THE ROLE OF PROTEIN PHOSPHATASE 5 (PP5) IN THE REGULATION OF HEAT SHOCK FACTOR 1 (HSF1) IN *XENOPUS LAEVIS* OOCYTES

A Thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master's of Science in the Department of Anatomy and Cell Biology
University of Saskatchewan
Saskatoon

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Spring 2004

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ABSTRACT

Cells are continuously exposed to a variety of physiological and environmental stresses that can lead to protein aggregation and/or denaturation, and eventually cell death. In order to ensure survival, cells have evolved a stress response that monitors, detects, and responds to changes within the cellular environment. The stress response is characterized by the up-regulation of heat shock protein (*hsp*) genes whose products can mediate the assembly and/or degradation of misfolded or aggregated proteins within the cell. This stress-induced upregulation of heat shock protein encoding genes is under the regulation of heat shock transcription factor 1 (HSF1) and its associated proteins that together form what is known as the HSF1 heterocomplex. In eukaryotic cells, HSF1 exists as a non-DNA binding monomer in the absence of stress. Upon exposure to stress, HSF1 undergoes trimerization and acquires the ability to bind heat shock elements (HSEs) located upstream of all *hsp* genes and after further modification, can become converted into a transcriptionally active form. Following prolonged stress or after removal of stress, HSF1 loses its ability to bind DNA and transcription ceases in a process termed attenuation.

Several studies have suggested that the DNA-binding and transcriptional activities of HSF1 are regulated by phosphorylation and dephosphorylation events and by chaperone-based folding mechanisms similar to those involved in the regulation of glucocorticoid receptors. Protein phosphatase 5 (PP5) has been identified as a member

of the glucocorticoid receptor chaperone complex and its phosphatase activity has been shown to regulate the maturation and activation of the receptor. It has been suggested that PP5 may regulate HSF1 in a manner similar to that of glucocorticoid receptors however it has not yet been determined how PP5 interacts with the HSF1 heterocomplex or if PP5 functions to regulate HSF1-DNA binding and/or HSF1 transactivation.

Utilizing the *Xenopus* model system, I tested the hypothesis that PP5 regulates the DNA binding and transcriptional activities of HSF1 through interactions with the HSF1 heterocomplex. Increasing the activity of PP5, either through the elevation of PP5 protein levels or by activating endogenous PP5, resulted in decreased HSF1-DNA binding as well as accelerated attenuation after the removal of stress. Conversely, inhibiting the phosphatase activity of PP5 using okadaic acid or by immunotargetting, where an antibody recognizing PP5 was microinjected into the nuclei of oocytes, resulted in delayed HSF1 attenuation. Transcription assays performed using activated PP5 also demonstrated that PP5 acts to decrease HSF1-mediated transcription. Immunoprecipitation and gel mobility supershift assays were also used to show that PP5 interacts with the HSF1 heterocomplex and PP5-HSP90 binding mutants illustrated that PP5 may exert its repressive effects independently of binding directly to HSP90.

ACKNOWLEDGMENTS

I would like to begin by thanking Dr. N. Ovsenek for welcoming me into his lab as a summer student many years ago and for introducing me to something even better then science, my future husband Dr. S. Bharadwaj! As well, I would like to thank all of the past and present members of the Ovsenek lab, especially Dr. I.J. Xavier and Dr. S. Bharadwaj for all of their technical support and advice throughout my stay in the Ovsenek lab, this work could not have been completed without you. I would also like thank Dr. P. Krone, Dr. C. Rasmussen, Dr. T. Harkness, Dr. D. Schreyer, and Dr. B. Rosseler for taking the time to serve as members of my Master's thesis committee, as well as everyone else in the Department of Anatomy and Cell Biology who have helped me throughout the years either technically or by listening to me vent about how well my experiments were going in the lab!

In addition to those listed above, I would like to thank K.C., Edna, Kitty, Julius, and Annie for all the laughs, fun, and headaches that they have lovingly given me over the past few years. And of course to my Steven, whom I could never thank enough for keeping me sane in the lab, happy at home, and by his side forever. You have made me the happiest and luckiest person ever!! (C.M. + S.B.) Finally, I would like to thank my family (Dad, Mom, Geoff, Greg, Morgan, Phil, and Michelle) for their lifelong support (and frequent visits). None of this would have been possible without you!

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LIST OF ABBREVIATIONS

ASK1- apoptosis signal-regulating kinase 1

APC- anaphase promoting complex

CAT- chloramphenicol acetyl transferase

CMV- cytomegalovirus

CyP40- cyclophilin 40

DBD- DNA binding domain

DNA- deoxyribonucleic acid

EDTA- ethylene-diaminetetra-acetic acid

EMSA- electrophoretic mobility shift assay

FKBP- FK506 binding protein

GR- glucocorticoid receptor

GSK3β- glycogen synthase kinase 3 β

Hip- Hsp70 interacting protein

Hop- Hsp90-Hsp70 organizing protein

HR- heptad repeat

HSE- heat shock element

HSF- heat shock transcription factor

HSP- heat shock protein

IκB- inhibitor of kappa β

MAPK- mitogen activated protein kinase

OR2- operation repair media

PCNA- proliferating cell nuclear antigen

PKC- protein kinase C

PP1- protein phosphatase 1

PP2A- protein phosphatase 2 A

PP5- protein phosphatase 5

PPP- phosphoprotein phosphatase

RD- regulatory domain

SDS-PAGE- sodium dodecylsulfate-polyacrylamide gel electrophoresis

TPR- tetratricopeptide repeat

TD- transactivation domain

YY1- Yin Yang 1

1.0 INTRODUCTION

1.1 The Stress Response

F. Ritossa first detected the stress response in 1962. After applying heat shock to the salivary gland cells of the fruit fly *Drosophila buschii*, Ritossa noted a new pattern of "puffing" within the polytene chromosomes (Ritossa, 1996). It has since been discovered that this puffing was a response elicited by the cell to increase the expression of specific genes encoding proteins that are needed to repair cellular constituents damaged by the stress of heat shock (Lindquist, 1986; Lindquist and Craig, 1988). Further studies have revealed that the stress response is activated by various physiological, environmental, and chemical stresses such as elevated temperatures, bacterial or viral infection, inflammation, ischemia, and exposure to toxic chemicals. Stress response proteins are also expressed under non-stress conditions including development and differentiation (Craig *et al*, 1993; Georgopolous and Welch, 1993; Parsell and Lindquist, 1993; Lis and Wu, 1993; Morimoto, 1993; Wu, 1995; Hartl, 1996). Under these conditions, proteins within the cell may unfold, misfold, or aggregate, causing the cell to function improperly. In order to counteract this effect and ensure cellular survival, cells have evolved a stress response that detects and responds to changes within the cellular environment. A cell exposed to stress will increase the

expression and synthesis of a family of cytoprotective proteins called heat shock proteins (HSPs).

HSPs function as molecular chaperones during both steady state and stressful conditions, and assist in the trafficking and folding of nascent polypeptide chains. Conversely, HSPs are also involved in the degradation of proteins that have become denatured or have been folded incorrectly. HSPs have been highly conserved throughout evolution and are arranged into five families based on molecular mass: small HSPs (sHSPs), HSP60, HSP70, HSP90, and HSP100 (as reviewed by Glover and Tkach, 2001, and Voos and Rottgers, 2002). sHSPs range in size from 15-30 kDa. They function to suppress the aggregation of proteins and assist in the folding of proteins in an ATP-independent manner. HSP60 also mediates the folding of proteins but does so in an ATP-dependent manner. HSP100 had been implicated in the prevention of and re-solubilization of protein aggregates under stress conditions. HSP70 and HSP90 family members have been found to be directly involved in the regulation of the stress response.

HSP expression is under the control of a family of transcription factors called heat shock transcription factors (HSFs). HSFs bind to heat shock elements (HSEs) located in the promoter region upstream of all HSP genes (Ananthan *et al*., 1986; Lis and Wu, 1993; Morimoto, 1993). Four distinct HSFs have been identified: HSF1, HSF2, HSF3, and HSF4. HSF1 has been shown to play an important role in development, however most research involving HSF1 has been centred around its involvement in the rapid transcriptional upregulation of HSP genes (Xiao *et al*., 1999; Christians *et al*., 2000; Pirkkala *et al*., 2001). It is known that in unstressed cells HSF1 exists as a

transcriptionally inert monomer that is unable to bind DNA. Upon exposure to stress, HSF1 trimerizes, becomes competent to bind DNA and gains the potential to induce the transcription of *hsp* genes. Following the removal of stress, HSF1 dissociates back into its monomeric form and is again incapable of binding DNA, a process known as attenuation. HSF2 is considered to be the nonstress-inducible member of the HSF family and is activated in response to signals associated with development and differentiation (Sistonen *et al*., 1992; Xiao *et al*., 1999). Relatively little is known about the regulation of HSF2. However, a recent study has shown that it becomes inactivated in cells exposed to heat shock (Mathew *et al*., 2001). The authors suggest that heat shock proteins upregulated as a result of HSF1 transactivation may act to negatively regulate the transcriptional activities of HSF2. HSF3 has also been shown to be responsible for the stress-inducible up-regulation of HSP genes, although unlike HSF1 which has been identified in several species, HSF3 is unique to avians (Sarge *et al*., 1993, Nakai and Morimoto, 1993; Nakai *et al*., 1995). The activation/deactivation mechanisms of HSF3 are similar when compared with HSF1, except that HSF3 exists as a dimer in its inactive state rather than as a monomer. As well, HSF3 is activated under more extreme stress conditions such as severe heat shock, and attenuation appears to be somewhat more delayed (Nakai 1999; Tanabe *et al*., 1998; Pirkkala *et al*., 2001). The most recently identified member of the HSF family is HSF4 (Nakai *et al*., 1997). Due to alternative mRNA splicing, there are two HSF4 isoforms (HSF4α and HSF4β) with contrasting functions (Tanabe *et al*., 1999). HSF4β, in a similar fashion to that of HSF1, has been shown to function as a transcriptional activator of genes encoding HSPs. Conversely, HSF4α acts to inhibit HSF1-mediated transcription by

interacting with and binding to the HSE; transcription does not occur as the HSF4α isoform lacks a transcriptional activation domain (Nakai *et al*., 1997; Nakai 1999; Tanabe *et al*., 1999; Zhang *et al*., 2001; Frejtag *et al*., 2001).

1.2 Heat Shock Factor 1(HSF1)

It has been well established that HSF1 is the major heat shock transcription factor involved in regulating the stress-inducible expression of HSP genes. The resultant nascent HSPs are available to stabilize aggregated or denatured proteins, thereby ensuring cellular survival. HSF1 has been studied intensively and although much is known about its structure and function, the mechanisms leading to HSF1 activation and regulation have yet to be fully elucidated.

1.2.1 HSF1 Function

Under non-stress conditions, HSF1 exists as a monomer and it was once believed to be located primarily within the cytoplasm (Baler *et al*., 1993; Sarge *et al*., 1993). Recent evidence however suggests that HSF1 is located in both the cytoplasm and the nucleus (Orosz *et al*., 1996). Interestingly, HSF1 in *Xenopus* oocytes resides entirely in the nucleus (Mercier *et al*., 1997). Upon exposure to stress, HSF1 trimerizes (Baler *et al*., 1993; Sarge *et al*., 1993) and acquires the ability to bind the conserved 5'-nGAAn-3" pentanucleotide repeat found in HSEs located upstream of all heat shock genes (Wu, 1995; Morimoto, 1998; Morano and Thiele, 1999). However, binding of HSF1 to the

HSE is not sufficient to induce the transcription of HSP-encoding genes. For example, it has been shown in *S. cerevisiae* that HSF1 is bound constitutively to the HSE and that further conformational changes are required before transcription is initiated in response to stress (Sorger *et al*., 1987; Sorger and Pelham, 1988; Jakobsen and Pelham, 1988). Certain chemical stressors such as salicylate are also known to induce DNA binding but do not activate transcription in mammalian cells. It therefore appears that HSF1 must undergo further modifications before becoming transcriptionally active (Jurivich *et al*., 1992; Lee, *et al*., 1995). After the removal of stress, HSF1 becomes deactivated by dissociation into monomers and the transcription of HSP genes ceases (Clos *et al*., 1990; Nakai *et al*., 1993). Studies utilizing tissue culture cells have also shown that HSF1 transcriptional activity ceases when a cell is exposed to a prolonged stress. HSF1 reverts to the monomeric state and becomes unable to bind DNA in a process referred to as attenuation (Clos *et al*., 1990; Rabindran *et al*., 1991; Nakai *et al*., 1993). Interestingly, studies involving *Xenopus* oocytes have shown that although the oocyte retains the mechanisms to undergo attenuation, HSF1 remains bound to the HSE during prolonged stress treatments and attenuation occurs only after cessation of stress-treatments (Gordon *et al*., 1997; Bharadwaj, S *et al*., 1999).

1.2.2 HSF1 Structure

The structure of HSF1 has been highly conserved across various species of plants and animals (Sorger and Pelham, 1988; Wiederrecht, *et al*., 1988; Clos, *et al*., 1990; Scharf, *et al*., 1990; Sarge, *et al*., 1991; Nakai and Morimoto, 1993; Treuter *et al*.,

1993; Nover *et al*., 1996; Nakai *et al*., 1997). HSF1 contains 2 hydrophobic heptad repeats (HR-A/B and HR-C) and has 3 major domains: the DNA binding domain (DBD), the regulatory domain (RD), and the transcriptional activation domain (TAD) (Appendix 1).

The helix-turn-helix DNA binding domain (Damberger *et al*., 1994) is located near the N-terminus of HSF1 and binds the HSE located upstream of all HSPs genes (Wu, 1995; Morimoto, 1998; Morano and Thiele, 1999). The regulatory domain is located within the central region and contains serine/threonine residues that can be both constitutively and inducibly phosphorylated. This domain has been shown to play an important role in determining the transactivation potential of HSF1 (Hensold *et al*., 1990; Sarge *et al*., 1993; Cotto *et al*., 1996; Kline and Morimoto, 1997; Chu *et al*., 1998; Holmberg *et al*., 2001; Soncin *et al*., 2003). The transcriptional activation domain (TAD) is located at the C-terminus of the HSF1 molecule and (Green *et al*., 1995; Zuo *et al*., 1995) it appears to be regulated by the regulatory domain (Hoj *et al*., 1994; Green *et al*., 1995; Shi *et al*., 1995; Zuo *et al*., 1995). The TAD is not particularly well conserved between species (Goodson *et al*., 1995; Green *et al*., 1995; Newton *et al*., 1996). There are two hydrophobic heptad repeats within the HSF1 protein. The HR-A/B heptad repeat, adjacent to the N-terminal DNA binding domain, contains leucine zipper motifs that are important for the trimerization of HSF1 (Sorger and Nelson, 1989; Clos *et al*., 1990; Peteranderl and Nelson, 1992; Rabindran *et al*., 1993; Harrison *et al*., 1994). The HR-C heptad repeat is found adjacent to the transactivation domain and is believed to negatively regulate the trimerization of HSF1 by interacting intramolecularly with the HR-A/B region (Rabindran *et al*., 1993). This

region is absent in HSF4 and is not well conserved in plants (Scharf *et al*., 1990; Nakai and Morimoto, 1997). Budding yeast also lacks the HR-C repeat which may explain why *S. cerevisiae* HSF is constitutively trimerized (Jakobsen and Pelham, 1988).

1.2.3 HSF1 Regulation

When a cell is exposed to stress, HSF1 monomers trimerize and become competent to bind DNA. However, binding of HSF1 to DNA does not alone result in the transcription of *hsp* genes (Clos *et al*., 1990; Rabindran *et al*., 1991; Sarge *et al*., 1991, 1993; Baler *et al*., 1993). A great deal of evidence suggests that the trimerization/DNA binding and transcriptional activation of HSF1 are uncoupled and may be regulated through different means. It has been shown in cell types other than *S. cerevisiae* that HSF1 is unable to bind the HSE until it has undergone trimerization. The formation of HSF1 trimers is dependent on two hydrophobic heptad repeats (HR-A/B and HR-C) located within the HSF1 molecule (Sorger and Nelson 1989; Clos *et al*., 1990; Peteranderl and Nelson, 1992; Rabindran *et al*., 1993; Harrison *et al*., 1994). During non-shock conditions, the HR-C heptad region is bound to the HR-A/B region, and acts to suppress trimerization (Rabindran *et al*., 1993; Orosz *et al*., 1996; Farkas *et al*., 1998). In order for trimerization to occur, the HR-C region separates from HR-A/B, exposing the HR-C region. HSF1 monomers are then able to interact through their HR-A/B repeats and do so forming a three stranded coiled-coil domain (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992). The resulting coiled-coil domain

acts to orient the three DNA binding domains, allowing the HSF1 homotrimer to bind the HSE (reviewed by Ahn *et al*., 2001).

The mechanism by which HSF1 senses stress is poorly understood. However, recent evidence suggests that a loop within the helix-turn-helix DNA binding domain (DBD) is essential for sensing heat shock and stabilizes intermolecular interactions between the HSF1 trimers. *In vitro* studies have shown that the DBD loop undergoes a conformational change in response to stress. As intramolecular interactions are weakened, the trimerization domain becomes exposed and trimerization is enhanced (Ahn *et al*., 2001).

In addition to intrinsic regulation, extrinsic mechanisms have been shown to regulate the DNA binding and transcriptional activities of HSF1. For example, HSP90 and HSP70 are molecular chaperones known to regulate HSF1. Pharmacological inhibition or artificial reduction in levels of HSP90 have been shown to enhance the DNA binding and transcriptional activation of HSF1 (Ali *et al*., 1998; Zuo *et al*., 1998; Bharadwaj, S *et al*., 1999). Several studies support the idea that HSP70 acts as a negative regulator of HSF1. For example, Abravaya *et al*. (1992) found that during heat shock, HSP70 binds to HSF1 and acts to enhance the attenuation phase of the heat shock response. Similarly, other studies have shown that overexpressed HSP70 negatively regulates HSF1 transcriptional activity by binding to the transactivation domain (Baler *et al*., 1992; Mosser *et al*., 1993; Rabindran *et al*., 1994; Shi *et al*., 1998). These results suggest that HSPs upregulated by the stress response act to regulate the activities of HSF1 in a negative feedback loop manner.

Recently an additional protein, heat shock factor binding protein 1 (HSBP1), has been found to bind HSF1 and regulate both DNA binding and transcriptional activities. *In vivo* experimentation has demonstrated that HSBP1 binds the HR-A/B repeats in trimers as HSF1 becomes competent to bind DNA (Satyal *et al*., 1998; Tai *et al*., 2002). During the attenuation phase of the heat shock response, newly synthesized HSP70 binds HSBP1, mediating the conversion of HSF1 trimers into inactive monomers (Satyal *et al*., 1998). *In vitro* studies have also shown that overexpression of HSBP1 decreases the DNA binding and transcriptional activities of HSF1, indicating that HSBP1 acts as a negative regulator of HSF1 (Satyal *et al*., 1998). Interestingly, attenuation of HSF1 was not observed in the presence of HSBP1 under severe heat shock conditions where HSP gene expression was not induced. This further suggests that HSP70 is required for proper HSF1 disassembly (Satyal *et al*., 1998).

Recently, two ubiquitin-like proteins, small ubiquitin-related modifier 1 and 2 (SUMO-1 and SUMO-2) have been identified as regulators of HSF1 activity (Hong *et al*., 2001; Hilgarth *et al*., 2003; Hietakangas *et al*., 2003). Sumoylation of HSF1 involves the covalent linkage of SUMO-1 or SUMO-2 proteins on lysine residues. As a result, HSF1 undergoes a conformational change allowing HSF1 to bind the HSE. The mechanisms underlying HSF1 regulation through sumoylation have not been extensively studied, but the following model has been put forth to explain the mode of action. New evidence suggests that HSF1 becomes inducibly phosphorylated within the regulatory domain on serine 303/307 residues (Hietakangas *et al*., 2003). This causes HSF1 to undergo a conformational change, permitting sumoylation at lysine residue 298. It is believed that the sumoylation of lysine 298 promotes dissociation of

the HR-A/B and HR-C domains and subsequent trimerization (Hong *et al*., 2001; Hietakangas *et al*., 2003; Hilgarth *et al*., 2003). It has also been suggested that further stress-inducible sumoylation positively regulates transactivation (Hong *et al*., 2001). The mechanisms underlying this transcriptional regulation, apart from DNA binding activities, remains controversial and unclear.

It has long been suggested that phosphorylation and dephosphorylation events play a pivotal role in the regulation of HSF1. Both the transcriptionally active and inactive forms of HSF1 are known to be heavily phosphorylated, however the role of phosphorylation in the regulation of HSF1 is complex and not well understood. Initial studies suggested that HSF1 remained transcriptionally inert through constitutive phosphorylation and that hyperphosphorylation was required for transcriptional activation (Sorger *et al*., 1987; Sorger and Pelham, 1988; Baler *et al*., 1993; Sarge *et al*., 1993). Jurvich *et al*. (1992) examined different forms of stress and observed the effects on HSF1. It was shown that cells tested with the anti-inflammatory drugs indomethacin and sodium salicylate activated HSF1 DNA-binding but not transactivation (Jurvich *et al*., 1992; Lee *et al*., 1995). Cotto *et al*., (1996) performed similar stress experiments but in addition focused on tryptic digests and phosphopeptide mapping. These studies revealed that the transcriptionally active form of HSF1 found in heat shocked HeLa cells was more heavily hyperphosphorylated then HSF1 in cells treated with indomethacin or salicylate. Subsequent studies have since produced evidence supporting the hypothesis that HSF1 must undergo additional phosphorylation to become transcriptionally active. Xia and Vollmey (1997) observed that HSF1 transactivation was reduced in heat shocked HeLa cells treated with serine/threonine

kinase inhibitors, and conversely, that transactivation was enhanced in cells treated with phosphatase inhibitors. They performed tryptic digests and repeatedly found four serine/threonine residues that became hyperphosphorylated during heat shock (Xia and Vollmey, 1997).

Although there appears to be a link between phosphorylation and HSF1 activity, it has proven relatively difficult to characterize the specific residues targeted for both constitutive and inducible phosphorylation. Recent research has identified residues that are inducibly phosphorylated and are required for the full activation of HSF1 transcriptional activity. Holmberg *et al*., (2001) identified a specific phosphorylation site on HSF1 that promotes stress-induced transactivation. Using specific phosphopeptide antibodies, they have shown that serine 230 is phosphorylated after heat shock, that this phosphorylation event is required for HSF1 transactivation, and that substitution of this site results in a reduction of HSP gene expression (Holmberg *et al*., 2001). More recently, *in vitro* experiments and phosphopeptide mapping suggest that threonine 142 becomes phosphorylated when HSF1 is incubated with casein kinase 2 (CK2) (Soncin *et al*., 2003). Mutational analysis has shown that the phosphorylation of threonine 142 is necessary for transactivation and is required for efficient binding of HSF1 to the HSE (Soncin *et al*., 2003). Another recent study using phosphoantibodies identified serine 303 and 307 as sites that are inducibly phosphorylated after heat shock. Phosphorylation at this site has also been shown to positively regulate HSF1 transactivation (Hietakangas *et al*., 2003).

It is now quite evident that phosphorylation is required for HSF1 transcriptional activation. However, the issue of phosphorylation is rather complex since constitutive

or inducible phosphorylation of specific serine residues within the TAD of HSF1 may act to repress its transcriptional activities. Studies using tissue culture cells and *Xenopus* oocytes have shown that phosphorylation of HSF1 on serine 303, 307, and 363 residues represses transcription (Chu *et al*., 1996 and 1998; Kline and Morimoto, 1997; Xavier *et al*., 2000). Kline and Morimoto (1997) have demonstrated, using mutational analysis, that HSF1 remains transcriptionally inert when serines 303 and 307 are constitutively phosphorylated under stress conditions and may offer an explanation as to why HSF1 remains transcriptionally inactive under non-shock temperatures. Chu *et al*., (1996 and 1998) suggested that transcriptional repression of HSF1 is partly regulated through a series of protein kinase cascades. *In vitro* and *in vivo* studies using transcriptional assays and kinase overexpression, have shown that the transactivation of HSF1 is repressed when serine 307 is phosphorylated by mitogen activated protein kinases (MAPKs) prior to serine 303 phosphorylation by GSK3β (Chu *et al*., 1996). Similar *in vitro* experiments also showed that phosphorylation of serine 363 by protein kinase C (PKC) represses HSF1 transcriptional activity (Chu *et al*., 1998). However, these authors mentioned that *in vivo* experiments inhibiting PKC activity did not markedly enhance transactivation, suggesting that PKC may play a less prominent role in regulating HSF1 transcription activation than GSK3β and MAPKs. Although these studies offer an explanation for the sequence of phosphorylation, the mechanisms involved in the repression or activation of HSF1 transactivation remain unclear**.**

1.3 Protein Phosphatase 5 (PP5)

Although the details are unclear, it is generally agreed that a number of phosphorylation events are involved in the regulation of HSF1. Phosphorylation has been shown to both negatively and positively regulate the transcriptional and DNA binding activities of HSF1. However, little is known about how and what protein phosphatases may regulate the activities of HSF1. Ding *et al*., (1998) have shown that protein phosphatases PP1 and PP2A have elevated activity in cells overexpressing HSP70 and it has been speculated that PP1 and PP2A may act to dephosphorylate HSF1 and negatively regulate its transcriptional activity. As well, Guo *et al*., (2001) presented evidence suggesting that the protein phosphatase PP5 may interact with HSF1, but it has not yet been determined if PP5 functions to regulate the DNA binding and transcriptional activities of HSF1.

PP5 has been identified as a member of the phosphoprotein phosphatase (PPP) family along with the protein phosphatases PP1, PP2A, PP2B, PP2C, PP4, PP6, and PP7 (Becker *et al*., 1994; Chen *et al*., 1994; Chinkers *et al*., 1994). Due to low protein levels and low basal phosphatase activity in many cells, PP5 has gone relatively undetected. Within the past nine years, the function, structure, and regulation of PP5 has begun to be elucidated.

1.3.1 PP5 Structure

The reversible phosphorylation of proteins by phosphatases is critical in the regulation of a wide variety of biological processes including metabolism, signal transduction, and cell cycle progression. As a result, phosphatases, including PP5, have been highly conserved across several species. PP5 has been identified as both a cytoplasmic and nuclear protein in all cell types examined to date (Becker *et al*., 1994; Chen *et al*., 1994; Chinkers *et al*., 1994; Cohen, 1997; Chinkers, 2001; Dobson *et al*., 2001).

The phosphatases have traditionally been grouped into five sub-families (PP1, PP2A (which includes PP4 and PP6), PP2B, PP5, and PP7) that are partially based on their sequence and biochemical properties (Cohen, 1997; Huang *et al*., 1998; Zuo *et al*., 1998). PP5, unlike the other members of the PPP family, is unique in that it does not contain separate catalytic and regulatory subunits. The subcellular localization signal, enzymatic activity, substrate specificity, and regulatory domains of PP5 are all contained within a single polypeptide chain (Chen *et al*., 1994; Chinkers *et al*., 1994).

PP5 is a 58 kDa protein composed of 499 amino acids and contains 5 major domains; a catalytic domain (CD), a regulatory domain (RD), and 3 tetratricopeptide (TPR) domains (see Appendix 2).

The catalytic domain, also known as the phosphatase domain, is centrally located and closely related to the catalytic subunits of PP1, PP2A, and PP2B, as the amino acid sequences in the respective catalytic regions are 42-43% identical (Barton *et al*., 1994; Chinkers, 2001).

The regulatory domain of PP5 is located at the extreme C-terminus of the peptide and is believed to have an autoinhibitory function (Chen and Cohen, 1997; Sinclair *et al*., 1999; Kang *et al*., 2001; Ramsey and Chinkers, 2002). This domain interacts with the N-terminal TPR domains and prevents the access of substrate proteins to the catalytic domain, thereby inhibiting phosphatase activity. Upon binding with an activator or substrate, the regulatory and TPR domains separate, thus exposing the phosphatase domain and relieving any inhibition (Chen and Cohen, 1997; Sinclair *et al*., 1999; Kang *et al*., 2001).

Another unique feature of PP5 is that it possesses three TPR domains located near its N-terminus (Chen *et al*., 1994; Chinkers *et al*., 1994; Fukuda *et al*., 1996). The TPR domains of PP5 belong to a family of heat shock protein 90 (HSP90)-binding TPR domains (Chinkers, 1994; Chinkers *et al*., 2001). The crystal structure of the PP5 TPR domains have recently been characterized (Das *et al*., 1998). Each domain is virtually identical in structure, containing a pair of antiparallel α-helices. The three TPR domains align in a parallel fashion in an arrangement that creates an amphipathic protein-binding groove (Das *et al*., 1998). Three amino acid residues within the groove (lysine 32, lysine 97, and arganine 101) are believed to be essential for the binding of PP5 to HSP90 and other TPR containing proteins (Das *et al*., 1998; Russell *et al*., 1999; Scheufler *et al*., 2000; Ramsey *et al*., 2000). Although the TPR domains are required for mediating protein-protein interactions, they also play an additional role in the activation/deactivation of PP5. In the absence of a substrate or activator, the TPR domain binds to the regulatory domain of the PP5 molecule which acts to inhibit the

catalytic domain. As the two domains separate, both become accessible allowing PP5 to bind substrates and initiate its phosphatase activity (Chinkers, 2001).

1.3.2 Regulation of PP5

As described above PP5, is composed of a single polypeptide chain with autoregulated phosphatase activity. Chen and Cohen (1997) described the autoinhibitory regulation of PP5 after removing the N-terminal TPR domain and observing a 25-fold increase in PP5 phosphatase activity. From these studies it was determined that the TPR domain acted to shield the active site of the catalytic domain. It was also suggested that the catalytic domain was only inaccessible to larger molecules as low basal activity of PP5 could still be detected. Studies by Kang *et al*., (2001) have identified two amino acid residues that are required for PP5 autoinhibition. Site-directed mutagenesis of PP5 was performed on several residues located within both the N-terminal (TPR) and C-terminal (regulatory) domains. It was determined that glu-76 (within the TPR domain) and gln-495 (within the regulatory domain) are necessary to maintain the autoinhibition of PP5. Mutation of these residues increased basal phosphatase activity by 10-fold over wild-type PP5 (Kang *et al*., 2001). Although glu-76 and gln-495 are important for autoinhibition, it was noted that mutation of these residues did not result in the full activation of PP5, therefore suggesting that other unidentified molecules/mechanisms may be involved in its regulation.

In order to better understand the biochemical and biological activities of PP5, researchers have searched for potential activators and inhibitors of PP5. Initial studies

focused on treating purified full length PP5 enzyme with various agents that had already been identified as inducers of general phosphatase activity. These included compounds such as protamine, spermine, ceramide, and arachidonic acid (Cohen, 1989; Gong *et al*., 1992; Dobrowsky *et al*., 1993). Polyunsaturated fatty acids, such as arachidonic acid, linoleic acid, oleic acid, and linolenic acid were found to activate PP5 phosphatase activity. However, the levels required to activate PP5 *in vitro* exceed physiological concentrations (Chen and Cohen, 1996; Skinner *et al*., 1996). Nevertheless, the mode of activation of PP5 by polyunsaturated fatty acids was partially characterized using site directed mutagenesis and proteolysis (Chen and Cohen, 1996; Sinclair *et al*., 1999; Kang *et al*., 2001). Chen and Cohen (1996) showed that the fatty acid component of phosphatidylinositol binds to the TPR domain of PP5 and derepresses phosphatase activity. Kang *et al*., (2001) discovered that asparagine 491 was required for arachidonic acid to bind and activate PP5. Sinclair *et al*., (1999) illustrated that removal of both the TPR and regulatory domains rendered PP5 unresponsive to arachidonic acid. Together these results suggest that PP5 is activated through releasing the autoinhibitory interactions between the N-terminal TPR and C-terminal regulatory domains.

Although polyunsaturated fatty acids act as activators of PP5, *in vitro* activation of phosphatase activity is only observed when fatty acids are present at supraphysiological concentrations. As a result, attempts have been made to find related compounds that may act as physiological activators of PP5. Previous research has revealed that long-chain fatty acyl-CoA esters play a role in regulating gene expression and signal transduction processes *in vivo* (Hertz *et al*., 1998; Black *et al*., 2000; Corkey

et al., 2000). This led to the hypothesis that long-chain CoA compounds may activate PP5 phosphatase activity. It was found that both unsaturated and saturated long-chain fatty acyl-CoA esters activated PP5 (Ramsey and Chinkers, 2002). Interestingly, not all saturated fatty acid moieties activate PP5 and the specificity of PP5 to polyunsaturated fatty acids is apparently due to the limited solubility of saturated compounds (Ramsey and Chinkers, 2002).

Since PP5 activation was known to require separation of the C-terminal-regulatory and N-terminal TPR domains, the next step was to identify proteins that bind the TPR domain of PP5 and determine what effects they may have on PP5 activation (Chen and Cohen, 1996; Sinclair *et al*., 1999; Kang *et al*., 2001). Using yeast two-hybrid analysis, Yamaguchi *et al*., (2002) identified two G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, that bind PP5 and discovered that its phosphatase activity increased 2.5 fold when the GTPase activity of these proteins was active. The mechanisms of activation were not determined, although it has been suggested that binding of G proteins to the TPR domain alleviated the autoinhibition of PP5. As PP5 contains a TPR domain that belongs to the family of heat shock protein 90 (HSP90)-binding TPR domains, Ramsey and Chinkers (2002) hypothesized that HSP90 may itself act as a physiological activator of PP5. Although native full-length HSP90 did not activate PP5 phosphatase activity *in vitro*, the 12kDa C-terminal domain of HSP90 stimulated PP5 activity 10 fold. The authors suggested that, although only the C-terminal region of HSP90 activated PP5 *in vitro*, full-length HSP90 may still act as a physiological activator of PP5. It was also suggested that the full-length HSP90 used in these experiments may

have had to undergo additional conformational changes in order to decrease possible steric hindrance by the amino-terminal region (Ramsey and Chinkers, 2002).

Additional studies focused on the identification of specific PP5 inhibitors. This has proven difficult as many of the known inhibitors act in a non-specific manner. Nevertheless, okadaic acid, nodularin, and fostreicin have been identified as potent inhibitors of PP5 when used within a specific concentration range. The IC_{50} values of okadaic acid, nodularin, and fostriecin for PP5 are 7nm, 2.5nm, and 700μm respectively (Chen *et al*., 1994; Walsh *et al*., 1997; Borthwick *et al*., 2001). However, at higher concentrations, each of these components inhibit other members of the PPP family such as PP1 and PP2A (Honkanen *et al*., 1991 and 1994; Walsh *et al*., 1997).

To date only one protein, the human blue-light photoreceptor cryptochrome2 (hCRY2), has been shown to specifically inhibit PP5 phosphatase activity (Zhao and Sancar, 1997). An interaction between the TPR domains of PP5 and hCRY2 was discovered using yeast two-hybrid analysis. Phosphatase assays showed that hCRY2 inhibits PP5 phosphatase activity by 75% when compared with the basal activity of PP5 however the mechanisms of inhibition have not yet been determined.

1.3.3 PP5 Function

Some of the published research on PP5 is beginning to focus on the identification of novel PP5-protein interactions and on determining its effects on a variety of substrates. One of the first studies to identify PP5 used yeast-two hybrid screening with the atrial natriuretic peptide receptor (ANP receptor) (Chinkers, 1994). Although it was

initially thought that there was direct interaction between PP5 and the ANP receptor, it has since been discovered that the interaction was mediated by HSP90 (Kumar *et al*., 2001). To date however, only an interaction between the two proteins has been established, and the effect of PP5 phosphatase activity on the regulation of the ANP receptor has yet to be determined.

Recently another protein has been shown to interact with PP5 via HSP90. The heme-regulated inhibitor of protein synthesis (HRI) is a kinase that co-ordinates the synthesis of globin with the availability of heme (as reviewed by Chen, 1993; Chen and London, 1995; Shao *et al*., 2002). HSP90 is known to associate with HRI intermediates and is required for the maturation of the HRI complex (Uma *et al*., 1997; Shao *et al*., 2002). In order to further characterize the composition of the chaperone complex associated with the HRI, immunoadsorptions were performed and the results identified PP5 as a member of the HSP90-HRI complex. Additional experimentation using activators and inhibitors of PP5 confirmed that PP5 acts to negatively regulate HRI maturation. Thus HRI has been established as one of the few known substrates of PP5 (Shao *et al*., 2002).

The binding of PP5 to proteins via the TPR domain of HSP90 was initially identified in studies involving glucocorticoid receptors (GRs) (Chen *et al*., 1996; Silverstein, 1997) which, after binding hormone, translocate to the nucleus and regulate the transcription of target genes. GRs acquire the ability to bind hormone through interactions with HSP90 heterocomplexes and must undergo conformational changes needed for conversion to a hormone binding form. The process of maturation involves the sequential binding and release of several proteins. Initially, HSP70, HSP70

interacting protein (HIP), and HSP40 interact with the non-hormone binding form of the receptor (Dittmar *et al*., 1998; Kosano *et al*., 1998). The HSP90-HSP70 organizing protein (HOP) then mediates the binding of an HSP90 dimer to HSP70 and the immature steroid receptor forming an intermediate complex (Johnson *et al*., 1998; Prodromou *et al*., 1999). Finally HSP70, HIP, and HSP40 dissociate from the HSP90-steroid receptor complex, allowing the binding of p23 and one of the immunophilins FKBP51, FKBP52, or CyP-40 (Johnson *et al*., 1994 and 1998). The receptor then dimerizes and translocates into the nucleus where it is capable of binding hormone and transcribing target genes.

HOP and the immunophilins associate with HSP90 via the TPR domain (Radanyi *et al*., 1994; Chen *et al*., 1996; Owens-Grillo *et al*., 1996; Ratajczak and Carrello, 1996). The interaction of PP5 with GR-complexes was confirmed with immunoprecipitations that were performed using PP5 and HSP90 (Chen *et al*., 1996). Further research showed, *in vitro*, that approximately 50% of GR-HSP90 heterocomplexes within the cytosol bind FKBP51 or FKBP52, 35% bind PP5, and an undetermined fraction appears to bind CyP-40 (Silverstein, 1997). To determine what effects PP5 may have on GR regulation, Chen *et al*., (1996) went on to use a PP5 dominant negative mutant and showed that the inhibition of PP5 resulted in a decrease GR-mediated transcription. However, this data was contradicted by a more recent study in which antisense oligonucleotides were used to suppress the expression of PP5. Suppression of PP5 led to an increase in both DNA binding and GR-regulated transcription suggesting that PP5 acts as a negative regulator of GR-DNA binding and transcriptional activity (Zuo *et al*., 1999). Additional research illustrated that the

increase in DNA binding is related to the mechanism by which PP5 regulates the nucleocytoplasmic shuttling of GRs. Dean *et al*., (2001) showed that the suppression of PP5 results in the nuclear accumulation of GRs and a consequent increase in GR-DNA binding. Further studies must be performed in order to determine exactly what effects PP5 has on the regulation GR activities.

Yeast two-hybrid screening has also identified other proteins that interact with PP5: protein phosphatase 2A (PP2A) (Lubert *et al*., 2001), apoptosis signal-regulating kinase 1 (ASK1) (Morita *et al*., 2001), and the anaphase-promoting complex (APC) (Ollendorff and Donoghue, 1997). The interaction between PP5 and PP2A has been confirmed *in vivo* using immunoprecipitation analysis. However, the biological purpose of this interaction has yet to be elucidated.

The apoptosis signal-regulating kinase 1 (ASK1) is a MAP kinase kinase kinase that activates the JNK and p38 pathways in response to several forms of stress (Xia *et al*., 1995; Kyriakis *et al*., 1996; Ichijo *et al*., 1997; Ono and Han, 2000; Davis, 2000; Tobiume *et al*., 2001). PP5 has been identified as a binding partner of ASK1 and was shown to regulate ASK1 by dephosphorylating a phospho-threonine residue within the activation domain of ASK1. This dephosphorylation acts to inactivate ASK1 activity suggesting that PP5 may function in an anti-apoptotic manner (Morita *et al*., 2001).

To identify cell cycle proteins with possible PP5 interactions, Ollendorff and Donoghue (1997) performed a yeast two-hybrid screen. It was determined that the anaphase promoting complex (APC) interacts with and binds to PP5 (Ollendorff and Donoghue, 1997). The APC is a multisubunit complex composed of 13 subunits and is required for sister chromatid segregation and exit from mitosis (Zachariae and

Nasmyth, 1999; Harper *et al*., 2002; Hall *et al*., 2003). Additional research has also shown that phosphorylation of three of the thirteen subunits is required for APC activation (Kotani *et al*., 1998; Rudner and Murray; 2000). However, *in vitro* studies have demonstrated that only two of the eight subunits, CDC16 and CDC27, bind to the TPR domain of PP5 (Ollendorff and Donoghue, 1997). Although an interaction between PP5 and two subunits of the APC has been confirmed, it has yet to be determined what effect the phosphatase activity of PP5 has on the regulation of the anaphase-promoting complex.

p53 has also been identified as a protein that is regulated in part by PP5, although an interaction between the two proteins has not been observed (Zuo *et al*., 1998). It has been demonstrated that by inhibiting PP5 expression using antisense oligonucleotides, p53 becomes hyperphosphorylated resulting in an increase in p53 mediated transcription (Zuo *et al*., 1998). Specifically, the regulation of $p21^{WAF/CiP1}$ by p53 was investigated and it was determined that hyperphosphorylation of p53 due to the inhibition of PP5 gene expression led to the induction of $p21^{WAF/CiP1}$ and to G_1 growth arrest. The authors suggest further investigation of PP5 inhibition may uncover the potential to act in the treatment of cancer. Interestingly, fostriecin, an inhibitor of protein phosphatases at varying concentrations has been investigated in clinical trials for use as an antitumour drug (de Jong *et al*., 1999).

1.4 The *Xenopus* Oocyte Model System

The *Xenopus* oocyte is an excellent model system that offers unique opportunities for research in both cell and developmental biology. One of the benefits of the system is that large numbers of oocytes can be easily extracted from the animal during a single surgical procedure. As well, oogenesis is continuous throughout the frog's reproductive cycle, therefore all stages of oocytes (I-VI) are accessible and readily available at any given time. Ovulation can also be induced or oocytes may be fertilized *in vitro* to allow developmental studies with embryos. Stage VI oocytes (used throughout this thesis) are extremely large (1.3 mm in diameter) making manipulation of the oocyte relatively simple. For example, DNA, mRNA, and proteins can be microinjected into the nucleus or cytoplasm of the oocyte with relatively little difficulty (Gurdon *et al*., 1971; Gurdon and Wickens, 1983). The nucleus can also be separated intact from the cytoplasm allowing for studies to be carried out within isolated cellular compartments (Gurdon and Wickens, 1983). The oocyte has a large synthetic capability possessing the same volume as $2x10^5$ tissue culture cells with ability to synthesize 20 ng of total RNA and 400 ng of total protein in one day (Gurdon and Wickens, 1983).

Another benefit of the oocyte model system is that the stress response has been well characterized. Certain features of the response vary between the oocyte and tissue culture models. The major difference is that HSF1 within the oocyte does not undergo the process of attenuation when exposed to ongoing heat stress. Instead, HSF1 retains its DNA binding activity until heat shock has been removed (Gordon *et al*., 1997;

Bharadwaj, S *et al*., 1998, 1999). It has also been determined that an increase in HSP levels as a result of HSF1 transcriptional activation is not detectable in oocytes (Horrell *et al*., 1987; King and Davis, 1987). This is most likely due to the fact the oocyte DNA/cytoplasm ratio is at least 10,000 times lower than in most cells.

2.0 EXPERIMENTAL OBJECTIVES

It has been well established that HSF1 is the main transcription factor responsible for the upregulation of HSP gene transcription during the heat shock response. However, the mechanisms involved in the regulation of HSF1-DNA binding and transcriptional activation remain largely unknown. Recent studies have begun to investigate the regulation of HSF1 by means of post-translational modification, more specifically the effects of phosphorylation and sumoylation. The hypothesis to be tested here is that HSF1 is regulated by a protein phosphatase. The present study focuses on PP5, due to previous findings of HSP90 in HSF1 complexes and PP5 in HSP90 complexes.

The aim of this study was to examine the effects of PP5 on both HSF1-DNA binding and transactivation. The approach taken was to 1) activate endogenous PP5 or increase PP5 protein levels by microinjection of expression vectors, and 2) inhibit PP5 phosphatase activity using a potent inhibitor of PP5, okadaic acid. Next I set out to determine if PP5 interacts with HSF1. To accomplish this, immunoprecipitations and gel mobility supershift assays were performed.

3.0 MATERIALS AND METHODS

3.1 Oocyte Extraction

Xenopus laevis frogs were purchased from Xenopus I, Ann Arbor Michigan. Oocytes were surgically removed from frogs anaesthetized in 0.2% (w/v) Tricaine. Extracted oocytes were agitated in a calcium-free OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES, 10 mg/L benzyl penicillin, 10 mg/L streptomycin sulphate ;Wallace *et al*., 1973) containing 0.2% (w/v) collagenase for 3-4 hours until follicular cells were removed. After removal of follicle cells, oocytes were washed extensively and placed in petri dishes containing OR2 (as above with 1 mM $CaCl_2$ added). Healthy stage VI oocytes were selected under a dissecting microscope and staged according to Dumont (1972). Oocytes were then kept in OR2 at 18^oC until further experimentation.

3.2 Protein Extract Preparation

Following experimental treatments, oocytes were placed in 1.5 ml microcentrifuge tubes and excess OR2 buffer was removed. Buffer C (50 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.1 mM EDTA (pH 8.0), 20% (v/v) glycerol, 2

mM dithiothreitol, 10 µg/ml aprotinin, 10 µg/ml leupeptin ;Dignam *et al*., 1983) was added to microcentrifuge tubes at a final volume of 10 µl per oocyte. Samples were homogenized using a micropipetter and centrifuged at 15,000xg for 5 min (4^{o}C). The supernatant was removed and transferred to a new microcentrifuge tube and stored at -80^{o}C. Extracts from oocytes injected with CMV-CAT or *hsp*-70 plasmids were made using 10 µl per oocyte 0.25 mM Tris-HCl (pH 7.5) containing aprotinin and leupeptin instead of Buffer C (Landsberg *et el*., 1995). All oocyte extracts were stored at –80^{o}C.

Nuclear and cytoplasmic extracts were made as follows. After experimental treatments, oocytes were scored on the centre of the animal pole with Dumont No. 5 forceps. Oocytes were then gently squeezed with the forceps, forcing out the nucleus. The remaining contents of the oocyte (minus the nucleus) were retained for cytoplasmic extracts. Nuclear and cytoplasmic fractions were transferred to separate 1.5 ml microcentrifuge tubes and excess OR2 buffer was removed. 2 µl of Buffer C was added per nuclei and 10 µl was added per cytoplasmic fraction. The nuclear-buffer/cytoplasmic-buffer mixtures were homogenized and centrifuged as above and the samples were stored at –80^{o}C.

3.3 Stress treatments

Stage VI oocytes were exposed to various stressors and treatments. For heat shock, oocytes were placed in sealed petri dishes containing pre-warmed

OR2 buffer and immersed in a 33^{o}C water bath for times indicated in the figures. For recovery experiments, oocytes were heat shocked as above, removed from the 33^{o}C water bath and allowed to recover at 18^{o}C for 0, 5, 15, 30, and 60 minutes. As a control for each experiment, unshocked oocytes were incubated in OR2 buffer and kept at 18^{o}C. For chemical stresses, chemicals were diluted directly into the OR2 buffer (18^{o}C) to yield the final required concentrations (Gordon *et al.*, 1997). After stress treatments, oocytes were rinsed and placed in fresh OR2 buffer and allowed to recover at 18^{o}C until extracts were made (as indicated in the figures).

3.4 Antibodies and Enzymes

Antibodies used in this thesis are as follows: polyclonal anti-HSP90 (gift from S. Hartson and B. Matts, Oklahoma State University, OK.), polyclonal anti-PP5 (gift from M. Chinkers, University of South Alabama, AB.), monoclonal anti-p23 (clone JJ3 ;gift from D. Toft, Mayo Graduate School, MN.), polyclonal antibodies anti-FKBP52 (clone Hi52c), anti-Hip (clone 2G6), and anti-Hop (clone f5 ;gift from D. Smith, University of Nebraska, NE.), polyclonal anti-HSF1 (gift from S. Fox, Northwestern University, IL.), polyclonal anti-PP1 (539517-Calbiochem), polyclonal anti-PP2A (05-421-Upstate Biotech.), monoclonal anti-IκB (sc-1643), polyclonal anti-YY1 (sc-372G), monoclonal anti-PCNA (sc-56 ;Santa Cruz Biotechnology, Santa Cruz, CA.), and horseradish peroxidase-conjugated goat anti-rabbit and goat anti-

mouse immunoglobulin G (BioRad). Enzymes used for microinjection were PP1 (539527-Calbiochem) and PP2A (539508-Calbiochem).

3.5 Fostriecin and Okadaic Acid Treatments

Fostriecin was purchased from Calbiochem and okadaic acid was purchased from Sigma. Fostriecin was dissolved in H_2O and okadaic acid was dissolved in dimethyl sulfoxide (DMSO) (1:500 v/v). In all experiments, oocytes were pretreated for 2 hours at 18^{o}C in OR2 buffer containing 4 μM of fostriecin or 100 nM of okadaic acid and then heat shocked at 33^{o}C for times indicated in the figures. For controls, oocytes were pretreated at 18^{o}C for 2 hours in OR2 containing DMSO at a dilution of 1:500 (v/v) and heat shocked as described above.

3.6 Microinjections

Stage VI oocytes were injected with antibodies, enzymes, or plasmids (Gurdon and Wickens, 1983) using a Narishige IM 300 microinjector. All injections were done directly into the nucleus of the oocyte. Oocytes receiving double injections (plasmid and/or antibody and enzyme) were first injected with plasmid and incubated at 18^{o}C for 12 hours followed by injections with either antibody or enzyme. The oocytes were then allowed to recover for 30 minutes at 18^{o}C before exposure to chemical stress or heat shock.

3.6.1 Antibody and Enzyme

Prior to injection, antibodies and enzymes were diluted 1:1 in sterile water. A volume of 15 nl was injected into each oocyte nucleus and then oocytes were allowed to recover for 30 minutes at 18^{o}C prior to experimental treatments.

3.6.2 Plasmid DNA

For overexpression experiments, pCMV-PP5 (Chinkers 1994), pCMV-PP5flag, pET30-K97A, pET30-R101A, and pCMV-C-90 (Russel *et al*., 1999) plasmids were diluted to a final concentration of 20 ng/µl in sterile water. A volume of 15 nl was injected directly into the nucleus of each oocyte and incubated for 12 hours at 18^{o}C prior to stress treatments to allow for expression.

Plasmids microinjected for CAT assays were the CMV-CAT and *Xenopus hsp70*-CAT (Landsberg *et el*., 1995) reporter constructs. In these constructs, the cytomegalovirus (CMV) or *Xenopus hsp70* promoters are linked to the chloramphenicol acetyl transferase gene (CAT) and were used as described under CAT assays. Plasmids were diluted in sterile water to a final concentration of 2 ng/µl and equal amounts of DNA were injected into the nuclei of stage VI oocytes. Following injection, oocytes were incubated at 18^{o}C for 12 hours to allow for proper chromatin assembly (Landsberg *et al*., 1995). In some experiments, CMV-CAT and *hsp70*-CAT were co-injected with

expression vectors for PP5, C-90, K97A, or R101A or with antibodies and enzymes (as indicated in the figures). The final injection volume was 15 nl. Following injection, oocytes were allowed to recover for 12 hours at 18^{o}C then were heat shocked at 33^{o}C for one hour or chemically stressed at 18^{o}C for 2 hours.

3.7 Cell Transformation

DH5α cells were made competent using the $CaCl_2$ method. DH5α cells were grown overnight (shaking) at 37^{o}C in YT media (0.5% (w/v) NaCl, 1 % (w/v) tryptone, 0.5% (w/v) yeast extract). 100 µl of these cells were taken and added to 5 ml fresh YT media and grown (shaking) for an additional 5 hours at 37^{o}C. Cells were then pelleted by centrifugation at room temperature for 10 minutes at 2500 xg. The supernatant was discarded and the pellets were resuspended in 2.5 ml of 50 mM $CaCl_2$ and incubated on ice for 1 hour. Cells were centrifuged again at room temperature for 10 minutes at 2500 xg and the resulting pellets were resuspended in 500 µl of 50 mM $CaCl_2$ and transferred to a 1.5 ml microcentrifuge tube.

Transformations were subsequently carried out by adding 50-100 ng of DNA, 9 µl of sterile water and 20 µl of TCM buffer (10 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)) to 100 µl of competent cells. The resulting mixture was incubated on ice for 1 hour, then heat shocked at 42^{o}C for 2 minutes and allowed to recover for 15 minutes at room temperature. 300 µl of

YT media (37^{o}C) was added to the mixtures and incubated at 37^{o}C for another 30 minutes. Transformed cells were then plated onto YT/agar plates (15 g agar for 1 L of YT media) containing the appropriate antibiotics and kept overnight at 37^{o}C.

Single colonies were chosen from each transformation for plasmid isolation. Plasmids preparations were carried out using the Wizard Prep method available from Promega. Isolated DNA was quantified by gel electrophoresis using 1% agarose gels stained with 1 μg/ml ethidium bromide in 1X TAE buffer (1 mM EDTA (pH 8.0), 40 mM Tris-acetate). Plasmid DNA was compared with 1 μg of λDNA digested with HindIII enzyme.

3.8 Electrophoretic Mobility Shift Assay (EMSA)

Mobility shift assays were performed using double stranded Heat Shock Element (HSE) oligonucleotides end-labelled with ^{32}P-dCTP (Bharadwaj *et al*, 1999). The HSE sequence used is indicated below (bold letters indicate the end-filled nucleotides):

5'-GGGCGT CATAGAATATT CTCGAATTCTAAATCAGG-3'

3'-**CCCGCAGTATCTTATAA**GAGCTTAAGATTTAGTCC-5'

To end-label HSE oligonucleotides, the following reaction mixture was incubated at 37^{o}C for 20 minutes: 100 ng of HSE oligonucleotides, 1 mM of each dGTP, dATP, and dTTP, 1X Klenow buffer (New England Biolabs), 4 μl ^{32}P-dCTP (New England Nuclear), and 1 μl of Klenow (New England Biolabs)

to a final volume of 12 µl. After the 20 minute incubation period, 1 mM of dCTP was added to the mixture and incubated for another 5 minutes at 37^{o}C. The reaction was stopped using 12 µl of stop buffer (1% (w/v) SDS, 20 mM EDTA (pH8.0)). The labelled oligonucleotides were separated by centrifugation (5 minutes at 2000 xg) using a DNA grade Sephadex (G-50) mini-column and stored at -20^{o}C until further use.

For gel mobility shift assays, 10 µl of stage VI oocyte protein extract (approximately 20 µg of protein) was added to 10 µl of binding buffer (10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA (pH 8.0), 5% (v/v) glycerol) containing 1 µg of poly dI-dC and 50 cps of radiolabelled HSE. Reaction mixtures were incubated at room temperature for 30 min and immediately loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed in TNANA buffer (6.7 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 3.3 mM sodium acetate (pH 5.0)) for 2 hours at 150 volts. Gels were dried and exposed to X-ray film (Kodak X-omat) overnight at –80^{o}C. Quantitation of DNA-binding activity was performed on the resultant autoradiographs using NIH Image Software (Version 1.6.1) and was expressed in arbitrary densitometry units.

For supershift analysis, HSP90, PP5, p23, FKBP52, Hip, Hop, PP1, PP2A, YY1, or PCNA antibodies were added directly to DNA-binding reactions (final dilution of 1:20 for each) prior to the 30 min incubation at room temperature. For super-supershift assays PP5 and FKBP52 antibodies were added to separate reactions as above and incubated for 30 min at room temperature. FKBP52 and

PP5 antibodies were then added to the PP5 and FKBP52 reactions respectively, and incubated for another 30 min again at room temperature.

3.9 Immunoblotting

Protein extracts were separated using 10% SDS-gel electrophoresis (Laemmli, 1970). Proteins were transferred onto Nitrocellulose membranes (BioRad) using the semi-dry transfer method (BioRad). Acrylamide gels and membranes were placed between 3 mm filter paper (BioRad) and proteins were transferred for 30 minutes using a 20 volt. Membranes were blocked for 2 hours while shaking at room temperature in TBST buffer (20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% (w/v) blocking powder (BioRad). Primary antibodies PP5, PP1, PP2A, YY1, IκB, and PCNA were diluted directly into the TBST/blocking solution containing the blot and left shaking overnight at 4^{o}C (a dilution of 1:5000 was used for all antibodies). Membranes were rinsed 3 times at room temperature for 5 minutes each with fresh TBST/blocking solution. Blots were then incubated at room temperature for 2 hours in fresh TBST/blocking solution containing the appropriate HRP-conjugated secondary antibodies diluted to a final volume of 1:5000 (HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG, BioRad). Proteins were detected using chemiluminescence (Renaissance, Dupont NEN) and autoradiography (XAR, Kodak x-ray film) after blots were rinsed at room temperature 3 times for 5 minutes in TBST. One stage VI oocyte

equivalent was used for all blots unless otherwise indicated in the figure legends.

3.10 Immunoprecipitation

Immunoprecipitations were performed using the method described by Firestone and Winguth (1990). Oocyte cell extracts were made in TETN250 (25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 250 mM NaCl, 1% (v/v) Triton X-100) containing 10 μg/μl of both leupeptin and aprotinin. Heat killed *Staphylococcus aureus* (*Staph. A*) cells in formaldehyde were used as an immunoabsorbent in order to isolate immunoprecipitated complexes (BioRad). Prior to use in the immunoprecipitations, 1.5 ml of Staph A. cells were washed with TETN250 and centrifuged at 10,000 xg for 30 seconds. This tube will be referred to as the washed Staph A. cells. Another 1.5 ml of Staph A. cells were pre-absorbed using oocyte extracts to decrease non-specific antibody complexes. This was done by washing the Staph A. cells with TETN250 and centrifuging at 10,000 xg for 30 seconds. The cells were then resuspended in 250 μl of whole cell oocyte extracts (made using TETN250 as the buffer) and left at room temperature for 15 minutes. This tube is referred to as the pre-absorbed sample.

10 μl of washed Staph A. cells were then added to 250 μl of whole cell oocyte extracts and the mixture was incubated at room temperature for 15 minutes. The Staph A. cells (bound to non-specific proteins) were pelleted for 3

minutes at 10,000 xg and the remaining supernatant was added to fresh microcentrifuge tube containing 100 µl of 50 mg/ml BSA in TETN250 and either 2 µl of HSF1, PP5, YY1, or IκB antibodies. The binding reactions were performed overnight at 4^{o}C. 10 µl of pre-absorbed Staph A. cells were then added to each reaction and incubated at room temperature for 15 minutes to bind antibody/antigen complexes. The reactions were then layered onto 400 µl of sucrose cushions (1.0 M sucrose/TETN250) and centrifuged for 3 minutes at 10,000 xg to separate the antibody/antigen complexes from the non-specific bound proteins absorbed by the Staph A. cells. The resultant pellets were then resuspended in 1 ml of TE (10 mM Tris-Cl (pH 7.5), 5 mM EDTA (pH 8.0)) and centrifuged again for 3 minutes at 10,000 xg. The supernatants were discarded and the resultant pellets, containing the antibody/antigen complexes, were resuspended in 25 µl of SDS-PAGE 1X sample buffer (New England Biolabs) and subjected to SDS-PAGE. Immunoprecipitated material was analyzed by Western blotting.

3.11 CAT Assay

CAT assays using reporter constructs were performed to determine the transcriptional activity of HSF1 (as described by Gorman *et al*, 1982). CMV-CAT was used as the control. 10 µl of protein extracts made from oocytes injected with CMV-CAT or *hsp*70-CAT plasmid constructs (as described under oocyte microinjections) was combined with 0.2 µl 100 mM Acetyl CoA and 0.5

µl ^{14}C-Chloramphenicol. Reaction mixtures were incubated at 37^{o}C for 20 minutes, extracted with 250 µl cold ethyl alcohol, and centrifuged at 14,000xg for 4 minutes at room temperature. The organic phase was removed and placed in a fresh 1.5 ml microcentrifuge tube and lyophilized. The remaining pellet was resuspended in 10 µl ethyl acetate and spotted onto Whatman silica gel TLC plates. The acetylated products were separated in a TLC chamber containing 95% chloroform and 5% methanol. Plates were dried and exposed to X-ray film (Kodak X-OMAT) overnight at room temperature.

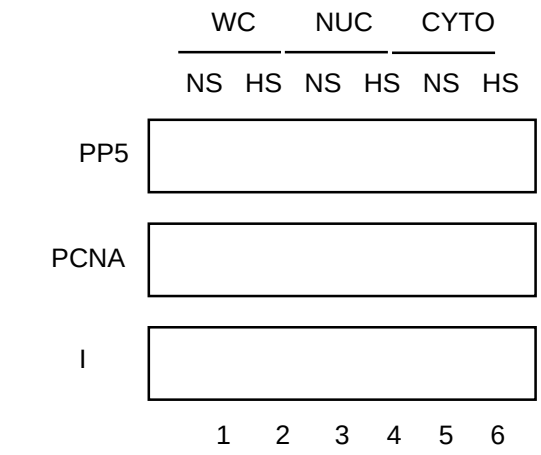
4.0 RESULTS

4.1 Subcellular localization of PP5.

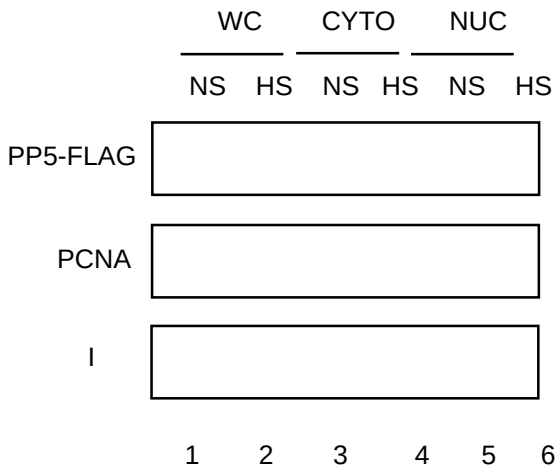
PP5 is involved in regulating the activity of glucocorticoid receptors (GRs) (Chen *et al*., 1996; Silverstein *et al*., 1997; Zuo *et al*., 1999; Ramsey *et al*., 2000), and many of the same chaperones involved in regulating GRs appear to act as regulators of HSF1 (Zuo *et al*., 1998; Bharadwaj *et al*., 1999). This lead to the hypothesis that PP5 may play a role in the regulation of HSF1. It is known that HSF1 exists as a nuclear protein in *Xenopus* oocytes (Mercier *et al*., 1997). In order to examine the possible involvement of PP5 in the regulation of HSF1, the first step was to determine the subcellular location of PP5. Western blot analysis was performed using whole cell, nuclear, and cytoplasmic extracts from unshocked or heat shocked stage VI oocytes (figure 1.A). The majority of PP5 was found in the cytoplasm (lanes 5 and 6) although PP5 was also detected in the nucleus (lanes 3 and 4). Interestingly, the amount of nuclear PP5 increased slightly after heat shock indicating that translocation of PP5 may occur. To demonstrate the purity of the nuclear and cytoplasmic fractions, appropriate control blots were performed: PCNA was used as a nuclear marker to determine that nuclear fractions were not present in cytoplasmic extracts, and IκB was used to show that cytoplasmic proteins did not contaminate nuclear extracts. It is important to note that the decrease and increase observed in heat shocked whole cells oocytes and heat

Figure 1. Immunoblots showing the subcellular localization of PP5 in non-shocked and heat shocked stage VI oocytes. Oocytes were incubated at 18^{o}C (non-shocked=NS) or 33^{o}C (heat shocked=HS) for 30 minutes. Whole cell (WC), nuclear (NUC), or cytoplasmic (CYTO) extracts were subjected to SDS-PAGE and Western blotting. **(A)** Lanes 1-2 contain WC extracts (NS/HS), lanes 3-4 contain NUC extracts (NS/HS), and lanes 5-6 contain CYTO extracts (NS/HS). PP5 was detected using a polyclonal PP5 antibody (as indicated on the left). **(B)** To determine the subcellular localization of exogenous PP5, a PP5 Flag-tagged expression vector was microinjected into oocytes and was allowed to express for 12 hours at 18^{o}C before extracts were made from NS or HS oocytes. Flag-tagged PP5 was detected using a monoclonal FLAG antibody. Lanes 1-2 contain WC extracts (NS/HS), lanes 3-4 contain CYTO extracts (NS/HS), and lanes 5-6 contain NUC extracts (NS/HS). IκB monoclonal and PCNA polyclonal antibodies were used in both figures 1.A and 1.B as controls to show purity of the nuclear and cytoplasmic extracts. All experiments were performed a minimum of 3 times.

A. Western Blot



B. Western Blot



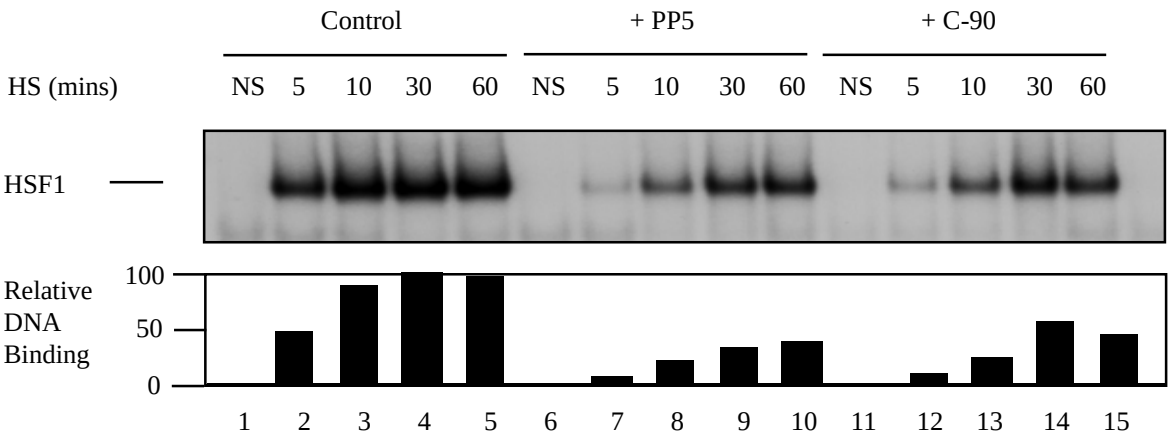
shocked cytoplasmic extracts (lane 2 and lane 6 respectively), were not a consistent result and is due to experimental error. As an additional control, the subcellular location of exogenously expressed PP5 was tested (figure 1.B). FLAG-tagged PP5 was expressed by microinjection of pCMV-FLAG-PP5 and was detected in the cytoplasm under non-shock and heat shock conditions (lanes 3 and 4). However it was only present in the nucleus after heat shock (lanes 5 and 6). This further supports the finding that PP5 undergoes nuclear translocation after heat shock. Controls using PCNA and IκB confirmed the efficiency of the nuclear isolation technique after microinjection of vectors encoding FLAG-tagged PP5 (figure 1.B).

4.2 Increasing the activity of PP5 reduces HSF1-HSE binding.

In order to determine whether PP5 is involved in the regulation of HSF1, PP5 protein levels were increased either by microinjecting oocytes with a CMV expression vector encoding PP5, or endogenous oocyte PP5 was activated by microinjecting pCMV-C90 plasmid encoding the C-terminus of HSP90, a known activator of PP5 (Ramsey and Chinkers, 2002) (figure 2.). The subsequent effects on HSF1-DNA binding was determined in comparative gel mobility shift assays. In lanes 1 (control), 5 (+PP5), and 11 (+C-90), HSE-binding activity was undetectable under non-shock conditions, whereas heat shock 33^{o}C for 5, 10, 30 and 60 minutes resulted in HSF1-HSE complex formation (lanes 2-5 (control), 7-10 (+PP5), and 12-15 (+C-90)). However, oocytes containing elevated levels of PP5 (lanes 7-10), or activated PP5 (lanes 12-15) showed a substantial decrease in the levels of HSF1-HSE complex

Figure 2. Increased levels of or activation of PP5 decreases the level of HSF1-DNA binding. Oocytes were left uninjected (lanes 1-5) or were microinjected with pCMV-PP5 (lanes 6-10) or pCMV-C-90 (lanes 11-15) and incubated for 12 hours at 18^{o}C. Oocytes were then heat shocked (HS) at 33^{o}C for 5 mins (lanes 2, 7, 12), 10 mins (lanes 3, 8, 13), 30 mins (lanes 4, 9, 14), or 60 mins (5, 10, 15). Control oocytes were incubated at 18^{o}C (non-shocked=NS; lanes 1, 6, 11). Extracts were analyzed using gel mobility shift assays and ^{32}P labelled HSE. The HSF1-HSE complex is indicated on the left and densitometry measurements are shown below. All experiments were performed a minimum of three times.

Gel Shift


Control
+ PP5
+ C-90
HS (mins)
NS 5 10 30 60 NS 5 10 30 60 NS 5 10 30 60
HSF1
Relative DNA Binding
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

formation compared to the control, uninjected oocytes (lanes 2-5). A comparison of HSF1-HSE complexes after 5 min of heat shock (lanes 2, 7 and 12) suggest that elevating PP5 activity through both the activation of endogenous PP5 or the introduction of exogenous PP5, delays or inhibits the onset of HSF1-DNA complex formation during the induction stage of heat shock. The levels of HSF1-DNA binding in control samples (lanes 4 and 5) are considerably higher at later points in the heat shock treatment compared to extracts containing elevated PP5 protein levels (lanes 9 and 10) or activated PP5 (lanes 14 and 15). This indicates that activated or increased levels of PP5 decrease the amount of HSF1-HSE binding not only at the onset of heat shock but also at later phases of heat shock induction. Both methods used to increase PP5 activity within the cell had the same negative effect on HSF1-DNA binding. Together these results suggest that PP5 may negatively regulate the DNA-binding activity of HSF1 either by inhibiting HSF1 trimer formation or by promoting the rapid disassembly of HSF1 trimers. It is important to note that previous studies, as well as experiments throughout this thesis, have determined that the microinjection procedure does not affect the formation of HSF1-HSE complexes (data not shown).

4.3 Time course of PP5 effect on HSF1-HSE binding.

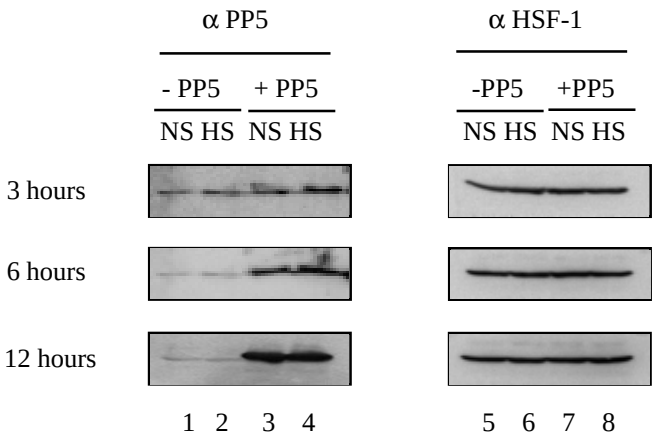
I next set out to determine the threshold of PP5 elevation needed to achieve a noticeable diminishment in HSF1-HSE complex formation. To control the increase in PP5 protein levels in this experiment, pCMV-PP5 was microinjected into the nuclei of stage VI oocytes and the oocytes were incubated at 18^{o}C for 3, 6 or 12 hours. Western

blot analysis was used to verify the increase in PP5 protein levels (figure 3.A). It should be noted here that to date, no specific phosphatase assay is available for *Xenopus* oocytes to ensure that the levels of PP5 were indeed increased. Lanes 3 and 4 show an elevation in PP5 protein levels at all time points compared to endogenous PP5 (lanes 1 and 2). The levels of PP5 increased progressively between the 3, 6 and 12 hour time points. As an additional control to show that the heat shock and microinjection procedures did not affect HSF1 protein levels, the same extracts used in lanes 1-4 were again analyzed by Western blotting utilizing an HSF1 antibody. No significant changes in HSF1 protein levels were detected (lanes 5-8).

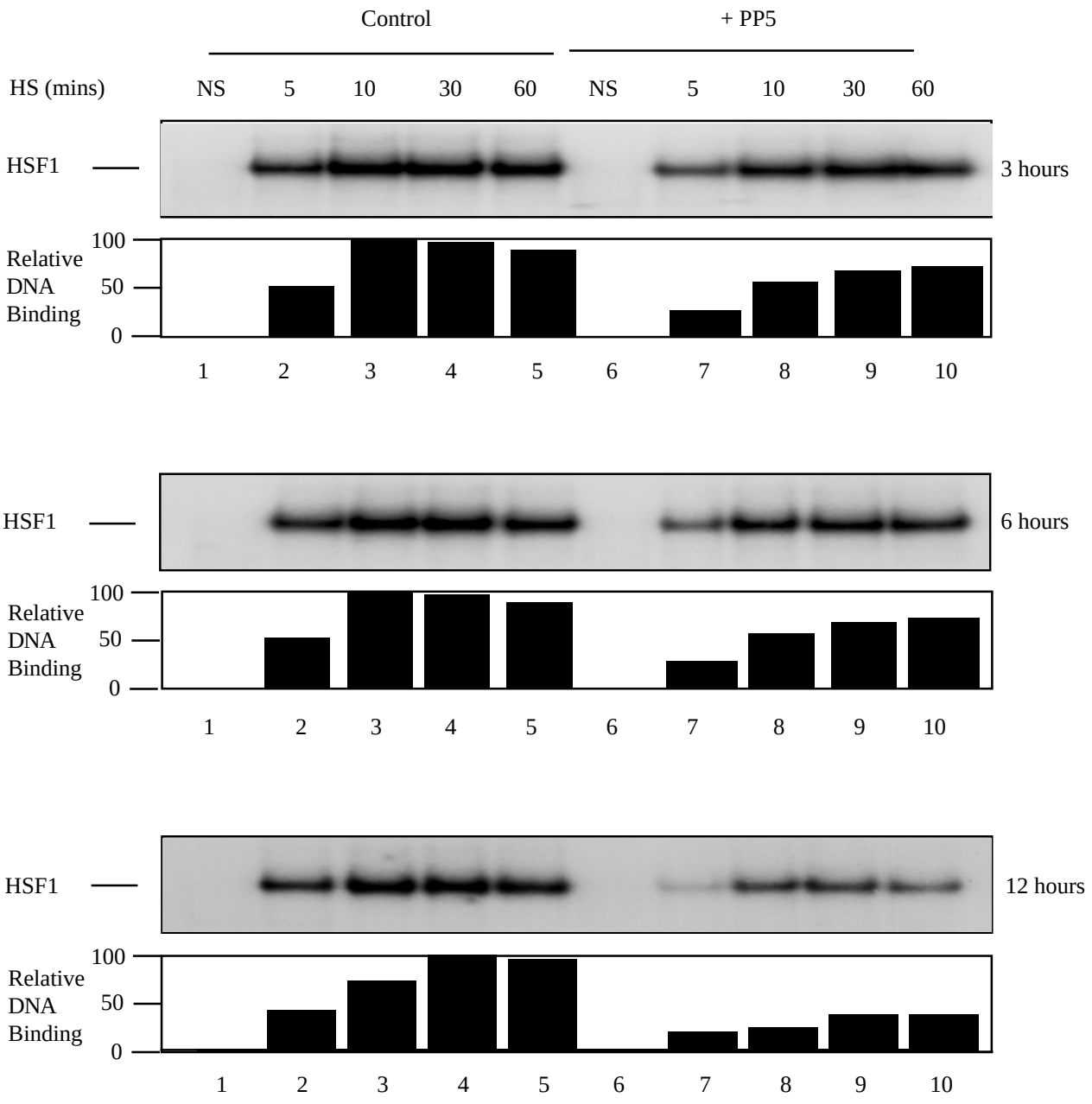
Following time to permit PP5 protein synthesis (3, 6 or 12 hours), oocytes were either heat shocked at 33^{o}C for 5, 10, 30 or 60 min or left unshocked at 18^{o}C (figure 3.B). Extracts were then subjected to electrophoretic mobility shift assay (EMSA) and the levels of HSF1 complex formation was observed. After allowing only 3 hours to increase PP5 protein levels, no significant decrease was seen in the amount of HSF1-HSE binding when compared to uninjected control samples (lanes 1-10, top panel). However, a decrease in complex formation became more evident when the time permitted for PP5 protein synthesis was extended to 6 hours (lanes 1-10, middle panel). A more substantial decrease in HSF1-DNA binding activity was seen as PP5 levels continued to increase for 12 hours (lanes 1-10, bottom panel). It is important to note that PP5 protein synthesis was permitted to continue for up to 18 and 24 hours and that

Figure 3. The effects of increased levels of PP5 on HSF1 protein levels and HSF1-HSE DNA binding. (A) Immunoblots using extracts of oocytes where PP5 levels were allowed to increase for 3, 6 or 12 hours. Oocytes were microinjected with pCMV-PP5 (+PP5/lanes 3, 4, 7 and 8) and incubated for 3, 6 or 12 hours at 18^{o}C or were left uninjected as controls (-PP5/lanes 1, 2, 5 and 6). Oocytes were then incubated at 18^{o}C (non-shocked=NS) or were heat shocked (HS) at 33^{o}C for 1 hour. The resulting extracts were subjected to SDS-PAGE and protein levels were detected by immunoblotting with a PP5 or HSF1 polyclonal antibody (lanes 1-4 and lanes 5-8 respectively). Increasing levels of PP5 and the microinjection process does not affect HSF1 levels within the oocyte. **(B)** Gel mobility shift assays using uninjected (control) or injected oocytes (+PP5) to determine what level of PP5 expression is required to elicit changes in HSF1-HSE binding. Extracts were made from uninjected oocytes (lanes 1-5) or those injected with pCMVPP5 (lanes 6-10) and incubated for 3 6, or 12 hours at 18^{o}C. Oocytes were then incubated at 18^{o}C (NS lanes 1 and 6) or heat shocked (HS) at 33^{o}C for 5 mins (lanes 2 and 7), 10 mins (lanes 3 and 8), 30 mins (lanes 4 and 9) or 60 mins (lanes 5 and 10). Extracts were analyzed using EMSA and ^{32}P radiolabelled HSE. The HSF1-HSE complex is indicated on the left and the densitometry measurements are shown below. **(C)** Extracts used in figure 3.B. above were subjected to EMSA using radiolabelled CCAAT and SP1 promoters. Increased levels of PP5 have no effect on CCAAT and SP1 DNA binding. CCAAT and SP1 complexes are indicated on the left and densitometry measurements are shown below. All of these experiments were performed a minimum of three times.

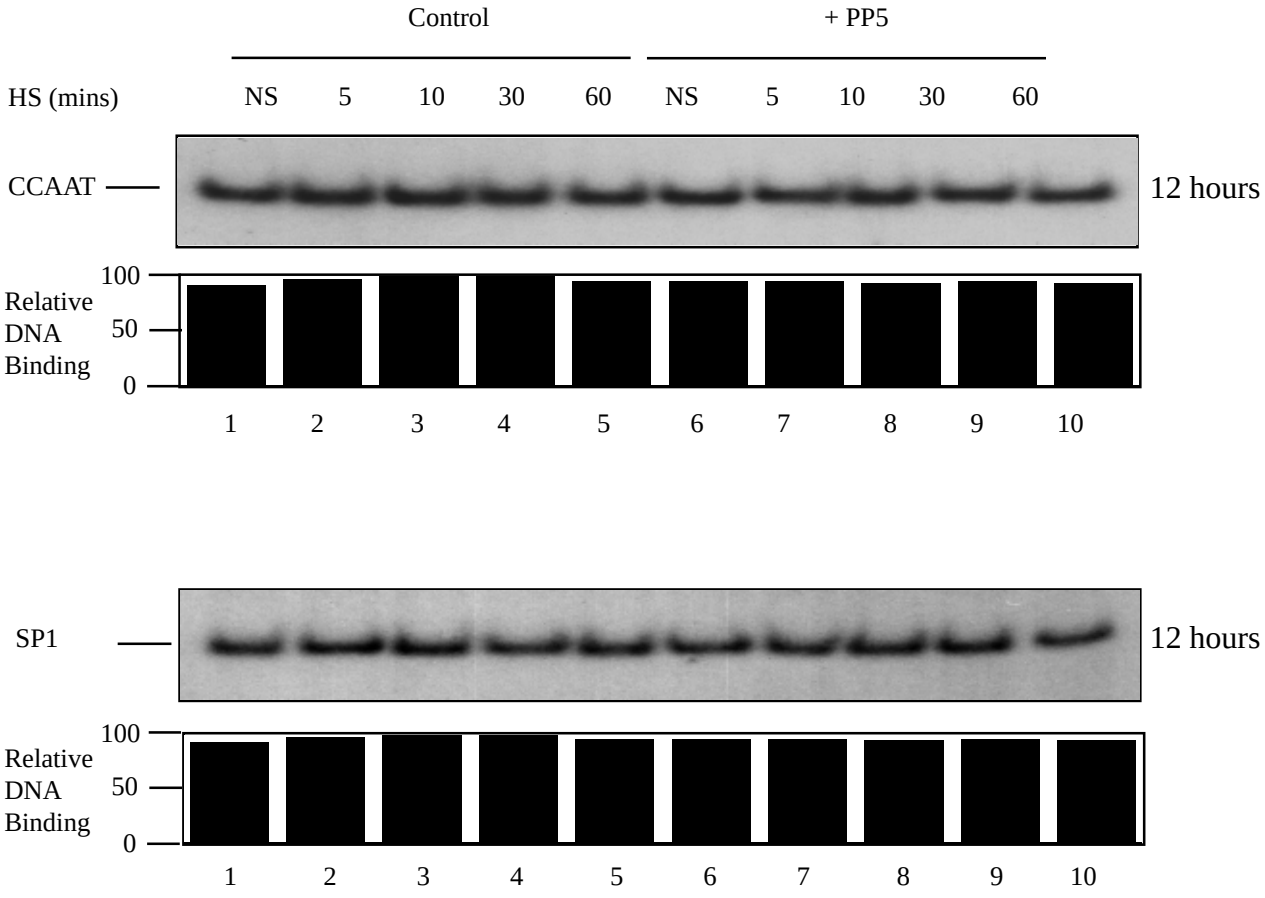
A. Western Blot



B. Gel Shift


Control
+ PP5
HS (mins)
NS
5
10
30
60
NS
5
10
30
60
HSF1
3 hours
Relative DNA Binding
100
50
0
1
2
3
4
5
6
7
8
9
10
HSF1
6 hours
Relative DNA Binding
100
50
0
1
2
3
4
5
6
7
8
9
10
HSF1
12 hours
Relative DNA Binding
100
50
0
1
2
3
4
5
6
7
8
9
10

C. Gel Shift


Control
+ PP5
HS (mins)
NS
5
10
30
60
NS
5
10
30
60
CCAAT
12 hours
Relative DNA Binding
100
50
0
1
2
3
4
5
6
7
8
9
10
SP1
12 hours
Relative DNA Binding
100
50
0
1
2
3
4
5
6
7
8
9
10

no further decrease in HSF1 complex formation was observed when compared to samples synthesising PP5 for 12 hours (data not shown). For consistency, all plasmids were microinjected into the nuclei of oocytes and incubated at 18^{o}C for 12 hours in all subsequent experiments throughout this thesis.

To ensure that PP5 specifically affected the DNA binding activities of HSF1, the same extracts used above (containing PP5 levels after 12 hours of protein synthesis), were subjected to gel mobility shift analysis using radiolabelled oligonucleotide probes with consensus-binding sites for other transcription factors (CCAAT and SP1) (figure 3.C). The relative levels of either CCAAT or SP1 complex formation was not affected by increased levels of PP5 in these experiments. This is evident in figure 3.C. as no change in CCAAT and SP1 binding activity was seen between lanes 1 and 10. Together these results indicate that PP5 specifically down-regulates the inducible DNA-binding activities of HSF1. It is noteworthy that all experiments throughout this thesis were repeated a minimum of three times using a minimum of 20 oocytes per extract to ensure consistency and validity.

4.4 Inhibition of PP5 does not affect HSF1-HSE binding during the induction of heat shock.

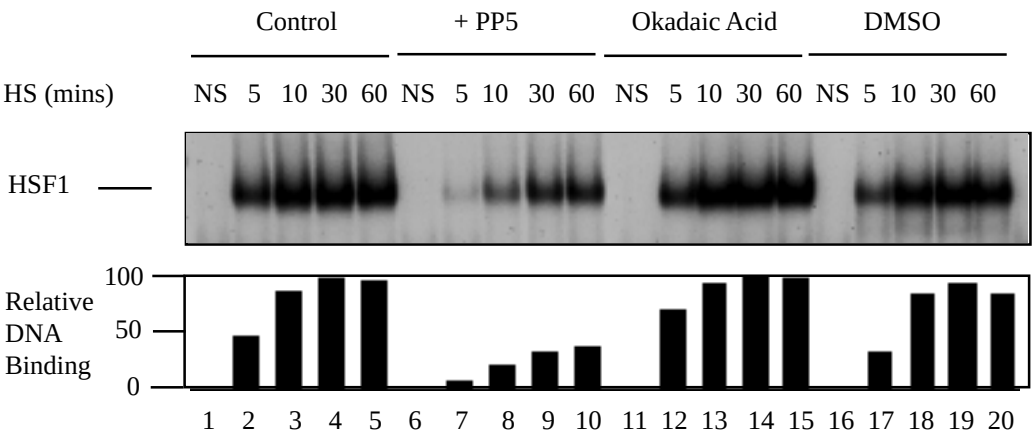
After observing that elevated levels of PP5 decreased the amount of heat induced HSF1-HSE complex formation in oocytes, I next determined the effects of inhibiting PP5 on HSF1-DNA binding activity. Oocytes were treated for 2 hours with 100 nM okadaic acid, a specific inhibitor of PP5 at nanomolar concentrations (Borthwick *et al*.,

2001), then either heat shocked at 33^{o}C for 5, 10, 30 or 60 minutes or left unshocked at 18^{o}C. The levels of HSF1-HSE complex formation were then analyzed by gel mobility shift assay (figure 4.A). No significant change was seen in HSF1-DNA binding between controls (lanes 2-5) and okadaic acid treated samples (lanes 12-15). Lanes 6-10 contain oocytes with elevated levels of PP5 to compare activation and inhibition treatments of PP5. The results show that although activation of endogenous PP5 decreased the amount of HSF1-HSE binding, inhibition of endogenous PP5 had little or no effect on DNA-binding. DMSO was used as the vehicle control for okadaic acid (lanes 16-20) and no significant change in HSF1 activity relative to untreated controls was observed.

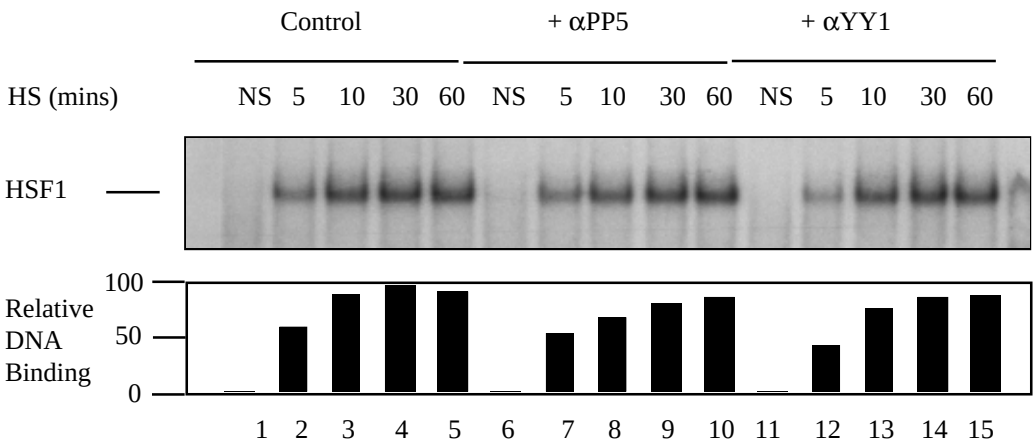
Another method used to examine the effects PP5 inhibition had on HSF1-DNA binding was immunotargetting (figure 4.B). An antibody specific to PP5 was microinjected directly into the nuclei of oocytes. After the microinjection procedure, oocytes were allowed to recover at 18^{o}C for 30 minutes before being subjected to heat shock. Control oocytes were left unshocked at 18^{o}C. Extracts were again analyzed using EMSA. Similar to results seen in figure 4.A, no increase in HSF1-HSE complex formation was observed when comparing controls (lanes 1-5) and PP5 antibody-injected samples (lanes 6-10). From these results it was determined that the inhibition or immunotargetting of PP5 had little or no effect on HSF1-HSE binding during the induction of heat shock. The non-specific antibody against Yin Yang 1 (YY1) was used as an injection control (lanes 11-15) to ensure the injection procedure had no effect on HSF1-HSE complex formation.

Figure 4. Chemically inhibiting endogenous PP5 has no effect on HSF1-HSE binding during the induction phase of the heat shock response. (A) Uninjected, untreated oocytes were used as controls (lanes 1-5). PP5 protein levels were increased as described in previous figures (lanes 6-10). Subsets of oocytes were chemically treated for 2 hours at 18^{o}C with 100 nM of the PP5 inhibitor okadaic acid (lanes 11-15), or the vehicle control DMSO (lanes 16-20). Oocytes were then incubated at 18^{o}C (non-shocked=NS; lanes 1, 6, 11 and 16) or heat shocked (HS) at 33^{o}C for 5 mins (lanes 2, 7, 12 and 17), 10 mins (lanes 3, 8, 13 and 18), 30 mins (lanes 4, 9, 14 and 19), or 60 mins (lanes 5, 10, 15 and 20). The resultant extracts were subjected to EMSA. **(B)** EMSA was performed on protein extracts made from oocytes that were microinjected with pCMV-PP5 and incubated at 18^{o}C for 12 hours (lanes 6-10), or injected with PP5 polyclonal (lanes 11-15) or YY1 polyclonal antibodies (lanes 16-20). Antibody injected oocytes recovered after the injection procedure for 30 minutes at 18^{o}C prior to heat shock treatment. Control oocytes were left uninjected as indicated in lanes 1-5. Oocytes were then subjected to heat shock as described above in figure 4.A. HFS1-HSE complexes are indicated on the left and the densitometry measurements are shown below. All experiments were performed a minimum of three times.

A. Gel Shift



B. Gel Shift



4.5 Effect of other protein phosphatases on HSF1-HSE binding activity.

Previous studies have shown that HSF1 is constitutively phosphorylated on serine and threonine residues and becomes hyperphosphorylated in response to heat shock (Baler *et al*., 1993; Sarge *et al*., 1993; Kline and Morimoto, 1997). Several kinases including glycogen synthase kinase 3β (GSK3β), protein kinase C (PKC), and mitogen activated protein kinases (MAPK), have all been implicated in the regulation of HSF1 (Chu *et al*., 1996; Xavier *et al*., 1999; Holmberg *et al*., 1997; Kim *et al*., 1997). Interestingly, although phosphorylations of HSF1 are reversible (as reviewed by Pirkkala *et al*., 2001) nothing is known about how specific phosphatases might regulate HSF1. One study described elevated activities of PP1 and PP2A in cells overexpressing HSP70 (Ding *et al*., 1998). The authors suggested that HSP70 inhibited HSF1 hyperphosphorylation by stimulating protein phosphatases and inhibiting protein kinases (Ding *et al*., 1998), but potential roles for PP1 and PP2A in the regulation of HSF1 were not identified. In addition, PP2A has been found to interact with HSF2 and PP5, although it has not yet been determined what effect if any, PP2A has on PP5 activity (Hong and Sarge, 1999; Hong *et al*., 2000, Lubert *et al*., 2001).

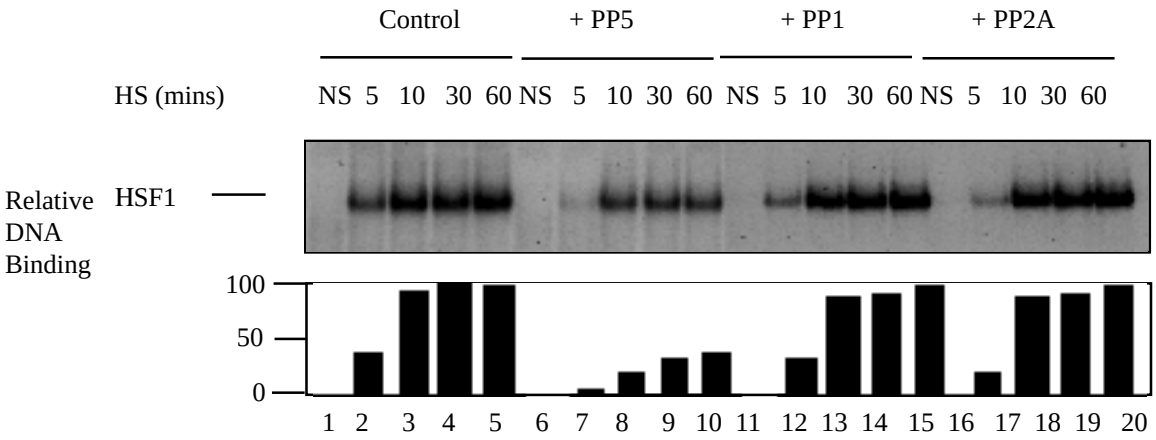
In light of this information, it was important to determine whether the inhibition of HSF1-HSE complex formation in oocytes was specifically due to the phosphatase activity of PP5 and not that of PP1 or PP2A. To accomplish this, diluted PP1 or PP2A enzyme (1:1) was microinjected directly into the nuclei of oocytes in order to increase their respective activities. Oocytes were allowed to recover after the microinjection procedure for 30 minutes and were then incubated at 18^{o}C as a control or heat shocked

at 33^{o}C for 5, 10, 30 or 60 minutes. The resulting extracts were used for gel mobility shift assays with radiolabelled HSE (figure 5.A). The level of HSF1-HSE complex formation in oocytes heat shocked for 5 minutes (lanes 2, 7, 12 and 17, figure 5.A) was lower in samples with elevated PP5 (lane 7). Lanes 2 (control) and 12 (PP1 injected) showed comparable levels of HSF1-DNA binding. Interestingly, lane 17 (PP2A injected) also showed a decrease in HSF1-HSE complex formation in the presence of PP2A as complex formation is approximately half that seen in control extracts (lane 2). In the samples at 10, 30 and 60 minutes of heat shock, HSF1 activity was decreased in cells containing elevated levels of PP5 (compare lanes 3-5 with lanes 8-10), but HSF1 activity in cells with elevated PP1 (13-15) and PP2A (18-20) levels, were the same as controls. These results indicate that increased levels of PP2A have only a slight effect on HSF1-HSE binding and suggest that the decreases seen in HSF1-HSE complex formation are specifically due to the phosphatase activity of PP5. To ensure the levels of microinjected PP1 and PP2A were elevated, nuclear extracts from microinjected oocytes were subjected to Western blot analysis and the results confirm that the levels of PP1 and PP2A were indeed increased significantly (figure 5.B).

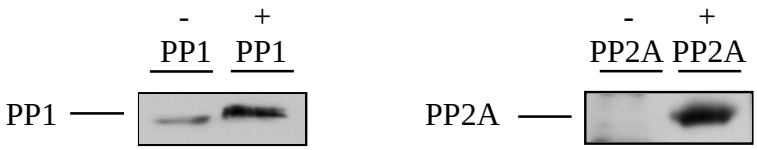
It was also necessary to demonstrate what happens to HSF1-HSE binding when PP2A is specifically inhibited as okadaic acid has also been shown to inhibit PP2A activity at higher concentrations (Cohen 1989; Cohen *et al*., 1990). As further control, oocytes were treated for 2 hours with 4 μM of fostriecin, a known inhibitor of PP2A (Walsh *et al*., 1997; Borthwick *et al*., 2001). Oocytes were left unshocked or heat shocked as described above and extracts were analyzed using EMSA (figure 5.C).

Figure 5. Other members of the protein phosphatase family do not affect the binding activities of HSF1 during the induction of heat shock. (A) PP1 and PP2A activities were increased by microinjecting purified enzyme into the nuclei of oocytes (lanes 11-15 and lanes 16-20 respectively). PP5 levels were increased by microinjecting pCMV-PP5 into oocytes and allowing 12 hours for plasmid expression. Protein extracts were made from injected or uninjected (control) oocytes as follows: oocytes were incubated at 18^{o}C (non-shocked=NS) or heat shocked (HS) at 33^{o}C for 5 mins (lanes 2, 7, 12 and 17), 10 mins (lanes 3, 8, 13 and 18), 30 mins (lanes 4, 9, 14 and 19), or 60 mins (5, 10, 15 and 20). The resultant extracts were subjected to EMSA. **(B)** Western blots of nuclear oocyte extracts confirming PP1 and PP2A elevated protein levels. **(C)** Gel mobility shift assay using protein extracts from uninjected oocytes (control lanes 1-5), oocytes that were injected with pCMV-PP5 and incubated for 12 hours (+PP5 lanes 6-10), or oocytes that were treated with 4 μM of the PP2A specific phosphatase inhibitor fostriecin (+fostriecin lanes 11-15). Oocytes were then incubated at 18^{o}C (NS) or heat shocked (HS) as above in figure 5.A. HSF1-HSE specific binding is indicated on the left and the densitometry measurements are shown below. All experiments were performed a minimum of three times.

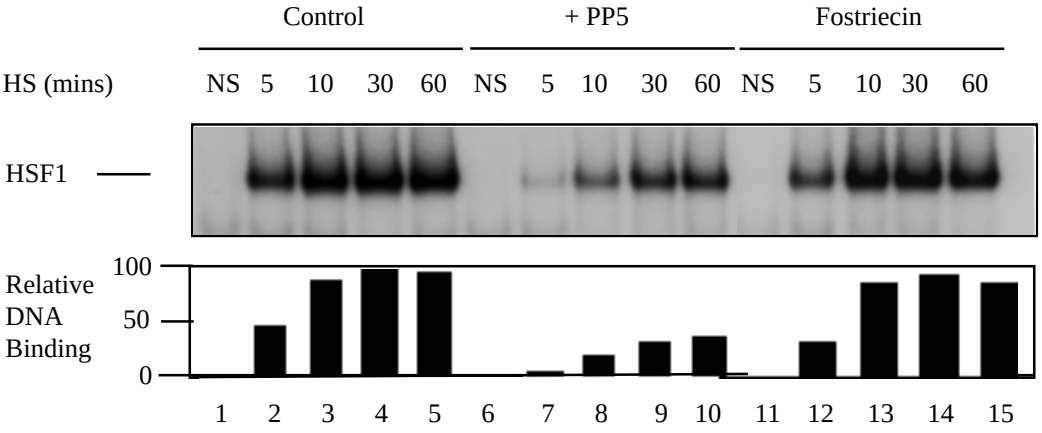
A. Gel Shift



B. Western Blot



C. Gel Shift



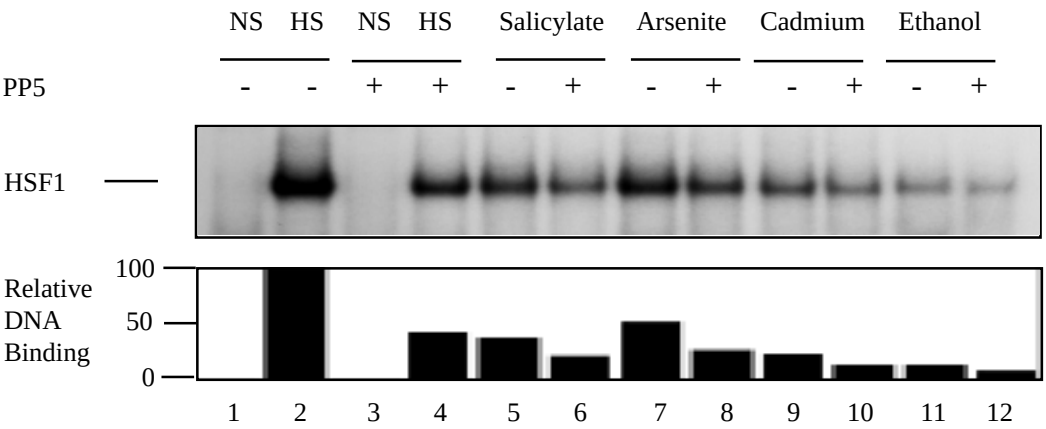
Oocytes containing elevated levels of PP5 (lanes 6-10) showed a significant decrease in HSF1-HSE complex formation when compared to control (lanes 1-5) and fostriecin treated oocytes (lanes 12-15). No change in HSF1-DNA binding was observed between controls (lanes 1-5) and fostriecin treated oocytes (lanes 11-15). This demonstrates that inhibition of PP2A had no effect on HSF1-HSE binding as observed in figure 5.A (lanes 16-20). These results illustrate that when endogenous PP2A is inhibited, the level of heat-induced HSF1-HSE complexes remains unchanged (figure 5.C). Therefore, the inhibitory effect on HSF1-DNA binding observed in previous experiments was likely due to the specific phosphatase activity of PP5.

4.6 PP5 decreases the amount of HSF1-HSE binding in response to stresses other than heat shock.

The previous experiments establish that elevated levels of activated PP5 suppress the formation of HSF1-HSE complexes under heat shock conditions. Next I wanted to determine if this phenomenon was restricted to heat shock, or if similar inhibitory effects could be observed using stress treatments other than heat shock. Studies using tissues culture cells and *Xenopus* oocytes have shown that HSF1-HSE binding can be activated by sodium salicylate, sodium arsenite, cadmium chloride, or ethanol (Jurivich *et al*., 1992, 1995; Bharadwaj *et al*., 1999). Control oocytes and oocytes with elevated PP5 levels were chemically treated for 2 hours with 70 mM sodium salicylate, 5 mM sodium arsenite, 20 mM cadmium chloride, or 10% ethanol. The resulting extracts were subjected to EMSA using radiolabelled HSE (figure 6). Lanes 2 and 4 compare

Figure 6. Increased levels of PP5 decrease HSF1-DNA binding during stresses other than heat shock. Protein extracts were prepared using oocytes uninjected (lanes 1-2, 5, 7, 9 and 11) or injected with pCMV-PP5 and incubated for 12 hours at 18^{o}C (lanes 3-4, 6, 8, 10 and 12). Oocytes were then treated at control (non-shocked=NS) temperature (18^{o}C) (lanes 1 and 3), heat shocked (HS) at 33^{o}C for 1 hour (lanes 2 and 4), or chemically stressed for two hours at 18^{o}C by treatment with 70 mM salicylate, 5 mM arsenite, 50 mM cadmium, or 10% ethanol (v/v) (lanes 5-6, 7-8, 9-10, 11-12 respectively). Extracts were subjected to EMSA using radiolabelled HSE. The HSF1-HSE complex is indicated on the left and densitometry measurements shown below. All experiments were performed a minimum of three times.

Gel Shift


NS
HS
NS
HS
Salicylate
Arsenite
Cadmium
Ethanol
PP5
- - + + - + - + - + - +
HSF1
Relative DNA Binding
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12

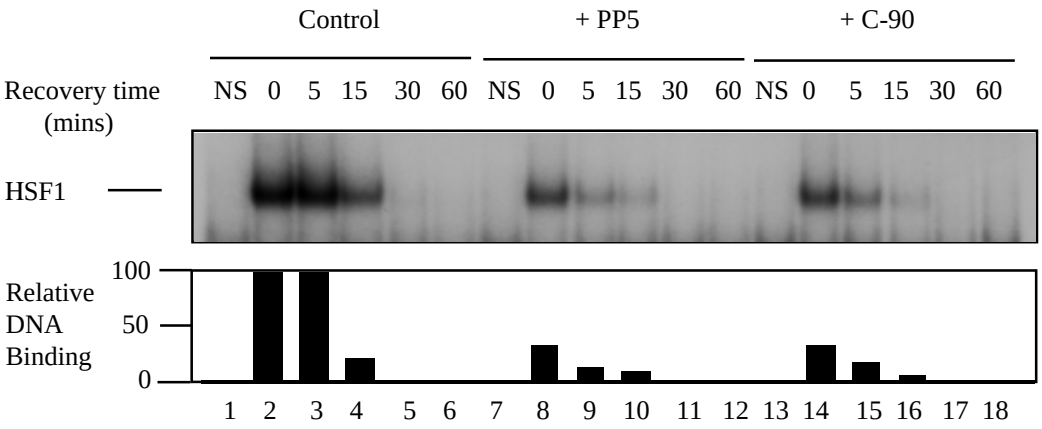
HSF1-HSE binding in heat shocked control (uninjected) and PP5-elevated samples. Approximately a two-fold reduction in HSF1-HSE complex formation was observed in PP5-expressing cells. Similarly, a two-fold reduction in HSF1-DNA binding was seen in salicylate (compare lanes 5-6), arsenite (lanes 7-8), cadmium (lanes 9-10), and ethanol (lanes 11-12) treated oocytes. These results show a consistent decrease in HSF1-HSE binding in chemically stressed oocytes brought about by elevated levels of PP5. It therefore appears that PP5 plays a similar negative-regulatory role in various stress regimes.

4.7 PP5 activation or upregulation increases the rate of HSF1-HSE deactivation.

I have demonstrated that by increasing the levels of PP5, either through activating endogenous PP5 or by plasmid-directed overexpression, HSF1-HSE binding is repressed during the induction phase of the heat shock response (figures 2 and 3). The recovery profile of HSF1-DNA binding has been investigated in *Xenopus* oocytes and it is known that HSF1 attenuates rapidly after the removal of heat shock (Bharadwaj *et al*., 1999). Knowing that PP5 acts to decrease HSF1-HSE binding during induction of heat shock, I wanted to determine if increased levels or activated PP5 would decrease HSF1-DNA binding or accelerate the attenuation phase of heat shock (figure 7). Oocytes containing activated PP5 or those injected with expression vectors encoding PP5 were heat shocked for 1 hour at 33^{o}C and allowed to recover at 18^{o} for 0, 5, 15, 30

Figure 7. Elevated PP5 protein levels decrease HSF1-DNA-binding during the attenuation phase of the heat shock response. Oocytes were left uninjected (control, lanes 1-6) or PP5 and C-90 levels were increased by injecting stage VI oocytes with pCMV-PP5 or pCMV-C-90 (lanes 7-12 and lanes 13-18 respectively). After allowing 12 hours for plasmid expression, oocytes were incubated at 18^{o}C (non-shocked=NS; lanes 1, 7 and 13) or were heat shocked (HS) at 33^{o}C and allowed to recover at 18^{o}C for 0 mins (lanes 2, 8 and 14), 5 mins (lanes 3, 9 and 15), 15 mins (lanes 4, 10 and 16), 30 mins (lanes 5, 11 and 17) or 60 mins (lanes 6, 12 and 18). The resultant protein extracts were subjected to gel mobility shift assay. HSF1-HSE complexes are indicated on the left and the densitometry measurements are shown below. All experiments were performed a minimum of three times.

Gel Shift


Control
+ PP5
+ C-90
Recovery time (mins)
NS 0 5 15 30 60 NS 0 5 15 30 60 NS 0 5 15 30 60
HSF1
Relative DNA Binding
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

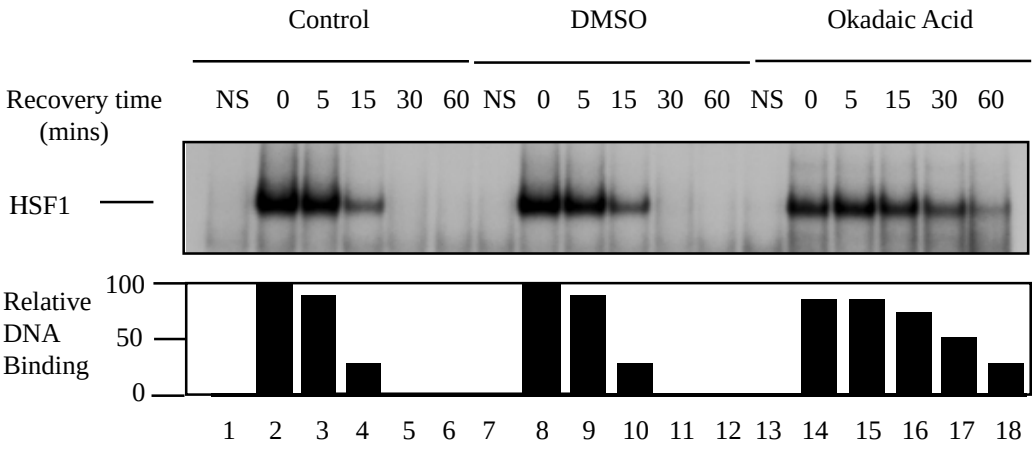
or 60 minutes. Control oocytes were incubated at 18^{o}C and extracts were subjected to EMSA. In control, uninjected oocytes (lanes 1-6), a high level of HSF1-HSE complex formation was observed immediately after heat shock, and complexes disappeared by 15-30 minutes of recovery. In contrast, there was less HSF1-HSE complex formation immediately after heat shock in PP5-expressing (lanes 7-12) or PP5-activated cells (lanes 13-18), and HSF1-HSE complexes disappeared more rapidly between 5 and 15 minutes, compared to 15-30 minutes in controls (compare lanes 4, 10 and 16). This suggests that PP5 may be involved in the negative regulation of HSF1-DNA binding activities during the attenuation phase of heat shock either by inhibiting HSF1 trimer formation or by accelerating the disassembly of HSF1 trimers.

4.8 The inhibition of PP5 prolongs HSF1-HSE binding.

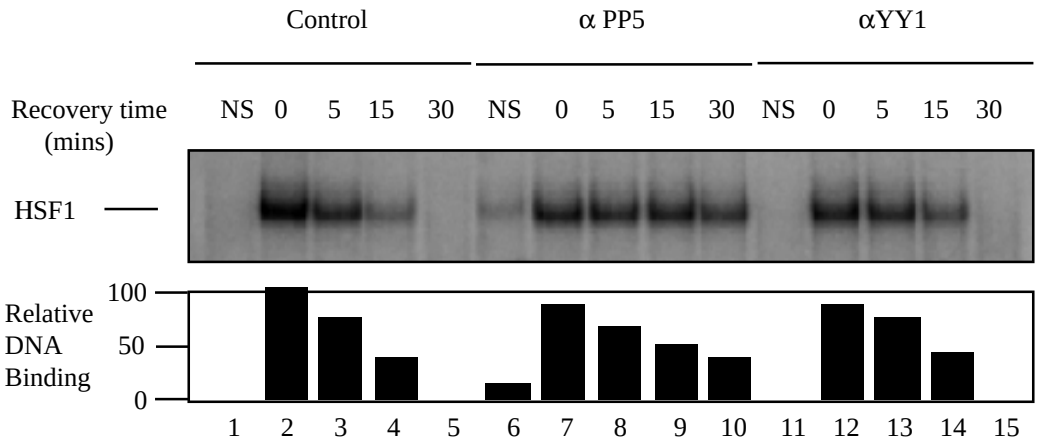
I have shown that activated or elevated levels of PP5 decreased HSF1-HSE complex formation during heat shock recovery (figure 7). These observations suggested that PP5 functions to repress HSF1 and the stress response. I next hypothesized that the inhibition of PP5 would either activate HSF1 trimer formation (and hence HSF1-HSE complex formation) or inhibit the dissociation of complexes, resulting in prolonged retention of HSF1-HSE complexes during recovery from heat shock (figure 8). In order to determine the effect of PP5 inhibition on recovery from heat shock, oocytes were treated with 100 nM of the PP5 inhibitor okadaic acid and left at 18^{o}C for 2 hours. Treated and untreated control oocytes were either left unshocked

Figure 8. Inhibiting endogenous PP5 prolongs DNA-binding in the attenuation phase of the heat shock response. (A) Gel mobility shift assays were performed with protein extracts made from oocytes that were untreated (control-lanes 1-6), or treated for two hours at 18^{o}C with the 100 nM of the PP5 inhibitor okadaic acid (lanes 7-12) or the vehicle control DMSO (lanes 13-18). Oocytes were left non-shocked (NS) at 18^{o}C (lanes 1, 7 and 13) or were heat shocked (HS) at 33^{o}C and allowed to recover at 18^{o}C for 0 mins (lanes 2, 8 and 14), 5 mins (lanes 3, 9 and 15), 15 mins (lanes 4, 10 and 16), 30 mins (lanes 5, 11 and 17), or 60 mins (lanes 6, 12 and 18). **(B)** A similar recovery experiment was performed using oocytes that were uninjected (lanes 1-5) or injected with PP5 or YY1 polyclonal antibodies (lanes 5-10 and lanes 11-15 respectively). Oocytes were left non-shocked (NS) at 18^{o}C (lanes 1, 6 and 11) or were heat shocked (HS) at 33^{o}C and allowed to recover at 18^{o}C for 0 mins (lanes 2, 7 and 12), 5 mins (lanes 3, 8 and 13), 15 mins (lanes 4, 9 and 14), or 30 mins (lanes 5, 10 and 15). The HSF1-HSE specific complexes are indicated at the left and the densitometry measurements are shown below. All experiments were performed a minimum of three times.

A. Gel Shift



B. Gel Shift



at 18°C or heat shocked at 33°C for 1 hour. Heat shocked oocytes were then allowed to recover at 18°C for 0, 5, 15, 30 or 60 minutes. The samples were analyzed by gel mobility shift assay to test for HSF1-HSE complexes (figure 8.A). In controls (lanes 2-4) there was a rapid decline in HSF1-HSE complex formation after removal of heat shock up until HSF1-DNA binding was no longer detectable in oocytes allowed to recover for 30 or 60 minutes after heat shock (lanes 5 and 6). However, oocytes treated with okadaic acid showed an extension of HSF1-HSE binding as binding was still detected after 60 minutes recovery (lanes 14-18). The differences detected in HSF1-HSE binding were particularly noticeable at the 15 and 30 minute time points. After 15 minutes, control samples (lane 4) showed approximately a 4-fold decline in HSF1-HSE complex formation compared to no significant decline in complex formation in okadaic acid treated samples (lane 16). After 30 minutes of recovery there were no detectable HSF1-HSE complexes in control samples (lane 5) whereas in comparison, okadaic acid treated oocytes (lane 17) retained a significant level of binding. This indicates that the dissociation of HSF1-HSE complexes is greatly prolonged when PP5 is inhibited and suggests that PP5 may normally act to promote the dissociation of HSF1 trimers (figure 8.A). HSF1-HSE binding was not observed in any of the non-shocked samples (lanes 1 and 13), therefore it may be unlikely that PP5 alone suppresses HSF1 trimer formation as inhibition of PP5 might then have resulted in the formation of HSF1 trimers. Oocytes treated with DMSO (lanes 7-12), the vehicle for okadaic acid, had similar amounts of HSF1-HSE complex formation when compared to control oocytes (lanes 1-6).

In addition to okadaic acid, immunotargetting was used to inhibit PP5. Polyclonal PP5 antibodies were microinjected directly into oocyte nuclei and samples were treated as above. The results observed were similar to the effects seen on HSF1-DNA binding when okadaic acid was used to inhibit PP5 activity (figure 8.B). HSF1-HSE binding was prolonged in PP5 inhibited oocytes (lanes 7-10) when compared to control, uninjected oocytes (lanes 2-5). More specifically, HSF1-HSE binding was present in both control and PP5-inhibited oocytes 15 minutes after removal from heat shock, however the binding in control oocytes (lane 4) was approximately half of that observed in PP5-inhibited oocytes (lane 9). DNA binding was still detectable 30 minutes after PP5-inhibited oocytes were removed from heat shock (lane 10) but was not detected in controls (lane 5). These results support the suggestion that PP5 functions to promote the dissociation of HSF1 trimers. Interestingly, there was no induction of HSF1-HSE binding in non-shocked oocytes treated with okadaic acid (figure 8.A lane 13), however, inhibition of PP5 by antibody injection showed slight, but reproducible DNA binding in non-shocked oocytes (figure 8.B lane 6). This may suggest that PP5 acts to suppress trimer formation as HSF1-HSE binding was not detected in control, non-shocked oocytes (lane 1). However, this effect appeared to be restricted to immunotargetting experiments only. A non-specific antibody against Yin Yang 1 (YY1) was used as an injection control (lanes 11-15) indicating that the presence of antibody did not result in prolonged attenuation or induction of DNA binding under non-shock conditions. Together, these results further support the previous conclusions that PP5 acts as a negative regulator of HSF1 and suggests that

rather then repressing trimer formation, PP5 may promote more rapid trimer disassembly.

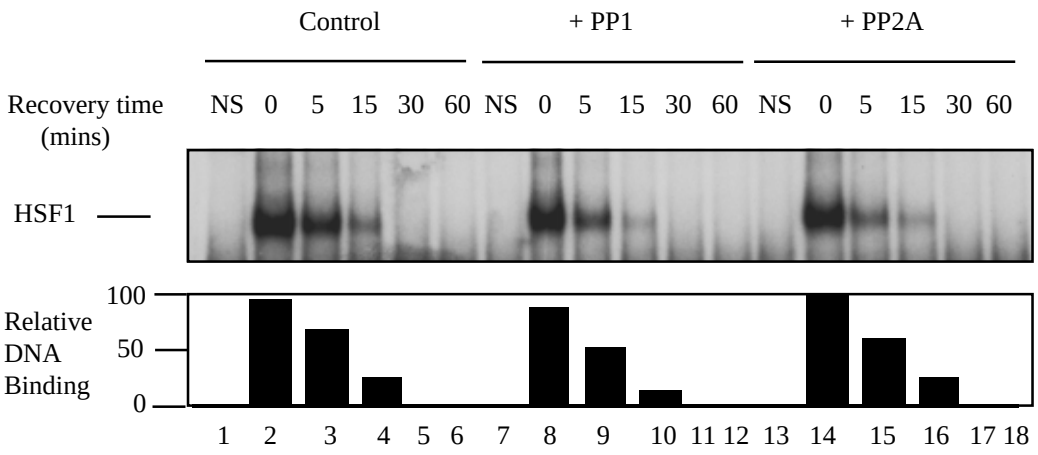
4.9 Other protein phosphatase family members do not affect HSF1-DNA binding during the attenuation phase of heat shock.

In order to determine if the rapid recovery of HSF1-HSE binding during the attenuation phase of heat shock was specifically attributable to the activities of PP5, the activities of two other members of the protein phosphatase family were increased. PP1 and PP2A enzyme was microinjected directly into the nuclei of oocytes as was done in Figure 5.A. Oocytes were incubated at control temperatures (18^{o}C) or were heat shocked for 1 hour at 33^{o}C and allowed to recover at 18^{o} for 0, 5, 15, 30 or 60 minutes. The resultant extracts were subjected to EMSA (figure 9.A). No difference in HSF1-HSE complex formation was observed in oocytes microinjected with PP1 (lanes 7-12) or PP2A (lanes 13-18) when compared to uninjected control oocytes (lanes 1-6). These results demonstrate that increased levels of PP1 and PP2A have no effect on HSF1-HSE binding and suggest that it is the specific phosphatase activity of PP5 and not PP1 or PP2A that negatively modulates HSF1-HSE complex formation during the attenuation phase of heat shock.

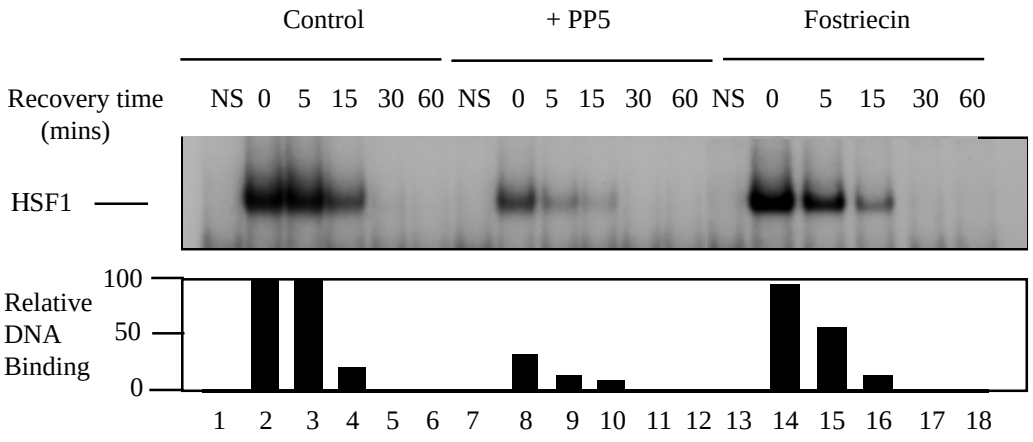
Endogenous PP2A was also inhibited as in figure 5 by treating uninjected oocytes for two hours with 4 μm fostriecin at 18^{o}C. Extracts were again subjected to EMSA (figure 9.B). Oocytes containing elevated levels of PP5 resulted in decreased HSF1-

Figure 9. Other members of the protein phosphatase family do not affect the binding activities of HSF1 during the attenuation phase of the heat shock response. (A) The levels of PP1 and PP2A were elevated by nuclear injection of purified enzyme (lanes 7-12 and lanes 13-18 respectively). Uninjected oocytes were used as a control (lanes 1-6). After allowing 30 minutes for the oocytes to recover after the injection procedure (at 18^{o}C), oocytes were incubated at 18^{o}C (non-shocked=NS) or were heat shocked (HS) at 33^{o}C and allowed to recover at 18^{o}C for 0 mins (lanes 2, 8 and 14), 5 mins (lanes 3, 9 and 15), 15 mins (lanes 4, 10 and 16), 30 mins (lanes 5, 11 and 17) or 60 mins (lanes 6, 12 and 18). The resultant protein extracts were subjected to EMSA. **(B)** Gel mobility shift assay with protein extracts made from uninjected oocytes (lanes 1-6), oocytes incubated with injected pCMV-PP5 for 12 hours at 18^{o}C (lanes 7-12), or oocytes treated with 4 μM of the PP2A phosphatase inhibitor fostriecin (lanes 13-18). Each treated subset of oocytes were then subjected to control (NS) temperatures (18^{o}C) or heat shocked (HS) at 33^{o}C as described above in figure 9.A. The HSF1-HSE complex is indicated on the left and the densitometry measurements are shown below. All experiments were repeated a minimum of three times.

A. Gel Shift



B. Gel Shift



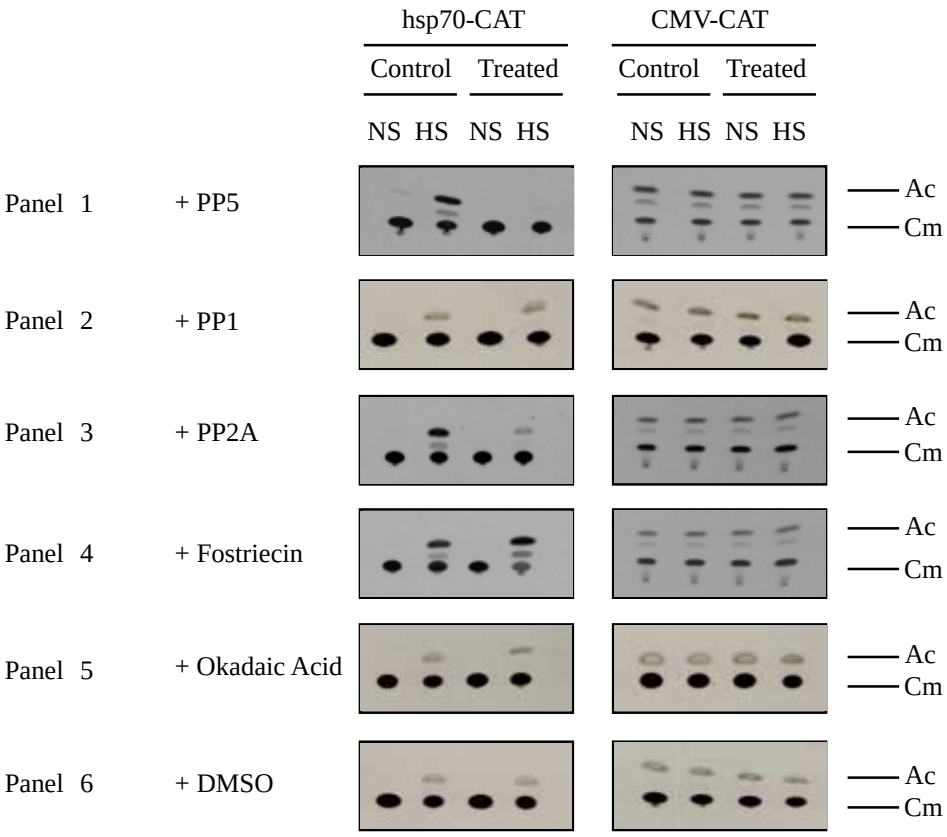
HSE complex formation immediately after heat shock (lane 8) compared to control (lane 2) and fostriecin treated oocytes (lane 14), and less DNA binding was detected after 5 and 15 minutes of recovery (compare lanes 3, 4 and 9, 10 and 15, 16) compared to controls (lanes 2 and 3) and fostriecin treated oocytes. However, a decrease in the amount of HSF1-HSE complex formation was seen when comparing control and fostriecin treated samples suggesting that inhibition of PP2A may effect HSF1-DNA binding (compare lanes 1-6 to lanes 14-18). This affect was minimal and it is most likely that the decrease in HSF1-HSE complex formation in these experiments is due to the phosphatase activity of PP5.

4.10 PP5 and HSF1-dependent transcription.

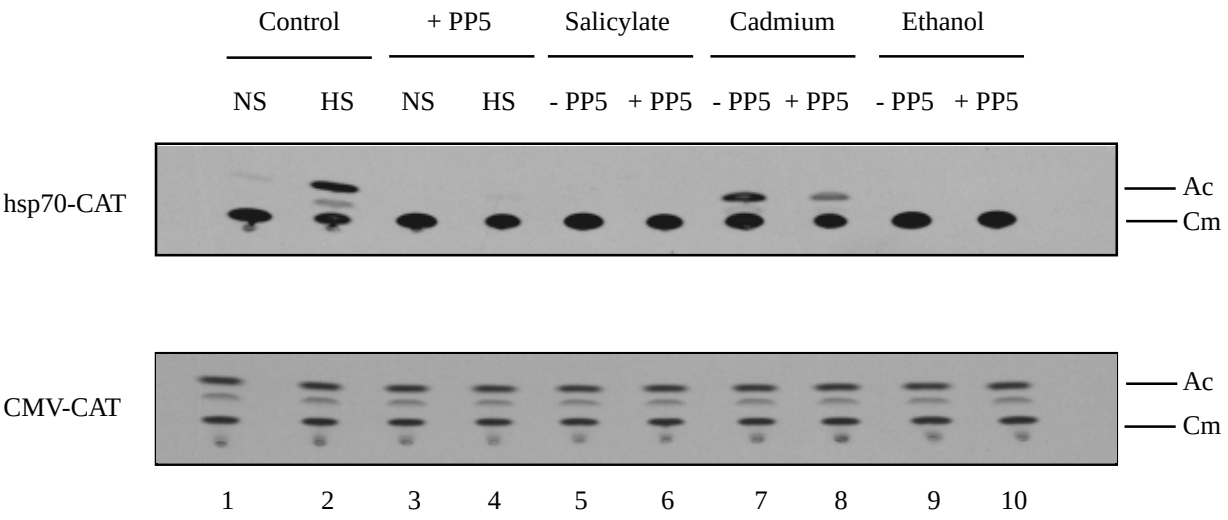
The DNA binding and transcriptional activities of HSF1 are known to be separately regulated (Hensold *et al*., 1990; Jurivich *et al*., 1992; Bharadwaj *et al*., 1999). Using gel mobility shift analysis, I have found that PP5 negatively regulates HSF1-DNA binding at the level of trimer formation/disassembly. To examine whether PP5 plays a role in controlling the transcriptional activity of HSF1, a series of transcription assays using CAT reporter constructs were performed (figure 10). *Hsp70*-CAT, containing the HSF1-dependent promoter of *hsp*70, was microinjected into stage VI oocytes. In order to control for potential effects of injected plasmids and enzymes or chemical treatments on general transcription, oocytes were also injected with the CMV-CAT construct containing a non-stress inducible promoter. To determine what effect PP5 activities had on HSF1-regulated transcription, reporter constructs were co-

Figure 10. Analysis of HSF1 transcriptional activity using *hsp*70-CAT reporter constructs. (A) Oocytes were microinjected with pCMV-PP5 and/or *hsp*70-CAT or CMV-CAT and allowed to incorporate plasmid DNA as described by Landsberg *et al*., (1995). CAT assays were performed using extracts from oocytes that were microinjected with the reporter plasmids *hsp*70-CAT or CMV-CAT and pCMV-PP5 (panel 1), PP1 (panel 2) or PP2A (panel 3) enzyme, or chemically treated for 2 hours at 18^{o}C with 4 μM fostriecin (panel 4), 100 nM okadaic acid (panel 5), or (v/v)% of the vehicle control DMSO (panel 6). Oocytes were then incubated at 18^{o}C for 12 hours and treated by non-shock (NS 18^{o}C) or heat shock (HS) at 33^{o}C for one hour. Thin-layer chromatography was used to separate the acetylated (Ac) and non-acetylated form of chloramphenicol (Cm). **(B)** CAT assays were performed using extracts from oocytes that were microinjected with the reporter plasmids *hsp*70-CAT or CMV-CAT. pCMV-PP5 was also injected into oocytes used in lanes 3-4, 6, 8 and 10 and incubated at 18^{o}C for 12 hours . Oocytes were then incubated at the non-shock temperature of 18^{o}C, heat shocked (HS) at 33^{o}C for one hour, or chemically treated at 18^{o}C for two hours with 70 mM salicylate (lanes 5 and 6), 50 mM cadmium (lanes 7 and 8), or 10% ethanol (lanes 9 and 10). Extracts were then subjected to CAT. The acetylated and non-acetylated forms of chloramphenicol are indicated on the right. All experiments were repeated a minimum of three times.

A. CAT Assays


hsp70-CAT
CMV-CAT
Control
Treated
Control
Treated
NS HS NS HS
NS HS NS HS
Panel 1
+ PP5
Panel 2
+ PP1
Panel 3
+ PP2A
Panel 4
+ Fostriecin
Panel 5
+ Okadaic Acid
Panel 6
+ DMSO
Ac
Cm

B. CAT Assays


Control
+ PP5
Salicylate
Cadmium
Ethanol
NS
HS
NS
HS
- PP5
+ PP5
- PP5
+ PP5
- PP5
+ PP5
hsp70-CAT
CMV-CAT
Ac
Cm
Ac
Cm
1
2
3
4
5
6
7
8
9
10

injected with expression vectors for PP5 or oocytes were treated with okadaic acid (PP5 inhibitor), DMSO (vehicle control), or fostriecin (PP2A inhibitor). PP1 and PP2A enzymes were also microinjected directly into the nuclei of oocytes to see what effect phosphatases other than PP5 had on HSF1 transactivation (figure 10.A). To minimize sample variability, all treatments in this experiment (10.A and 10.B) were performed using the same set of oocytes. The results in panel 1 illustrate that increased levels of PP5 significantly decrease HSF1-dependent transcription of the *hsp*70 promoter. Interestingly, PP5 completely inhibited heat shock-induced transcription, even though under the same conditions, residual HSF1-HSE complex formation was still detected in DNA binding assays (figures 2, 3 and 8). This suggests that PP5 acts to negatively regulate HSF1-DNA binding as well as HSF1 transcriptional activation. Inhibition of PP5 with okadaic acid had no effect on HSF1 transactivation (panel 5), consistent with the effects on DNA binding in okadaic acid treated oocytes (figures 4 and 8). Although PP5 inhibition delayed the attenuation of HSF1-DNA binding (figure 8), it did not affect *hsp*70 promoter activity. It is also important to note that C-90 or PP5 antibody could not be used to activate or inhibit PP5 in this assay as both were repeatedly found to interfere with general CMV-CAT expression (data not shown).

The results shown in Panel 2 illustrate that increased levels of PP1 had no effect on HSF1-mediated transcription of the *hsp*70 promoter in heat shocked oocytes. Again this agrees with previous experiments demonstrating that PP1 had no effect on HSF1-HSE binding (figures 5 and 9). Interestingly, panel 3 shows that increased levels of PP2A resulted in decreased *hsp*70 promoter activity. As expected, the *hsp*70 promoter activity increased when PP2A was inhibited using fostriecin (panel 4). Previous

experiments indicated that these treatments had no effect on HSF1-HSE binding suggesting that PP2A may play a role in regulating HSF1 at the level of transcription but not at the level of DNA binding.

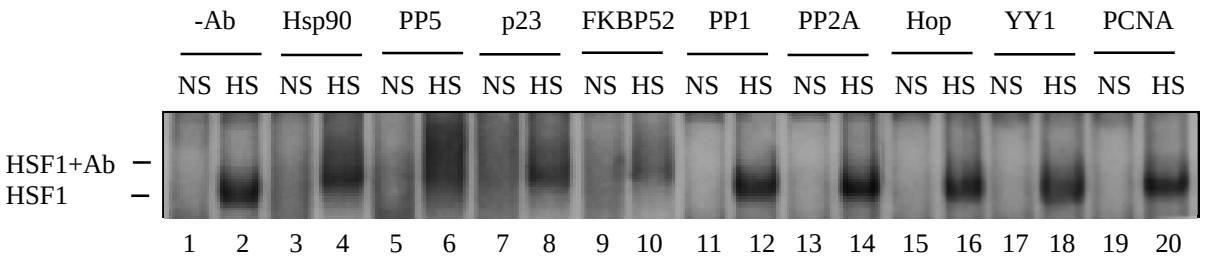
Next I wanted to determine if PP5 could suppress the transcriptional activity of HSF1 in response to stresses other than heat shock. Oocytes were microinjected with expression vectors encoding PP5 and reporter constructs then treated with heat shock, sodium salicylate, cadmium chloride, or ethanol. The results of the CAT assays illustrate that in addition to the suppression of HSF1-DNA binding (figure 6), elevated levels of PP5 also decreased the transcriptional activity of HSF1 (figure 10.B). HSF1-mediated transcription was reduced in response to heat shock (lanes 3, 4) and cadmium (lanes 7, 8) suggesting that PP5 acts to regulate the transcriptional activities of HSF1 in stress conditions other than heat shock. It is important to note that in *Xenopus* oocytes, not all stresses capable of activating HSF1-HSE binding activate HSF1 dependent transcription (Bharadwaj *et al*., 1999). Cadmium was the only stress used in this thesis other than heat shock that has been shown to activate HSF1 transcriptional activity (Bharadwaj *et al*., 1999). Consistent with these previous observations, salicylate (lanes 5, 6) and ethanol (lanes 9, 10) treatments did not induce *hsp*70 promoter activity. Parallel control experiments were performed using CMV-CAT injected oocytes to demonstrate that the treatments used in these experiments had no effect on general transcription (figure 10.B bottom).

4.11 PP5 interacts with HSF1 complexes.

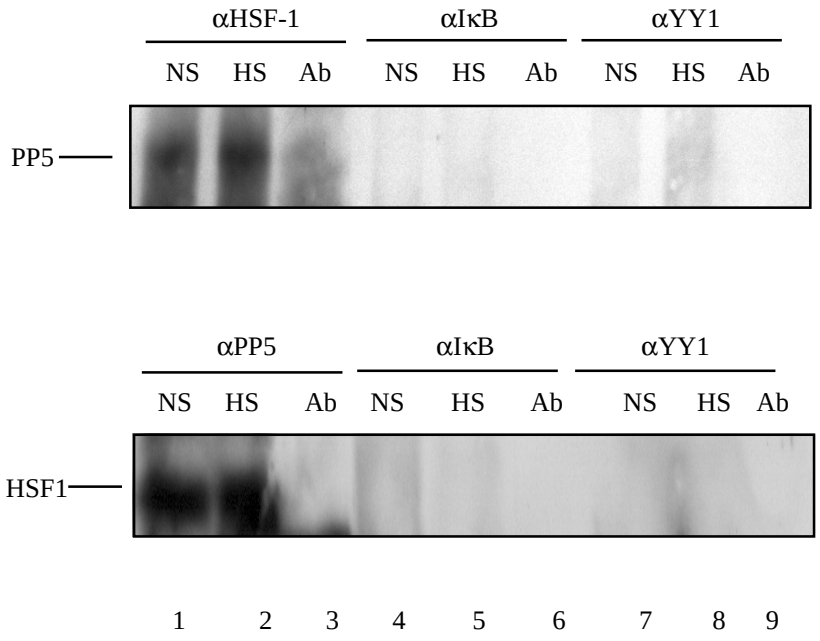
After observing that PP5 was present in oocyte nuclei and the apparent negative-regulatory role of PP5 on HSF1 DNA-binding and transcriptional activities, I next wanted to explore the possibility of a physical interaction between PP5 and the HSF1 heterocomplex. *In vitro* and *in vivo* studies have shown that the HSF1 complex interacts with the proteins HSP40, Hip (HSP70 interacting protein), Hop (HSP90 and HSP70 organizing protein), HSP90, HSP70, p23, and the immunophilins (FKBP51, FKBP52, or cyp40) (as reviewed by Morimoto, 1998; Bharadwaj *et al*., 1999; Guo *et al*., 2001). As well, Guo *et al*., (2001) have recently shown evidence, using co-immunoprecipitations, that PP5 may interact with the HSF1 heterocomplex. In order to determine whether PP5 interacts with the HSF1 complex in *Xenopus* oocytes, a gel mobility supershift analysis was performed using PP5 antibodies to test for effects on the HSF1-HSE complex (figure 11.A). Previous studies have shown that proteins found within the HSF1 complex can be identified when specific antibodies are incubated with oocyte extracts in DNA-binding reactions with radiolabelled HSE (Bharadwaj *et al*., 1999). Antibodies that recognize HSF1 heterocomplex constituents result in a supershift of the HSF1-HSE complex (Bharadwaj *et al*., 1999). Figure 11.A shows the results of a gel mobility supershift experiment using non-shocked and heat shocked oocyte extracts. HSP90, p23, and FKBP52 antibodies were used as positive controls for supershifts, and no antibody, or antibodies of proteins that are not in these complexes (Hop, YY1, and PCNA) were used as negative controls (lane 2). The DNA binding reactions with no antibodies served as a control to show the normal migration

Figure 11. Gel mobility supershift assay and co-immunoprecipitation illustrating interactions between PP5 and HSF1. (A) No antibody (lanes 1 and 2) or antibodies against HSP90, (lanes 3 and 4), PP5 (lanes 5 and 6), p23 (lanes 7 and 8), FKBP52 (lanes 9 and 10), PP1 (lanes 11 and 12), PP2A (lanes 13 and 14), Hop (lanes 15 and 16), YY1 (lanes 17 and 18), and PCNA (lanes 19 and 20) were incubated with radiolabelled HSE at room temperature for 30 minutes with non-shocked (NS-18^{o}C) or heat shocked (HS-33^{o}C for 1 hour) oocyte extracts. Incubated samples were electrophoresed on an acrylamide gel and HSF1-HSE complexes were detected (labelled HSF1). Antibodies that bind HSF1 show a supershift in the HSF1-HSE complex. **(B)** Immunoprecipitation using non-shocked (NS-18^{o}C lanes 1, 4 and 7) and heat shocked (HS-33^{o}C for 1 hour lanes 2, 5 and 8) oocyte extracts. Co-immunoprecipitations were performed with an HSF1 polyclonal antibody/immunoblotted with PP5 polyclonal antibody (top lanes 1-3) and PP5 antibody/immunoblotted with an HSF1 antibody (bottom lanes 1-3) to illustrate that PP5 interacts with HSF1. IκB and YY1 antibodies were used as negative controls (top and bottom lanes 4-6 and 7-9 respectively). Diluted HSF1, PP5, IκB, and YY1 antibodies were run as an additional control (lanes 3, 6 and 9). All experiments were performed a minimum of three times.

A. Gel Shift



B. Coimmunoprecipitation



of HSF1-HSE complexes in heat shocked oocytes. Lane 4 (HSP90), lane 8 (p23), and lane 10 (FKBP52) act as positive controls to show the retarded or supershifted migration pattern of the HSF1-HSE complex when antibody against a member of the heterocomplex is present in the DNA binding reaction. Similar migration to that found in lane 2, in which no apparent supershifting of the HSF1-HSE complex was observed, was seen with addition of antibodies to PP1 (lane 12), PP2A (lane 14), HOP (lane 16), YY1 (lane 18), and PCNA (lane 20). Therefore PP1, PP2A, HOP, and YY1 do not appear to interact with the HSF1 heterocomplex under the conditions used. Addition of PP5 antibody (lane 6) resulted in a supershifted migration pattern similar to that of the positive controls, HSP90 (lane 4), p23 (8), and FKBP52 (10). This indicates that PP5 may interact with the HSF1 heterocomplex. PP1 and PP2A antibodies did not supershift or diminish the HSF1-HSE complex (lanes 12 and 14).

Next I determined if PP5 could be co-immunoprecipitated with HSF1. HSF1 was immunoprecipitated from non-shocked and heat shocked oocytes using an HSF1 polyclonal antibody (figure 11.B (top) lanes 1 and 2) and the immunoprecipitated material was examined for the presence of PP5 by Western blotting. The results showed that PP5 was present in the HSF1-immunoprecipitated material under both non-shock and heat shock conditions. The reciprocal immunoprecipitation and Western blot was also performed using a PP5 polyclonal antibody (figure 11.B (bottom) lanes 1 and 2) and showed that HSF1 was present in the PP5-immunoprecipitated material, again under both non-shock and heat shock conditions. In order to demonstrate that the IgG portion of the antibody was not detected as bands on the Western blots, diluted HSF1 and PP5 antibody were added to an oocyte extract and subjected to Western blotting

(lane 3 top and bottom). The results indicate that the IgG portion of the antibody does not interfere with the immunoprecipitation results as no banding was detected on Western blots at the level of HSF1 or PP5. As an additional control, immunoprecipitations were also performed as above using IκB and YY1 antibodies. Neither PP5 or HSF1 were found in the control immunoprecipitations using these antibodies (lanes 4-6 and 7-9 top and bottom). These results along with the previous supershift (figure 11.A) confirm that PP5 interacts with and is a component of the HSF1-HSP90 heterocomplex. PP5 was identified with HSF1 in both non-shocked and heat shocked extracts indicating that PP5 interacts with both inactive HSF1 monomers and DNA binding HSF1 trimers under control and heat shock conditions respectively.

4.12 PP5 affects HSF1-HSE binding and HSF1 transactivation independently of HSP90.

It is known that PP5 contains a TPR domain that recognizes and binds HSP90, and it is through this interaction that PP5 associates with and regulates the activities of GRs (Chen *et al*., 1996; Silverstein *et al*., 1997). Since PP5 supershifted the HSF1-HSE complex (figure 11.A) and co-immunoprecipitated with HSF1 (figure 11.B), I next investigated whether PP5 exerts its effects on HSF1-DNA binding independently of being bound to HSP90. This was tested using full-length PP5 mutants (K97A and R101A) containing single alanine substitutions within the TPR binding domain. These PP5 mutants are known to retain full phosphatase activity and immunoprecipitation

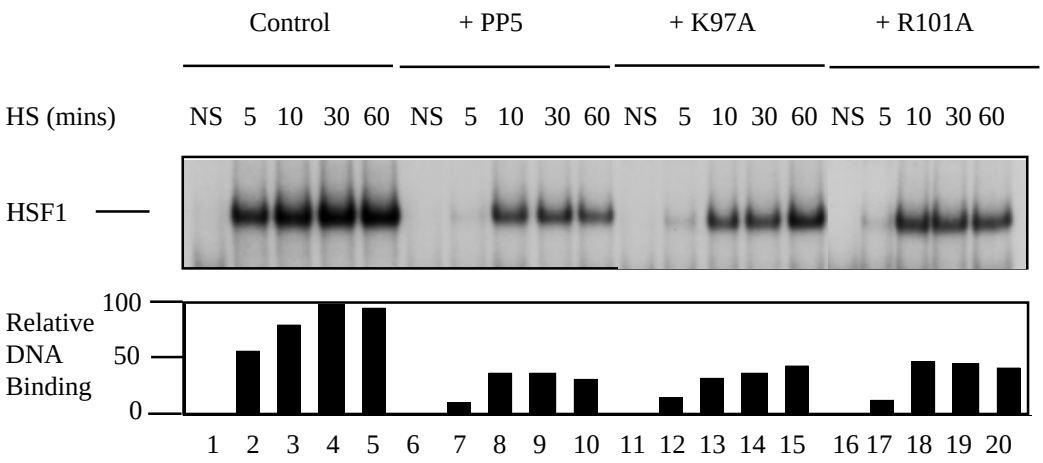
experiments using COS-7 cells have shown that each mutant is unable to bind HSP90 (Russell *et al*., 1999). Oocytes were microinjected with constructs encoding either wild-type PP5 or PP5 mutants K97A or R101A, and were incubated at the control temperature of 18^{o}C or were heat shocked at 33^{o}C for 5, 10, 30 or 60. The extracts were then analyzed using EMSA to determine if non-HSP90 binding mutants could affect the activity of HSF1 (figure 12.A). A similar reduction in the amount of HSF1-HSE complex formation was observed after expression of PP5 (lanes 6-10), K97A (lanes 11-15), and R101A (lanes 16-20) at each time point relative to the level of HSF1-HSE complex formation induced by heat shock in uninjected oocytes (lanes 1-5). This suggests that PP5 decreases HSF1-HSE binding during the induction stage of heat shock regardless of its capacity to bind HSP90. However, the decrease in HSF1-DNA binding was more pronounced in oocytes expressing wild-type PP5 in comparison to those expressing mutant PP5.

To confirm that the increases in protein levels of PP5 and the K97A and R101A mutants were relatively equal, and that their levels did not change under heat shock conditions, a Western blot was performed using extracts from figure 12.A. The results show that the elevated protein levels of wild-type and mutant PP5 are similar, approximately 10-fold higher than that of endogenous PP5, and do not change under heat shock conditions.

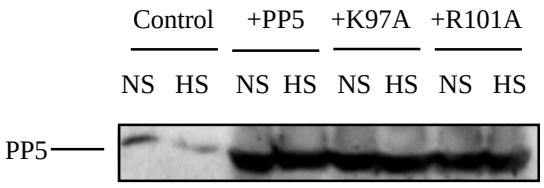
Oocytes were also injected as above and tested in recovery experiments in which a 1 hour 33^{o}C heat shock was followed by recovery for 0, 5, 15, 30 or 60 minutes at 18^{o}C. The extracts were again analyzed using EMSA to

Figure 12. PP5 interacts with and elicits its effect on HSF1-HSE complex independently of HSP90. (A) Wild-type PP5 and PP5 mutants (K97A and R101A) that are unable to bind HSP90 as a result of mutations to the TPR domain were injected into oocytes that were incubated for 12 hours at 18^{o}C. Uninjected (control lanes 1-5) or injected with pCMV-PP5 (lanes 6-10), pET30-K97A (lanes 11-15), or pET-R101A (lanes 16-20). Uninjected and injected oocytes were incubated at 18^{o}C (non-shocked=NS lanes 1,6,11, and 16) or were heat shocked (HS) at 33^{o}C for 5 mins (lanes 2, 7, 12 and 17), 10 mins (lanes 3, 8, 13 and 18), 30 mins (4, 9, 14 and 19), or 60 mins (lanes 5, 10, 15 and 20). **(B)** Western blot to ensure that the increased levels of wild-type and mutant PP5 are comparable in the above experiments. Non-shocked (NS) and heat shocked (HS at 33^{o}C for 60 minutes) protein extracts used in figure 12.A. above were used and immunoblotted with a polyclonal PP5 antibody. **(C)** Oocytes were injected as in figure 12.A. and treated at control (NS-18^{o}C lanes 1, 7, 13 and 19) temperatures or heat shocked at 33^{o}C for 1 hour and allowed to recover at 18^{o}C for 0 mins (lanes 2, 8, 14 and 20), 5 mins (3, 9, 15 and 21), 15 mins (4, 10, 16 and 22), 30 mins (5, 11, 17 and 23), and 60 mins (6, 12, 18 and 24). Extracts were subjected EMSA using radiolabelled HSE. The HSF1-HSE specific complex is indicated on the left and the densitometry is shown below. All experiments were performed a minimum of three times.

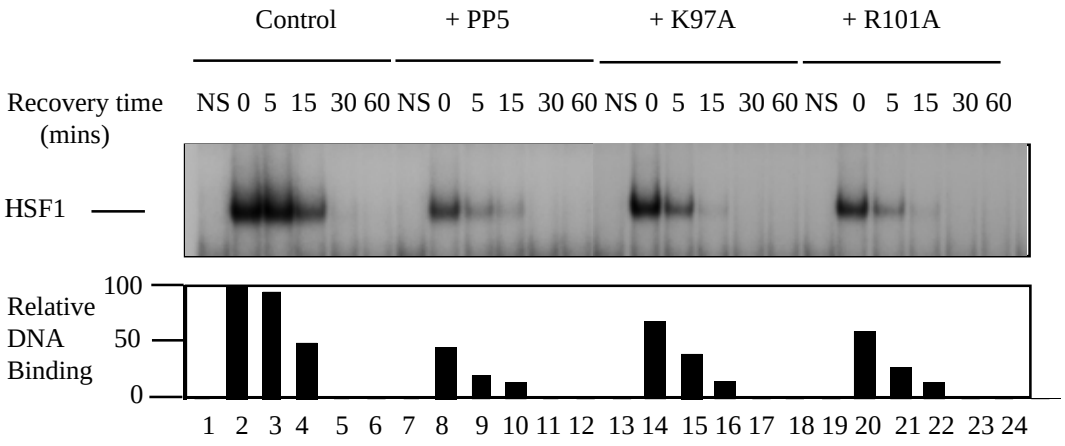
A. Gel Shift



B. Western Blot



C. Gel Shift



compare the effects on HSF1-HSE complex formation (figure 12.C). The level of HSF1-HSE complex formation was similar in oocytes injected with constructs encoding PP5 (lanes 7-12), K97A (lanes 13-17), and R101A (lanes 19-24), and each time point was less than that of control, uninjected oocytes (lanes 1-6). Again, slightly more HSF1-HSP90 complexes were formed in oocytes containing mutant PP5 when compared to those containing wild-type PP5. The results of this experiment illustrate that PP5 and the non-HSP90 binding mutants, exert the same inhibitory effects on HSF1-HSE binding in the oocyte.

4.13 PP5 mutants and HSF1 transcriptional activation.

Transcription assays were performed as described in figure 10, to determine if the PP5 mutants (K97A and R101A) also affected heat-induced HSF1 transactivation of the *hsp*70 promoter (figure 13). *Hsp*70-CAT or control CMV-CAT reporter constructs were co-injected with expression vectors for wild-type and mutant PP5 (K97A and R101A). The results presented in panel 1 indicate that elevated levels of PP5 completely inhibited the promoter activity of *hsp*70. Panels 2 and 3 show transcription assays for oocytes with increased levels of the PP5 mutants (K97A and R101A) and demonstrate a similar inhibition of HSF1-mediated transcription when compared to that observed in oocytes containing wild-type PP5. The mutants repressed HSF1 transcriptional activation, to the same extent as that of wild-type PP5. This implies that the phosphatase activity of PP5 could regulate HSF1-DNA binding and HSF1 transactivation independently of being bound to HSP90.

Figure 13. Analysis of HSF1 transcriptional activity using *hsp*70-CAT reporter constructs and PP5 mutants. Oocytes were microinjected with pCMV-PP5, pET30-K97A, or pET30-R101A and/or *hsp*70-CAT or CMV-CAT and incubated for 12 hours at 18^{o}C. Oocytes were then incubated at non-shock temperature (NS 18^{o}C) or heat shocked (HS) at 33^{o}C for one hour. Extracts were then subjected to CAT assay. Thin-layer chromatography was used to separate the acetylated (Ac) and non-acetylated form of chloramphenicol (Cm) (as indicated on the right). All experiments were performed a minimum of three times.

CAT Assays

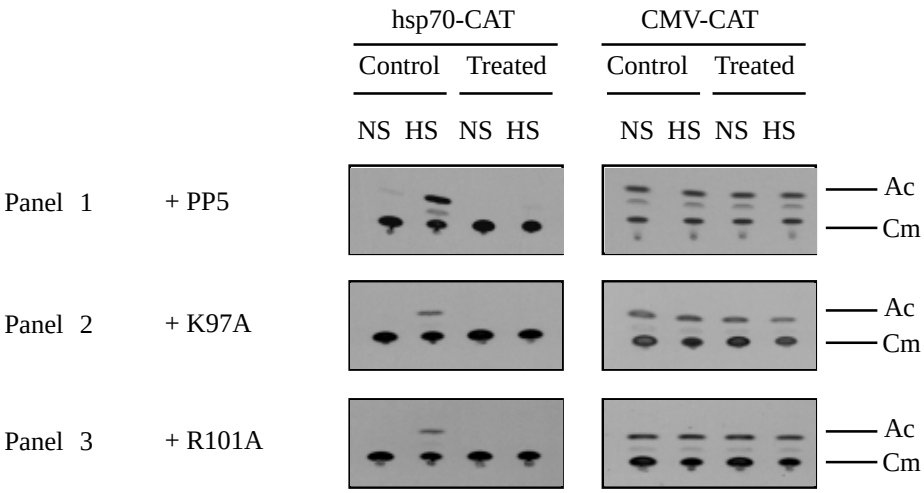
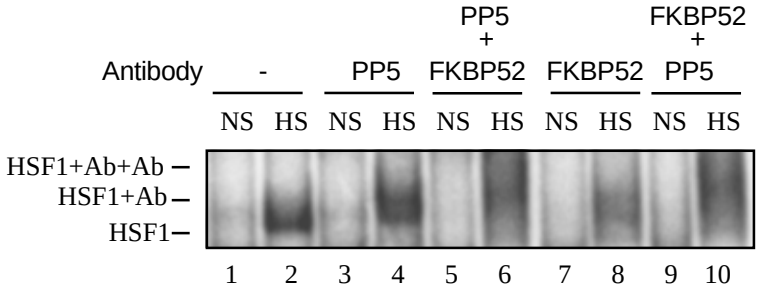

hsp70-CAT
CMV-CAT
Control
Treated
Control
Treated
NS HS NS HS
NS HS NS HS
Panel 1
+ PP5
Ac
Cm
Panel 2
+ K97A
Ac
Cm
Panel 3
+ R101A
Ac
Cm

Figure 4.14 PP5 is present in HSF1 heterocomplexes containing an immunophilin.

Previous studies involving glucocorticoid receptors and HSF1 illustrate that only one immunophilin interacts with the HSF1 heterocomplex at any given time (Ratajczak and Carrello, 1996; Chen *et al*., 1996; Silverstein *et al*., 1997; Owens-Grillo *et al*., 1998; Bharadwaj *et al*., 1999; Chinkers, 2001). Furthermore, additional studies have demonstrated that PP5 cannot bind GRs if an immunophilin is present, as they both bind HSP90 at the same location (Chen *et al*., 1996; Silverstein *et al*., 1997). Using co-immunoprecipitations and gel mobility supershifts, I have shown evidence that PP5 is a component of HSF1 heterocomplexes (figure 11). Figures 12 and 13 illustrated that PP5 may bind the HSF1 heterocomplex and affect HSF1 activity independently of binding to HSP90 suggesting that the interactions between HSF1 and PP5 are different than those between GRs and PP5. In order to investigate this hypothesis further, gel mobility supershift assays were performed to determine if PP5 and the immunophilin FKBP52 are present within the same HSF1 heterocomplexes, or if interactions between PP5 and FKBP52 are mutually exclusive (figure 14). Similar to the supershift assays performed in figure 11.A, non-shock (18^oC) and heat shocked (33^oC for 1 hour) oocyte extracts were used to detect migration changes in HSF1-HSE complexes. The migration of the HSF1-HSE complex in uninjected heat shocked extracts is seen in lane 2. Binding reactions shown in lanes 4 and 8 contain PP5 or FKBP52 antibodies. As also seen in figure 11, these antibodies supershifted the HSF1-HSE complex when compared to extracts without antibody (lane 2), suggesting that these proteins are

Figure 14. Super-supershifts showing the presence of both PP5 and FKBP52 in the HSF1-heterocomplex. Oocytes were incubated with radiolabelled HSE at room temperature for 30 minutes with non-shocked (NS-18^{o}C) or heat shocked (HS-33^{o}C for 1hour) oocyte extracts and no antibody (lanes 1 and 2) or antibodies against PP5 (lanes 3 and 4) and FKBP52 (lanes 7 and 8). Lanes 5 and 6 (PP5 and FKBP52) and lanes 9 and 10 (FKBP52 and PP5) were incubated as above with the antibody listed first and then incubated with the second antibody for another 30 minutes at room temperature. All samples were electrophoresed on an acrylamide gel and HSF1-HSE complexes were detected (labelled HSF1, HSF1+Ab, or HSF1++Ab). All experiments were performed a minimum of three times.

SuperShift


PP5
+
FKBP52
+
Antibody
-
PP5
FKBP52
FKBP52
PP5
NS HS NS HS NS HS NS HS NS HS
HSF1+Ab+Ab
HSF1+Ab
HSF1
1 2 3 4 5 6 7 8 9 10

present in the HSF1 heterocomplex. In order to determine if both proteins are present within the complex simultaneously, PP5 antibody was added to the binding reaction followed by the addition of an FKBP52 antibody (lane 6). The reciprocal experiment was also done where the addition of PP5 antibody followed the addition of the FKBP52 antibody (lane 10). The addition of a second antibody resulted in further retardation of the HSF1-HSE complex (lanes 6 and 10) when compared to the migration observed when only a single antibody was used in the reaction (lanes 4 and 8). The additional retardation brought about by sequential addition of the respective antibodies suggest that both PP5 and FKBP52 may be present in the heterocomplex at the same time. If this is indeed true and both proteins are present in the heterocomplex simultaneously, then PP5 may bind directly to HSF1 or a member of the heterocomplex other than HSP90, as previous studies have already shown that FKBP52 binds the complex via the TPR domain of HSP90. These results further support the hypothesis that PP5 may bind the HSF1 heterocomplex independently of HSP90.

5.0 DISCUSSION

The major finding of this thesis is that PP5 is a component of the HSF1-HSP90 heterocomplex, serving as a negative-regulator of DNA binding and transcriptional activation.

In this thesis I utilized the *Xenopus* oocyte model system to test the hypothesis that PP5 regulates the DNA binding and transcriptional activities of HSF1. The experimental approach taken was to manipulate the level of PP5 activity within the oocyte and determine the subsequent effects on HSF1 activities. To elevate intracellular PP5 phosphatase activity, expression vectors encoding PP5 or the HSP90 C-terminal region known to stimulate PP5, were microinjected into oocytes serving either to increase PP5 protein levels or to elevate the activity of endogenous PP5. PP5 was also inhibited using immunotargetting experiments and by treating oocytes with okadaic acid. As well, I attempted to determine if PP5 interacts with the HSF1 heterocomplex using a combination of gel mobility supershift assays and immunoprecipitations. Thus together, the results presented in this thesis provide evidence that PP5 interacts with the HSF1 heterocomplex and functions to negatively regulate the DNA binding and transcriptional activities of HSF1.

It has been hypothesized that the chaperone machinery required for GR assembly may be similar to that involved in the regulation of HSF1 (Nair *et al*., 1996). Using the *Xenopus* model system, Ali *et al*., (1998) and Bharadwaj *et al*., (1999)

confirmed that the chaperones involved in GR maturation resemble those present in the chaperone heterocomplex regulating the activities of HSF1. Interestingly, several studies have identified PP5 as a member of the chaperone complex involved in the maturation and activation of GRs. For example, Dean *et al*., (2001) demonstrated that in the absence of PP5, shuttling of GRs from the nucleus to the cytoplasm ceases, resulting in the nuclear accumulation of GRs and increased GR-DNA binding. Two contradicting studies have also shown that the inhibition of PP5 can result in either an increase or decrease in GR-mediated transcription (Chen *et al*., 1996; Zuo *et al*., 1999). As well, PP5 has recently been found to interact with the HSF1 heterocomplex (Guo *et al*., 2001), however no studies prior to this one had examined what role PP5 may play in the regulation of HSF1.

5.1 The Subcellular Localization of PP5

It has been established that HSF1 exists in the nucleus of *Xenopus* oocytes both under non-shock and heat stress conditions (Mercier *et al*., 1997). Therefore in order to examine a possible role for PP5 in the regulation of HSF1, I wanted to determine the subcellular localization of PP5. Previous studies have identified PP5 as both a nuclear and cytoplasmic protein in mammalian cells, but the subcellular localization of PP5 in *Xenopus* oocytes has not previously been examined. Using Western blot analysis on manually dissected oocyte fractions, I determined that PP5 is both a cytoplasmic and nuclear protein in *Xenopus* oocytes (figure 1). It should be noted that although the majority of PP5 appeared to be present in the cytoplasm, in this assay, the total protein

mass of the cytoplasmic fraction is much greater than that of the nuclear fraction therefore, there is actually more PP5 present in the nucleus than the results of the Western blot would suggest. It was determined that PP5 is a nuclear protein before and after heat shock and that it appears to undergo translocation in response to heat treatment (figure 1). Consequently, it is possible that PP5 has the potential to interact with and regulate the activities of HSF1 under both non-shock and heat shock conditions. For example, since PP5 is present in the nucleus before heat shock, it is possible that PP5 regulates the DNA binding and transcriptional activities of HSF1 by suppressing the formation of HSF1 trimers prior to heat shock.

Interestingly, PP5 has been identified as both a member of cytoplasmic and nuclear GR complexes and was found to play a key role in the nucleocytoplasmic shuttling of GRs (Dean *et al*., 2001). Since HSF1 is a purely nuclear protein in *Xenopus* oocytes, the reason for the translocation of PP5 can only be speculated. One possibility is that PP5 translocates with other members of the molecular chaperone complex required to repress HSF1-DNA binding and HSF1 transcriptional activation. For example, our lab has previously shown that HIP, HOP, and the immunophilin FKBP52 translocate from the cytoplasm to the nucleus in heat shocked oocyte extracts (Bharadwaj *et al*., 1999). Although interactions between PP5 and these proteins have not been investigated as of yet, HOP and FKBP52 each contain at least 3 TPR domains belonging to the HSP90-family of TPR domains and therefore may have the potential to interact with PP5 (Honore *et al*., 1992; Ratajczak *et al*., 1993; Kieffer *et al*., 1993; Owens-Grillo *et al*., 1996). Another possible reason as to why PP5 undergoes translocation after heat shock is that the additional nuclear PP5 may be required to

regulate the attenuation of HSF1. For example, PP5 could function to either increase the rate of trimer disassembly and promote HSF1 attenuation or further suppress the formation of HSF1 trimers. However, it has not been determined why the translocation of PP5 occurs after heat shock in *Xenopus* oocytes as this is the first study examining the role of PP5 as a regulator of HSF1 using this model system.

5.2 The Role of PP5 in HSF1 Oligomerization

The first major conclusion of these experiments is that PP5 regulates the DNA binding activity of HSF1 either by suppressing the conversion of HSF1 monomers into trimers or by promoting the disassembly of trimers. This could occur both during the induction and recovery phases of the heat shock response. The gel mobility shift assays performed throughout this thesis provide an indirect but efficient method of measuring the oligomeric status of HSF1 since it is known that HSF1 only binds the HSE after it has undergone trimerization, and that increases seen in HSF1-HSE binding are a direct result of HSF1 trimer assembly in response to stress (reviewed by Lis and Wu, 1993). Thus the formation of HSF1-HSE complexes are a direct reflection of the formation of HSF1 trimers. It has also been determined that HSF1 protein levels do not increase or decrease in response to heat shock (figure 3), therefore in these experiments, any changes seen in HSF1-DNA binding were due to the various treatments (increased PP5 levels, phosphatase inhibition) in the oocytes.

PP5 appears to function as a protein that represses HSF1-DNA binding, and hence trimer formation. In all experiments, activation of endogenous PP5 or elevation of PP5

protein levels using microinjected expression vectors, consistently resulted in a decrease in HSF1-HSE complex formation during the induction and recovery stages of heat shock (figures 2, 3, and 8). The decreased levels of HSF1-HSE binding demonstrate that after heat shock, lower levels of HSF1 trimers are present in oocytes containing activated or elevated levels of PP5 when compared to control, uninjected oocytes. This suggests that PP5 may suppress the DNA binding activity of HSF1 by two possible mechanisms. The first possibility is that PP5, through the dephosphorylation of HSF1 itself or components of the HSF1-HSP90 heterocomplex, inhibits trimerization or stabilizes the monomeric state of HSF1. Interestingly, inhibition of PP5 activity by immunotargeting or treatment of oocytes with okadaic acid, had no apparent effect on the magnitude of trimer formation during the induction stage of heat shock (figure 4). Since PP5 appears to repress the trimerization of HSF1, it might have been expected that the inhibition of PP5 activity would increase the amount of trimers formed during heat shock. However, this did not occur and is most likely due to the fact that at maximal or optimal stress conditions used in these experiments (33^{o}C in *Xenopus* oocytes), all of the HSF1 present within the nucleus trimerizes and additional HSF1-HSE binding could not be observed (DiDomenico *et al*., 1982; Mosser *et al*., 1988; Strauss *et al*., 1990; Abrayava *et al*., 1991; Gordon *et al*., 1997). Therefore, an increase in HSF1 trimer formation was not observed when PP5 was inhibited even though this presumably functioned to promote the formation of HSF1 trimers.

It also might have been expected that the inhibition of PP5 would have promoted the formation of trimers independently of heat shock in the absence of stress, and that

HSF1-HSE complexes would have been detected in non-shocked okadaic acid treated oocytes. This was not observed (figures 5 and 8), but HSF1-HSE binding in the absence of stress was detected in attenuation studies where PP5 was inhibited by immunotargeting accomplished through injection of PP5 antibodies. Therefore it remains possible that PP5 may regulate HSF1 by suppressing trimer formation (figure 8.B). However, since DNA binding was not induced in all non-shocked oocytes containing inhibited PP5, it is more likely that PP5 promotes the disassembly of HSF1 trimers and that the diminished amount of HSF1-HSE complexes seen in these experiments was due to an increased rate of trimer disassembly rather than a decreased rate of trimer assembly.

The most likely mechanism by which PP5 acts to reduce the level of HSF1-HSE trimers is by accelerating the rate at which HSF1 trimers are converted into inactive monomers, as elevated levels of PP5 phosphatase activity decreased the detectable level of HSF1-HSE complex formation (figure 2, 3, and 7). It follows that if PP5 were inhibited, then a delay in HSF1 timer disassembly and therefore prolonged HSF1-HSE binding would be observed in cells recovering from heat shock. Attenuation studies performed on oocytes containing inhibited PP5 activity indeed resulted in prolonged retention of HSF1-HSE complexes after removal from heat shock conditions (for at least 15 minutes longer) (figure 8).

It has been determined that the level and activation of HSF1-DNA binding and HSF1-mediated transcription varies according to the type of stress. For example, salicylate has been shown to induce the formation of HSF1-HSE complexes without inducing the transcriptional activation of HSF1 (Jurivich *et al*., 1992 and 1995).

Therefore, since the activities of HSF1 vary according to the type of stress, it was possible that the mechanisms of HSF1 regulation also vary and that PP5 may not function to decrease HSF1-HSE binding in stresses other than heat shock. To determine what effect PP5 had if any on HSF1 trimer formation in stresses other than heat shock, oocytes with increased levels of PP5 were treated with salicylate, arsenite, cadmium, and ethanol (figure 6). It was determined that elevation of PP5 indeed decreased the amount of HSF1-HSE complex formation in salicylate; arsenite; cadmium; and ethanol-treated oocytes to approximately the same degree as in heat shock treated oocytes. This suggests that PP5 may regulate the DNA binding activities of HSF1 in a similar manner in response to a variety of stresses. Again, in the case of chemical stressors, it is possible that PP5 represses formation of trimers or promotes trimer disassembly.

5.3 The Role of PP5 in HSF1-mediated Transcription

The second major conclusion of this thesis is that PP5 also acts to negatively regulate the transcriptional activities of HSF1. Several studies have demonstrated that the transcriptional activation and DNA binding activities of HSF1 are separately regulated (Hensold *et al*., 1990; Jurivich *et al*., 1992; Bharadwaj SHOE *et al*., 1999). Therefore, it was possible that PP5 could inhibit or suppress HSF1-HSE complex formation, but not affect the transactivation of HSF1. Transcription assays using reporter constructs were performed to test this (figure 10.A). It was observed that elevated levels of PP5 acted to significantly decrease or, in fact, completely repress

HSF1-dependent transcription of the *hsp*70 promoter. These results correlate well with the HSF1-DNA binding studies demonstrating that elevation of PP5 decreases HSF1-HSE complex formation (figures 2,3, and 7) and suggests that PP5 acts to negatively regulate HSF1-DNA binding as well as HSF1 transcriptional activation. It is possible that PP5 affects both the DNA binding and transcriptional activities of HSF1 by repressing the formation of HSF1 trimers however, because the degree of transcriptional inhibition (no *hsp*70 promoter activity) is much greater than that observed in DNA binding reduction (approximately 2-3 fold), it is possible that PP5 functions not only to inhibit the formation of HSF1 trimers, but also to inhibit the transcriptional activation domain of any HSF1 trimers that do form in oocytes containing elevated levels of PP5.

Oocytes that were treated with okadaic acid showed results similar to those found in HSF1-HSE binding assays; inhibition of PP5 did not result in increased HSF1-mediated transcription (figures 4 and 8). Although PP5 inhibition was shown to prolong the attenuation of HSF1-DNA binding (figure 8), this did not enhance *hsp*70 promoter activity and could again suggest that PP5 may regulate HSF1-DNA binding differently than HSF1 transactivation as the two events are separately regulated.

It was also necessary to determine if the effects seen on HSF1 transactivation were due specifically to the activities of PP5 and not that of other protein phosphatases. Experiments in which PP1 enzyme levels were increased in oocytes demonstrated that this phosphatase had no significant effect on HSF1 transcriptional activation (figure 10.A). This was consistent with experiments demonstrating that PP1 had no effect on HSF1-HSE binding (figures 5 and 9). Interestingly, it was found that increased levels

of PP2A resulted in a significant decrease in *hsp*70 promoter activity (figure 10.A). The converse was true when PP2A was inhibited using fostriecin, since enhanced transcriptional activity. Previous experiments indicated that these treatments had a minor effect on HSF1-HSE binding, therefore it may be that PP2A acts to regulate HSF1 at the level of DNA binding and transactivation or that PP2A stimulates the phosphatase activity of PP5 *in vivo* and indirectly inhibits the activities of HSF1. Although it has been established that PP2A and PP5 interact *in vitro*, it has not been determined if PP2A regulates the activities of PP5 *in vivo*. It remains possible that PP2A itself effects the transactivation and DNA binding of HSF1. Ding *et al*., (1998) observed previously that the phosphatase activity of PP2A was elevated in cells overexpressing HSP70, and suggested that PP2A may function to dephosphorylate HSF1 and negatively regulate its transcriptional activities. However, no further studies have been performed to determine how PP2A may regulate HSF1. Future studies should be carried out using PP1 and PP2A expression vectors and additional inhibitors to investigate exactly how PP2A may regulate HSF1-HSE binding and HSF1 transcriptional activation.

It has been demonstrated that stresses other than heat shock activate HSF1-DNA binding but not HSF1-mediated transcription. Bharadwaj *et al*., (1999) showed in *Xenopus* oocytes that cadmium chloride activates both the DNA binding and transcriptional activities of HSF1. It is important to note that in *Xenopus* oocytes, not all stresses capable of activating HSF1-HSE binding activate HSF1 dependent transcription (Bharadwaj *et al*., 1999). Cadmium was the only stress used other than heat shock that activates HSF1 transcriptional activity (Bharadwaj *et al*., 1999).

Transcription assays illustrated that elevated PP5 protein levels decreased the transcriptional activity of HSF1 in response to cadmium (figure 10.B). Thus the inhibitory effect of PP5 on transcription is not restricted to heat shock.

5.4 PP5 interactions with HSF1

Previous studies have identified PP5 as a member of the GR-HSP90 heterocomplex that regulates the maturation and activation of GRs (Chen *et al*., 1996; Silverstein *et al*., 1997; Zuo *et al*., 1999; Dean *et al*., 2001). It has been well established that the chaperone complex associated with the regulation of HSF1 shares many similar structural and functional features with the heterocomplexes that regulate GRs (Ali *et al*., 1998; Zuo *et al*., 1999; Bharadwaj *et al*., 1999). This gave rise to the hypothesis that PP5 may interact with and somehow regulate the DNA binding and transcriptional activities of HSF1 in *Xenopus* oocytes. Guo *et al*., (2001) have recently shown, using tissues culture cells and immunoprecipitation experiments, that PP5 interacts with the HSF1 heterocomplex, although the function of PP5 within the complex was not investigated. This thesis has provided evidence that the phosphatase activity of PP5 negatively regulates the DNA binding and transcriptional activation of HSF1. It is possible that PP5 exerts its effects on HSF1 through direct physical interactions with the heterocomplex or indirectly, perhaps by removal of phosphate groups from auxiliary regulating proteins.

Showing retardation of the HSF1-HSE complex with PP5 antibodies provided evidence for a direct physical association between PP5 and HSF1 (figure 11.A). Co-

immunoprecipitations confirmed that PP5 interacts with HSF1 trimers and that PP5 also interacts with the inactive monomeric form of HSF1 that exists under non-shock conditions (figure 11.B). Together these results strongly suggest that PP5 interacts with the HSF1 heterocomplex during both non-shock and heat shock conditions. These results are consistent in all experiments performed throughout this thesis, and when combined, indicate that PP5 negatively regulates the activities of HSF1 *in vivo*. This finding is significant because it sheds light on a potential mechanism for PP5 function in the HSF1 heterocomplex. Apparently, PP5 remains associated with HSF1 in monomeric and trimeric states and the association of PP5 with HSF1 appears not to be affected by heat shock since it co-immunoprecipitated equally in control and stressed samples (figure 11.B). Thus PP5 may function as a key regulatory phosphatase throughout the activation/deactivation cycle of HSF1 before, during, and after stress.

PP5 has been shown to interact with GRs by binding to HSP90 through its TPR domain, and immunoprecipitation studies suggest binding of an immunophilin or PP5 to the GR complex is mutually exclusive. One could suggest then, that if PP5 interacts with the HSF1 heterocomplex via similar HSP90-TPR dependent binding, it should exert its regulatory effects on HSF1 dependent on HSP90, and might not co-exist within the heterocomplex along with one of the immunophilins. However, supershift assays indicate that both PP5 and FKBP52 are present in HSF1 complexes simultaneously (figure 14). Since several studies involving GR complexes have shown that these two proteins compete for binding to HSP90 and display exclusive binding to GR-HSP90 complexes, the results shown here suggest that PP5 interacts differently with HSF1-HSP90 heterocomplexes. It is possible that PP5 binds the heterocomplex at

a different location than immunophilins, therefore, unlike the immunophilins, PP5 may exist within the HSF1 heterocomplex throughout the activation and deactivation cycle. Indeed, co-immunoprecipitation experiments showed that PP5 interacts with both the monomeric and trimeric forms of HSF1.

To further investigate how PP5 may bind the HSF1 heterocomplex, expression vectors encoding PP5 mutants unable to bind HSP90 were microinjected into oocytes. Increased levels of the mutated forms of PP5 were still able to exert similar repressive effects on HSF1-HSE complex formation as wild-type PP5, since reduced levels were seen during both the induction and recovery stages of heat shock (figure 12) and suggests that PP5 may act to decrease HSF1-HSE binding independent of its ability to bind HSP90. Transcription assays using reporter constructs also illustrated that the PP5 mutants, similarly to wild-type PP5, decreased HSF1-mediated transcription (figure 14). This could suggest that unlike in GR regulation, PP5 interacts with HSF1 directly or with heterocomplex members other than HSP90. It is possible though that the findings observed are the result of PP5 mutants exerting phosphatase activity on HSF1-HSP90 complexes independently of direct stable binding as the PP5 mutants may retain the ability to interact with *Xenopus* HSP90, however this is unlikely as HSP90 is a highly conserved protein. A third possibility is that the PP5 exerts its effects on HSF1 independently of being bound to an existing HSF1 heterocomplex. Again however this is probably unlikely as immunoprecipitation and supershift assays experiments have indicated that PP5 interacts with the HSF1 heterocomplex (figure 11.A and B).

Unfortunately, it was not possible to directly assess the interactions of the PP5 mutants with the HSF1 complex (using supershift or immunoprecipitation assays), because there was no way to distinguish the mutated forms of PP5 from endogenous PP5 using antibodies. Future experiments may include performing an immunoprecipitation with PP5 and HSP90 to further determine how PP5 may bind the HSF1-HSP90 heterocomplex.

5.5 Mechanisms of HSF1 Regulation by PP5

Many studies suggest that phosphorylation events regulate HSF1 at the level of DNA binding and transcriptional activation. However, the mechanisms and sequences of phosphorylation/dephosphorylation events remain unclear, and the majority of the previous research has focused on how hyperphosphorylation might regulate the transactivation of HSF1. HSF1 remains transcriptionally inert through constitutive phosphorylation and HSF1 transcriptional activation is associated with hyperphosphorylation (Sorger *et al*., 1987; Sorger and Pelham, 1988; Baler *et al*., 1993; Sarge *et al*., 1993). Subsequent studies have since identified specific residues, such as serine 230, 303, 307, and threonine 142, that must be inducibly phosphorylated for full transcriptional activation of HSF1 (Holmberg *et al*. 2001; Soncin *et al*., 2003; Hietakangas *et al*., 2003). Conversely, several studies have illustrated that the constitutive or inducible phosphorylation of HSF1 acts to repress HSF1 transcriptional activities. Phosphorylation of serines 303, 307, and 363 have all been shown to repress the transcriptional activation of HSF1 (Chu *et al*., 1996 and 1998; Kline and Morimoto,

1997). It has also been demonstrated that HSF1 transactivation is repressed when the enzyme level of several kinases such as MAPK, PKC, and GSK3β are increased (Chu *et al*., 1996 and 1998; Xavier *et al*., 2000). Except for the observations by Ding *et al*., (1998) that suggest the activities of PP1 and PP2A increase in response to elevated HSP70 protein levels, none of the previous studies have identified a phosphatase that might be responsible for the regulation of HSF1.

The results presented in this thesis demonstrate that PP5 negatively regulates both HSF1-HSE complex formation and HSF1 transactivation. Since previous studies have shown that serines 230, 303, and 307, and threonine 142 become hyperphosphorylated on transcriptionally active HSF1, and since elevated levels of PP5 were shown to decrease the level of HSF1-mediated transcription, it is possible that PP5 may exert its regulatory effects on HSF1 by dephosphorylating one or more of these residues. Phosphorylation of threonine 142 may also be required for the efficient binding of HSF1 to the HSE (Soncin *et al*., 2003). Therefore, since a decrease in HSF1 complex formation was seen in oocytes containing activated or elevated levels of PP5, it could be that PP5 specifically dephosphorylates threonine 142 and negatively regulates the DNA binding activities of HSF1. From the current data, the precise mechanism by which PP5 regulates the DNA binding and transcriptional activities of HSF1 is not yet known.

In summary, it has been shown that PP5 interacts with both the monomeric and trimeric forms of HSF1 before and after heat shock, that activated or increased levels of PP5 decrease both the level of HSF1-HSE complex formation and HSF1 transactivation, and that cells containing inhibited PP5 result in prolonged HSF1-HSE

binding in response to heat shock. From these results it can be concluded that PP5 acts to down-regulate HSF1.

6.0 REFERENCES

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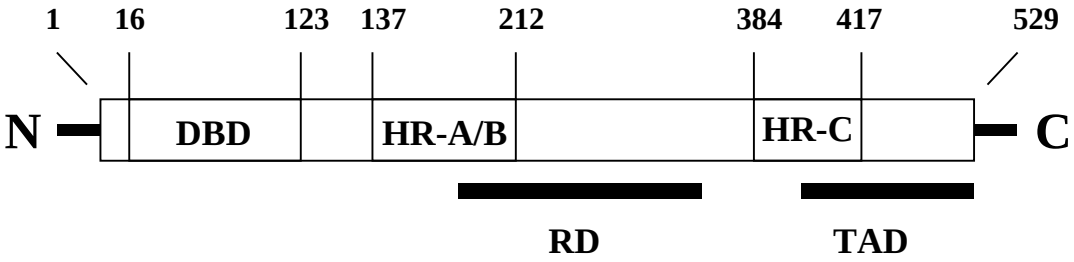
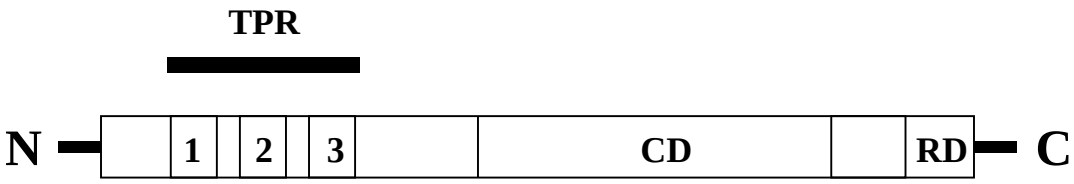
APPENDIX 1. Structure of HSF1



Appendix 1: Structure of human heat shock transcription factor 1 (HSF1). HSF1 is composed of three major domains, a DNA binding domain (DBD), a regulatory domain (RD), and a transcriptional activation domain (TAD). HSF1 also contains two heptad repeat regions (HR-A/B and HR-C) that are involved in the regulation of HSF1 trimerization.

APPENDIX 2. Structure of PP5



Appendix 2: The structure of human protein phosphatase 5 (PP5). PP5 is composed of three tetratricopeptide repeat domains (TPR) labelled 1, 2, and 3, a catalytic domain (CD), and a C terminal regulatory domain (RD).