 10). Testing the titre of sera against BSA conjugated peptides eliminated the contribution of antibodies generated to KLH that would potentially have been visualized in the ELISA.

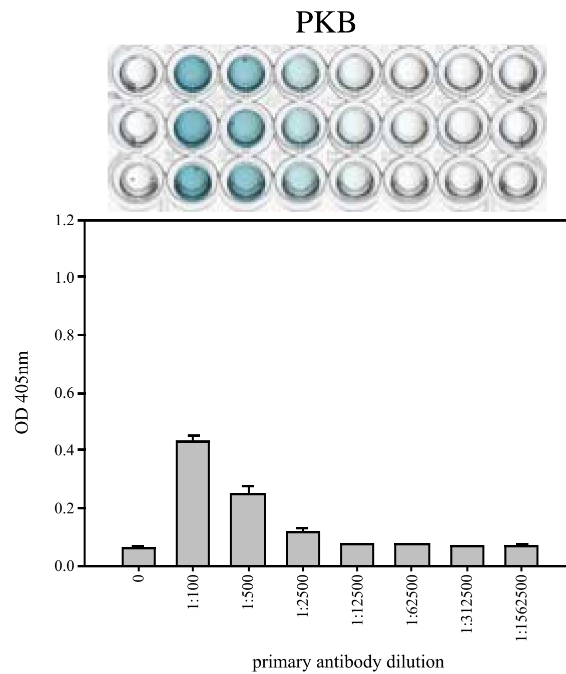
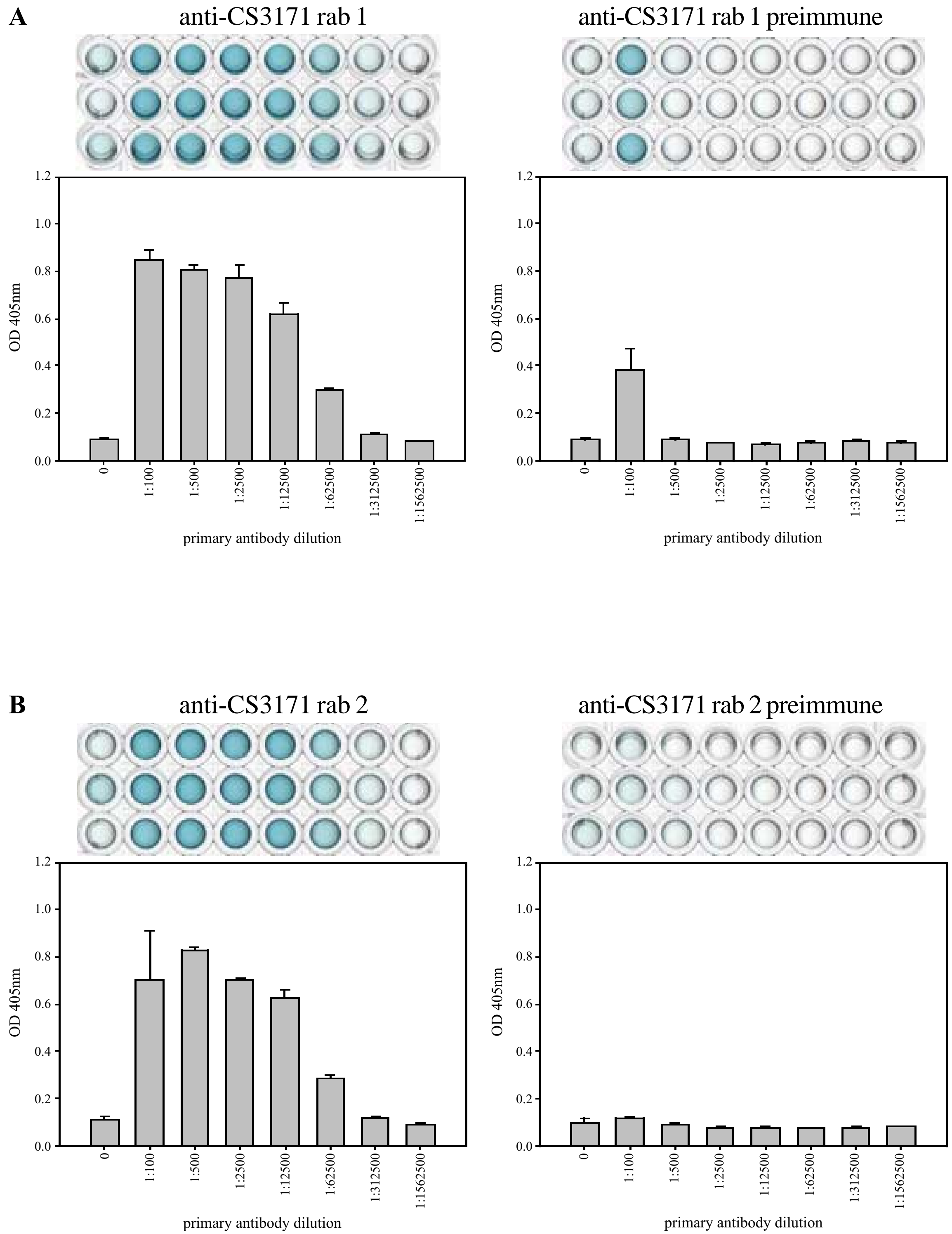



**Figure 9. Validation of ELISA protocol.** The titre of anti-PKB antibody against the peptide to which it was produced as measured by ELISA. The ELISA plate is depicted along with the quantitation of each sample set below. Each bar represents the average OD 405nm obtained from three samples for the indicated primary antibody dilutions. Included also are standard error bars.



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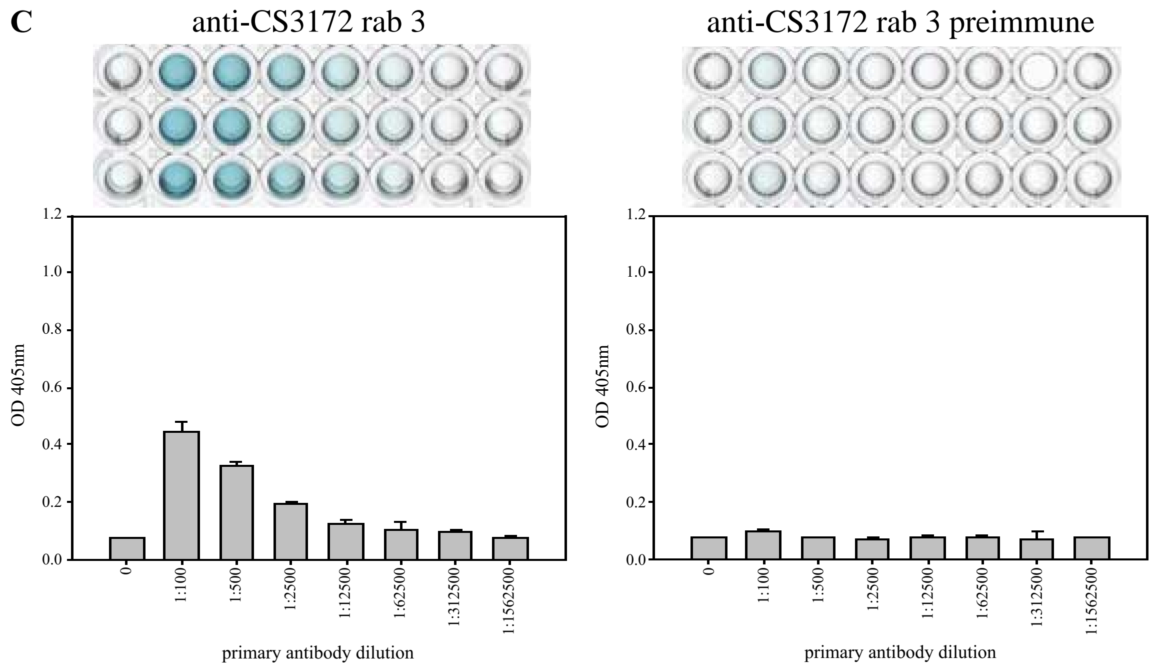


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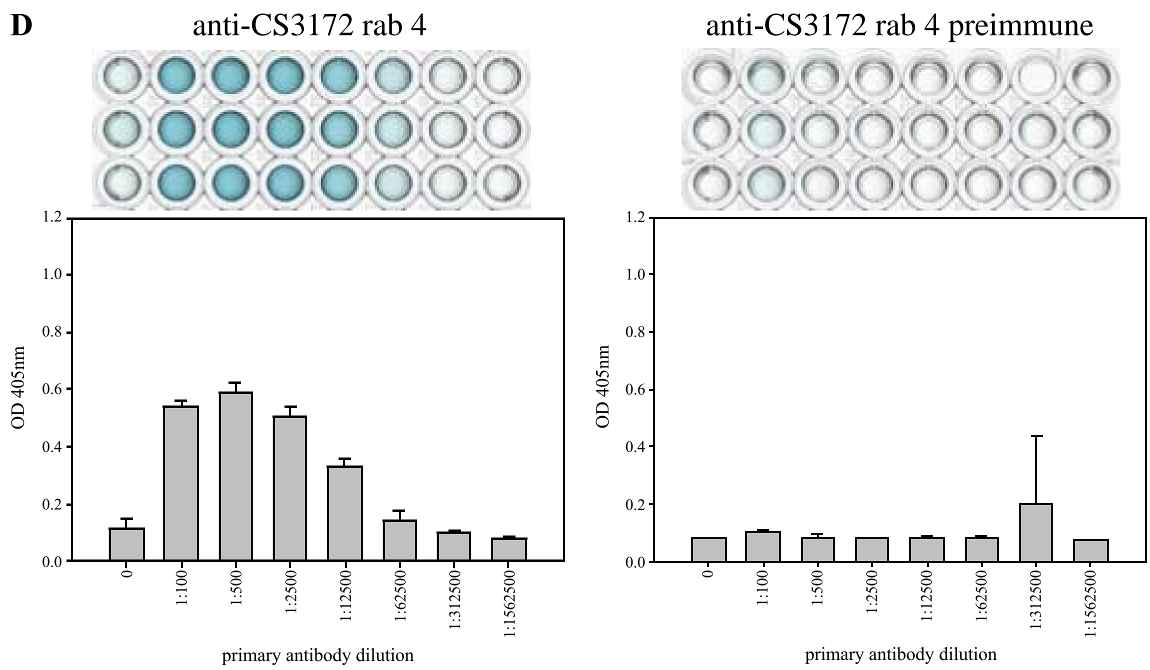




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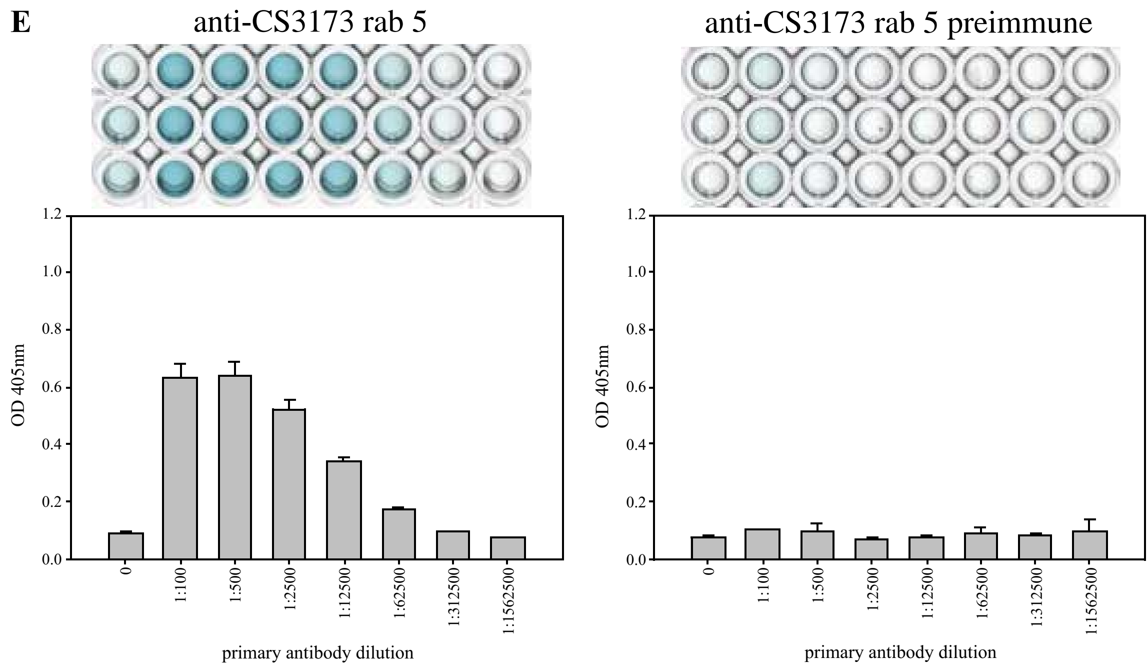


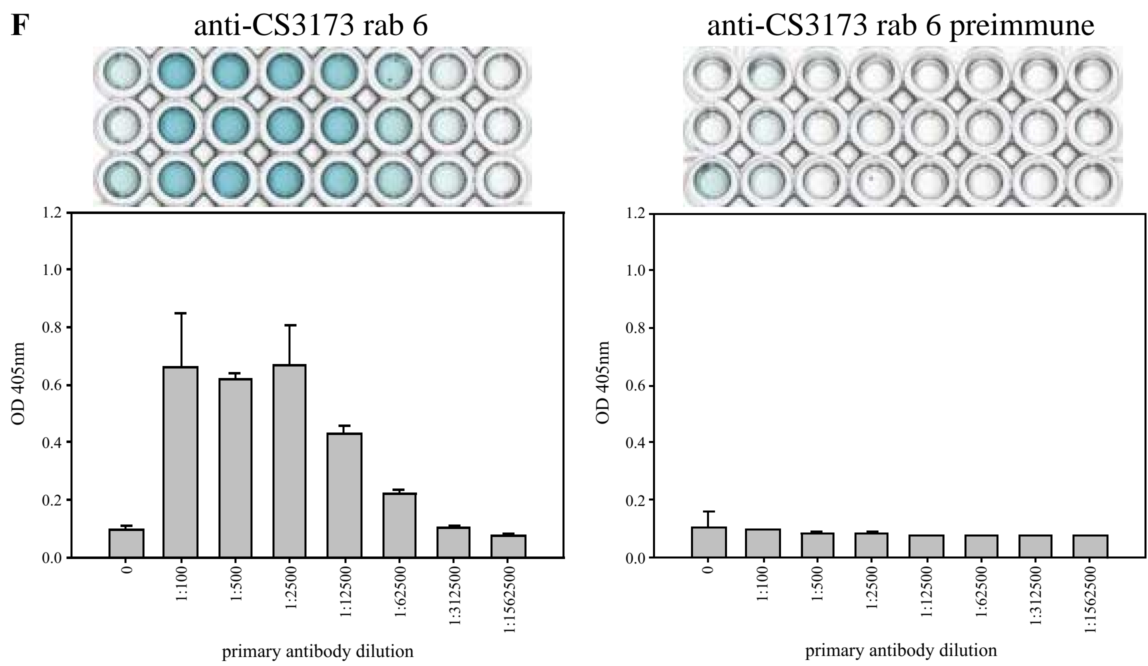




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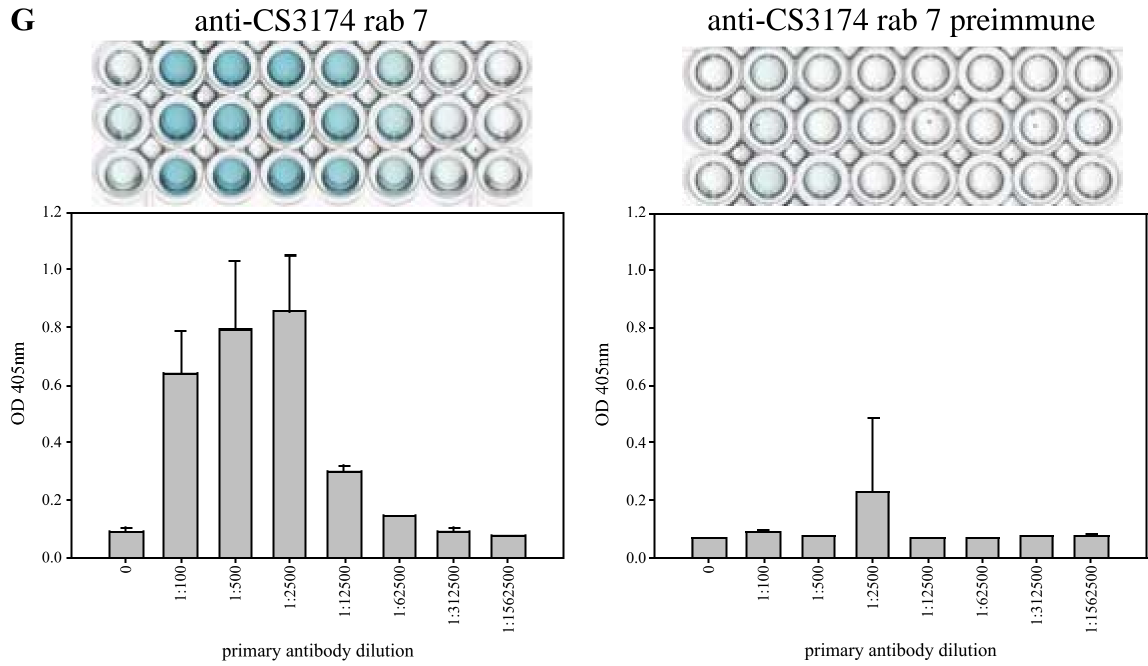


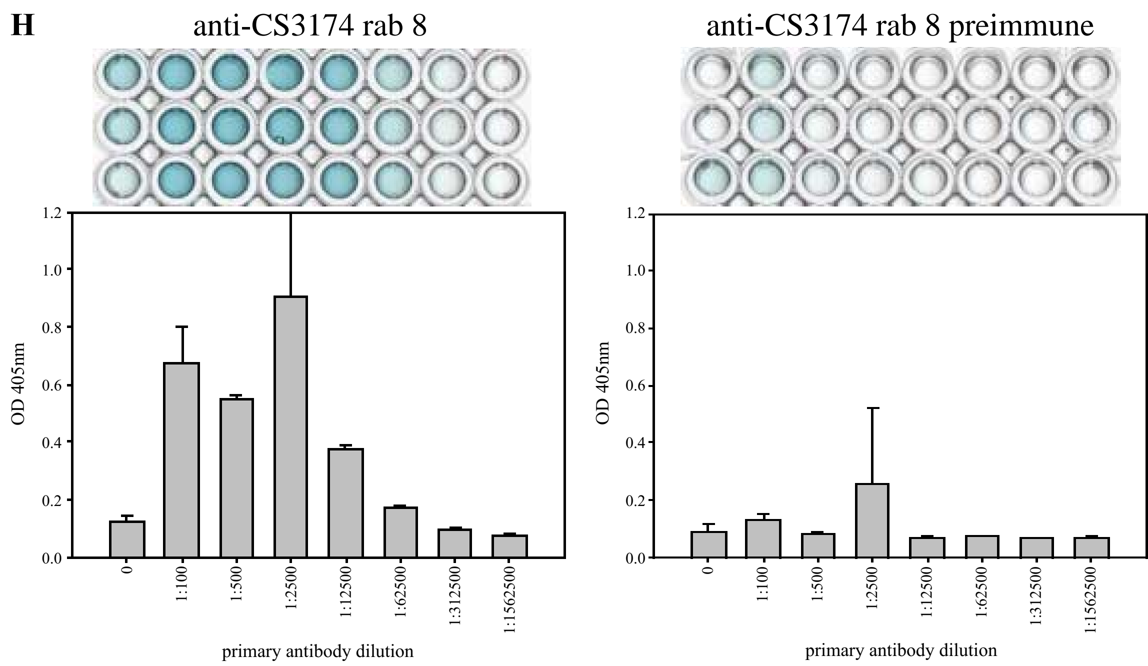




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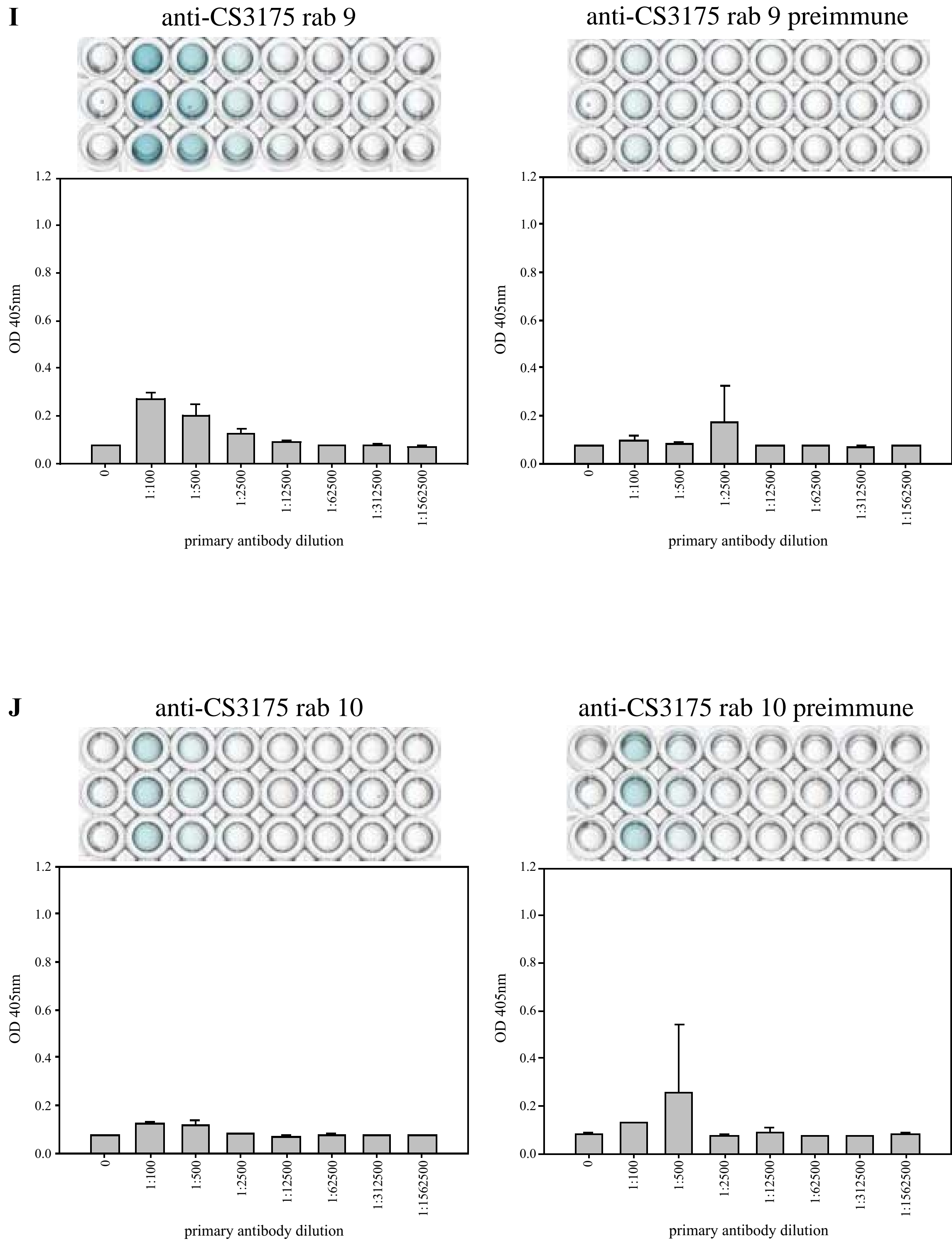



**Figure 10. Titre of antibodies to XHSF1.** The titre of the sera obtained from each injection of KLH conjugate of CS3171 (rabbit 1**(A)**, rabbit 2**(B)**), CS3172 (rabbit 3**(C)**, rabbit 4**(D)**), CS3173 (rabbit 5**(E)**, rabbit 6**(F)**), CS3174 (rabbit 7**(G)**, rabbit 8**(H)**), CS3175 (rabbit 9**(I)**, rabbit 10**(J)**) was measured against the corresponding BSA conjugate by ELISA. Each immune sample was compared directly with that rabbits' preimmune sera. The ELISA plate is depicted along with the quantitation of each sample set below. Each bar represents the average OD 405nm obtained from three samples for the indicated primary antibody dilutions. Included also are standard error bars.

The presence of preexisting antibodies to each peptide was also determined by measuring the titre of antibodies in the preimmune sera that reacted to the peptides. The absence of appreciable reactions for preimmune sera from each rabbit against each peptide suggests that all rabbits did not contain preexisting antibodies to the various antigens (Figure 10). Generally, positive reactions above background were observed for immune sera from all rabbits immunized with KLH-CS3171, 3172, 3173,3174 peptides. A qualititative measure of titre was established by determining the primary antibody dilution that represented the point at which the reaction had decreased to half the maximal level. For the purposes of this study titre was only measured to determine if antibodies produced were specific to the antigens, thus a more accurate measure of titre was not required. Rabbits immunized with KLH-CS3171, 3172, 3173,3174 all produced antibodies with approximately equal or higher titre to BSA-CS3171, 3172, 3173, 3174 than the commercially available PKB antibody tested in Figure 9. Rabbits immunized with KLH-CS3175 did not produce CS3175-specific antibodies. As discussed in the Methods and Materials the KLH-CS3175 peptide was extremely difficult to solubilize in saline solution. As suggested by the Alberta Peptide Institute, harsh conditions were employed to solubilize the KLH-CS3175 antigen that involved solubilization in glacial acetic acid. This treatment may have damaged the peptide antigen. In addition, much of KLH-CS3175 had re-precipitated following dialysis of the material possibly contributing to a diminished immune response.

### 4.2.2 Specificity of immune sera for XHSF1 in immunoblot analysis

One of the purposes of creating antibodies to XHSF1 was to detect the presence of XHSF1 in a protein sample. Thus, each antisera was tested for its ability to bind XHSF1 in immunoblot analysis of unshocked and heat shocked *Xenopus* oocyte extracts (Figure 11). As a control, the presence of antibodies in the rabbit that could potentially cross-react with *Xenopus* proteins was first assessed by immunoblotting unshocked and heat shocked extracts with preimmune sera. Less stringent conditions were used in immunoblots probed with preimmune sera in order to visualize any cross reactivity that could interfere with the detection of XHSF1. The preimmune immunoblot was performed using a higher concentration of sera than immune blots (1:2000 versus 1:4000). In addition immunoblots probed with preimmune sera were exposed overnight as opposed to 2 minutes for the immune blots. Preimmune sera from each of the 10 rabbits displayed varying degrees of reactivity to *Xenopus* proteins (Figure 11). Proteins of a particular molecular weight that reacted with preimmune sera for each rabbit were disregarded as non-specific antibody binding proteins in immunoblots probed with immune sera from the same rabbit. Immune sera from each of the 10 rabbits displayed varying degrees of reactivity to *Xenopus* proteins (Figure 11). Sera was determined to bind XHSF1 if it reacted with a protein with the same molecular mass as XHSF1 which has been determined by two separate labs to be 67kDa (Mercier et al., 1997; Stump et

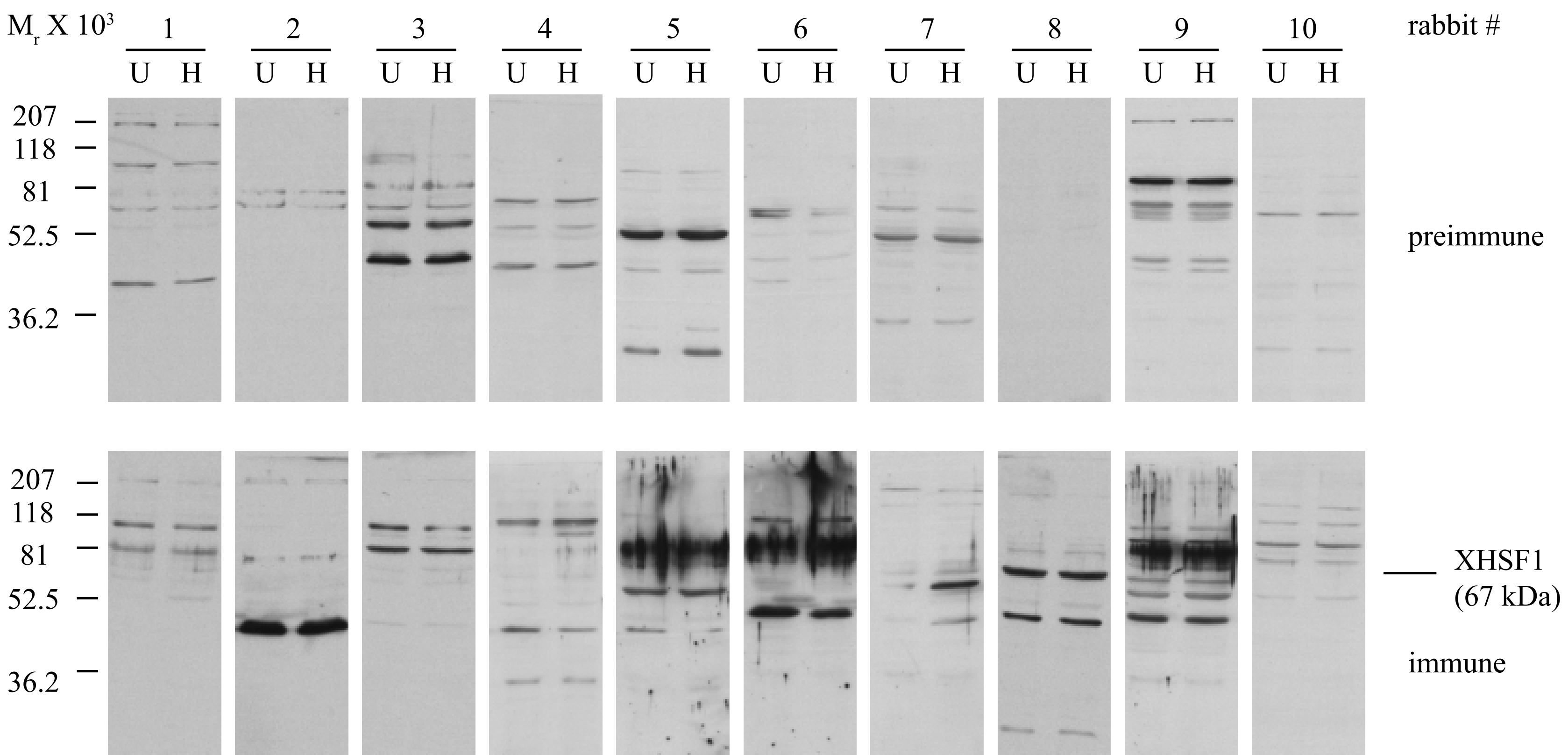
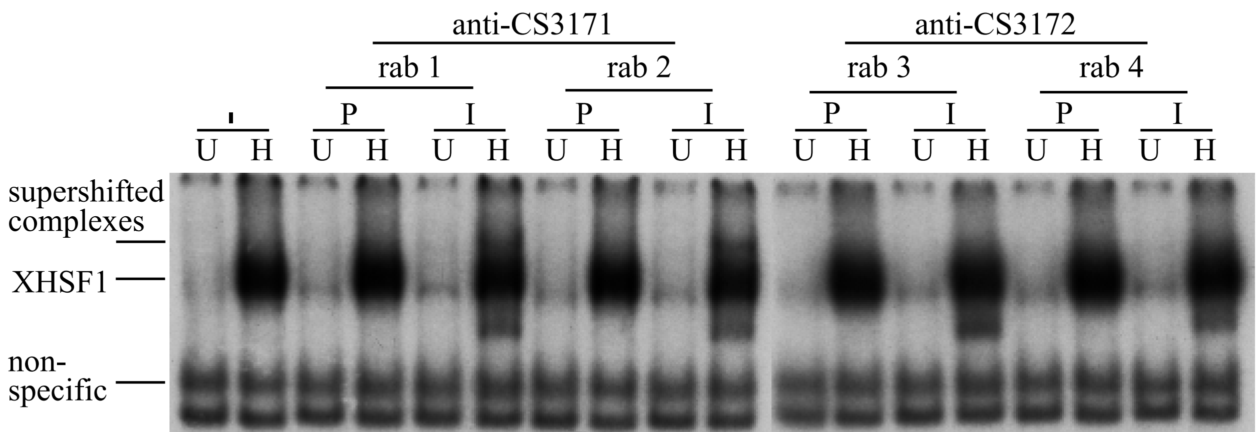



**Figure 11. Detection of denatured XHSF1 by peptide antisera.** Extracts from *Xenopus* oocyte treated as unshocked (U) or heat shocked (H, 33°C for 30 minutes) were separated on SDS-PAGE and blotted onto nitrocellulose. Blots were then tested for reactivity to preimmune sera from rabbits 1-10 diluted at 1:2000 or peptide antisera from rabbits 1-10 diluted at 1:4000 where indicated. Positions of molecular weight markers are indicated on the left. Sera were tested for the ability to recognize a protein of the same molecular weight of XHSF1 (67 kDa (Stump et al., 1995)), indicated at the right side of the panel.

al., 1995). The sera from rabbit 8 (immunized against CS-3174) provided an antibody that most specifically bound a protein of 67kDa.

### 4.2.3 Specificity of immune sera to XHSF1 in EMSA

The reason antigens were designed to several regions of XHSF1 was to obtain antibodies that could potentially bind different regions of intact native XHSF1. The antisera were tested for binding to native XHSF1 in EMSA (Figure 12). Antibodies were mixed with extracts of unshocked and heat shocked *Xenopus* oocytes *in vitro* prior to addition of the radiolabeled HSE. Antibody was added to the extract prior to the HSE to detect interaction of antibody to any domain of XHSF1 that would potentially be masked by the HSE (i.e. antibodies to CS-3173 were designed to specifically bind the DNA-binding domain of XHSF1). The effect of each antibody on unshocked extracts was tested because some antibodies to HSF1s have been shown to activate trimerization and DNA-binding activity of HSF1 in unshocked extracts *in vitro* (Zimarino et al., 1990). Antibody that reacted with XHSF1 would be expected to supershift the DNA-bound XHSF1 trimer or eliminate the ability of XHSF1 trimers from heat shocked *Xenopus* extracts to bind the HSE. Addition of preimmune sera from any of the rabbits to unshocked or heat shocked extracts had no effect on the level of XHSF1 DNA-binding (Figure 12). This result confirms that there were no preexisting antibodies to XHSF1. Addition of immune sera from any of the rabbits had no effect on the amount of XHSF1:HSE complex formation in unshocked controls in heat shocked samples



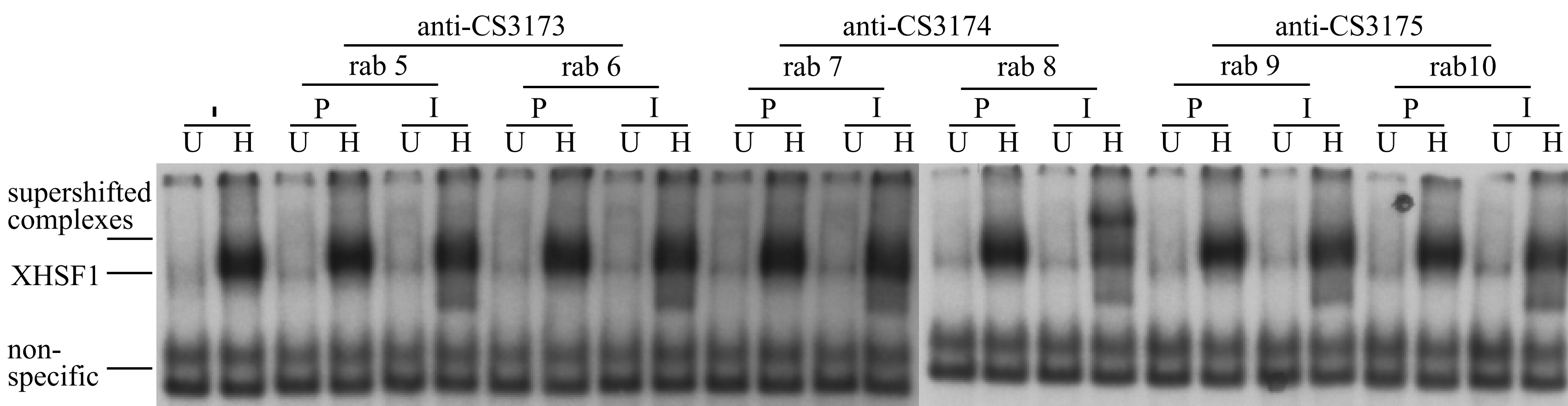




**Figure 12. Detection of native XHSF1 by peptide antisera.** Extracts from *Xenopus* oocytes treated as unshocked (U) or heat shocked (H, 33°C for 30 minutes) were subjected to EMSA using radiolabeled HSE. The preimmune (P) or immune (I) sera from the indicated rabbit immunized with the indicated antigen were added to the protein extract prior to the addition of the HSE. The positions of supershifted XHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

(Figure 12). Antisera raised against KLH-CS3171 from rabbits 1 and 2 induced a slight but perceptible supershift of XHSF1 (Figure 12). Antisera raised against CS-3174 from rabbit 8 significantly supershifted the XHSF1 (Figure 12). Immune antisera from all other rabbits did not supershift the XHSF1:HSE complex (Figure 12). Based on its ability to bind both denatured forms (Figure 11) and native forms of XHSF1 (Figure 12) antibody to CS3174 from rabbit 8 was used in all subsequent experiments.

## *4.3 High temperature heat shock prevents Hsp transcription in an HSF1 dependent manner*

### 4.3.1 Attenuation of XHSF1 DNA-binding is delayed following recovery from a high temperature heat shock

As was observed in Figure 7, there was a significant delay in the recovery of HSF DNA-binding after oocytes were heat shocked at high temperature. In order to characterize the extent to which recovery of HSF1 DNA-binding was impaired, it was first important to determine if HSF1 protein levels were affected by high temperature heat shock. In order to test this, the DNA-binding activity of HSF and the levels of XHSF1 were examined in oocytes treated at different temperatures (Figure 13). As shown previously, XHSF1 DNA-binding activity was optimal at 33°C - 36°C, but XHSF1 was also active in oocytes treated at 39°C and 42°C. Western blot analysis of these samples with XHSF1 antibody show that XHSF1 protein levels were the same in

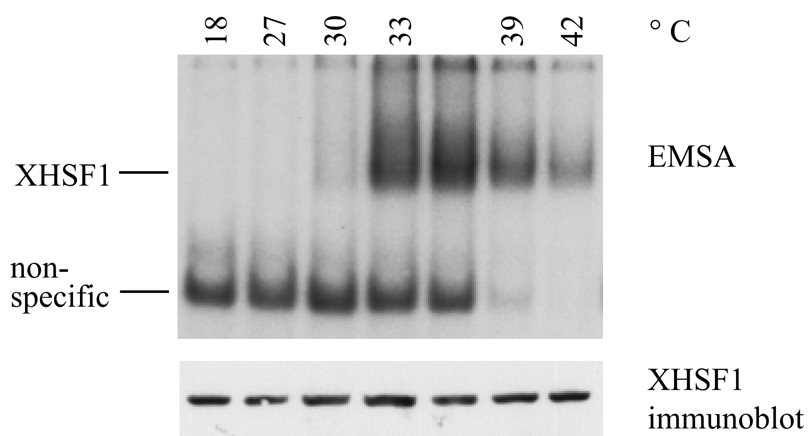



**Figure 13. XHSF1 protein levels in oocytes treated at different temperatures.** *Xenopus* oocytes were treated at the indicated temperatures for 20 minutes except for oocytes treated at 42°C, which were heat shocked for 10 minutes. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The position of the XHSF1:HSE, and non-specific DNA binding protein:HSE complexes are indicated. The same extracts were also run on SDS-PAGE and immunoblotted for XHSF1.

each extract (Figure 13), indicating that the differences observed in XHSF1 DNA-binding activity at different temperatures is a result of differential activation of XHSF1.

To determine how long recovery of XHSF1 DNA-binding was delayed after high temperature heat shock, oocytes were treated at 39°C for 20 minutes and subsequently allowed to recover at 18°C for up to 24 hours. Samples were taken periodically and the DNA-binding activity of XHSF1 was tested by EMSA. As shown in Figure 14 XHSF1:HSE complexes were still detectable even 24 hours after a high temperature 39°C heat shock. XHSF1:HSE complexes were detected until the oocytes died (data not shown). Thus the mechanism that regulates recovery of XHSF1 DNA-binding is permanently deactivated upon a high temperature heat shock.

The persistence of HSF1 DNA-binding during recovery from heat shock had not previously been described in any other cell type. A possible explanation for this phenomenon was that high temperature heat shock altered the DNA-binding specificity such that XHSF1 could bind DNA non-specifically. To confirm that XHSF1 maintained its sequence specificity for the HSE, a competition assay was performed wherein different unlabeled DNA fragments were added to a 39°C treated oocyte extract before addition of the labeled HSE (Figure 15). As a control, the DNA-binding specificity of XHSF1 from 33°C treated oocyte extracts was also compared. Addition of unlabeled sonicated *E.coli* DNA or YY1BE did not affect the binding of XHSF1 to the HSE from either 33°C or 39°C treated cells. The only competitor capable of competing with the labeled HSE was unlabeled (cold) HSE for both 33°C and 39°C activated XHSF1. This confirms that XHSF1 activated by a 39°C heat treatment maintains its binding specificity for the HSE sequence.

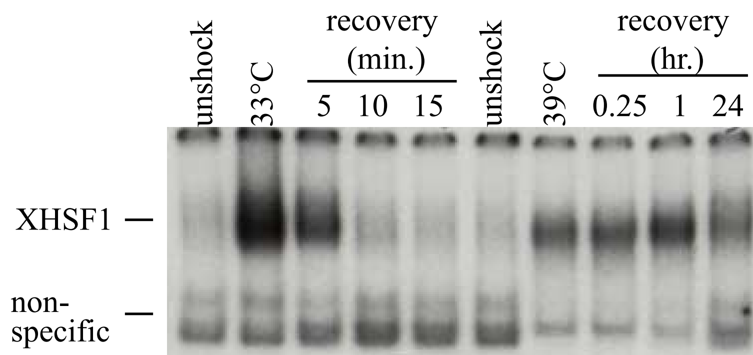



**Figure 14. Attenuation of XHSF1 DNA binding is delayed following recovery from a 39°C heat shock.** *Xenopus* oocytes were treated as unshocked (U, 18°C) or heat shocked at 39°C for 20 minutes where indicated. Following heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

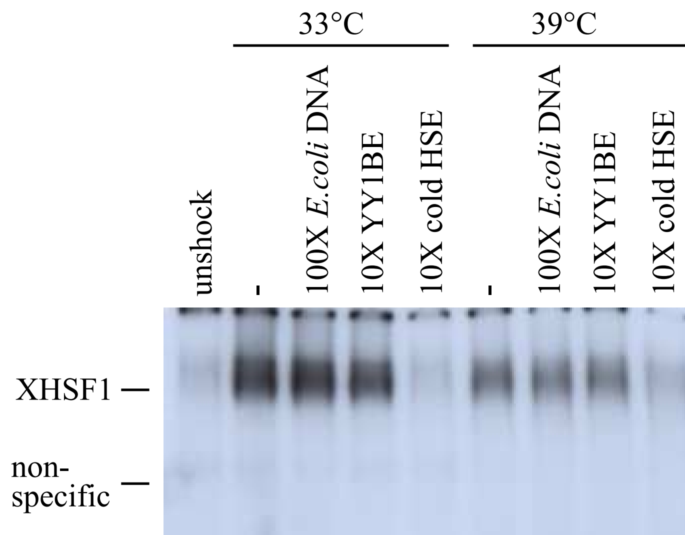



**Figure 15. Sequence specificity of XHSF1 activated by high temperature.** *Xenopus* oocytes were treated as unshocked (U, 18°C) or heat shocked at 33°C or 39°C for 20 minutes. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. Competitor DNA including sonicated *E.coli* DNA, YY1 binding element (YY1BE) or cold HSE were added to oocyte extracts in molar excess as indicated prior to addition of the radiolabeled HSE. The positions of the XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

### 4.3.2 Mechanism that regulates recovery of HSF1 DNA-binding

One of the models of how cells regulate HSF1 activation and deactivation involves a feedback loop whereby HSF1 is kept in a monomeric state by the action of molecular chaperones present in unshocked cells, particularly Hsp90 and Hsp70 (Abravaya et al., 1991a; Ali et al., 1998; Craig and Gross, 1991; DiDomenico et al., 1982; Guo et al., 2001; Voellmy, 1996; Xia et al., 1998). Cellular stress such as heat stress is thought to cause the partial unfolding of an abundance of cellular proteins that require chaperones to prevent aggregation and to recover their proper 3-dimensional structure (Lindquist and Craig, 1988; Morimoto et al., 1997; Morimoto, 1994). HSF1, now free of the negative regulatory effects of an Hsp90 and/or Hsp70 complex, forms homotrimers which can bind the HSE upstream of heat shock genes. Once normal conditions resume or the intracellular pool of free Hsps is increased, an Hsp90 and/or Hsp70 chaperone complex disassembles HSF1 trimers. Since there is no detectable increase in the cellular content of Hsps in *Xenopus* oocytes during stress (Gordon et al., 1997), it is possible that a high temperature heat shock creates damage that cannot be repaired by the existing pool of Hsps. This model could help explain why HSF1 does not recover from a high temperature heat shock.

The first step required to test this model was to determine the threshold temperature that could cause sufficient damage to completely inhibit attenuation of XHSF1 DNA-binding. Oocytes were treated at 33°C, 37°C, 38°C, and 39°C for 20

minutes and allowed to recover at 18°C. Protein extracts were made and XHSF1 DNA-binding was tested by EMSA. XHSF1 DNA-binding recovers within 5 minutes from a 33°C heat shock (Figure 16A). A 37° heat shock delayed the attenuation of XHSF1 DNA-binding beyond 30 minutes. When tested 24 hours later, XHSF1 heat shocked at 37°C had recovered. Similarly, a 38°C heat shock also delayed the attenuation of XHSF1 DNA-binding beyond 30 minutes. When tested 24 hours later XHSF1 heat shocked at 38°C had recovered. A 39°C heat shock delayed XHSF1 recovery beyond 24 hours. Thus heat shock treatments of 37°C and 38°C for 20 minutes can significantly delay XHSF1 attenuation but a 39°C heat treatment for 20 minutes prevents XHSF1 attenuation during recovery. The duration of heat shock at 39°C required for this effect was also examined (Figure 16B). Initially, oocytes were treated at 39°C and tested for XHSF1 DNA-binding activity at 5,10, 15, and 20 minutes. XHSF1 DNA-binding is activated after 5 minutes of heat treatment. Prolonged treatment at 39°C does not further increase XHSF1 DNA-binding. The attenuation of XHSF1 DNA-binding following a 10 minutes 39°C was examined (Figure 16B). Heat shock at 39°C for 10 minutes delayed XHSF1 recovery beyond 2 hours but XHSF1 DNA-binding eventually recovered by 24 hours. These results indicate that a threshold amount of cellular damage that can be caused by a 39°C heat shock for 20 minutes must occur in order to permanently disable the mechanism that drives attenuation of XHSF1 DNA-binding during recovery.

It was possible that repeated heat shocks at a lower temperature, or prolonged heat shock at lower temperatures, could mimic the inhibition of recovery seen in 39°C-20 minute heat shocked oocytes. In the next experiment oocytes were repeatedly heat

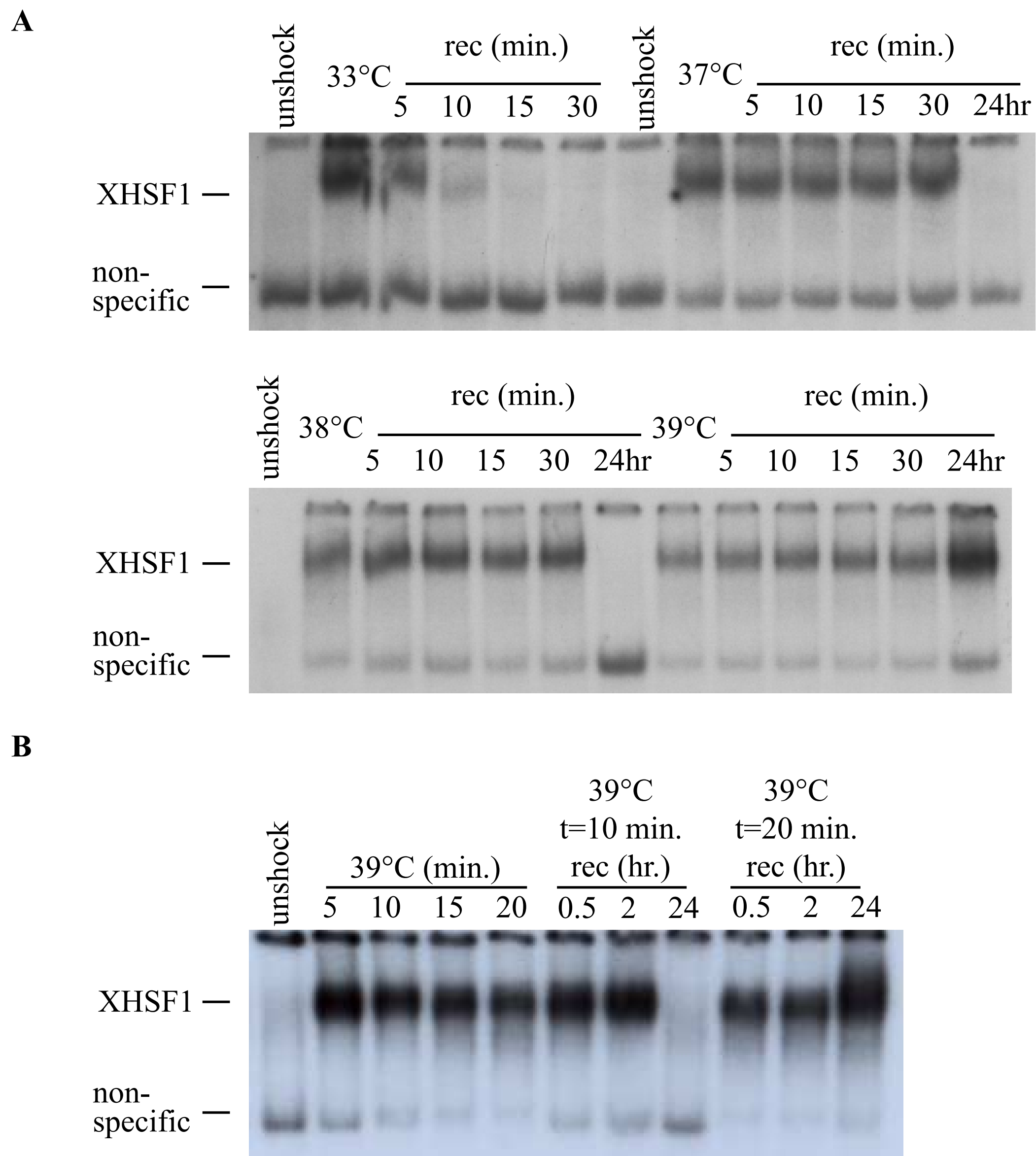



**Figure 16. Attenuation of XHSF1 DNA binding is delayed following recovery from a high temperature heat shock. (A)** Xenopus oocytes were treated as unshocked (18°C) or heat shocked at 33°C, 37°C, or 39°C for 20 minutes where indicated. Following heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of the XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** Xenopus oocytes were treated as unshocked (18°C) or heat shocked at 39°C, for the indicated time. Following heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of the XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

shocked at 33°C for 30 minutes, allowing only 15 minutes for recovery at 18°C between heat shocks. DNA-binding of XHSF1 was tested by EMSA on samples taken immediately following heat shock or following full recovery (Figure 17). DNA-binding was repeatedly activated and completely recovered during this procedure, indicating that the mechanism driving attenuation of XHSF1 DNA-binding can function despite repeated heat shocks and that it can quickly cycle between on and off states. To study the effects of long term heat shock on attenuation of XHSF1 DNA-binding, oocytes were treated at 31°C or 33°C for 4 hours and subsequently allowed to recover (Figure 18). DNA-binding recovered within 5 minutes following a 4 hour 31°C heat shock. XHSF1 DNA-binding recovered within 5-10 minutes following a 4 hour 33°C heat shock, which is similar to the kinetics of recovery as observed in short term 33°C heat shocked oocytes (Figure 16). These results demonstrate that the XHSF1 recovery mechanism functions after exposure of cells to long term heat shock at low temperatures (31, 33°C). This is probably because the oocyte contains enough chaperones to repair the cellular damage as it occurs. It is possible that higher temperature heat shock causes protein denaturation that cannot be rectified by existing chaperones. After 20 minutes at a 39°C, the existing level of chaperones might be permanently sequestered by the damaged proteins and are unavailable to disassemble XHSF1 trimers.

The lack of attenuation following a 20 minute heat shock at 39°C could also be explained by a failure of the cell to activate the XHSF1 recovery mechanism. The recovery mechanism might require a modest stress stimulus at temperatures lower than 39°C. To examine this possibility, oocytes were heat shocked at 39°C for 20 minutes, allowed to recover for 3 hours at 18°C, then heat shocked at 33°C for 20 minutes and

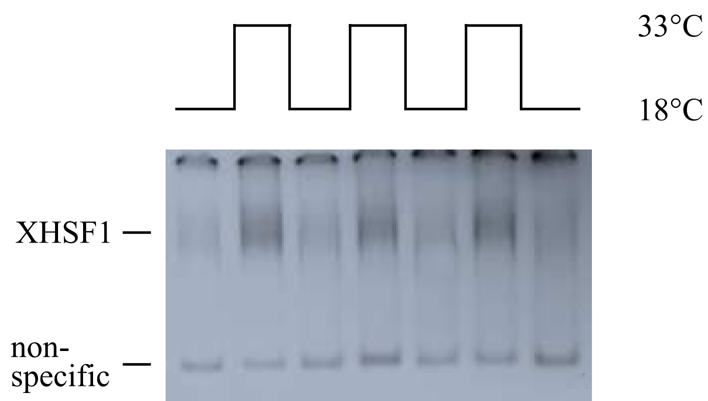



**Figure 17. Rapid sequential induction, recovery, and reinduction of XHSF1.** *Xenopus* oocytes were treated as unshocked (18°C), or repeatedly heat shocked at 33°C for 30 minutes followed by placement of oocytes in 18°C media for 15 minutes. Protein extracts of oocytes were made immediately following heat shock or following recovery and subjected to EMSA using radiolabeled HSE. The positions of the XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

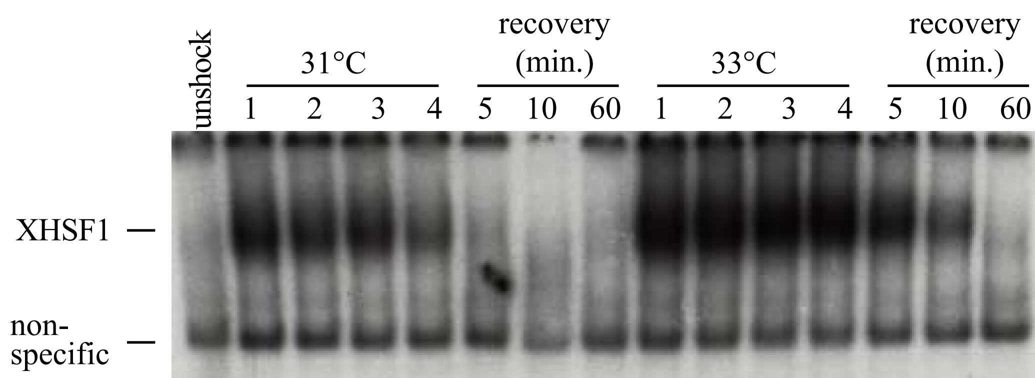



**Figure 18. Attenuation of XHSF1 DNA binding following recovery from long term heat shock.** *Xenopus* oocytes were treated as unshocked (18°C) or heat shocked at 31°C, or 33°C for the indicated times. Following 4 hours of heat shock, oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

allowed to recover again (Figure 19). XHSF1 DNA-binding in oocytes treated in this manner failed to recover within 60 minutes. Therefore, it appears that failure of XHSF1 to recover after a severe heat shock is not due to the absence of a putative signal arising from a moderate stress required to activate the XHSF1 recovery mechanism.

It was hypothesized that there are cellular components comprising an XHSF1 recovery mechanism, and that these components could be isolated and identified by conventional chromatography. In an effort to purify the components required for XHSF1 recovery by conventional chromatography, development of an *in vitro* recovery system was attempted. Unshocked and freshly recovered oocyte extracts were tested for their ability to attenuate XHSF1 DNA-binding *in vitro* following addition to stressed oocyte extracts (33°C for 30 minutes, 39°C for 20 minutes, and 39°C for 20 minutes followed by recovery at 18°C for 24 hours) (Figure 20). Since the depletion of cellular ATP has been proposed as a possible method by which HSF1 DNA-binding is activated (Winegarden et al., 1996), ATP was supplemented to some samples. BSA was also added to some samples to equalize the amount of total protein. Addition of unshocked and recovered extracts to 33°C heat shocked, 39°C heat shocked, and 39°C/24 hour recovered extracts did not affect DNA-binding of activated XHSF1 even in the presence of excess ATP.

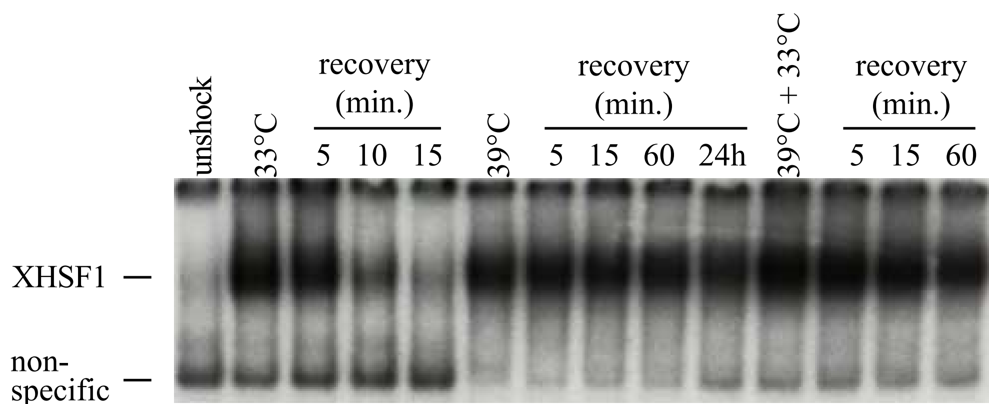



**Figure 19. Attenuation of XHSF1 DNA binding following recovery from sequential 39°C and 33°C heat shock.** *Xenopus* oocytes were treated as unshocked (18°C), or heat shocked at 33°C, or 39°C for 20 minutes, or heat shocked at 39°C, placed in 18°C for 15 minutes and subsequently treated at 33°C for 20 minutes (39°C + 33°C). Following treatment oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

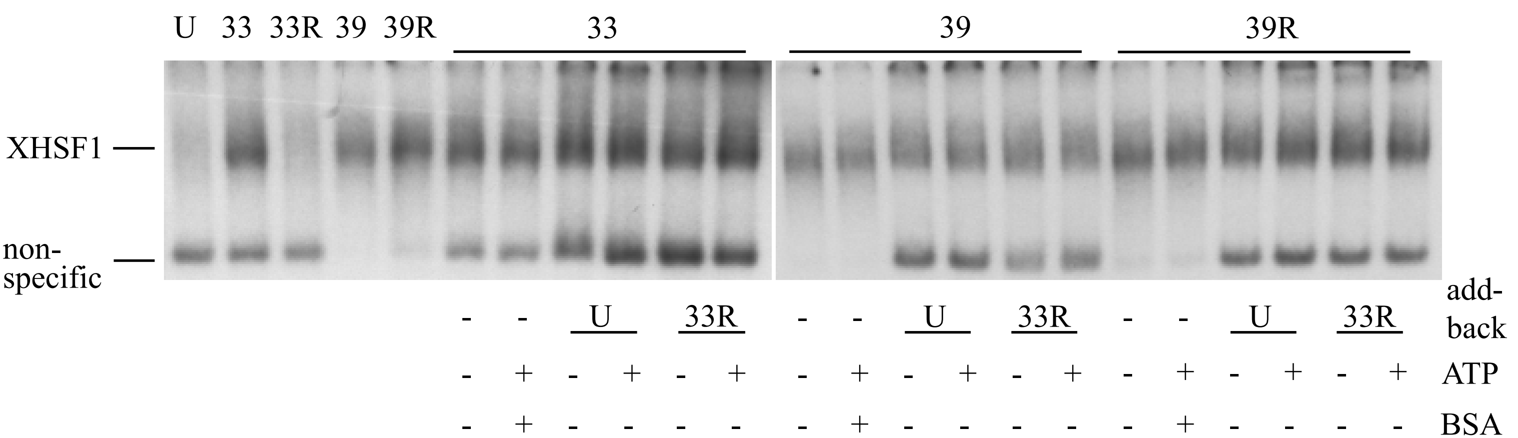



**Figure 20. Attenuation of XHSF1 DNA binding *in vitro*.** *Xenopus* oocytes were treated as unshocked (U, 18°C), or heat shocked at 33°C for 30 minutes (33), 33°C for 30 minutes followed by placement in 18°C media for 15 minutes (33R), 39°C for 20 minutes (39), or 39°C followed by placement in 18°C media for 24 hours (39R). Protein extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. Where indicated 10 µg BSA, ATP to a final concentration of 0.1 mM, or equal volumes of unshocked (U) or 33°C heat shocked and recovered extracts (33R) were added to 33°C, 39°C, and 39°C heat shocked and recovered extracts prior to addition of the radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

### 4.3.3 XHSF1 activated by high temperatures is transcriptionally incompetent

Prolonged activation of XHSF1 DNA-binding after removal of the stress has been observed previously after treatment of oocytes with sodium arsenite (Bharadwaj et al., 1998). However, it was not possible to assess the transcriptional competence of XHSF1 (activated to bind DNA) due to inhibition of the general transcriptional machinery by arsenite. Treatment of cells with high temperature heat shock combined with the experimental flexibility of the large manipulatable oocyte afforded us a unique opportunity to assess the transcriptional competence of HSF1 in the absence of persistent or ongoing stress. Oocytes were injected with either CMV:CAT or HSP70:CAT reporter constructs, incubated overnight at 18°C and heat shocked at either 33°C for 30 minutes or 39°C for 20 minutes (Figure 21B). Following heat shock, oocytes were incubated at 18°C overnight. Extracts of oocytes were made and subjected to CAT assays. As reported previously, 33°C heat shock increased CAT expression from the hsp70 promoter when compared to similarly injected unshocked oocytes (Landsberger and Wolffe, 1995b). Although treatment of oocytes at 39°C induced XHSF1 DNA-binding, it did not increase expression from the Hsp70 promoter. Therefore, XHSF1 DNA-binding and transcription were uncoupled following a 39°C heat shock. Uncoupling of DNA-binding and transcription has been observed in oocytes using different stresses such as mercury, ethanol, methanol, and salicylate (Bharadwaj et

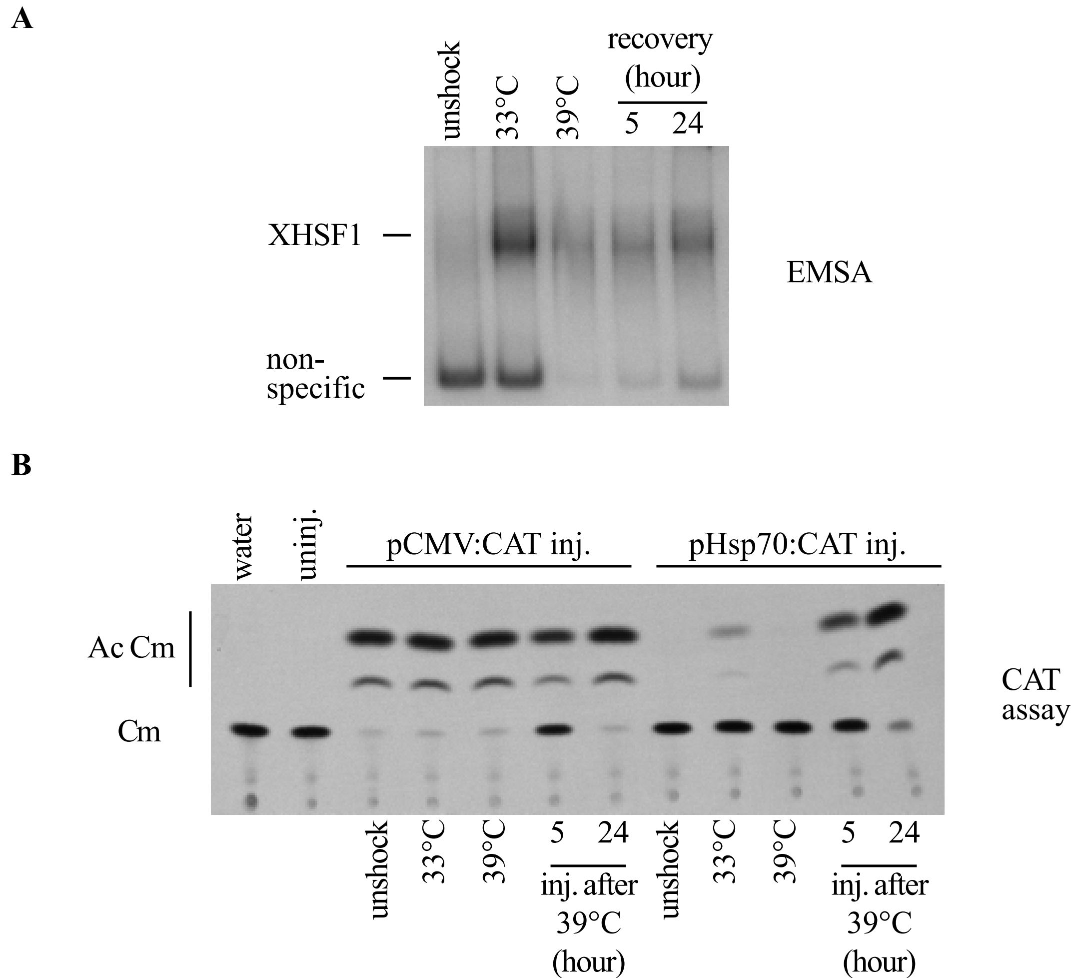



**Figure 21. Transcriptional activity of XHSF1 activated by high temperature heat shock. (A)** *Xenopus* oocytes were treated as unshocked (18°C) or heat shocked at 33°C or 39°C for 20 minutes where indicated. Following heat shock, 39°C treated oocytes were subsequently placed in 18°C media where indicated. Extracts of oocytes were made immediately following heat treatment or at the indicated times following placement in 18°C media and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** *Xenopus* oocytes injected with pCMV:CAT or pHsp70:CAT were incubated for 3 hours at 18°C and subsequently treated as unshocked (18°C) or heat shocked at 33°C or 39°C for 20 minutes where indicated. Where indicated *Xenopus* oocytes were injected with pCMV:CAT or pHsp70:CAT following treatment at 39°C for 20 minutes and placement in 18°C media for 5 or 24 hours. Protein extracts of oocytes were made 24 hours following pCMV:CAT or pHsp70:CAT injection. Extracts of oocytes were subjected to CAT assay. To control for any background CAT activity uninjected oocytes and water were also subject to CAT assay. The positions of the chloramphenicol (Cm) and acetylated chloramphenicol (Ac Cm) are indicated on the left.

al., 1998). To assess the transcriptional competence of 39°C-activated HSF in the absence of ongoing stress, oocytes were treated at 39°C, allowed to recover for either 5 or 24 hours and then injected with either CMV:CAT or Hsp70:CAT plasmids (Figure 21B). Expression from the hsp70 promoter was detected in oocytes injected 5 hours after 39°C heat treatment. The level of expression was higher in oocytes that were injected with HSP70:CAT plasmid 24 hours after 39°C heat treatment. Therefore HSF1 induced transcription from the Hsp70 promoter in 39°C treated oocytes could be achieved depending on when the construct was introduced. The CMV promoter was equally expressed in all experimental groups, under all conditions used in these experiments (Figure 21B). This set of controls indicated that any changes in transcription observed from the Hsp70 promoter were due to specific changes in the activity of that promoter, and not global changes in transcription. EMSA was performed on oocytes from the same female treated at 33°C for 30 minutes, 39°C for 20 minutes, and oocytes heat shocked for 20 minutes at 39°C then allowed to recover at 18°C for 5 and 24 hours (Figure 21A). These results confirm that XHSF1 was competent to bind DNA under the conditions used in the above experiments.

To determine if the severe 39°C heat shock induced permanent transcriptional incompetence of XHSF1, oocytes were injected with either CMV:CAT or Hsp70:CAT reporter plasmid as above, incubated at 18°C overnight and treated at 39°C for 20 minutes, incubated at 18°C for 15 minutes and further heat shocked at 33°C for 20 minutes (Figure 22B). No CAT activity was detected from the Hsp70 promoter in the oocytes heat shocked at 39°C and subsequently heat shocked at 33°C, indicating that the inability of XHSF1 to activate transcription after a severe heat shock was not due to the

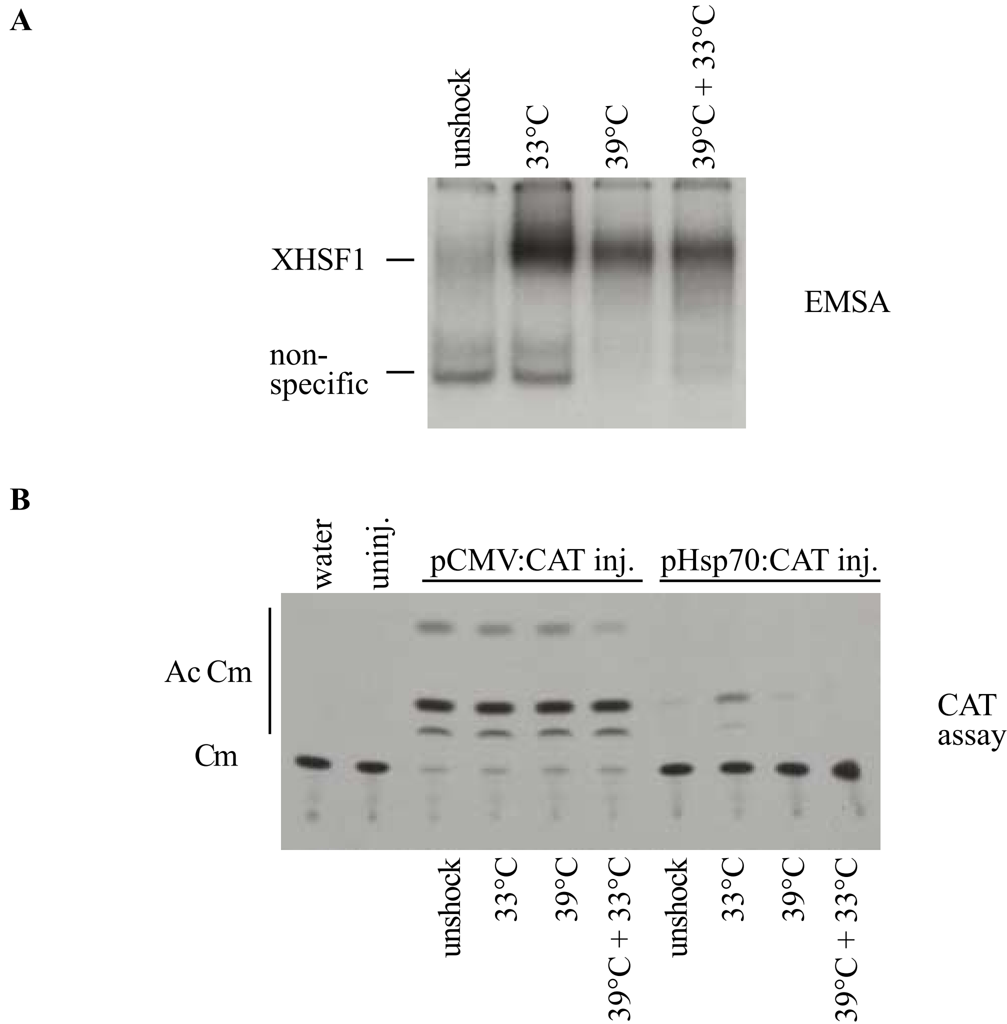



**Figure 22. Transcriptional activity of XHSF1 following subsequent 39°C and 33°C heat shock. (A)** *Xenopus* oocytes were treated as unshocked (18°C), or heat shocked at 33°C, or 39°C for 20 minutes, or heat shocked at 39°C for 20 minutes, placed in 18°C for 15 minutes and subsequently treated at 33°C for 20 minutes (39°C + 33°C). Extracts of oocytes were made immediately following heat treatment and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** *Xenopus* oocytes injected with pCMV:CAT or pHsp70:CAT were incubated for 3 hours at 18°C and subsequently treated as unshocked (18°C) or heat shocked at 33°C, 39°C for 20 minutes, or heat shocked at 39°C for 20 minutes, placed in 18°C for 15 minutes and subsequently treated at 33°C for 20 minutes (39°C + 33°C) where indicated. Protein extracts of oocytes were made 24 hours following pCMV:CAT or pHsp70:CAT injection. Extracts of oocytes were subjected to CAT assay. To control for any background CAT activity uninjected oocytes and water were also subject to CAT assay. The positions of the chloramphenicol (Cm) and acetylated chloramphenicol (Ac Cm) are indicated on the left.

lack of a signal that is only activated at milder temperatures. The CMV promoter was equally expressed in all experimental groups, under all the conditions used in these experiments (Figure 22B). EMSA was performed on oocytes from the same batch treated at 33°C for 20 minutes, 39°C for twenty minutes, or heat shocked at 39°C for 20 minutes, incubated at 18°C for 15 minutes and further heat shocked at 33°C for 20 minutes (Figure 22A). These results confirm that XHSF1 was capable of DNA-binding under the conditions used in the above experiment.

It appears that the putative post-translational modification that activates transcription cannot occur if XHSF1 is bound to DNA. One model that can be proposed which accounts for these observations is that XHSF1 which is activated by a severe heat shock is able to bind to HSEs within heat shock promoters, but remains transcriptionally incompetent for a period of time. XHSF1 bound to these promoters cannot dissociate from DNA, either because the attenuation ability of XHSF1 is disabled, or because the cellular attenuation machinery responsible for the DNA dissociation is disabled. Thus when the oocyte is treated at 39°C after the injection of Hsp70:CAT plasmid, no CAT activity is observed. It is possible that in oocytes not injected with Hsp70:CAT, in the relative absence of HSEs (there is only one set of chromosomes in a very large cell), XHSF1 remains soluble and was slowly modified to become transcriptionally competent. Once the HSE-containing promoters were introduced by microinjection, XHSF1 may have been competent to bind to promoters and activate transcription. This implies that soluble XHSF1 was modified following 5 hours of recovery from 39°C such that DNA-binding was not inhibited, but transcriptional competence was attained.

The activation of XHSF1 by severe heat shock was similar to activation by salicylate, in that both stresses induced DNA-binding but not transcriptional competence (Jurivich et al., 1995; Jurivich et al., 1992; Lee et al., 1995; Winegarden et al., 1996). The characteristics of these two inducers on the heat shock response in oocytes were compared. As discussed previously, treatment of cells with 80 mM salicylate for 1 hour activates XHSF1 DNA-binding (Figure 6A, 23A). However, in no case does salicylate treatment induce the same amount of XHSF1-HSE complex formation as a 33°C heat shock (Figure 6A, 23A). Treatment of oocytes with 80 mM salicylate for 1 hour concurrent with a heat shock at 33°C for the final 20 minutes of salicylate treatment did not increase XHSF1 DNA-binding beyond the level achieved with 80 mM salicylate treatment for 1 hour (Figure 23A). Therefore salicylate appears to inhibit or limit the maximal amount of HSF1 activity seen with 33°C heat shock. To assess the transcriptional competence of salicylate-activated XHSF1, oocytes were injected with CMV:CAT or Hsp70:CAT plasmids, incubated overnight at 18°C, then treated at 33°C for 20 minutes, with 80 mM salicylate for 1 hour, or with 80 mM salicylate concurrent with a 33°C heat shock for the final 20 minutes of salicylate treatment. Oocytes were then incubated overnight at 18°C. Oocytes extracts were made and analyzed by CAT assay (Figure 23B). As reported earlier in our laboratory (Bharadwaj et al., 1998) treatment of oocytes with salicylate did not induce transcription from the Hsp70 promoter (Figure 23B). Treatment of oocytes with 80 mM salicylate concurrent with a 33°C heat shock did not induce transcription from the Hsp70 promoter. Activity from the CMV promoter was not affected by these conditions (Figure 23B). Thus it appears as if salicylate treatment inhibits heat induced transcriptional activation. A less likely

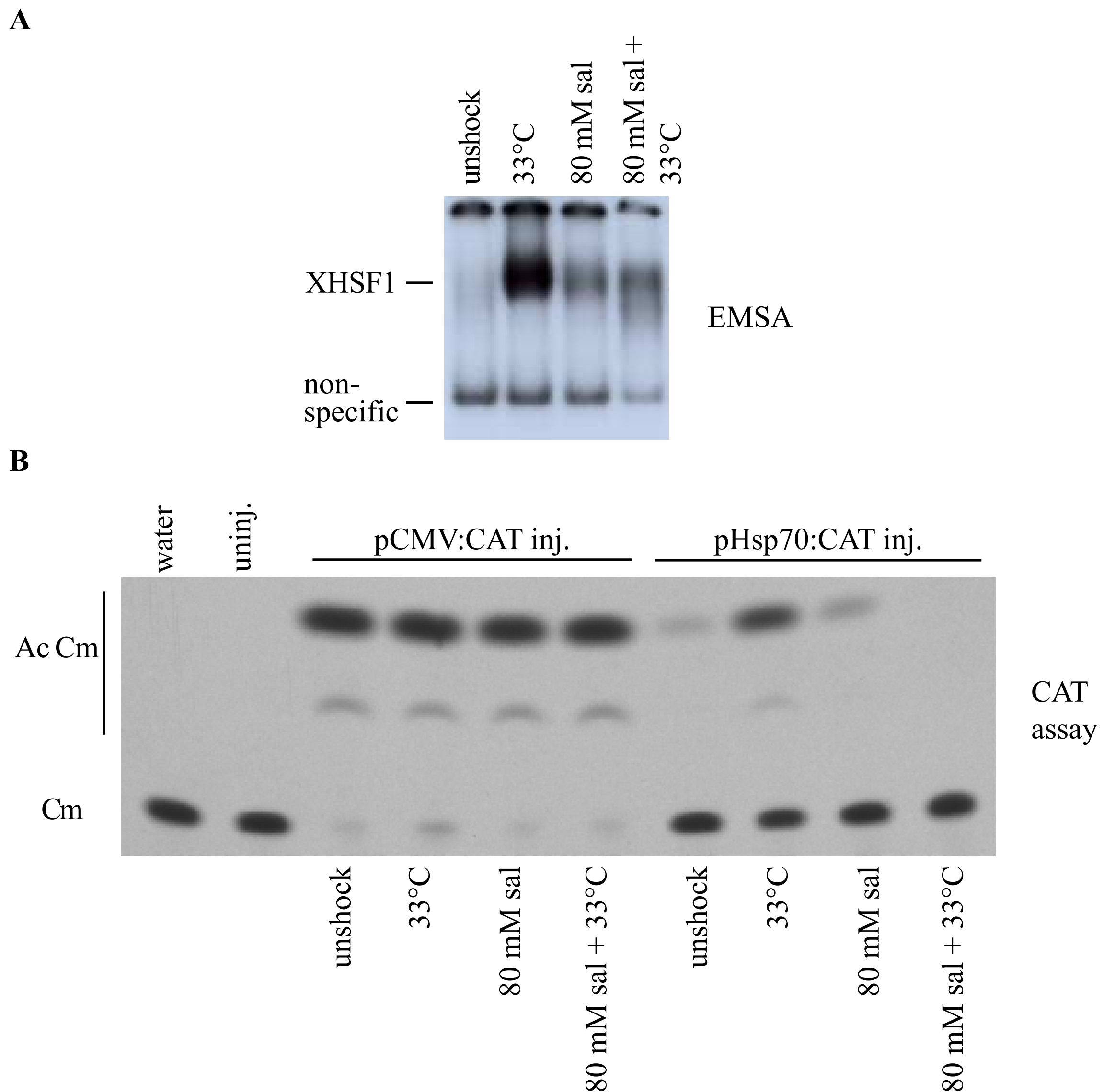



**Figure 23. Transcriptional activity of XHSF1 following salicylate and heat treatment.** **(A)** *Xenopus* oocytes were treated as unshocked (18°C), heat shocked at 33°C for 20 minutes, incubated in media containing the 80 mM salicylate for 1 hour, or incubated in media containing the 80 mM salicylate for 1 hour with incubation at 33°C for the final 30 minutes. Extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** *Xenopus* oocytes injected with pCMV:CAT or pHsp70:CAT were incubated for 3 hours at 18°C and subsequently treated as unshocked (18°C), heat shocked at 33°C for 20 minutes, incubated in media containing the 80 mM salicylate for 1 hour, or incubated in media containing the 80 mM salicylate for 1 hour with incubation at 33°C for the final 30 minutes. Protein extracts of oocytes were made 24 hours following pCMV:CAT or pHsp70:CAT injection. Extracts of oocytes were subjected to CAT assay. To control for any background CAT activity uninjected oocytes and water were also subject to CAT assay. The positions of the chloramphenicol (Cm) and acetylated chloramphenicol (Ac Cm) are indicated on the left.

explanation is that salicylate treatment abrogates the cell stress-sensing mechanism that elicits transcriptional activation of XHSF1.

## *4.4 HSF1 binding partners and post-translational modifications associated with HSF1 DNA-binding and transcriptional activities*

### 4.4.1 Molecular chaperones associated with XHSF1 at different stages in the activation/deactivation cycle

Our laboratory and others have shown that Hsp90 and a number of associated cochaperones interact with XHSF1 and can affect XHSF1 DNA-binding and transcriptional activities (Ali et al., 1998; Bharadwaj et al., 1999; Guo et al., 2001; Xia et al., 1998). The current model of HSF1 regulation suggests that HSF1 is regulated by interaction with a series of molecular chaperone and cochaperone complexes, perhaps containing different subsets of chaperones (Craig and Gross, 1991; Morimoto, 2002; Voellmy, 1996). Different treatments that produce forms of HSF1 at discrete intermediates in the activation-deactivation profile had been characterized. These treatments would potentially be useful in discovering binding partners and/or post-translational modifications that are key in determining the activity of XHSF1. It is possible that different components of the Hsp70/Hsp90 chaperone complex of different XHSF1 post-translation modifications could be detected at discrete stages of the

activation/deactivation cycle. Unshocked cells contain XHSF1 at the beginning of the activation cycle. Cells treated with 80mM salicylate for 1 hour, or heat shocked at 39°C for 20 minutes contain XHSF1 trimers that can bind DNA-but are not transcriptionally active. Cells heat shocked at 33°C for 30 minutes, or heat shocked at 39°C for 20 minutes followed by 24 hours of recovery at 18°C contain XHSF1 that can bind DNA and is transcriptionally active. Finally, cells heat shocked at 33°C for 30 minutes followed by 15 minutes of recovery at 18°C contain XHSF1 at the end of the deactivation cycle.

In order to examine the binding partners of XHSF1 it was first important to determine the size of complexes at each stage of the activation deactivation profile. Cells were treated as described above and subjected to size exclusion chromatography under non-denaturing conditions. Fractions were run on SDS-PAGE and western blots were performed using anti-XHSF1 antibodies (Figure 24). Most of the XHSF1 eluted in fractions 12 and 13 representing a molecular range of 200-400 kDa in unshocked, 33°C, 39°C/recovered, 33°C/recovered, and 80mM salicylate treated cells. XHSF1 from 39°C-treated oocytes was concentrated in fractions 11 and 12, representing a molecular weight of 350-600 kDa. Although this molecular weight determination is not precise, it suggests that XHSF1 is found in a large complex that is similar in size in all of the above treated cells. This could suggest that XHSF1 in unshocked, 33°C, 39°C/recovered, 33°C/recovered, and 80 mM salicylate treated cells may be present in similar high molecular weight complexes and that any changes in XHSF1 activity that occur throughout these treatments are not brought about by changes in the identity of the proteins in the complex. Potential events that could account for differential XHSF1

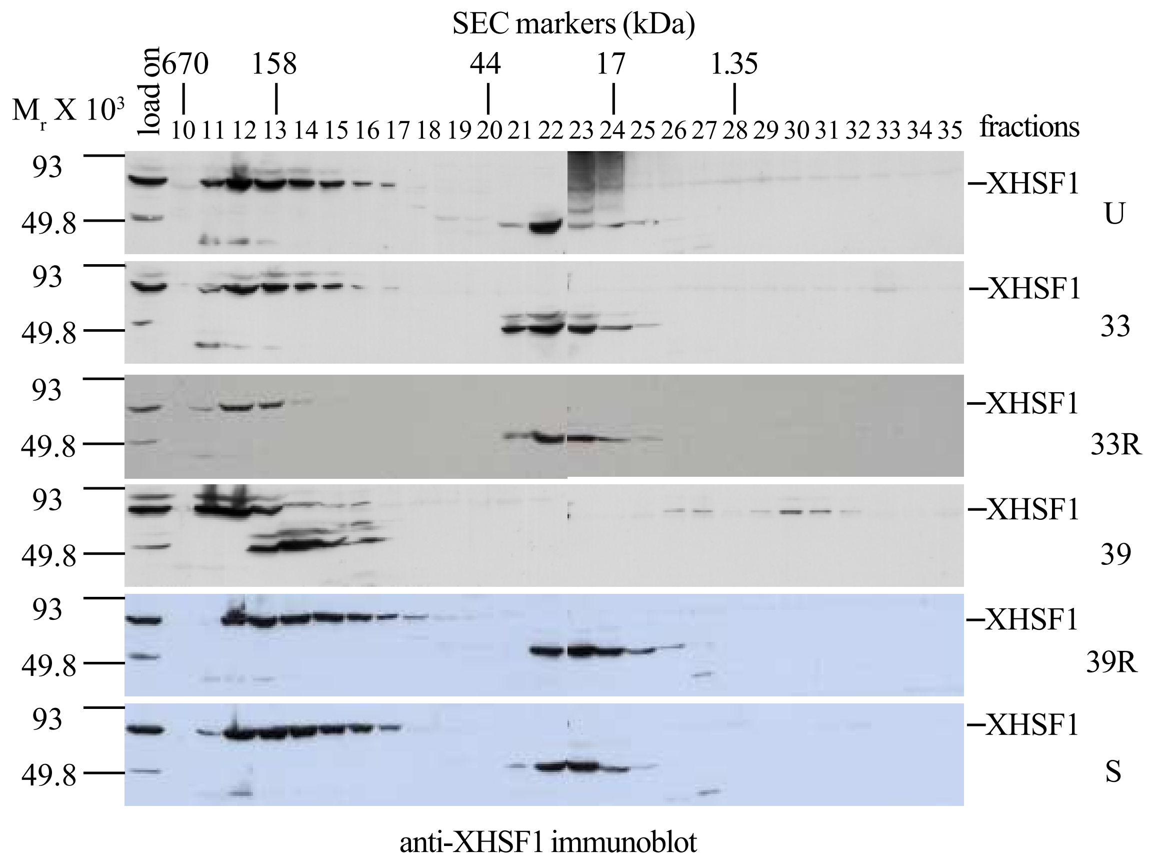



**Figure 24. Molecular weight of XHSF1 containing protein complexes.** *Xenopus* oocytes were treated as unshocked (U, 18°C), heat shocked at 33°C for 30 minutes (33), 33°C for 30 minutes followed by treatment at 18°C for 15 minutes (33R), 39°C for 20 minutes (39), 39°C for 20 minutes followed by treatment at 18°C for 24 hours (39R), or incubated in media containing the 80 mM salicylate for 1 hour (S). Protein extracts of oocytes were made and subjected to SEC (size exclusion chromatography). Fractions were precipitated, subject to SDS-PAGE and immuno-blotted using anti-XHSF1 antibodies. A sample of the load on was also run on the same gel. SEC molecular weight markers are indicated at the top of the figure. SDS-PAGE molecular weight markers are indicated on the left. The position of XHSF1 is indicated on the right.

activity include post-translational modification of XHSF1 such as phosphorylation, SUMO modification, or XHSF1 conformational changes driven by the action of XHSF1-associated molecular chaperones. Another possibility is that there are differences in the components of XHSF1 hetercomplexes that are not detectable by size-exclusion chromatography because the protein-protein interactions are unstable. Surprisingly, a minor quantity of XHSF1 from 39°C cells eluted late with small molecular weight complexes (<1.35kDa). Because this is much smaller than even a single monomer of XHSF1, this may represent XHSF1 molecules that have gained affinity for the resin in the size exclusion column.

Of interest was the mobility of a smaller (approx. 50kDa) protein that cross-reacted with the anti-XHSF1 peptide antibody in fractions of cell extracts passed through the size exclusion column. In unshocked, 33°C, 39°C/recovered, 33°C/recovered, and 80 mM salicylate treated cells, this protein eluted mostly in fractions 22 and 23 representing 20-30 kDa (Figure 24). In 39°C treated oocytes this protein eluted mainly in fraction 14 representing a molecular weight of approximately 150 kDa. The apparent increase in size of the 50 kDa protein only after a 39°C heat shock as assessed by size exclusion column may have been a reflection of cellular damage and protein aggregation that occurs after severe heat stress (Glover and Lindquist, 1998).

It was important to rule out the possibility that the presence of XHSF1 in high molecular weight complexes was not due to non-specific protein aggregation elicited by various treatments. The level of protein aggregation in unshocked, 33°C, 39°C, 39°C/recovered, 33°C/recovered, and 80 mM salicylate treated oocytes was assessed by size exclusion chromatography under non-denaturing conditions as above. Fractions

were run on SDS-PAGE and western blots were performed using anti-PCNA antibodies. It was expected that spurious protein aggregation would be detected by a change in elution profile of PCNA through the column. In all samples the majority of PCNA eluted in fractions 19 and 20 representing a molecular weight range of 40-60kDa, similar to the molecular weight for PCNA observed on SDS-PAGE (Figure 25). Although the identity of the 50 kDa protein that cross-reacted with anti-XHSF1 peptide antibodies remains unknown, the shift in size (molecular weight) of complexes containing this protein in cells treated at 39°C is probably not reflective of large-scale protein aggregation during the various cell treatments.

Several components of the XHSF1 complex have been identified (Bharadwaj et al., 1999). The presence of these components was tested by supershift analysis of cell lysates from 33°C, 39°C, 39°C/recovered, and 80mM salicylate treated oocytes (Figure 26A). Antibodies against XHSF1, Hsp90, p23, FKBP52, Hip, Hop, Cyp40, SUMO-1, ubiquitin, and PCNA were added individually to cell lysates *in vitro* prior to the addition of labeled HSE. Binding reactions were then analyzed by EMSA (Figure 26B). XHSF1 antibodies supershifted XHSF1 from all treatments providing an example of a supershifted XHSF1:HSE complex. Antibodies to Hsp90, p23, FKBP52, Hip, and Hop supershifted HSE bound XHSF1 activated by all treatment regimes. Antibodies to Cyp40, SUMO-1, and ubiquitin failed to supershift HSE bound XHSF1 activated by all treatment regimes. This suggests Hsp90, p23, FKBP52, Hip, and Hop are associated with trimeric XHSF1 activated in cells treated at 33°C, 39°C, 39°C/recovered, or with salicylate. In a previous study, Bharadwaj (Bharadwaj et al., 1999) had observed that antibodies to Hsp90, FKBP52, and p23 supershifted XHSF1 activated by a 33°C heat

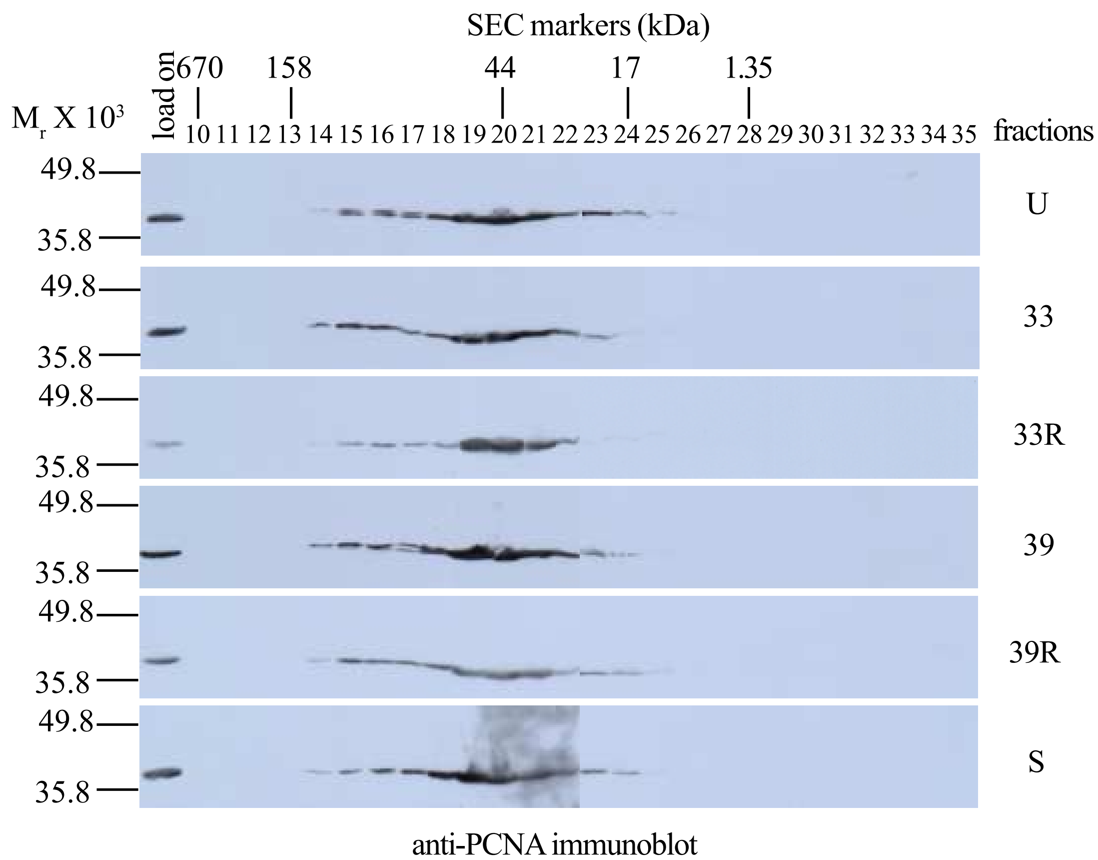



**Figure 25. Protein aggregation in heat shocked *Xenopus* oocytes.** *Xenopus* oocytes were treated as unshocked (U, 18°C), heat shocked at 33°C for 30 minutes (33), 33°C for 30 minutes followed by treatment at 18°C for 15 minutes (33R), 39°C for 20 minutes (39), 39°C for 20 minutes followed by treatment at 18°C for 24 hours (39R), or incubated in media containing the 80 mM salicylate for 1 hour (S). Protein extracts of oocytes were made and subjected to SEC. Fractions were precipitated, subjected to SDS-PAGE and immunoblotted using anti-PCNA antibodies. A sample of the load on was also run on the same gel. SEC molecular weight markers are indicated at the top of the figure. SDS-PAGE molecular weight markers are indicated on the left.

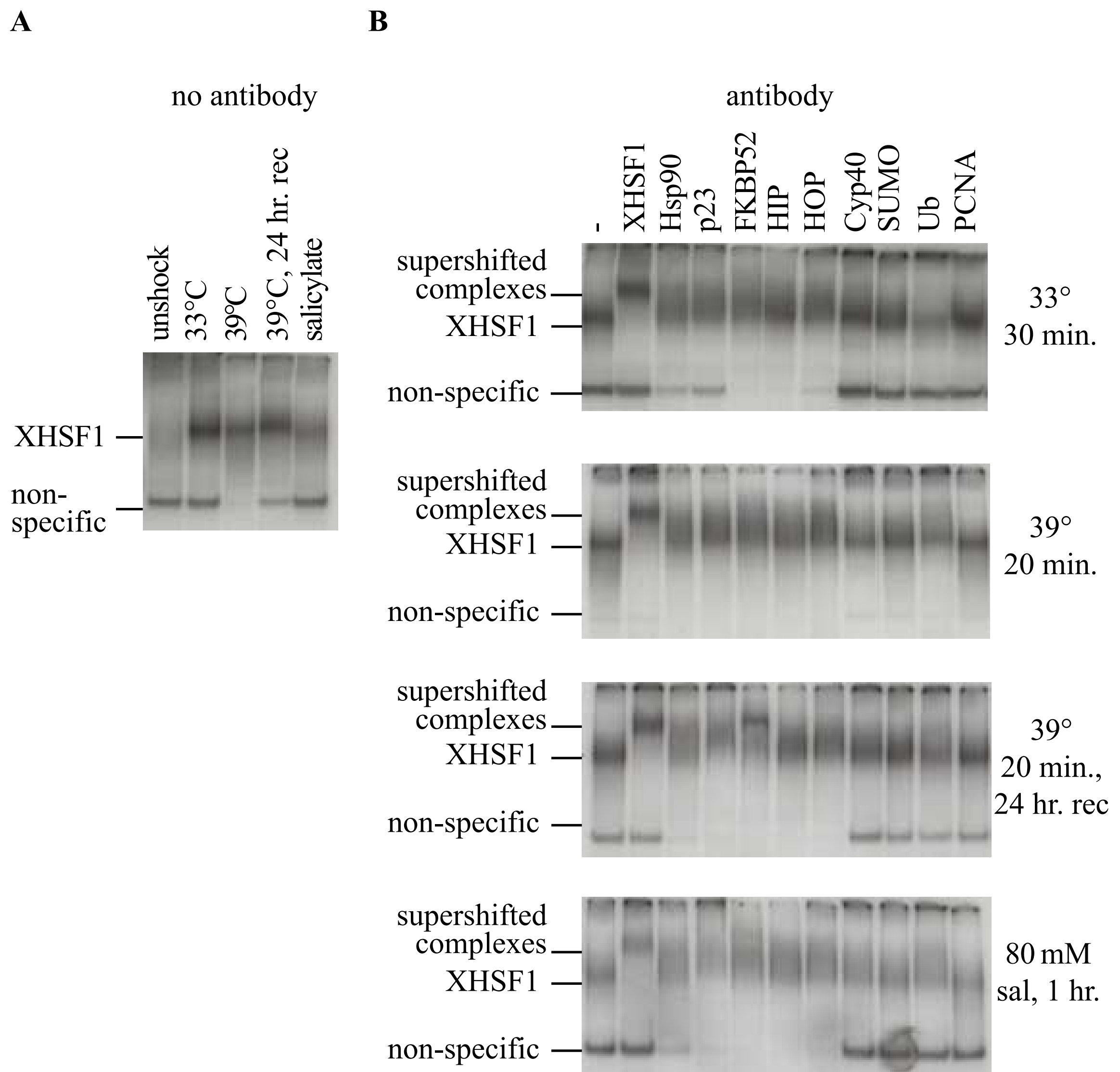



**Figure 26. XHSF1 interacting proteins that recognize DNA bound XHSF1**. **(A)** *Xenopus* oocytes were treated as unshocked (18°C), heat shocked at 33°C for 30 minutes, 39°C for 20 minutes, 39°C for 20 minutes followed by treatment at 18°C for 24 hours, or incubated in media containing the 80 mM salicylate for 1 hour. Protein extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The positions of XHSF1:HSE and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** *Xenopus* oocytes were heat shocked at 33°C for 30 minutes, 39°C for 20 minutes, 39°C for 20 minutes followed by treatment at 18°C for 24 hours, or incubated in media containing the 80 mM salicylate for 1 hour. Protein extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. The indicated antibodies were added to the heat shocked protein extracts prior to addition of HSE. The positions of supershifted XHSF1:HSE, XHSF1:HSE, and the non-specific DNA binding protein:HSE complexes are indicated on the left.

shock or by salicylate treatment. Bharadwaj (Bharadwaj et al., 1999) had also observed that antibodies to Cyp40, Hip, Hop FKBP51, Hsp70 caused minor shifts in the mobility of XHSF1 from cells heat shocked at 33°C or treated with salicylate. The results presented here confirm that Hsp90, p23, FKBP52, Hip, and Hop are associated with trimeric XHSF1 activated by several different treatments. Significant supershifts were not detected in this study when antibodies to Cyp40 were added to cell lysates, despite the use of gradient gels which were used to more precisely differentiate between XHSF1 complexes of different size. The present analysis does not exclude the possibility that other proteins besides those tested may be present in each XHSF1 complex or that different quantities of each chaperone is present in different XHSF1 complexes. These results strengthen the observations from size-exclusion chromatography and also suggest that the complex of proteins associated with XHSF1 are similar in XHSF1 complexes that have different activities.

### 4.4.2 Post-translational modifications associated with XHSF1 at different stages in the activation/deactivation cycle

HSF1 is known to be a heavily phosphorylated molecule both before and after heat shock (Cotto et al., 1996; Fritsch and Wu, 1999; Kline and Morimoto, 1997; Knauf et al., 1996; Xia et al., 1998). Phosphorylation is thought to influence the activity of HSF1 by affecting monomer-trimer transitions and/or transcriptional activity, however a clear role for HSF1 phorphorylation has not been identified (see Introduction). Since phosphorylation of XHSF1 had not been studied, it was important to develop techniques

to visualize XHSF1 phosphorylation. For the first experimental strategy, XHSF1 phosphorylation sites were labeled with $^{32}P$ by incubating oocytes with $^{32}P$ orthophosphate or $^{32}P$-γATP. Following heat shock, XHSF1 would then be purified from the cell lysates by immunoprecipitation using XHSF1 antibodies and phosphorylation would then be visualized by SDS-PAGE electrophoresis, autoradiography, or by tryptic phosphopeptide mapping (Boyle et al., 1991), or HPLC of tryptic fragments of XHSF1 (Juhl and Soderling, 1983; Scott et al., 1993). Labeling of oocyte intracellular proteins was accomplished by incubating oocyte in media containing 1mCi/ml $^{32}P$-orthophosphate overnight, or directly injecting oocytes with undiluted $^{32}P$-orthphosphate or $^{32}P$-γATP. Lysates were made of unshocked or heat shocked (33°C, 60 minutes) oocytes and the proteins were separated and visualized by SDS-PAGE followed by autoradiography. Intracellular proteins were weakly labeled using these techniques (data not shown). XHSF1 was immunoprecipitated from $^{32}P$-labeled cell lysates and subject to SDS-PAGE followed by autoradiography (data not shown). A band corresponding to the molecular weight of XHSF1 was not visible from either unshocked and heat shocked cells. This method was unsuccessful in labeling XHSF1 either in unshocked or heat shocked conditions.

Since direct labeling of XHSF1 proved difficult, indirect means of studying phosphorylation were undertaken. Extracts from unshocked, 33°C, 39°C, and 39°C/recovered cells were treated with calf intestinal phosphatase (a non-specific phosphatase) and subjected to EMSA (Figure 27). Potential dephosphorylation of XHSF1 by the phosphatase could be visualized in EMSA as a change in mobility of DNA-bound XHSF1 or possibly as a change in DNA-binding activity.

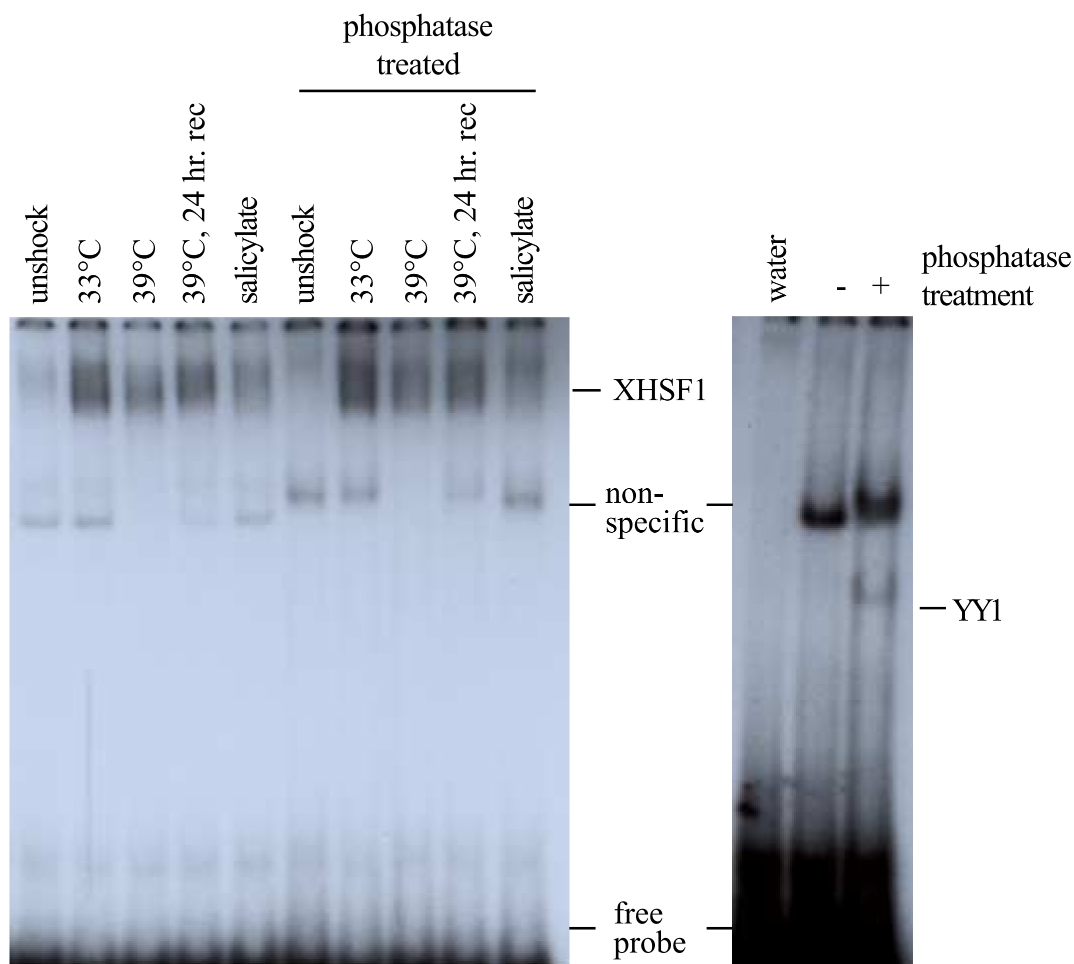



**Figure 27. Mobility of phosphatase treated XHSF1 in non-denaturing gels. (A)** *Xenopus* oocytes were treated as unshocked (18°C), heat shocked at 33°C for 30 minutes, 39°C for 20 minutes, 39°C for 20 minutes followed by treatment at 18°C for 24 hours, or incubated in media containing the 80 mM salicylate for 1 hour. Protein extracts of oocytes were made and subjected to EMSA using radiolabeled HSE. Where indicated, CIP was added to the protein extracts and incubated for 20 minutes at 4°C prior to addition of the radiolabeled HSE. The positions of XHSF1:HSE and the non-specific DNA binding protein:HSE complexes are indicated on the left. **(B)** Protein extracts of *Xenopus* oocytes were made and subjected to EMSA using radiolabeled YY1BE. Where indicated, CIP was added to the protein extract and incubated for 20 minutes at 4°C prior to addition of the radiolabeled YY1BE. In order to visualize the position of the unbound YY1BE water was used to dilute the radiolabeled YY1BE and subjected to EMSA.

Either a change in the mobility of DNA-bound XHSF1 or a change in activity would indicate a difference in the phosphorylation of XHSF1 from differentially treated cells. Since YY1 DNA-binding ability is dramatically increased upon treatment of stage VI oocytes with calf intestinal phosphatases, it was used as positive control (Ficzycz et al., 1997). Treatment of heat shocked extracts with calf intestinal phosphatase did not shift the mobility of XHSF1:HSE complexes nor were there any changes in the level of XHSF1:HSE complexes compared to untreated controls. These results indicated that either XHSF1 is not significantly phosphorylated in oocytes, or that differences in XHSF1 phosphorylation do not alter its mobility on a non-denaturing gel, or that the phosphatase did not dephosphorylate XHSF1 in this experiment.

A final method to study XHSF1 phosphorylation was attempted that involved purification of XHSF1 from cells and digestion of XHSF1 with trypsin followed by mass spectrometry to identify phosphorylated peptides. With a large supply of XHSF1 specific antibodies in hand, the purification of XHSF from cell extracts was attempted using an anti-XHSF1 column. This method of HSF1 purification would have advantages over all published methods that rely on the affinity of HSF1 for the HSE (Rabindran et al., 1991) because it could purify HSF1 from unshocked cells and heat shocked cells. XHSF1 antibodies were conjugated to high pressure Protein-A-Affigel (BIORAD). The titre of the XHSF1 antibody was tested before and after Affigel conjugation (Figure 28). These results indicate that the antibodies successfully bound the Affigel. In order to stabilize the interaction of antibody to Protein-A, the proteins were subject to a coupling reagent (dimethylpimelimidate) that covalently cross-links proteins in a close proximity. XHSF1 was purified from extracts from unshocked, 33°C,

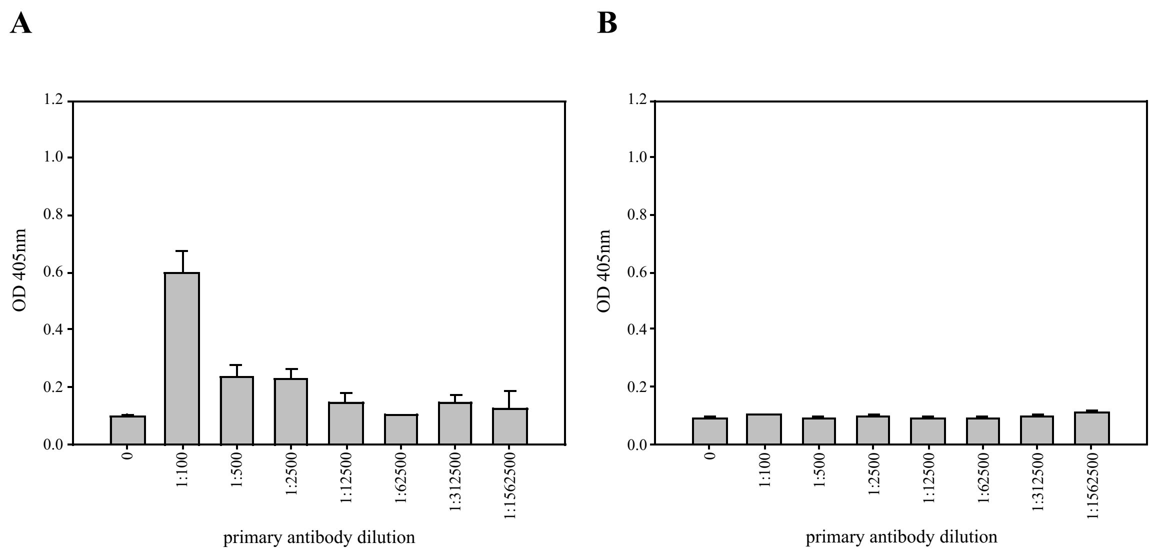



**Figure 28. Production of Protein-A Affigel-anti-XHSF1 column**. **(A)** The titre of XHSF1 antibody solution to BSA-CS3174 prior to conjugation with protein-A Affigel. Quantitation of the ELISA plate is depicted. Each bar represents the average OD 405nm obtained from three samples for the indicated primary antibody dilutions. Included also are standard error bars. **(B)** The titre of XHSF1 antibody solution to BSA-CS3174 following conjugation to Protein-A Affigel. The ELISA plate is depicted along with the quantitation of each sample set below. Each bar represents the average OD 405nm obtained from three samples for the indicated primary antibody dilutions. Included also are standard error bars. ELISA of antibody subsequent to incubation with Protein-A Affigel.

39°C treated cells using the anti-XHSF1 column (Figure 29). Fractions were tested for the presence of XHSF1 by running the fractions on SDS-PAGE and western blotting using XHSF1 antibodies. XHSF1 from unshocked cells eluted in fractions 21-23. XHSF1 from 33°C and 39°C heat shocked cells eluted in fractions 18-21. This may indicate that XHSF1 from unshocked and heat shocked cells has different affinity for the XHSF1 antibody. As harsh conditions were employed to elute XHSF1 from the column, the eluates were tested for the possibility that XHSF1 antibodies had eluted from the column during chromatography despite the previous chemical cross-linking procedure. Unshocked oocyte extracts were applied to the column and the eluate was tested for antibody content by western blotting using goat anti-rabbit secondary antibody (Figure 29). Some of the later fractions (24-26) contained antibody as visualized by blotting with a secondary antibody. This indicates that the elution conditions employed could elute significant quantities of antibody that was presumably not covalently bound to the affigel. XHSF1 could however be separated from contaminating secondary antibody because it eluted earlier (fractions 18-22). Staining of parallel SDS-PAGE gels with sypro stain revealed that several fractions contained XHSF1 at approximately 80-95% purity (data not shown) however XHSF could not be purified in significant quantities to be used for mass spectrometry. In order to increase the yield of XHSF1, purification was attempted in batch (Figure 30), however only a minor improvement in yield was realized. This line of investigation was not subsequently pursued.

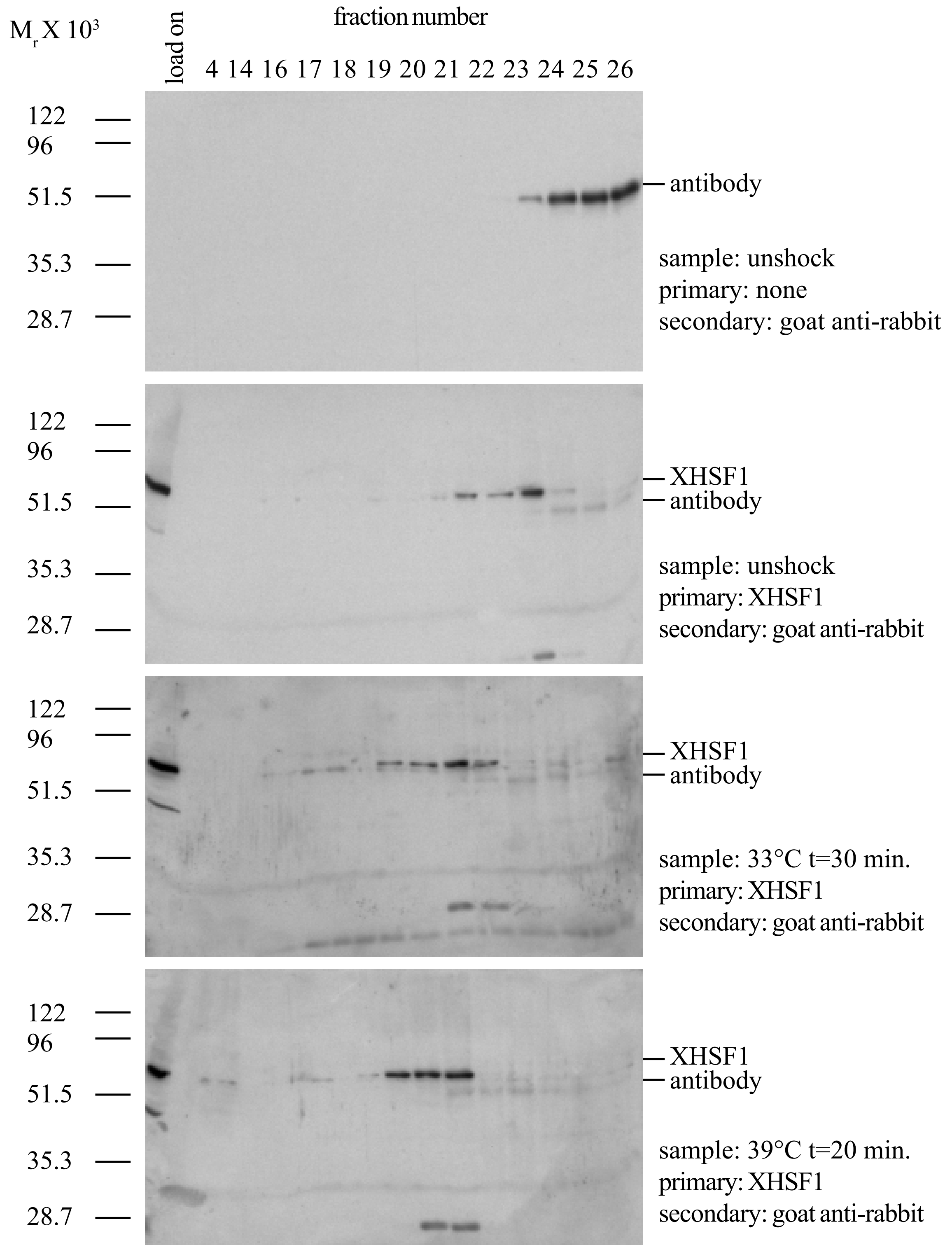



**Figure 29. Purification of XHSF1 using an anti-XHSF1 column.** *Xenopus* oocytes were treated as unshocked (18°C) or heat shocked at 33°C for 30 minutes, or 39°C for 20 minutes. Protein extracts of oocytes were made immediately following treatment and subject to anti-XHSF1 chromatography. Fractions were precipitated, subjected to SDS-PAGE and immuno-blotted with antibodies as indicated. A sample of the load on was also run on the same gel. SDS-PAGE molecular weight markers are indicated on the left.

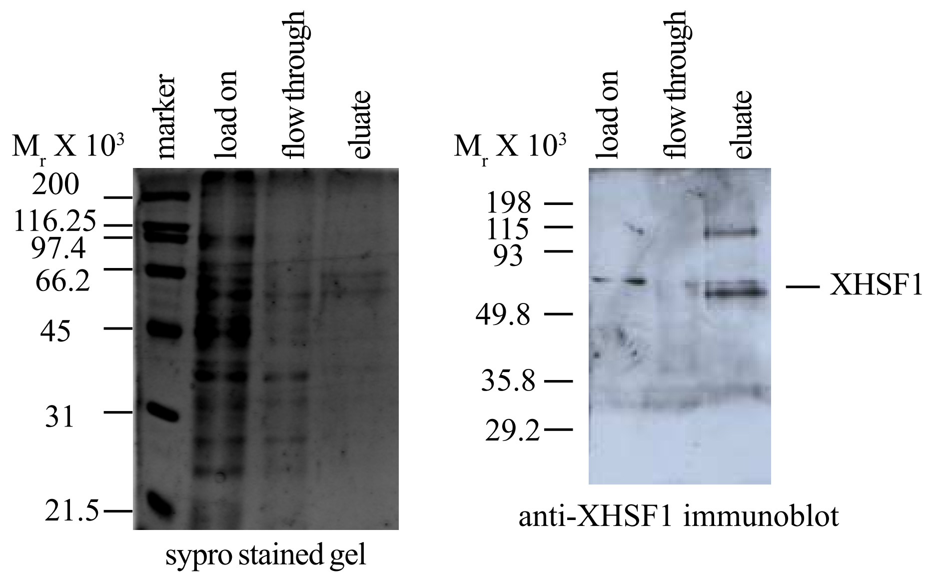



**Figure 30. Purification of XHSF1 using anti-XHSF1 Protein A-Affigel in batch.** Protein extracts of unshocked (18°C) Xenopus oocytes were made and used in batch purification of XHSF1 using anti-XHSF1 protein A-Affigel. Load on, flow through, and eluate of were subjected to SDS-PAGE and stained with sypro orange (left) or subject to SDS-PAGE and immuno-blotted using anti-XHSF1 antibodies (right). SDS-PAGE molecular weight markers are indicated on the left. The position of XHSF1 is indicated on the right.

# 5 Discussion

The focus of this project was the mechanism of regulation of HSF1. Individual projects were designed around the use of the *Xenopus* oocyte model system that affords unique methods of investigation to address issues surrounding HSF1 regulation. Use of the *Xenopus* oocyte system also allowed me to build upon contemporary theories concerning the role of a chaperone heterocomplex in HSF1 regulation that had been developed by our laboratory. Specifically, this research was aimed at studying the regulatory mechanisms of HSF1 activation that is characterized by a monomer to trimer conversion with a concomitant increase in DNA-binding ability upon stress, acquisition of transcriptional competence, and eventual deactivation following the resumption of normal conditions. The data presented in this thesis increase the understanding of the mechanisms of HSF1 regulation.

The major findings of the thesis are:

1. The activation set-point of HSF1 DNA-binding in response to heat is determined both by inherent properties of the HSF1 molecule and interaction with the cellular environment.

2. Exogenous HSF1 molecules expressed in oocytes retain their activation set-points in response to salicylate.

3. Attenuation of XHSF1 DNA-binding during recovery from a high temperature heat shock (39°C) is delayed.

4. HSF1-dependent transcription is inhibited during a high temperature heat shock in an HSF1-dependent manner.

5. The transcriptional competence of HSF1 trimers is established prior to DNA-binding.

6. The components of the HSF1-chaperone heterocomplex did not change appreciably during all phases of the activation/deactivation pathway.

7. Antibodies specific to XHSF1 were produced during the course of these studies that will be instrumental in future studies of the stress response in *Xenopus*.

## 5.1 Activation of HSF1 DNA-binding

A major finding reported in this thesis is that both interactions with the cellular environment and innate properties of the molecule allow HSF1 to set its DNA-binding activation set-point in response to heat. The set-point of heat activation of hHSF1 DNA-binding in oocytes was lower compared to the heat activation set-point of hHSF1 DNA-binding in human cells (39°C versus 42°C) indicating that activation of hHSF1 *in vivo* is

not a simple function of the absolute environmental temperature and that the cellular environment can modulate the HSF1 activation set-point in response to heat (Figure 5). If activation of HSF1 DNA-binding was a direct function of temperature, then one would expect hHSF1 to retain the temperature activation profile observed in human cells when introduced into a different environment. Importantly, the activation profile of hHSF1 DNA-binding due to heat stress in oocytes was not lowered to the activation set-point of XHSF1 in oocytes (Figure 5). This implies that although the cellular environment plays a role in determining the activation set-point of hHSF1, it is not the sole determining factor and cannot completely reprogram hHSF1 to the activation profile of the endogenous factor. Only by observing the DNA-binding activity of hHSF1 in *Xenopus* oocytes has this observation been made. It is important to note that these conclusions were formulated based on the assumption that HSF1s from human and *Xenopus* did not form heterotrimers. Experiments described in Figure 8 could not confirm or deny this assumption.

Some studies have suggested that the primary determinant of HSF1 activation in response to heat was the absolute temperature while others suggested that the cellular milieu determined the HSF1 activation set-point. DNA-binding of purified HSF1 could be activated by similar temperatures as HSF1 *in vivo* suggesting that direct sensation of heat by HSF1 may be the dominant mechanism of HSF1 induction in cells (Farkas et al., 1998; Goodson and Sarge, 1995; Larson et al., 1995; Zhong et al., 1998). Conversely, hHSF1 expressed in *Drosophila* cells (Clos et al., 1993) and tobacco protoplasts (Treuter et al., 1993) adopted the temperature activation profile of the endogenous factors in response to heat, suggesting that the cellular environment was the primary determinant of the activation set-point of HSF1 in response to heat. The degree to which activation

of hHSF1 DNA-binding is regulated by cellular factors varies depending on the cellular milieu (*Xenopus* oocytes versus *Drosophila* or tobacco protoplasts).

Exogenous HSF1s expressed in oocytes retained their activation set-points in response to salicylate (Figure 6). Zhong et al. (Zhong et al., 1998) have observed that salicylate cannot activate purified HSF1 and suggested that salicylate activated HSF1indirectly through cellular factors. A model that can account for these observations is that the cellular factors that affect HSF1 DNA-binding are activated by salicylate with similar kinetics and concentration dependence in *Xenopus* oocytes as they are in *Drosophila* and human cells. The ability of exogenous HSF1s to retain their activation set-points in response to salicylate represents a difference in the ability of these cellular factors to activate HSF1s from different species.

## *5.2 High temperature heat shock prevents synthesis of Hsps in an HSF1 dependent manner*

Attenuation of HSF1 DNA-binding during recovery from a high temperature heat shock is delayed. XHSF1 DNA-binding is optimally activated in cells heat shocked at 33°C-36°C (Figure 5). Following heat shock at these temperatures XHSF1 DNA-binding recovers within 5-10 minutes (Figure 7). Heat shock treatment at 37°, 38°, and 39°C all delayed attenuation of XHSF1 DNA-binding during recovery (Figure 16). XHSF1 DNA-binding did not attenuate following 24 hours of recovery or before the eventual death of the oocyte following a 20 minute 39°C heat shock. To my knowledge, a delay in HSF1 recovery following high temperature heat shock has not been reported

in any system. The observation that HSF1 DNA-binding from a 39°C followed by a subsequent 33°C heat shock did not recover indicates that the delay in recovery of HSF1 DNA-binding during recovery from a 39°C heat shock is not due to the lack of a signal normally activated by a 33°C, but represents an inhibition in the recovery mechanism that normally functions to deactivate HSF1 DNA-binding.

These results are consistent with the model in which Hsps operate in a feedback loop to limit their own production following heat stress by down-regulating HSF1 as originally proposed by DiDomenico (DiDomenico et al., 1982). Increases in the severity of stress such as increases in the temperature of heat shock to 37°, 38°, and 39°C, or increases in the length of time of heat shock at 39°C, all increase the delay of attenuation of XHSF1 DNA-binding (Figure 16). Increases in stress may cause increased protein damage and aggregation that sequesters Hsps for longer periods of time. These Hsps would therefore be unavailable to attenuate XHSF1 DNA-binding. Such a delay in the attenuation of XHSF1 is not observed following recovery from long term (4 hour) continuous heat shocks at 31° and 33°C, suggesting that low temperature heat shocks may induce cellular damage that may be more easily repaired by existing molecular chaperones than the damage elicited by a higher temperature heat shock.

HSF1 activated by a high temperature heat shock was not transcriptionally active (Figure 21,22). Similarly, in human cells high temperatures (44-46°C compared to the optimal 42°C heat shock) activate HSF1 DNA-binding but Hsp expression is not increased (Samali et al., 1999). Furthermore, initial activation of HSF1 at 39°C prevents HSF1-dependent expression from a subsequent 33°C heat shock (Figure 22). Thus,

during a high temperature heat shock Hsp synthesis is prevented and Hsp expression is inhibited during recovery until the eventual death of the cell.

What is the physiological relevance of producing an HSF1 trimer that cannot activate transcription, cannot dissociate from DNA even following the resumption of normal conditions, and prevents the upregulation of Hsps following a high temperature heat shock? Hsp induction may be inhibited during severe stress to ensure that the cell commits to death. Hyperthermia has been characterized as a mutagen in microorganisms, plants, insects, and mammalian cells (Hahn, 1982; Kato, 1980; King and Wild, 1983; Lindgren, 1972; Speit, 1980). It would therefore be advantageous for a multicellular organism for cells sustaining a high temperature heat shock to be eliminated. Inhibiting Hsp synthesis would abort any attempt of the cell to repair cellular damage.

High temperature heat shock promotes apoptosis by inhibiting the production of Hsp70, 90 and 27 that all have anti-apoptotic activities (reviewed in (Beere, 2001; Beere and Green, 2001; Garrido et al., 2001; Jolly and Morimoto, 2000)). Hsp70 can inhibit apoptosis at several points in the apoptotic pathway. Hsp70 inhibits caspase processing by interacting with Apaf-1, preventing the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Saleh et al., 2000). Hsp70 also has anti-apoptotic effects downstream of the caspase-3 protease (Jaattela et al., 1998). Hsp70 also inhibits JNK activation, which when activated promotes apoptosis by altering the half-life of p53 (Fuchs et al., 1998a; Fuchs et al., 1998b), phosphorylates c-Myc (Noguchi et al., 1999) and more importantly causes the release of cytochrome c from mitochondria (Tournier et al., 2000). Activated JNK can also neutralize Bcl-2 and Bcl-XL, two anti-apoptotic proteins (Fan et al., 2000; Maundrell et al., 1997; Yamamoto et al., 1999). Hsp70 has

been shown to modulate JNK by inhibiting stress-induced dephosphorylation (Meriin et al., 1999) or by directly inhibiting is association with SEK1, an upstream kinase (Park et al., 2001). Hsp70 also inhibits apoptosis in a Apaf-1-independent manner by binding to and inhibiting AIF (apoptosis inducing factor), a pro-apoptotic factor released by mitochondria in response to stress (Loeffler et al., 2001; Ravagnan et al., 2001; Susin et al., 1999). Hsp70 can also suppress PKR, a pro-apoptotic kinase (Pang et al., 2002). Hsp90 directly associates with Apaf-1 and prevents its oligomerization (Pandey et al., 2000), binds to and stabilizes the active form of Akt, an anti-apoptotic kinase (Sato et al., 2000), stabilizes RIP (receptor interaction protein) which is a component of the tumor necrosis factor receptor 1 complex that activates NF-κB and promotes cell survival (Lewis et al., 2000), and stabilizes Raf-1 and its interaction with Ras which can modulate cellular survival by modulating the Ras-Raf-MAPK pathway (Schulte et al., 1995; Schulte et al., 1996) . Hsp27 sequesters cytochrome c and prevents its association with Apaf-1 (Bruey et al., 2000), and suppresses Daxx-Ask1 dependent Fas-induced cell death by inhibiting the association of Daxx to Fas ligand (Mehlen et al., 1996).

Inhibition of Hsp production by the genetic deletion of HSF1 also suggests that Hsps are protective. Deletion of HSF1 in human, *Drosophila*, and yeast sensitizes cells to heat-induced apoptosis and prevent cells from becoming thermotolerant (Jedlicka et al., 1997; McMillan et al., 1998; Smith and Yaffe, 1991).

Inhibition of HSF1 at high temperatures may have evolved to prevent the fruitless repair of a severely damaged cell and also prevent the production of anti-apoptotic factors in a situation in which it would be advantageous for that cell to die. Results from experiments in human cells suggest that cells die when exposed to higher heat shock

temperatures. HSF1 is activated in response to moderate heat stress (42°C in human cells), Hsps are expressed and the cell survives. In response to higher temperatures (44-46°C) Hsps are not expressed and cells begin to die (Samali et al., 1999). A 44°C heat shock induced cell death primarily by apoptosis and at 46°C most cells died by necrosis (Samali et al., 1999).

The most significant result in this thesis is that no transcriptional activity is observed when pHsp70:CAT is injected into oocytes prior to a 39°C heat shock, but if pHsp70:CAT is injected in oocytes 5 hours after a 39°C heat shock, HSF1 transcriptional activity is detected (Figure 21). My interpretation of this observation is that a 39°C heat shock induces a transcriptionally inactive trimer that tightly binds the HSE. Thus, if pHsp70:CAT is injected into oocytes prior to heat shock, no transcriptional activity from this construct is detected. During recovery following a 39°C heat shock, soluble HSF1 not bound to DNA is produced in a transcriptional active HSF1 form. This step may either involve the conversion of transcriptionally inactive trimers to active ones by an unknown mechanism or the trimerization of monomeric HSF1 not activated by the original 39°C heat shock. Thus, if pHsp70:CAT is introduced during the recovery phase, transcriptionally active HSF1 can bind DNA and activate transcription. This model suggests that HSF1 trimers produced by a 39°C heat shock that are bound to DNA cannot be modified to a transcriptionally active form. During a high temperature heat shock, Hsp expression is inhibited by the production of a transcriptionally inactive trimer that prevents the action of transcriptionally active HSF1 that is formed *in vivo* during the recovery period. This model also suggests that HSF1

trimers are formed in transcriptionally active or inactive conformations prior to DNA-binding.

These results are not necessarily exclusive of a model of 2-stage activation of HSF1 wherein HSF1 gains DNA-binding activity and subsequently acquires transcriptional competence. As discussed previously, the 2-stage model of HSF1 activation was developed primarily by studies performed on yeast HSF1. Evidence that HSF1 is activated in two discrete steps comes from observations of human cells in which HSF1 transcription was activated by heat in cells pretreated with salicylate (Cotto et al., 1996; Lee et al., 1995). These observations suggested that the transcriptional activity of HSF1 could be induced subsequent to DNA-binding. However, in yeast, *Drosophila*, and rat cells heat cannot activate HSF1 transcriptional activity when pretreated with salicylate (Giardina and Lis, 1995; Locke and Atance, 2000; Winegarden et al., 1996). In addition, pretreatment of oocytes with salicylate inhibited HSF1-dependent transcription following heat shock (Figure 23). Whether the difference between yeast, *Drosophila*, rat, and *Xenopus* with human cells is due to differential effects of salicylate on these cells or genuine differences in how HSF1 is regulated is not known. It is possible that under some circumstances, such as high temperature heat shock, HSF1 trimers are produced that are locked in a transcriptionally inactive state. Other stresses activate HSF1 trimers that have the potential to become transcriptionally active and undergo a step-wise activation process in which HSF1 binds DNA and then gains transcriptional competence.

### 5.3 HSF1 binding partners and post-translational modifications associated with HSF1 DNA-binding and transcriptional activities

During the course of this thesis a number of cell treatments in which HSF1 could be analyzed were characterized at different stages of the activation/deactivation cycle. These treatments were used to compare the properties of HSF1 at different stages of the activation/deactivation cycle in an effort to determine if specific HSF1 binding partners or post-translational modifications were observed at these stages of activation. Inactive monomeric HSF1 was studied from untreated oocytes maintained at 18°C. Treatment of cells with either 80 mM salicylate for 60 minutes, or 39°C heat shock for 20 minutes produced HSF1 that can bind DNA-but not activate transcription (Figures 21, 22, 23). Treatment of cells at 33°C for 30 minutes or 39°C for 20 minutes followed by an incubation at 18°C for 24 hours produced HSF1 that is transcriptionally active (Figures 21, 22, 23). Recovered HSF1 can be studied from oocytes treated at 33°C for 30 minutes followed by incubation at 18°C for 15 minutes (Figure 17).

Size exclusion chromatography of HSF1 from each of the described treatments indicates that HSF1 is found in a high molecular weight complex that is similar in size (350-600 kDa) at each discrete stage during activation and deactivation. Nunes has also observed that unshocked and heat shocked HSF1 can be found in a high molecular weight complex (Nunes and Calderwood, 1995). This is consistent with the hypothesis that a variety of molecular chaperones are involved in the regulation of the monomeric form of HSF1 and at each stage of activation and deactivation.

Supershift analysis determined that transcriptionally active and inactive HSF1 are all associated with the same molecular chaperones including Hsp90, p23, FKBP52, Hip and Hop (Figure 26). Salicylate treated and 33°C activated *Xenopus* HSF1 has been previously shown to interact with Hsp90, p23, and FKBP52 (Bharadwaj et al., 1999). The difference in these studies may be due to the gradient gels used in this study that served to accentuate slight mobility shifts upon addition of antibody. These results suggest that the presence or absence of these molecular chaperones are not responsible for the transition of HSF1 from a transcriptionally inactive to active form. This does not exclude the possibility that differential activities of these chaperones can promote the transcription of HSF1 from transcriptionally inactive to active form. It is also a possibility that a phosphorylation event is the trigger that activates the transcriptional activity of HSF1.

## *5.4 Model of HSF1 regulation*

Despite the increase in the literature concerning the regulation of HSF1 by a molecular chaperone heterocomplex, a comprehensive model of the regulation of HSF1 activation and deactivation has not been developed. I propose a speculative model of HSF1 regulation by molecular chaperones, kinases and phosphatases using current literature addressing the regulation of HSF1 and steroid receptors (Figure 31). Knowledge of the regulation of steroid receptors can be applied to HSF1 because of the similarities in the interactions and physiological roles of many molecular chaperones with these transcription factors. The purpose of a comprehensive model of HSF1

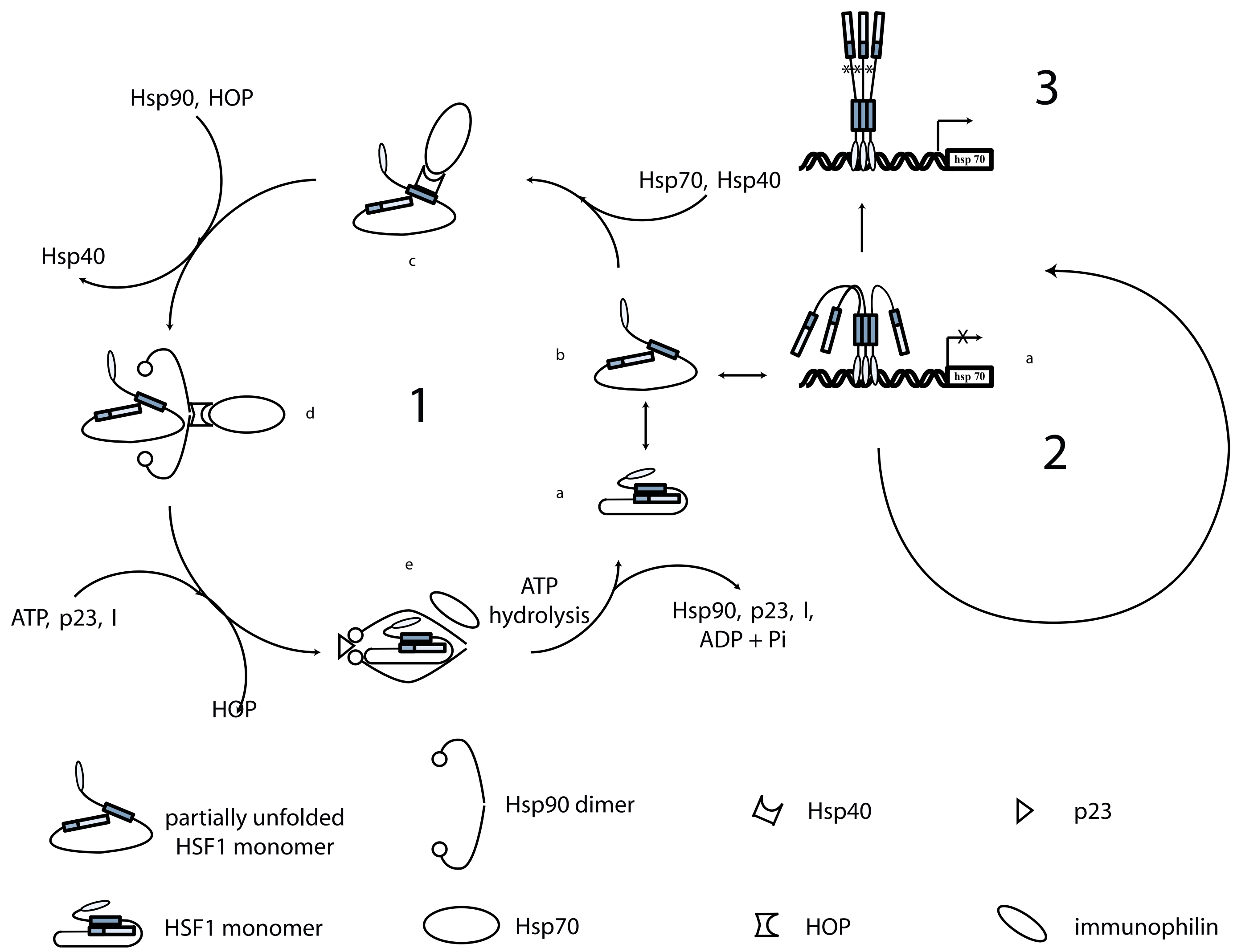



**Figure 31. Proposed model of HSF1 regulation.** For a description of the model refer to the text (Discussion, section 5).

regulation is to provide a context for which contemporary findings presented in this thesis can be applied. In summary, HSF1 regulation involves cooperation of three distinct stress sensing mechanisms, including HSF1 itself, members of the chaperone machine that interacts with HSF1, and one or a number of stress sensing signal transduction pathways. I hypothesize that each one of these systems participates in the sensing of stress and the upregulation of stress proteins.

This model makes no distinction between cognate and inducible forms of molecular chaperones. The activities of cognate and inducible forms of Hsps are indistinguishable, and HSF1 can be properly regulated in cells that do not display an appreciable increase in inducible Hsps, indicating that either the cognate or inducible forms of the chaperones can contribute to the negative regulation of HSF1.

This HSF1 regulatory model was developed under the presumption that the mechanism of HSF1 regulation in metazoans is universal. However, the model does not exclude cell type specific variation in the quantity and quality of the stress response. Phosphorylation and dephosphorylation events that affect HSF1 activity could easily alter intramolecular and intermolecular HSF1 interactions that alter the equilibrium and kinetics of HSF1 at any point in the regulatory cycle. Additionally, any difference in the cellular concentrations of any of the members of the HSF1 regulatory cycle could also alter the equilibrium and kinetics of HSF1 in the regulatory cycle (discussed in (Nollen and Morimoto, 2002)).

Studies of the initial stages of HSF1 activation i.e. trimerization and acquisition of DNA-binding ability, using purified HSF1, have suggested that there must be a minimum of two mechanisms that activate HSF1 DNA-binding, a mechanism in which HSF1 directly senses the stress, and at least one other indirect mechanism that involves

other cellular factors that sense stress and activate HSF1. Stresses such as heat, hydrogen peroxide, and low pH can all directly activate purified HSF1 DNA-binding in the physiological range (Farkas et al., 1998; Goodson and Sarge, 1995; Larson et al., 1995; Zhong et al., 1999; Zhong et al., 1998). That DNA-binding activity of purified HSF1 can be induced by heat in the physiological range with similar kinetics as the HSF1 *in vivo* suggests that direct sensation of heat by HSF1 may be the dominant mechanism of HSF1 induction in cells by heat shock. Results presented in this thesis suggest that both interaction with the cellular environment and innate properties of HSF1 determine the activation set-point in response to heat *in vivo*. Stresses such as salicylate, dinitrophenol, ethanol, and arsenite, however, cannot activate purified HSF1 DNA-binding, indicating that these stresses activate HSF1 *in vivo* by one or several indirect mechanisms that must involve cellular factors (Zhong et al., 1998).

Results presented in this thesis have led me to propose a model of how the trimerization and DNA-binding activity of HSF1 is regulated in unstressed cells and during stress (Figure 31 (1)). The critical premise of this model is that monomeric naked HSF1 has a tendency to unfold and will form trimers that would be stabilized by HSEs within the genome (Figure 31 (1a-b)). Such an idea has been proposed by studies using purified HSF1. In the absence of HSEs, purified HSF1 assumes a monomeric state (Zhong et al., 1998). If HSEs are added to a purified solution of HSF1 the trimeric DNA-binding form of HSF1 is stabilized in the absence of heat (Zhong et al., 1998) as the model predicts. An Hsp70/Hsp90 based chaperone system is constantly required to refold HSF1 into a stable monomeric form (Figure 31 (1c-e)).

I propose that the Hsp70/Hsp90 based chaperone functions in a stepwise manner to negatively regulate HSF1 DNA-binding in unstressed cells in a mechanism similar to

that which has been established for steroid receptors (reviewed in (Morimoto, 2002; Nollen and Morimoto, 2002; Pratt and Toft, 1997) and discussed in the Introduction). Initially (Figure 31 (1c)) Hsp70 is recruited to nascent HSF1 or free partially unfolded monomeric HSF1 (Figure 31 (1b)) by direct interaction with Hsp40. Hsp70 is recruited to HSF1 in an ATP bound form. Upon HSF1 binding, Hsp40 stimulates the ATPase activity of Hsp70, stabilizing the interaction of Hsp70 with HSF1. Exchange of ADP for ATP allows Hsp70 to repeatedly bind and release HSF1. At a certain point HSF1 is transferred from Hsp70 to Hsp90 by Hop (Figure 31 (1d)). Hop and a dimer of nucleotide free Hsp90 are recruited by direct interaction of Hop with both Hsp70 and Hsp90. Hop displaces Hsp40 from Hsp70 and Hsp70 dissociates from the complex. Hop then dissociates from the complex, followed by ATP binding of Hsp90 and the association of p23 and an immunophilin with Hsp90 (Figure 31 (1e)). ATP bound Hsp90 forms a molecular clamp around the substrate that stabilizes the interaction of HSF1 with Hsp90. HSF1 is released from molecular chaperones following the hydrolysis of ATP by Hsp90. Monomeric HSF1 (Figure 31 (1a)) reenters the chaperone cycle as it slowly dissociates, revealing Hsp40 binding regions.

Such a model is supported by a number of observations. Results presented in this thesis and observations of Nunes (Nunes and Calderwood, 1995) indicate that inactive HSF1 is found in a high molecular weight complex. It has long been hypothesized that Hsps negatively regulate their own synthesis by negatively regulating HSF1, particularly at the level of oligomerization (Abravaya et al., 1991a; Craig and Gross, 1991; DiDomenico et al., 1982). Any condition that inhibits the interaction of HSF1 with chaperone, whether by specific inhibition of Hsp40, Hsp70, Hsp90 or increasing the concentration of chaperone substrate by agents that increase the cellular concentration of

misfolded proteins, results in the activation of HSF1 (Ali et al., 1998; Bharadwaj et al., 1999; Guo et al., 2001; Marchler and Wu, 2001; Shi et al., 1998; Zou et al., 1998). Hsp40-HSF1 interactions have been detected and Hsp40 has been shown to be a negative regulator of HSF1 DNA-binding activity (Marchler and Wu, 2001; Shi et al., 1998). Direct interaction of HSF1 monomers with Hsp70 have been reported although discrepancies concerning the ATP-dependence of these interactions and the difficulty in detecting these interactions have lead some to propose that Hsp70-HSF1 interactions are transient (Abravaya et al., 1992; Baler et al., 1992; Baler et al., 1996; Rabindran et al., 1994; Shi et al., 1998). Over-expression of Hop in *Xenopus* oocytes has been shown to decrease HSF1 DNA-binding in response to heat (Bharadwaj et al., 1999). Direct interaction between Hsp90 and HSF1 have been reported (Ali et al., 1998; Guo et al., 2001; Nadeau et al., 1993; Nair et al., 1996; Xia et al., 1998), and Hsp90-HSF1 interactions are more easily detected than the interaction of HSF1 with other chaperones and cochaperones, indicating that this interaction is probably more stable (Ali et al., 1998; Guo et al., 2001; Nadeau et al., 1993; Nair et al., 1996; Xia et al., 1998). Immunotargeting of p23 in *Xenopus* oocytes has been shown to activate HSF1 DNA-binding in the absence of stress indicating that p23 is a negative regulator of HSF1 (Bharadwaj et al., 1999). Maintenance of HSF1 in a monomeric state may be ATP-dependent which could explain why agents that inhibit oxidative metabolism or reduce the intracellular concentration of ATP like salicylate (Winegarden et al., 1996) also activate HSF1 trimerization and DNA-binding. This model also allows for the possibility that HSF1 might detect stress directly to a certain degree. Agents such as heat and hydrogen peroxide that can activate DNA-binding of purified HSF1 (Liu et al., 1998) could act directly on HSF1, promoting unfolding of the monomer and trimer

formation faster that the chaperone machine could reform the monomer. That these agents also increase the level of misfolded proteins suggest that these agents activate HSF1 by two separate mutually supportive mechanisms.

This model does not exclude that possibility that other chaperones and co-chaperones may play a role in the regulation of HSF1 DNA-binding. Hip has been implicated in the regulation of HSF1 DNA-binding (Bharadwaj et al., 1999). Over-expression of Hip accelerated the recovery of DNA-binding during recovery from heat shock and inhibition of Hip by injection of antibodies in *Xenopus* oocytes delayed the recovery of HSF1 DNA-binding following stress (Bharadwaj et al., 1999). Thus, Hip is hypothesized to be a negative regulator of HSF1 DNA-binding. Hip is an Hsp70 co-chaperone that increases the chaperone activity of Hsp70 by stabilizing ADP-bound and substrate bound state (Hohfeld et al., 1995). In the context of the model presented above, Hip would be expected to be a negative regulator of HSF1 DNA-binding by stabilizing the interaction of Hsp70 with HSF1 that is known to negatively regulate HSF1 DNA-binding.

In some cases, chaperones could have different effects on target molecules dependent on their concentration that could lead to the misinterpretation of the regulatory role of these molecules. For example, the physiological BAG-1:Hsc70 ratio is approximately 0.03 in rabbit reticulate lysates. At low BAG-1:Hsc70 ratios BAG-1 can promote the release of Hop from the Hsp90-steroid receptor heterocomplex without inhibiting steroid receptor-Hsp90 formation (Kanelakis et al., 1999). At high BAG-1:Hsc70, BAG-1 can result in a concentration-dependent inhibition of steroid receptor-Hsp90 formation and inhibition of proper steroid receptor folding (Kanelakis et al., 1999). In physiologically relevant concentrations, BAG-1 modulates Hop release from

the assembly complex and does not inhibit correct folding of the glucocorticoid receptor or formation of an Hsp90-glucocorticoid receptor complex (Kanelakis et al., 1999). Thus, the role of BAG-1 in the regulation of steroid receptor regulation could have been misinterpreted had the participants in the above study had simply relied on experiments in which BAG-1 was significantly over-expressed. In another situation, increasing the concentration of a chaperone that is normally minimally expressed could promote non-physiological interactions that occur rarely *in vivo*. For example, although Hip has been detected in HSF1 complexes in some systems and Hip over-expression can alter HSF1 activity, it is possible that Hip never reaches those intracellular concentrations and never actually interacts with HSF1. Over-expression of chaperones could also lead to the remodeling of existing chaperone heterocomplexes and indirectly alter the chaperone heterocomplex associated with the target of interest. Correct interpretation of the role of chaperones in the regulation of HSF1 must regard studies in which chaperones are over-expressed or inhibited in the light of normal physiological levels of the proteins and the spatial and temporal characterization of HSF1 chaperone interactions *in vivo*.

There is little evidence to indicate that maintainence of HSF1 in the monomeric state requires immunophilins, and this is the only aspect of the model of regulation of the monomeric form of HSF1 presented here that is unsupported. The only study that has examined the interaction of unshocked monomeric HSF1 with immunophilins suggests that HSF1 does not bind FKBP52 (Guo et al., 2001). However, FKBP51, FKBP52, and Cyp40 have all been shown to affect HSF1 DNA-binding activity. Over-expression of Cyp40, FKBP52, or FKBP51 in *Xenopus* oocytes delayed recovery of HSF1 DNA-binding following heat shock (Bharadwaj et al., 1999). Immunotargeting of FKBP52 or FKBP51 delayed the recovery of HSF1 DNA-binding following heat shock

(Bharadwaj et al., 1999). Immunotargeting of Cyp40 had no effect on HSF1 DNA-binding (Bharadwaj et al., 1999). These results suggest that immunophilins can regulate HSF1 DNA-binding but may exert their effects primarily during the deactivation phase.

Results presented in this thesis suggest that HSF1 trimers are formed in either transcriptionally competent or incompetent forms before DNA-binding. Transcriptionally active HSF1 trimers must become activated in a step-wise manner, involving DNA-binding and subsequent acquisition of transcriptional activity. The role of molecular chaperones as negative regulators of the process has been established by several key observations: agents that increase the level of misfolded proteins can activate HSF1 transcriptional activity, and the transition of DNA-bound HSF1 to transcriptionally competent of HSF1 is coincident with the release of HSF1 by Hsp90, FKBP52, and p23 (Guo et al., 2001). It is hypothesized that this stage begins with the binding of a transcriptionally inactive HSF1 trimer to HSEs (Figure 31 2a). This species is negatively regulated by a similar stepwise association and action of Hsp70 and Hsp90 chaperones as described for maintenance of the monomer. Biochemical data supports the supposition that during this stage, interaction of Hsp70 and Hsp40 with HSF1 is transient, and final association of Hsp90, FKBP52, and p23 with HSF1 is stable. Hsp90, p23, and FKBP52 are found in association with DNA-bound HSF1 (Figure 26). In addition, Hsp70, Hip and Hop can also be detected on DNA-bound HSF1 (Bharadwaj et al., 1999), (Figure 26). Transformation of HSF1 to a transcriptionally active form could involve the hydrolysis of ATP by Hsp90 and dissociation of all molecular chaperones from HSF1, hyperphosphorylation of HSF1, followed by the interaction of HSF1 with the SWI/SNF chromatin remodeling machine and the transcriptional machinery as described above. Which of these events could be the trigger that allows HSF1 to adopt a

transcriptionally active state? Evidence suggests that each of these events is necessary for HSF1 to adopt a transcriptionally active state. Study of salicylate as an HSF1 inducer indicate that hyperphosphorylation is required for HSF1 to gain transcriptional competence as discussed above. Over-expressed HSF1 that is DNA-bound in unstressed cells can be made transcriptionally active by treatment of cells with a phosphatase inhibitor (Xia and Voellmy, 1997). That salicylate-activated HSF1 is DNA-bound but not hyperphosphorylated indicates that DNA-binding preceeds hyperphosphorylation. Whether HSF1 hyperphosphorylation triggers chaperone release or if these two events are separable is not known. As discussed previously, the identity of the phosphorylation event(s) and kinases or phosphatases involved during the activation of HSF1 transcriptional activity have not been determined.

Molecular chaperones have been implicated in the mechanism that is involved in HSF1 attenuation both during continuous heat shock and during recovery from heat shock. The amount of time that the cell requires to resume basal levels of Hsp synthesis is dependent on the severity of the heat shock and on the level of chaperones produced during the stress (DiDomenico et al., 1982). Attenuation of HSF1 during continuous heat shock and during recovery is coincident with the increased level of Hsps (Abravaya et al., 1991a; Welch and Suhan, 1986). In cells that do not upregulate significant levels of Hsps during long term heat shock, HSF1 does not attenuate (Gordon et al., 1997; Welch and Suhan, 1986). Hsp70, Hsp90 and their co-chaperones (Hsp40, p23, FKBP52, FKBP51, Hip, Hop, Cyp40) can all modulate HSF1 DNA-binding during recovery (Ali et al., 1998; Bharadwaj et al., 1999; Kim et al., 1995; Marchler and Wu, 2001; Mosser et al., 1993; Rabindran et al., 1994; Shi et al., 1998), consistent with a role for a chaperone heterocomplex as positive regulators of HSF1 recovery. I hypothesize that these

chaperones may all play a role in the deactivation and disassembly of HSF1 trimers during recovery from heat shock, similar to the step-wise manner proposed to maintain HSF1 in the monomeric form in unshocked cells. This model represents a departure from what is known about steroid receptor deactivation and eventual recycling. Both Hsp90 and p23 have been proposed to be main chaperones involved in the deactivation of hormone bound steroid receptors and eventual recycling of the receptor following dissociation of the hormone, but the process of steroid receptor deactivation is not completely understood (Freeman and Yamamoto, 2002). HSF1 phosphorylation/dephosphorylation events have also been shown to modulate HSF1 DNA-binding during recovery from heat shock (discussed in the Introduction) and may be included in the model for HSF1 deactivation and disassembly.

## 5.5 Production of Antibodies to XHSF1

At the start of this project our lab had no access to antibodies to XHSF1 that could specifically recognize XHSF1 in immunoblots. Previously, I had developed antibodies to XHSF1 that could recognize the native form of the protein (Mercier et al., 1997) but these antibodies could not be used to detect the levels of XHSF1 from protein samples. Here, I report the production of an antibody raised against a peptide corresponding to 15 amino acids in the C-terminal region of the XHSF1 that can be used to measure XHSF1 from protein extracts by western blotting. This antibody can also recognize the native form of the protein and could potentially be used in future *in vivo* studies. This antibody

represents an indispensable tool that will be used in a number of projects in our laboratory and potentially others.

A number of peptide antibodies to different regions of XHSF1 were also raised. Although these antibodies were not specific for XHSF1 in immunoblots or EMSA they may prove useful in future studies such as studies of purified XHSF1. Alternatively, steps could be taken to increase their specificity such as purification of specific antibodies on antigen columns as outlined by (Harlow and Lane, 1988).

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