Examination of the expression of the heat shock protein gene, *hsp110*, in *Xenopus laevis* cultured cells and embryos

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Prokaryotic and eukaryotic organisms respond to various stressors with the production of heat shock proteins (HSPs). HSP110 is a large molecular mass HSP that is constitutively expressed in most adult mammalian tissues. In the present study, we have examined for the first time the expression of the *hsp110* gene in *Xenopus laevis* cultured cells and embryos. The *Xenopus hsp110* cDNA encodes an 854 amino acid protein, which shares 74% identity with mice and humans. In *Xenopus* A6 kidney epithelial cells *hsp110* mRNA was detected constitutively and was heat inducible. Enhanced *hsp110* mRNA levels were detected within 1 h, and remained elevated for at least 6 h. A similar accumulation of *hsp70* mRNA was observed, but only in response to stress. Treatment of A6 cells with sodium arsenite and cadmium chloride also induced *hsp110* and *hsp70* mRNA accumulation. However, while ethanol treatment resulted in the accumulation of *hsp70* mRNA no effect was seen for *hsp110.* Similarly, HSP110 and HSP70 protein increased after a 2 h heat shock and 12 h sodium arsenite treatment. The elevation in HSP110 and HSP70 protein in response to heat was detectable for up to 6 h. Recent studies with mice suggest an important role for HSP110 during development. Analysis of *Xenopus* embryos revealed that *hsp110* mRNA was present in unfertilized eggs, indicating that it is a maternal mRNA, unlike the *hsp70* message which was only detectable in response to heat shock. Heat shock-induced *hsp110* mRNA accumulation was developmentally regulated, similar to *hsp70,* since it was not detectable until after the midblastula stage of development. Enhanced *hsp110* mRNA accumulation was evident with heat shock at the blastula stage, and levels continued to increase reaching a maximum at the late tailbud stage. Message for the small heat shock protein, *hsp27,* was not detectable until the early tailbud stage, indicating that this *hsp* was not present maternally and was developmentally regulated. *In situ* hybridization analysis revealed that *hsp110* mRNA was present in control embryos in the lens placode, spinal cord and somites, but increased upon heat shock in the anterior and posterior region, the lens placode, as well as in the somites and spinal cord. A similar

distribution was observed for the *hsp27* message, although it was not detectable until the early tailbud stage in control or heat-shocked embryos. The intracellular localization of HSP110 protein in response to stress was also investigated. HSP110 was detected predominantly in the cytoplasm in either a diffuse pattern or in long spindle-shaped fibres. Additionally, HSP110 was present in the nucleus. In heat shocked *Xenopus* A6 cells, HSP110 localized in distinct patterns surrounding the nucleus and was enhanced in the nucleus after prolonged heat stress. Sodium arsenite-treated cells displayed a similar pattern in which HSP110 localized on opposite ends of the nucleus. In contrast, in response to stress HSP30 was homogeneously distributed in the cytoplasm, moving into the nucleus only upon intense stress. This study presents, for the first time, a characterization of HSP110 in *Xenopus laevis,* adding to the growing knowledge of HSPs in this important model organism.

Acknowledgements

As can be imagined, a great many people have contributed in their own way to the ultimate completion of this thesis. I most certainly did not accomplish this entirely on my own. I have several people to thank, too many to list here, but I will of course mention a few.

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Not surprisingly, I have saved the best for last; my family. My parents have been unbelievably supportive and understanding through the duration of my graduate studies. I always knew I could go home and be taken care of, pampered, and loved, any time I needed it. A better family there could not be. And last, but totally not least, my husband, Luke Coleman. We were lucky (or unlucky) enough to endure our PhD's together. Luke is a brilliant academic with impressive enthusiasm for his research, which I find absolutely admirable. He has believed in me when I thought I could not continue; he somehow sees things in me that I can not. He gives me the strength and confidence and motivation to want to be a better scientist, and a better person.

I am very lucky to leave the University of Waterloo as a better person than when I arrived. I have only to thank all of the wonderful people that I have encountered along the way. I take with me very fond memories, and many wonderful friends.

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Chapter 1: Introduction

The biochemical processes that sustain higher organisms are, in general, temperature dependent (Katschinski, 2004). Responses to temperature variation are diverse; ranging from behavioural to molecular modifications. One mechanism used by the cell to maintain homeostasis, when faced with the devastating effects of stress, is the heat shock response. The heat shock response is a transient response which is triggered by a variety of stressful stimuli, ultimately providing protection for vital cellular proteins against damage inflicted by stress (Katschinski, 2004). Specifically, organisms respond to temperature stress with the rapid induction of heat shock protein (HSP) synthesis (Lindquist and Craig, 1988). The HSPs are highly conserved, existing in almost all organisms. Furthermore, it has been suggested that the heat shock response is the most highly conserved genetic system known (Lindquist and Craig, 1988). However, while several aspects of the heat shock response are universal, certain features may vary between organisms. Briefly, HSPs function to protect cellular proteins against irreversible damage, such as aggregation and denaturation induced by stress. Upon return to normal conditions, denatured proteins are refolded to their native state by members of the HSP superfamily or degraded. The focus of this thesis will be one HSP in particular, HSP110, and its involvement in the heat shock response of *Xenopus laevis*.

1.1 *Xenopus laevis*

Xenopus laevis laevis (Daudin) is one of the most widely used non-mammalian laboratory research animals. *Xenopus* has long been a useful amphibian model organism for development, and its cell and molecular biology have also been extensively examined. There

are 7 species within the *Xenopus* genus, and 7 subspecies of *Xenopus laevis*. The South African clawed frog is indigenous to temperate Southern and Western Africa. These animals are entirely aquatic, spending little if any time on land (Deuchar, 1975). *Xenopus* generally prefer stagnant pools of water and gulp air for respiration. In such an environment, prone to drastic temperature changes, *Xenopus* would be exposed to a broad temperature range, rendering *Xenopus* an interesting organism for heat shock research. In fact, the majority of heat shock research in amphibians has involved *Xenopus,* and as such the cell and molecular nature of its heat shock response has been well-characterized.

Xenopus laevis is a common model organism for early vertebrate development. Several features render *Xenopus* useful for this purpose; ease of laboratory maintenance, the production of a very large number of eggs, large egg size, the ability to induce egg production repeatedly, rapid development of embryos, external fertilization (enabling *in vitro* fertilization), embryo growth at room temperature, and also the availability of an extensive amount of literature (Deuchar, 1975; Sive *et al.*, 2000). The ability to stimulate egg induction hormonally, and fertilize eggs *in vitro,* provides a great deal of control to the researcher, making *Xenopus* a convenient model organism. The large egg size yields a copious amount of protein and nucleic acids for analysis, allows visual observation of developmental progression, and facilitates microinjection. In addition, the wealth of literature available regarding *Xenopus* embryogenesis provides researchers with a solid framework upon which to build. Although *Xenopus laevis* are tetraploid organisms, they have served very well as model amphibian organisms in both cellular and genetic studies (Graf and Kobel, 1991).

Several important discoveries in cellular and molecular biology have been made using the *Xenopus laevis* model system. For example, maturation promoting factor, was discovered by Masui and Markert (1971), using *Xenopus laevis* oocytes. This cytoplasmic factor was discovered to direct the induction of meiosis following hormone exposure. Ultimately, this initial finding in *Xenopus* facilitated the understanding of the overall eukaryotic cell cycle apparatus. Another *Xenopus* contribution was the discovery of the Spemann organizer by Spemann and Mangold, significantly increasing our knowledge of the control of vertebrate development (Gilbert, 1994). The concept of nuclear transfer was popularized with *Xenopus* embryos, and in specifically, this pioneering work demonstrated that transfer of the dorsal organizer region resulted in ectopic neural induction. In addition, the work of Gurdon using *Xenopus* has made substantial contribution to the literature (Gilbert, 1994). For example, research on the direction of development by differentiated nuclei, as well as the use of *Xenopus* oocytes to express exogenous proteins have led to many other discoveries. In summary, many very important milestone experiments have utilized the model organism *Xenopus laevis* and they remain equally important today.

Finally, *Xenopus* continuous cell lines are useful tools for *in vitro* molecular analyses. While not as popular as mammalian cell lines, several *Xenopus* cell lines have been established: A6, B3.2, KR, XF, XL2, XL110, XL-177 and XTC-2 (Smith and Tata, 1991), among several others. The most popular *Xenopus* cell line used today is the A6 somatic cell line, which was used in this study. This cell line was initiated in 1965 (Rafferty), by a primary culture derived from normal adult male kidney cells.

1.1.1 *Xenopus laevis* embryogenesis

Xenopus males and females are sexually mature by 10 to 12 months of age, at which point females produce oocytes capable of being fertilized. In the laboratory *Xenopus* females can produce eggs for fertilization approximately every 3 months, but they naturally mate in the spring. The numerous oocytes present in the ovaries of an adult female are at varying stages of maturity, since *Xenopus* oogenesis is asynchronous. Eggs must first be released from the ovary to prepare the vitelline membrane for sperm penetration. During oocyte maturation, precursors of RNA, DNA and protein, enzymes, energy stores, tRNA, mRNA and other factors accumulate (Gilbert, 1994). Together, these components allow the egg to survive initial developmental stages, during which the embryonic genome remains quiescent.

Early development of *Xenopus* is initiated and directed by the egg. Prior to fertilization eggs display an animal-vegetal polarity, whereby the pigmented animal half is darker than the unpigmented vegetal half (Keller, 1991). Fertilization allows the less dense pigmented half to move to the top of the embryo, and the cortex rotates, establishing the dorsal axis opposite the sperm entry point (stage 1; Figure 1). The first embryonic cleavage is initiated approximately 100 minutes after fertilization (stage 2), and rapid, synchronous cleavage continues throughout early development. To achieve such rapid cell division, the cell cycle is shortened to allow only DNA synthesis and mitosis (Newport and Kirchner, 1982a). Any protein produced during this time is translated from maternally inherited mRNA. It is not until stage 8.5, the midblastula transition (MBT) that transcription can occur in the newly activated embryonic genome. At this point in development, the cell cycle lengthens, cells

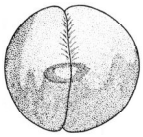
acquire motility and cell division becomes asynchronous (Brown and Littna, 1964; Newport and Kirchner, 1982b).

Following MBT, the embryos proceed to blastula (stage 9), at which point the blastocoel forms, and the embryo prepares for gastrulation. In blastula embryos, the primary germ layers of ectoderm, mesoderm and endoderm form, which will eventually give rise to all organs and tissues (Nieuwkoop and Faber, 1994). The initiation of gastrulation (stage 10) occurs at the dorsal marginal zone, triggering a complex program forming the basic embryo body plan. Extensive cellular rearrangement within the embryo accompanies the gastrula stage, shifting endoderm inside, and resulting in the formation of the archenteron, or primitive gut. The ectoderm remains on the surface, and the mesoderm lies between. Interaction between the mesoderm and overlaying ectoderm triggers organogenesis. Ultimately, the mesoderm gives rise to muscle, heart, bone, cartilage and the urogenital system. Endoderm becomes the digestive and respiratory lining. Ectoderm forms the brain, spinal cord, neural crest cells and skin (Keller, 1991).

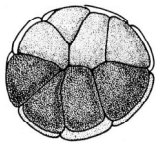
Figure 1. An overview of the early developmental stages of *Xenopus laevis.*

(Modified from Nieuwkoop and Faber, 1994).

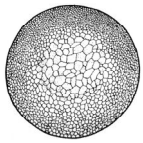
Fertilized Egg
(stage 1)



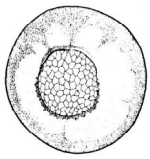
Early Cleavage
(stage 2)



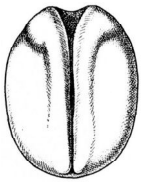
Late Cleavage
(stage 5)



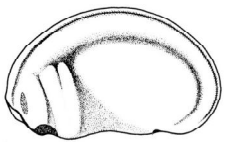
Blastula
(stage 9)



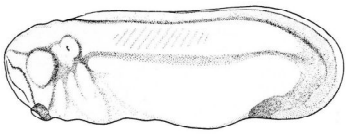
Gastrula
(stage 11)



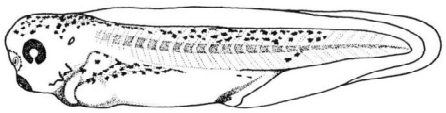
Neurula
(stage 17)



Early tailbud
(stage 23)



Mid tailbud
(stage 27)



Late tailbud
(stage 35)

Soon after gastrulation, neurulation (stage 19) occurs, at which point the chordamesoderm underlying the ectoderm signals neural tube formation and closure from the neural plate. The neural plate edges form the neural folds and neural crest (Baker and Bronner-Fraser, 1997). The ectodermally derived neural plate becomes more dominant on the dorsal side, where neural crest cells originate, and eventually give rise to neurons and glial cells of the nervous system (Nieuwkoop and Faber, 1994). Toward the end of the neurula stage, somites form from remaining mesoderm. The somites, located laterally on each side of the notochord, are distinct, but transient structures giving rise to vertebrae, ribs, dermis and skeletal muscle (Gilbert, 1994).

Once past the neurula stage, the embryos progress through the tailbud stages (early – stage 23, mid – stage 27, and late – stage 35). During this time the organs form, and commence function. The first of which is the cement gland, a temporary mucus-producing organ, which facilitates attachment of developing embryos to solid supports. During the tailbud stages the embryos hatch, becoming mobile, and quickly become full-fledged free-living tadpoles (stages 42-50).

1.1.2 *Xenopus laevis* gene expression during embryogenesis

Embryogenesis is a critical process for all organisms, comprising a tightly regulated series of events which are regulated by proteins. During *Xenopus* embryogenesis, there occurs a significant and abrupt transition in macromolecular synthesis, with no apparent accompanying morphological consequence, which is known as the midblastula transition. MBT is characterized by the initiation of transcription by RNA polymerase, a decline in the

rate of the cell cycle, asynchronous divisions, and cell motility (Newport and Kirschner, 1982a). These events activate the previously quiescent zygotic genome. Until MBT, the embryo is sustained and controlled by components present in the oocyte prior to fertilization. This developmental event is not unique to *Xenopus* as it has been described in *Drosophila* (Edgar *et al.*, 1986), zebrafish and mouse (Davidson, 1986), albeit with different timing.

The mechanism responsible for the repression of transcription of embryonic genes in pre-MBT embryos remains unclear, although several theories have been proposed. Newport and Kirschner (1982a) suggested that a general maternal repressor, eventually titrated out with regards to increasing levels of DNA, was responsible. Alternatively, experimental manipulation demonstrated that transcription was possible in pre-MBT embryos, and a hypothesis involving a repressively high mitotic rate was proposed (Kimelman *et al.*, 1987; Shiokawa, 1991). A later theory put forward by Hair *et al.* (1998) proposed that the unusually high ratio of histones to DNA in the early embryo was inhibitory, ultimately competing with basal transcription machinery. The concentration of factors originally present in the oocyte does not vary during embryogenesis, however, the stoichiometric ratio of DNA to nuclear protein does change, from 1:1 to 30 000:1 by the gastrula stage. Taken together, the authors suggested that chromatin structure, DNA accessibility and transcription complex-chromatin interactions are all important factors in early embryonic transcriptional regulation (Hair *et al.*, 1998). In summary, while strong evidence for potential mechanisms governing early gene repression in the *Xenopus* embryo grows, it is clear that the story is not yet complete.

1.2 The heat shock response

The heat shock response encompasses a number of processes including *hsp* gene activation, accumulation of HSPs and the acquisition of thermotolerance. This response was initially observed as a localized distortion or puffing of salivary glands in *Drosophila* (Ritossa, 1962). This observation was eventually attributed to heat shock-induced *hsp* gene transcription (Nover, 1991; Morimoto *et al.*, 1994b; Morimoto, 1998). The magnitude of the heat shock response of cells depends on a number of variables including the final heat shock temperature, the rate of temperature elevation, and the length of heat exposure. Early events of the heat shock response include *hsp* gene activation and cell cycle block. Later heat shock events include mRNA degradation, HSP accumulation, repression of HSP synthesis and induced thermotolerance (Nover, 1991; Parsell and Lindquist, 1993).

Although HSPs were aptly named after their discovery in response to heat shock, they were later shown to respond to other factors including chemical exposure and other environmental changes. As such, HSPs are often referred to generally as 'stress proteins'. Stress can be broadly defined as a disruption of equilibrium, and physiologically, a disruption to homeostasis. A stress may occur at many levels, from the cell to the whole organism. Chemical and environmental stressors include sodium arsenite, hydrogen peroxide, heavy metals (e.g. cadmium, copper, mercury and zinc), ethanol, hypoxia/anoxia and pH (Bernstam and Nriagu, 2000). Additional stressors include unfolded/abnormal proteins, amino acid analogues, gene expression inhibitors, steroid hormones (estrogen), viruses, teratogens, and ultraviolet irradiation (Nover, 1991). Many of the aforementioned stressors can act in concert yielding synergistic effects. For example, *Xenopus* A6 cells exposed to a moderate heat

shock while being incubated with sodium arsenite exhibit increased levels of *hsp* genes (Heikkila *et al.*, 1987).

Additional experimentation revealed that HSPs are fundamentally involved in cellular activity, and are found in a wide variety of organisms from bacteria to man (Morimoto *et al.*, 1994b). Some HSPs are expressed constitutively, indicating roles in normal cell function. In fact, HSPs are vital for cellular survival, regardless of stress. Under normothermic conditions, HSPs function as molecular chaperones in protein transport, translation of proteins and protein folding (Katschinski, 2004). In stressful situations they inhibit the irreversible aggregation of denatured protein and aid in its refolding (Gething and Sambrook, 1992). Eukaryotic HSPs are present in all major subcellular compartments including the cytoplasm, nucleus, mitochondria and endoplasmic reticulum (Nover, 1991). The endoplasmic reticulum (ER) contains a unique set of molecular chaperones known as glucose regulated protein (GRP) or immunoglobulin binding protein (BiP). These proteins were initially discovered in cells subjected to glucose starvation. GRPs are closely related to HSPs in structure and function, but are induced primarily by treatments that cause an unfolded protein response (UPR) in the ER (Easton *et al.*, 2000).

HSPs comprise several families related by sequence, which are classified by molecular size (kDa). They include: the small heat shock proteins (sHSPs) (10-30 kDa), the HSP40 family, the HSP60 family, the HSP70 family, the HSP90 family and the HSP110/SSE family (Parsell and Lindquist, 1993). Until recently, very little research has been carried out with the HSP110/SSE family, which will be the focus of this thesis.

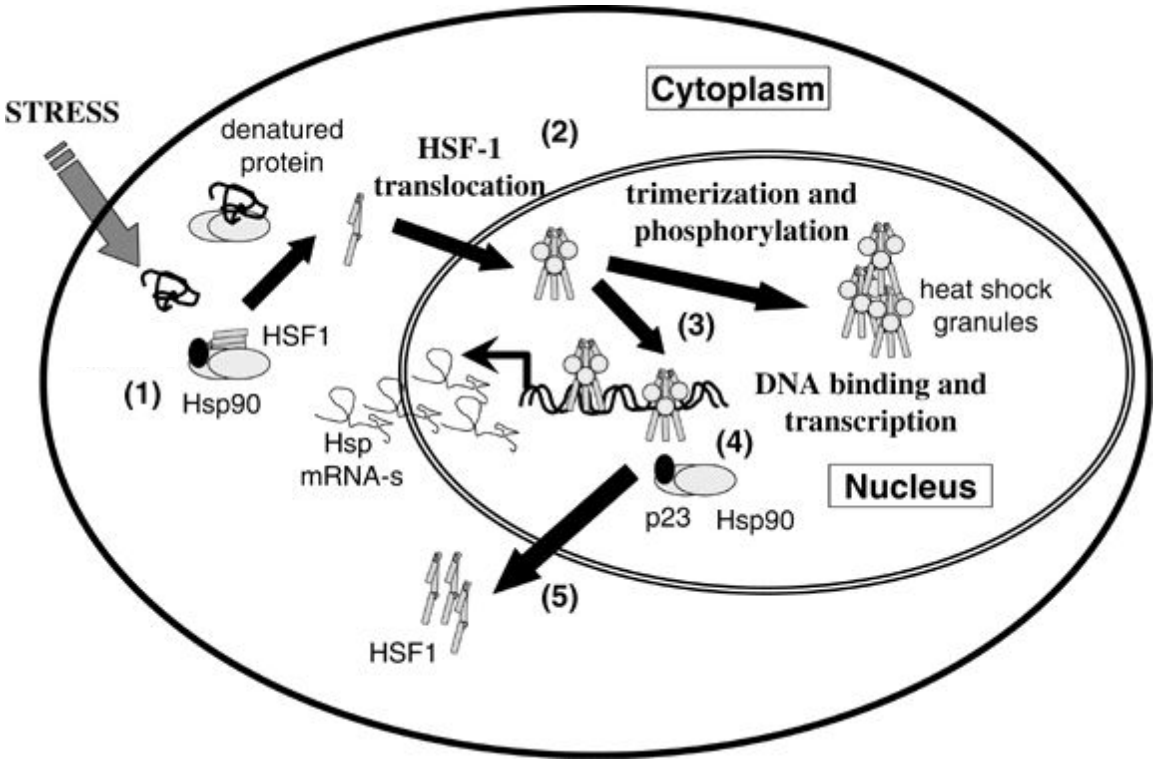
1.2.1 *Hsp* gene regulation

Hsp gene expression is regulated at several levels; mRNA synthesis, mRNA stability and translation efficiency (Katschinski, 2004). The synthesis of *hsp* mRNA is regulated by transcription factors known as heat shock factors (HSFs) that are activated in response to stress. Although some organisms possess only one HSF (yeast and *Drosophila*), higher eukaryotes possess multiple HSFs (Morimoto *et al.*, 1994a; Cotto and Morimoto, 1999). The HSF gene family includes HSF1, HSF2, HSF3 and HSF4 which are activated under different conditions. HSF1, the vertebrate homolog to the HSF found in yeast and *Drosophila,* is activated by heat shock and other stressors. Alternatively, HSF2 does not demonstrate stress-inducible heat shock transcription, but rather is activated during embryogenesis, spermatogenesis and erythroid differentiation (Morimoto *et al.*, 1994a). HSF3, an avian-specific HSF, responds to severe and persistent stress (Pirkkala *et al.*, 2001). Finally, HSF4, unlike the other HSFs, constitutively binds to DNA, and has been suggested to be a negative regulator of the heat shock response (Nakai *et al.*, 1997).

In metazoans, HSF is constitutively expressed and is present in the cell as an inactive monomer with a very weak affinity for DNA (Fernandes *et al.*, 1994; Cotto and Morimoto, 1999). Although a detailed mechanism for HSF activation remains unclear, Bharadwaj *et al.* (1999) proposed that HSP90 and p23 may function as a semi-permanent core heterocomplex maintaining HSF1 monomers inactive but competent for rapid trimerization (Figure 2). Stress causes monomeric HSF to trimerize, which greatly increases its DNA-binding affinity (Westwood *et al.*, 1991). The active HSF homotrimer now recognizes and binds to *cis*-acting

Figure 2. Current model for regulation of *hsp* gene transcription by HSF1

(1) Cytoplasmic complex of HSF1 and HSP90; (2) HSF1 translocates to the nucleus; (3) HSF1 intranuclear activation and *hsp* gene transcription regulation; (4) HSF1/HSP90/p23 intranuclear complex; (5) HSF1 returns to the cytoplasm. (Modified from Sõti *et al.*, 2005)


STRESS
denatured protein
Cytoplasm
HSF-1 translocation
(2)
trimerization and phosphorylation
heat shock granules
HSF1
(1)
Hsp90
(3)
DNA binding and transcription
Hsp mRNA-s
(4)
Nucleus
p23
Hsp90
(5)
HSF1

upstream DNA sequence elements, known as heat shock elements (HSEs) (Morimoto *et al.,* 1994a).

The HSE is a highly conserved domain, consisting of [nGAAn] inverted repeats, present in the 5' upstream region of *hsp* genes (Fernandes *et al.*, 1994). *Hsp* genes contain at least one upstream HSE, and the binding of HSF to HSE regulates transcription of the downstream *hsp* gene. An HSE is present in all heat shock promoters, mediating the response to heat shock as well as other stressors, including agents that alter protein conformation (Mosser *et al.*, 1990), and other chemicals such as sodium arsenite (Mosser *et al.*, 1988). The ability of such diverse stimuli to trigger the activation of HSF suggests a common feature shared by all. The common denominator shared by 'stressful conditions' is the potential for protein unfolding (Voellmy, 2004). The presence of stress-induced unfolded protein elsewhere in the cell competes for members of the multichaperone complex that represses HSF1, resulting in free HSF1 monomers. The monomers, no longer negatively regulated, rapidly trimerize and become transcriptionally competent (Voellmy, 2004).

The regulation of *hsp* gene transcription in *Xenopus* is controlled by HSF1 (Stump *et al.*, 1995) and HSF2 (Hilgarth *et al.*, 2004). Stump *et al.* (1995) isolated the *Xenopus* HSF1 cDNA and reported that it shared a high level of similarity with human HSF1. Furthermore, *Xenopus* HSF1 accumulated in the nucleus and activated transcription of the *hsp70* gene in response to heat shock. *Xenopus* HSF2 was recently identified and characterized by Hilgarth *et al.* (2004), and was also found to share strong similarity to its homolog in other organisms. Data from this study suggested the existence of at least 2 other HSFs in *Xenopus*, but these remain to be investigated.

1.2.2 Developmental expression of *hsp* genes in *Xenopus*

The expression of *hsp* genes during *Xenopus* development has been relatively well characterized (Heikkila *et al.*, 1997; Heikkila, 2003; Heikkila, 2004). In general, several *Xenopus hsp* mRNAs are present throughout early embryogenesis and are heat-inducible following MBT, while others are not stress-inducible until later stages (Heikkila *et al.*, 1997). The patterns of expression of representative *Xenopus hsp* genes *(hsp30, hsc70, hsp70, BiP, hsp47,* and *hsp90)* during development have been summarized below.

The *Xenopus hsp30* gene family displays a unique developmental pattern of expression compared to other *hsp* genes, such as *hsp70* and *hsp90*. While the known members of the *Xenopus hsp30* gene family include *hsp30A-E, hsp30B* and *E* are likely *pseudogenes (*Bienz, 1984a; Krone *et al.*, 1992*).* Constitutive expression of the *hsp30* gene family has not been detected during early *Xenopus* development except for the cement gland in early tailbud embryos (Lang *et al.*, 1999). Heat shock-induced accumulation of *hsp30* mRNA was first detected at the late neurula/early tailbud stage of development. This was in contrast to other *hsp* genes including *hsp70* which was heat-inducible immediately after the MBT (Krone and Heikkila, 1988; Lang *et al.*, 1999). A more detailed analysis revealed that although *hsp30A* and *hsp30C* were first heat-inducible at the late neurula/early tailbud stage, *hsp30D* was not heat-inducible until the midtailbud stage, one day later in development (Ohan and Heikkila, 1995). A recent study from our laboratory suggested that the state of chromatin conformation may play a role in the developmental regulation of *hsp30* gene expression (Ovakim and Heikkila, 2003). For example, treatment of *Xenopus* embryos with histone deacetylase inhibitors, which loosen the structure of repressed chromatin domains, resulted in heat-

inducible *hsp30* gene expression at the gastrula stage rather than late neurula/early tailbud. Finally, whole mount *in situ* hybridization revealed that heat shock-induced accumulation of *hsp30* mRNA was enriched in selected tissues of tailbud embryos (Lang *et al.*, 1999; Ovakim and Heikkila, 2003). However, the mechanism responsible for this enrichment is not known.

The *Xenopus hsp70* gene family consists of the stress-inducible *hsp70*, the constitutively expressed *hsc70* and the ER family member, *BiP*. In a study of *hsc70* mRNA accumulation in *Xenopus* embryos, Lang *et al.* (2000) observed that this message was present in unfertilized eggs, indicative of its maternal origin. *Hsc70* mRNA was detected at all stages of development with increasing relative levels at neurula and late tailbud stages. Unlike other *hsps*, *hsc70* mRNA was not heat-inducible at any stage of development (Ali *et al.*, 1996a). In contrast, *hsp70* mRNA was not detected constitutively at any stage of development, but was heat-inducible at all stages after MBT (Bienz, 1984a; Krone and Heikkila, 1988). Finally, *BiP* mRNA accumulation was detected at relatively constant levels from unfertilized egg to neurula, increasing slightly by tailbud (Miskovic and Heikkila, 1999). *BiP* message was also heat-inducible, but not until the gastrula stage.

HSP47 is an ER resident molecular chaperone involved in collagen production. Recently, Hamilton and Heikkila (2006) reported that *Xenopus hsp47* mRNA levels were present constitutively throughout development. The message was present in unfertilized eggs and cleavage stage embryos, indicating that the mRNA for this gene was maternally inherited. Heat treatment of embryos resulted in an increase in mRNA accumulation starting at the late blastula stage. Whole mount *in situ* hybridization analysis determined that *hsp47* mRNA was

enriched in tailbud embryonic tissues associated with collagen production including notochord, somites and head region.

In a study of *hsp90* gene expression during early *Xenopus* development, Ali *et al.* (1996b) observed that *hsp90* mRNA was present constitutively at all stages of embryogenesis. The presence of this message in unfertilized eggs and early cleavage stage embryos indicated that the *hsp90* mRNA was maternal in origin. After MBT and the onset of zygotic gene transcription *hsp90* mRNA levels were enhanced in response to heat shock treatment.

In summary, some *Xenopus hsp* mRNAs are constitutively expressed during development, including *hsp47, hsc70, BiP,* and *hsp90* while others are primarily stress-inducible, such as *hsp30* and *hsp70.* Heat-inducibility of *hsps* generally occurs immediately following MBT, as seen for *hsp47, hsp70, hsp90* and *BiP,* whereas *hsp30* is not heat-inducible until later stages of development. Finally, unlike other *hsps, hsc70* remains non-heat-inducible throughout early development. The presence of some *hsps* during early development, and some as maternally-derived messages, suggests an important role for these proteins in *Xenopus* embryogenesis.

1.3 Small heat shock proteins

SHSPs are stress-inducible molecular chaperones that range in size from 12 to 43 kDa and include the α-crystallins based on their physical and functional properties (Arrigo, 1998; Ehrnsperger *et al.*, 1997; MacRae, 2000; van Montfort *et al.*, 2002). With the exception of the α-crystallin domain, a low degree of conservation is shared among sHSPs. Although the sequences of sHSPs are divergent, the structural properties of these proteins have been

conserved (Lindquist and Craig, 1988). Most sHSPs form high molecular weight aggregates under conditions of cellular stress that are necessary for chaperone activity (Arrigo and Landry, 1994; Ehrnsperger *et al.*, 1997; Leroux *et al.*, 1997). In general, sHSPs bind to unfolded protein, maintaining the solubility and folding competence that permits refolding by other ATP-dependent molecular chaperones (Ehrnsperger *et al.*, 1997; MacRae, 2000; van Montfort *et al.*, 2002).

1.3.1 Xenopus sHSPs

The majority of the literature on sHSPs has focused on *Drosophila*, plant or mammalian model systems. Less information was available regarding sHSPs in amphibians. Until now, the only amphibian sHSP genes that have been studied with respect to their expression and molecular chaperone function were *Xenopus hsp30C* and *hsp30D* (Heikkila, 2004). During *Xenopus* early development the *hsp30* genes were repressed and were not heat-inducible until the late neurula/early tailbud stage. This is in contrast to other *hsp* genes, including *hsp70*, which were inducible at MBT (Lang *et al.*, 1999; Heikkila, 2004). Additionally, *hsp30* genes in tailbud and tadpole embryos were enriched preferentially in selected tissues in response to heat shock. Studies employing recombinant HSP30 proteins have demonstrated their ability to act as molecular chaperones. These proteins, which form multimeric complexes, inhibited heat-induced aggregation of target enzymes by maintaining them in a soluble form (Fernando and Heikkila, 2000; Abdulle *et al.*, 2002; Heikkila, 2004).

As mentioned previously, prior to this study no information was available for *hsp27* in *Xenopus laevis*. The typical mammalian and avian HSP27 has a well-conserved C-terminal

domain, and shares similarity with α-crystallin from the vertebrate eye. The N-terminal domain contains a hydrophobic motif which is necessary for oligomerization (Ciocca *et al.*, 1993). As with most other sHSPs, *hsp27* is transcriptionally regulated; however, post-translational modification in the form of phosphorylation is essential to its function as a molecular chaperone (Norris *et al.*, 1997; Wieske *et al.*, 2001; Thériault *et al.*, 2004). HSP27 in other organisms was constitutively expressed, and increased in response to stress. Accumulation of *hsp27* mRNA was induced by a number of naturally occurring components including retinoic acid, estrogen, tumor necrosis factor α, interleukin 1, and thrombin (Ciocca *et al.*, 1993). In other organisms, *hsp27* was developmentally regulated, and its expression varied depending on developmental stage and tissue. In *Drosophila*, the *hsp27* gene was activated along with the hormone, β-ecdysone (Ciocca *et al.*, 1993), and was implicated in *Drosophila* developmental regulation. Mammalian, avian and *Drosophila* HSP27s were shown to have molecular chaperone activity. For example, *Drosophila*, hamster and chicken members of the HSP27 sHSPs were shown to inhibit thermal aggregation of citrate synthase in a dose-dependent manner (Haslbeck and Buchner, 2002; Thériault *et al.*, 2004; Morrow *et al.*, 2006). In mammalian systems, a number of studies have shown an involvement of HSP27 in cell differentiation, modulation of redox parameters, inhibition of apoptosis and actin capping/decapping (MacRae, 2000; van Montfort *et al.*, 2002; Arrigo *et al.*, 2002; 2005).

1.4 The HSP70 family

The HSP70 family includes several highly conserved 68-74 kDa stress-related proteins that regulate protein folding under both non-stressful and stressful conditions. This protein was reported to be the most conserved protein in evolution, present from archaebacteria to humans (Daugaard *et al.*, 2007). Additionally, the HSP70 'superfamily' includes the HSP110/SSE family. HSP70 members are important molecular chaperones involved in the protection of nascent or denatured proteins from aggregation and the proper folding or refolding of cellular proteins (Katschinski, 2004). This gene family has been extensively studied and includes several functional homologs, including cytosolic stress-inducible HSP70, cytosolic constitutively expressed HSC70, mitochondrial p75 and ER-resident BiP (Morimoto, 1998). The high identity within and among species has facilitated identification of *hsp70* genes from several organisms. Eukaryotic HSP70s share roughly 50-98% amino acid identity and roughly 50% identity with prokaryotic counterparts (Craig *et al.*, 1983). The functional domains for HSP70 include: a conserved ATPase domain, a protease sensitive region, a peptide binding domain and a carboxyl terminal domain responsible for intracellular localization and substrate interaction (Morimoto and Milarski, 1990).

1.4.1 HSP70 function

This section does not attempt to summarize the vast literature concerning the role of HSP70, but rather to introduce the reader to some of the diverse functions of this protein. HSP70 has been implicated in roles as diverse as protein transport, degradation of unstable and misfolded protein, prevention and dissolution of protein complexes, folding and

refolding proteins, uncoating of clathrin-coated vesicles and regulatory protein control (Daugaard *et al.*, 2007). HSP70 synthesis has been reported in response to stressful conditions including elevated temperature, chemical and heavy metal exposure. In general, during periods of stress, HSP70 is synthesized and protects the cell from aggregation of denatured proteins and directs the refolding of misfolded proteins (Boorstein *et al.*, 1994). HSP70 does not fold proteins alone, but requires the help of co-chaperones including HIP, HOP, HSP40 and BAG-1 plus ATP (Katschinski, 2004). In addition to functioning as molecular chaperones, HSP70 family members are important in assembly and transport of cellular proteins between cellular compartments. HSP70 proteins are localized in different areas of the cell; HSC70 and HSP70 are both found in the cytoplasm, whereas BiP resides in the endoplasmic reticulum (Feige and Polla, 1994). Stress initiates translocation of HSC70 and HSP70 to the nucleus, and the presence of a nuclear localization signal (NLS) supports this (Ali *et al.*, 1996a). A role in cellular transport was demonstrated by Shi and Thomas (1992), whereby HSP70 and HSC70 were required for the transport of nucleoplasmin protein into the nucleus.

The most well-known function of HSP70 is as a chaperone. HSP70 has been shown to repair thermally denatured target enzymes in conjunction with other co-chaperones (Hartl *et al.*, 1994; Freeman and Morimoto, 1996). HSP70 proteins have also been shown to influence the organization and function of microtubules, microfilaments, and intermediate filaments (Liang and MacRae, 1997). A role for HSP70 in apoptosis has also been clearly established. In general, HSP70 plays an inhibitory role in stress kinase pathways (Sreedhar and Csermely, 2004) and in apoptosis (Mosser *et al.,* 2000). The ability of HSP70 to prevent apoptosis has

been reported by several authors (Beere, 2001; Parcellier *et al.*, 2003; Garrido *et al.*, 2006), although the ability of HSP70 to prevent apoptosis has been reported to be dependent on the type of stress (Anderson, 1998). Finally, in Xenopus embryos, elevated expression of *hsp70* genes in response to heat shock was correlated to an increase in thermoresistance (Ali *et al.*, 1996a; Heikkila *et al.*, 1985).

1.4.2 *Xenopus hsp70* developmental regulation

Five *Xenopus laevis* HSP70 members have been reported, including 2 inducible *hsp70* genes, 2 constitutive *hsc70* genes and the ER-resident *BiP* (Ali *et al.*, 1996a; b; Bienz, 1984a; b; Miskovic *et al.*, 1997). *Hsc70* mRNA was constitutively present throughout *Xenopus* development, and was detectable globally (Ali *et al.*, 1996a; Lang *et al.*, 2000). *Hsp70* message was not detectable until after MBT in *Xenopus* embryos, and beyond this stage, *hsp70* gene expression was developmentally and spatially regulated (Heikkila *et al.*, 1987; Krone and Heikkila, 1988; 1989; Lang *et al.*, 2000). *Hsp70* mRNA was detected in the head region, the lens placode, the cement gland, proctodeum, and somitic regions of heat shocked tailbud embryos (Lang *et al.*, 2000). Finally, *BiP* gene expression increased during development, and was heat-inducible in *Xenopus* embryos (Miskovic and Heikkila, 1999). This gene was also spatially regulated in *Xenopus* embryos, and displayed preferential accumulation in the neural plate and neural fold regions of neurula stage embryos and in dorsal regions at the tailbud stage.

1.5 Large molecular mass (LMM) HSPs

Several reports in the early 1990's suggested a large relative of the HSP70s, which was much more diverged than other family members (Subjeck *et al.*, 1983; Fathallah *et al.*, 1993; Mukai *et al.*, 1993). In 1995, a mammalian *hsp110* was cloned, and strong similarities to the HSP70 family in sequence and structure were observed (Lee-Yoon *et al.*, 1995). Although LMM-HSPs shared similarity with the HSP70 proteins, they were designated a separate HSP family. This relatively new HSP group is referred to as the HSP110/SSE family, which belongs to the HSP70 'superfamily', based on sequence similarity (Easton *et al.*, 2000).

The argument for a new HSP family outside of the HSP70 family is as follows: the molecular mass is substantially larger, there are six regions of high identity that are not found in HSP70 but are shared among the LMM-HSPs, there is a region between amino acids 500-700 of LMM-HSPs is absent from HSP70, and the cysteine content of this family in the amino-terminal region is high compared to the HSP70 family (Lee-Yoon *et al.*, 1995). There are several members of the HSP110/SSE family, including: APG-1/OSP94, APG-2, IRP94, HSP70RY, HSP105, SSE1, SSE2, PSS1, HSP91 and HSP88. It is worth noting that yeast or bacterial HSP104 does not belong to the HSP110/SSE family, since it displays several differences from HSP110 and exists only in lower eukaryotes, whereas HSP110 exists in organisms from yeast to humans, but has not been seen in prokaryotes (Easton *et al.*, 2000).

1.6 Mammalian *HSP110/105* gene organization

In 1983, Subjeck *et al.* published one of the first investigations of HSP110, prompted by previous hyperthermia and thermotolerance studies (Subjeck *et al.*, 1982a; b and c). This

paper characterized a 110 000 dalton (Da) mammalian heat shock protein using an immunological approach, and determined that this protein was analogous to a 107 kDa protein in hepatoma cells (Landry *et al.*, 1982) and a 112 kDa protein in mammary adenocarcinoma cells (Tomosovic *et al.*, 1983). The hamster *hsp110* cDNA was eventually cloned by Lee-Yoon *et al.* (1995) by screening a CHO expression library with an HSP110 antibody. The hamster *hsp110* gene was determined to be 3257 base pairs (bp) in length, with an open reading frame of 858 amino acids (aa), and a molecular weight of 96.1 kDa.

Four major domains of HSP110 were identified: the N-terminal ATP-binding domain (1-394 aa), the peptide-binding domain (β-sheet) (394-509 aa), the acidic loop domain (509-607 aa), and the carboxyl end (α-helical) (608-858 aa) (Oh *et al.*, 1997; Oh *et al.*, 1999). First, the N-terminal ATP-binding domain, although shared with HSP70, was found to display more identity with other HSP110 proteins than with HSP70 family members (Lee-Yoon *et al.*, 1995). However, murine ATP-binding experiments indicated that HSP105 was unable to bind ATP (Yasuda *et al.*, 1995). Second, a very highly conserved region present in all HSP70s, part of a peptide binding domain, was absent from HSP110-like proteins. Third, the acidic loop domain appeared to be different from any HSP70 proteins, and also unique among HSP110-like sequences. The major size difference between HSP70 and HSP110 appears to be within the α-helical loop/lid region. This acidic loop is connected to the β-sheet domain by a 100 amino acid segment, and the 'lid' itself is quite a bit larger than the corresponding DnaK structure (Oh *et al.*, 1997). Fourth, several C-terminal regions were identified that shared a very high level of similarity among all HSP110-like proteins (residues 421-450, 476-494, 627-644, 671-689, 698-712 and 776-781). In addition, the

predicted amino acid sequence indicated five putative N-glycosylation sites at positions 45, 61, 280, 302 and 466, as well as a nuclear localization consensus sequence (NLS), KKPK, at amino acid 586 (Lee-Yoon, *et al.*, 1995). From the full-length sequence obtained they identified a GCC sequence flanking the initiation codon in the 5' untranslated region (UTR), for optimal initiation of translation, but the G residue predicted at the +4 nt position for optimal translation initiation was replaced by a T. They also determined that there were two termination codons (2603 nt and 2633 nt) and a polyadenylation signal, AATAAA, at 3235 bp, in the 3' UTR.

A full-length murine *hsp105* cDNA (the mouse homologue of hamster *hsp110*) was cloned by screening a library from mouse FM3A cells treated at 42°C for 2 h (Yasuda *et al.*, 1995). Two full-length clones were obtained, a 3345 bp sequence with an open reading frame of 858 aa, and a 3268 bp sequence with an open reading frame of only 814 aa. The larger message, *hsp105*α, was constitutively expressed and also responded to temperature stress and chemical treatment. The smaller message, *hsp105*β, was identical to *hsp105*α but lacked a 131 bp segment. Interestingly, *hsp105*β was exclusively heat-inducible at 42°C (Yasuda *et al.*, 1995). Other treatments, including temperatures other than 42°C, sodium arsenite exposure, or exposure to other chemicals did not induce *hsp105*β gene expression. Western blot analysis revealed a 105 kDa band (HSP105α), and a 90 kDa band for 42°C only (HSP105β). In addition, both HSP105α and HSP105β were composed of two isoforms, an acidic and a basic, which resulted from post-transcriptional modification (Hatayama *et al.*, 1994).

A complete genomic cloning and promoter analysis study was carried out by Yasuda *et al.* (1999) for the mouse *hsp105* gene. Briefly, a mouse BALB/c genomic library was screened with an *hsp105* cDNA fragment. This study revealed that the gene was roughly 22 kb in length, containing 17 exons and 18 introns. All exons were less than 200 kb in length except for the last one (exon 18) which was composed of 867 bases. On the other hand, intron size ranged from 0.1 to 2.8 kb. Of interest was exon 12, extending from nt 1642 to nt 1773, which was present in the *hsp105*α isoform but completely lacking in the *hsp105*β isoform. They determined that this alternative splicing was due to an inability of exon 11 to splice to exon 12 when cells are exposed to a 42°C stress, and only to exon 13, resulting in the *hsp105*β isoform. In addition to determining that *hsp105* exists as a single copy in mice, Yasuda *et al.* (1999) also obtained 1.2 kb of the 5' UTR, enabling promoter analysis for this gene. With this information they determined that the transcription initiation site lied 165 nt upstream of the initiation codon. Both constitutive and heat inducible promoters were revealed in the 5' UTR. Constitutive promoter elements included a TATA box (at -33 nt), a CAAT box (at -210 nt), an inverted CAAT box (at -141 nt), and 2 GC boxes (at -248 and -166 nt). In addition to these, 2 HSE consensus sequences were identified (from -46 to -64 nt, and from -99 to -128 nt). This study also determined that both of the HSEs were required for sufficient heat-inducibility of this gene. Human *hsp105*α and *hsp105*β were cloned from a cDNA library prepared from HeLa cells (Ishihara *et al.*, 1999). The characteristics of human *hsp105*were sufficiently similar to murine *hsp105* that another description is not warranted.

1.6.1 Other HSP110/SSE family members

Hsp70RY, a cDNA isolated from transformed lymphocytes (Fathallah *et al.*, 1993), was discovered in Genbank as having identity with *hsp110* (Lee-Yoon *et al.*, 1995). Efforts to obtain a full length clone of *hsp70RY* have proven unsuccessful (Nonoguchi *et al.*, 1999), and it has been suggested that this may have been an artificial product, or anomaly (Easton *et al.*, 2000).

APG-1 and APG-2 (A: ATP-binding domain, P: peptide-binding domain, G: germ cell-derived) have been observed in both mice and humans. *Apg-1* message was present in all mouse tissues, but was predominantly expressed in the testes, and is likely involved in spermatogenesis. The *apg-1* mRNA was also heat-inducible, but displayed a different pattern of heat-induced accumulation than *hsp70*, implying it should not be grouped with the HSP70 family (Kaneko *et al.*, 1997; Nonoguchi *et al.*, 1999). Kojima *et al.* (1996) reported an osmotic stress protein isolated from mouse kidney, *osp94,* which was inducible by heat as well as hyperosmotic stress. This protein appears to be the same as APG-1, but was originally named differently as a result of the conditions under which it was observed (Easton *et al.*, 2000). Both *apg-1* and *apg-2* have been identified in humans as well. The *apg-2* transcript was identified in all human tissues, similar to mice, but was present at higher levels in gonadal tissue, and demonstrated a pattern of heat-inducibility unique from *apg-1* (Nonoguchi *et al.*, 1999). Gotoh *et al.* (2004) have demonstrated chaperone activity for *apg-2 in vitro,* and also observed that the *apg-2* transcript (in addition to *hsp110*) was overexpressed in hepatocellular carcinoma.

The ischemia-responsive gene (*irp94*), another member of the HSP110/SSE family, was abundant in neurons in the rat brain, and was enhanced following transient forebrain ischemia (Yagita *et al.*, 1999). Two different sized transcripts were observed for *irp94,* differing only in 3'UTR length. The second, shorter transcript, for *irp94* appeared in the testes. The longer length of the brain-resident *irp94* contained two stabilization motifs. The different location and stabilities of these transcripts suggest a different function in each organ (Yagita *et al.*, 1999).

Hsp110 homologs have been identified for several invertebrate species as well, including *C. elegans, Drosophila,* and sea urchin, although the *C. elegans* and *Drosophila* genes have not been fully characterized. Foltz *et al.* (1993) and Mauk *et al.* (1997) have reported the 'sea urchin egg surface receptor for sperm' in two different sea urchin species. These proteins shared 45% and 48% identity to hamster HSP110. Members of the HSP110/SSE family have also been identified in two yeast species, *sse*1 from *Saccharomyces cerevisiae* (Mukai *et al.*, 1993) and *pss1* from *Schizosaccharomyces pombe* (Chung, *et al.*, 1998). Yeast stress proteins follow a different nomenclature, and are referred to as stress seventy (SS) proteins. The SSEs (SSE1 and SSE2) of *S. cerevisiae* share sequence identity with HSP110 group members albeit low (25% identity to hamster HSP110), but are included on the basis of gene organization (Easton *et al.*, 2000). In addition, SSE1 possesses chaperone activity, and its genetics have been well-studied (Shaner *et al.*, 2004). In *S. pombe,* the *pss1* transcript was constitutively expressed, and was also induced by heat shock and nitrogen starvation (Chung *et al.*, 1998). An HSP110/SSE family member has been reported in *Arabidopsis thaliana,* and is referred to as HSP91. Recently, other *Arabidopsis* candidates have been proposed as

members of this family as well (Lin *et al.*, 2001). HSP88, in the mold *Neurospora crassa,* is a constitutively expressed, heat-inducible protein, which has been shown to bind preferentially to HSP30 (Plesofsky-Vig and Brambl, 1998). Although it differs in size, as several of the family members do, the structure and organization of the gene is very similar to others in the HSP110/SSE family.

1.6.2 HSP110 tissue and intracellular localization

The cellular localization of HSP110 has been previously characterized in mammalian systems. HSP110 was observed in all hamster tissues but was most abundant in the brain, followed by the liver (Lee-Yoon *et al.*, 1995). Similarly, HSP105 was present in all adult mouse tissues, and was also enriched in the brain (Hatayama *et al.*, 1997). This data was also supported by mouse embryo studies, although HSP105 was not only enriched in the brain, but also liver, dorsal root ganglion and chondrocytes. The lowest levels were observed in the heart and lung.

Subjeck *et al.* (1983) observed a heterogeneous nuclear localization as well as a nucleolar association for HSP110 in chinese hamster ovary (CHO) cells, C3H mouse embryo 10T½ cells and murine tissues. In later studies, Lee-Yoon *et al.* (1995) reported that in mouse 3T3 and CHO cells HSP110 was primarily cytoplasmic, but also associated with peripheral regions of nucleoli. Similar studies in mice revealed that HSP105 was mainly localized in the cytoplasm and nuclei of several mouse cell lines, similar to HSP110 in hamster cells (Hatayama *et al.*, 1994; Hatayama *et al.*, 1997; Yasuda *et al.*, 1995). However, mouse HSP105 was not found in the nucleoli under any conditions. Furthermore, HSP110

intracellular localization was not significantly affected by heat shock (Lee-Yoon *et al.*, 1995). This observation was supported in mice as well, since a similar pattern of HSP105 localization was observed under both control and stressed conditions (Hatayama *et al.*, 1997). These findings were in contrast to HSP70, which responded to heat shock by localizing to the nucleus (Lee-Yoon *et al.*, 1995). These observations are in agreement with observations in human systems as well. In human HeLa cells, HSP105 localized primarily in the cytoplasm in control cells, and accumulated around nuclei following heat shock, but was not found in the nucleoli (Ishihara *et al.*, 1999). This was also in direct contrast to HSP70 in HeLa cells, which localized in the cytoplasm of non-stressed cells, but accumulated in nucleoli and around the nuclei following heat shock.

The cellular function of of HSP110/105 is not clear. Subjeck *et al.* (1983) treated cells with ribonuclease, which resulted in the release of HSP110 from the nucleus. The authors suggested that this was because HSP110 was bound to RNA either directly or indirectly, and speculated that HSP110 might be involved in ribosome assembly (Subjeck *et al.*, 1983). Also mouse HSP105 may be involved in apoptosis as suggested by studies with mouse embryos which will be outlined in the next section.

Previously, a differentiation between the localization of mouse HSP105α and HSP105β was not possible, since the HSP105α antibody detected both proteins. Recently, Saito *et al.* (2007) produced antibodies that were able to distinguish between HSP105α and HSP105β. This study revealed that HSP105α and HSP105β localized in different subcellular compartments, with HSP105β predominantly in the nucleus of monkey COS-7 cells. Both

HSP105α and β contain functional NLS and nuclear export signal (NES) signals (Saito *et al.*, 2007). Since both of these elements were functional, how was HSP105α maintained in the cytoplasm and HSP105β in the nucleus? The difference between the two proteins lies in the 44 amino acid region that was alternatively spliced out of HSP105α. Studies with deletion mutants of this region showed that with increasing deletions in this region, HSP105α was less able to localize in the cytoplasm (Saito *et al.*, 2007). The ability of HSP105β to move in to the nucleus also appeared to be importin-β dependent. The NLS is recognized by importin α in the cytoplasm and a heteromeric complex is formed with importin β (Kohler *et al.*, 1999). This HSP105-importin α-importin β complex interacts with the nuclear pore complex and is transported through a nuclear pore (Gorlich *et al.*, 1995). Although this study provides insight into the difference in subcellular localization of HSP105, the roles of α and β in the different areas of the cell are still unclear.

1.6.3 HSP110/105 developmental regulation and involvement in apoptosis

Support for a role for HSP110/105 in development was provided by mouse embryo studies. Differential levels of *hsp105* mRNA during mouse embryo development were reported by Hatayama *et al.* (1997). Relatively high message levels were observed between gestational day (GD) 8-12, and declined by GD17. HSP105 protein was low in mice at GD8, increased to maximal expression levels at GD9-11, and then declined to half of the maximal level by GD17. This was contrasted by HSC70 levels which were unaltered throughout development. In light of the observed variation in the amount of HSP105 protein during embryogenesis, the

authors speculated that HSP105 may play an important role in organogenesis (Hatayama *et al.*, 1998). They also observed that the expression of HSP105 was particularly high in condensed cells and apoptotic bodies at the interdigital region of limbs implying a role in apoptosis (Hatayama *et al.*, 1997).

In order to further investigate the pro-apoptotic effect in mouse embryos, the mouse embryonal F9 cell line was established which constitutively over-expressed HSP105 (Yamagishi *et al.*, 2002). The overexpression of HSP105 was found to enhance caspase-3 activation, cytochrome *c* release, and p38 activation, ultimately enhancing stress-induced apoptosis. Evrard *et al.* (1999) demonstrated a localized expression of HSP110 in mouse embryo mesectodermal and ganglionic cells that were undergoing apoptosis. A more detailed analysis revealed that *hsp110* mRNA was elevated within 12 h of all-trans-retinoic acid exposure, and was depleted prior to the detection of the majority of apoptotic cells (Evrard *et al.*, 2000). Given this information, the authors hypothesized that HSP110 played a role in the early stages of apoptosis, but did not offer a mechanism. This study was supported by the findings of Gashegu *et al.* (2007), who observed that HSP110 was expressed before caspase-3 during development of the mouse eye, and suggested a pro-apoptotic role for HSP110 in caspase activation. Although several studies agree that HSP110 is involved at some level in apoptosis in the developing embryo, its exact role has not yet been clarified.

A protective role for HSP105 in neuronal PC12 cells was proposed by Hatayama *et al.* (2001). They reported that HSP105 protected neuronal cells against stress-induced apoptosis by inhibiting the activation of both caspase-3 and JNK. A similar protective role was suggested by Yamagishi *et al.* (2006) who reported that staurosporine-induced apoptosis was

suppressed by overexpression of HSP105. In an attempt to establish a mechanism by which HSP105 protected against apoptosis in HeLa cells, they determined that HSP105 suppressed the translocation of Bax to mitochondria, which prevents the downstream activation of caspases. In conclusion, it appears that HSP110/105 is able to function as either an enhancer or a suppressor of apoptosis, depending on the cell system being observed.

1.6.4 Molecular chaperone activity of HSP110

HSP110 has long been implicated in thermotolerance (Subjeck *et al.*, 1982). The first description of the protective properties of HSP110 was reported by Oh *et al.* (1997). They demonstrated that Rat-1 and HeLa cell lines overexpressing HSP110 were more resistant to lethal heat exposures. HSP110 alone provided a 25-33% full thermotolerant effect, as compared to pre-heat treated thermotolerant cells in which multiple HSP family members are induced (Oh *et al.*, 1997). HSP110 was also efficient in preventing heat-induced aggregation of luciferase *in vitro* in an ATP-independent manner. Coprecipitation of HSP110 with luciferase demonstrated its ability to selectively recognize denatured proteins *in vitro*. HSP110 was able to maintain luciferase in a folding competent state at a 1:1 (HSP110: luciferase) ratio, whereas HSC70 required a ratio of 4:1 to achieve the same result (Oh *et al.*, 1997). Targeted deletion studies revealed that the protective characteristics of HSP110 were conferred through the peptide binding domain and two of the conserved C-terminal domains (Oh *et al.*, 1999). Although HSP110 was unable to refold heat-denatured proteins alone, when added in conjunction with HSC70 and Hdj-1 to a denatured protein complex, protein refolding was increased to 25% of original activity (Oh *et al.*, 1997). In summary, HSP110

possesses potent chaperone capabilities, but is unable to refold heat-denatured protein alone, and has thus been described as an efficient 'holdase'. A role for HSP110 in the HSP70 folding complex was supported by the results of Dragovic *et al.* (2006). They showed that HSP110 functioned as a nucleotide exchange factor (NEF) in the HSP70 folding complex, and regulated its kinetics by enhancing the rate of HSP70 folding. A special role for HSP110 in folding complex multidomain proteins was proposed by Dragovic *et al.* (2006) based on the ability of HSP110 to function as both a chaperone and a NEF. This proposed role would be useful for the prevention of untoward reactions between the different domains of a folding protein.

Alternatively, a role for HSP110 as an 'RNA chaperone' was proposed (Henics *et al.*, 1999). In this study, HSP70, HSC70 and HSP110 were shown to bind to AU-rich 3'UTR RNA sequences (instability elements of proto-oncogene and lymphokine mRNAs). RNA binding was shown to occur in the N-terminal domain of HSP110. What is the purpose of this RNA-binding? Perhaps the binding facilitates the unwinding of complex secondary structures, exposing *cis*-acting elements for other proteins to bind. Alternatively, these HSPs may play a 'RNA chaperone' function enabling the proper folding of RNA to expose critical motifs for translation or degradation (Henics *et al.*, 1999).

While it is tempting to speculate that HSP110 shares a common chaperone role in all organisms, a recent study by Raviol *et al.* (2006) demonstrated otherwise. In this study, the chaperone abilities of two HSP110 homologues, human APG-2 and yeast SSE1, were compared. The results indicated that these two proteins have diverged substantially in function and although they both demonstrated ATPase activity, biochemically they were

quite different. As such, conclusions based on the activity and function in other species should be made with caution.

Objectives

The aim of the present study was to examine the expression of the *hsp110* gene in *Xenopus laevis*. HSP110 has not been studied as intensively as other HSPs including HSP90, HSP70, and the sHSPs. Most of the available information on HSP110 was derived from mammalian and yeast systems. Prior to my thesis research, no information was available on the expression of the *hsp110* gene in amphibians.

The main objectives of my thesis are as follows:

1. Obtain a full-length sequence of the *Xenopus laevis hsp110* cDNA.
2. Sequence analysis of the *hsp110* cDNA and comparison with *hsp110/105* genes in other organisms.
3. Northern blot analysis of the relative levels of *hsp110* mRNA in control, heat shocked and sodium arsenite-treated A6 cells or embryos.
4. Whole mount *in situ* hybridization analysis to examine the pattern of *hsp110* mRNA accumulation in control and heat shocked *Xenopus* embryos.
5. Western blot analysis of HSP110 protein levels in control, heat shocked and sodium arsenite-treated A6 cells.
6. Intracellular localization of HSP110 protein in control, heat shocked and sodium arsenite-treated A6 cells employing immunocytochemistry coupled with confocal laser scanning microscopy.

Chapter 2: Materials and Methods

2.1 Maintenance of *Xenopus laevis* A6 cells and embryos

Xenopus laevis A6 kidney epithelial cells (CCL-102; American Type Culture Collection (ATCC), Rockville, MD) were maintained at 22°C in T75 cm^2 tissue-culture flasks (BD Falcon, Oakville, ON). Leibovitz (L)-15 media (Sigma-Aldrich, Oakville, ON) diluted to 55% (v/v), and supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), 100 units(U)/mL penicillin (Sigma-Aldrich) and 100 μg/mL streptomycin (Sigma-Aldrich) was used for routine culturing. A6 cells were detached from the flask upon 90% confluency by first aspirating spent media, a brief treatment with 1X versene (2.68 μM KCl, 0.137 mM NaCl, 1.47 μM KH2PO4, 8.1 μM Na2HPO4, 10.7 μM EDTA, pH 7.2) followed by a brief treatment with 1X trypsin (Sigma-Aldrich) and then evenly redistributed into new tissue culture flasks. Cells were passaged in this manner 2 to 3 times per week, and all cells were used between passage numbers 12 and 80.

2.1.1 Treatment of *Xenopus* A6 cells

Cell treatments were performed upon cell confluency (80-90%), generally 2 days following sub-culturing. Heat-shock cell treatments were carried out in the original flask secured with parafilm and immersed in a water bath at the temperatures and times indicated. Chemical cell treatments were carried out in fresh media at 22°C at the indicated concentrations and for the indicated times. Following a treatment or recovery period cells were harvested. Cells destined for RNA isolation were immediately subjected to the following harvesting

procedure while cells destined for protein isolation were first allowed to recover at 22°C for 2 h and then were subjected to the harvesting procedure. The spent media was removed by aspiration and the cells were then rinsed in 2 mL of 65% Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich) which was subsequently aspirated as well. Finally, the cells were physically removed from the surface using cell scrapers, and suspended in 1 mL of 100% HBSS. This cell suspension was pelleted by centrifugation in an Eppendorf 5415D microcentrifuge (Brinkmann Instruments Ltd., Mississauga, ON) for 1 min at 13 200 revolutions per min (rpm). The supernatant was discarded and the cell pellet frozen at -80°C. Also, unless otherwise noted, all experiments were carried out multiple times, using separately treated cell extracts, and a representative result is presented.

2.1.2 Maintenance of *Xenopus* embryos

Isolation and maintenance of embryos was according to methods previously described in Sive *et al.* (2000). *Xenopus* female frogs (Boreal, St. Catharines, ON) were injected with 50 U human chorionic gonadotropin (hCG; Sigma-Aldrich) in sterile 0.65% NaCl (w/v) in the dorsal lymph sac with a 26 gauge needle 5 days prior to fertilization to prime for ovulation. Frogs were then injected with 1000 U hCG 9 h prior to egg collection to induce ovulation. Eggs were collected, by applying gentle pressure to the abdomen of the frogs, into 1X Modified Barth's Saline (MBS; 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1mM $MgSO_4$, 5mM HEPES-KOH, 2.5 mM $NaHCO_3$, pH 7.8).

Sperm for *in vitro* fertilization was obtained by removing the testes of a male *Xenopus* frog and the testes were stored in 1X MBS. Fertilization was carried out manually *in vitro* by first

removing the MBS bathing the eggs, and touching each egg with a portion of the testes. Eggs were then immersed in 0.1X MBS and left to fertilize for 30 min on an orbital shaker. Fertilization was determined by observing cortical rotation of fertilized eggs, which results in the animal pole of the eggs oriented upward. Following fertilization the jelly coat of the embryo was removed by agitation in 2% L-cysteine (Sigma-Aldrich) in 0.1X MBS (w/v), pH 8.0. Once embryos were de-jellied they were immediately washed 6 times in 0.1X MBS to remove the L-cysteine.

Embryos were subsequently maintained in 0.1X MBS at 22°C throughout development. Embryos were regularly monitored and any dead or deformed individuals were removed, and media was replaced periodically. External morphologies, described previously by Nieuwkoop and Faber (1967), were used for developmental staging.

2.1.3 Treatment of *Xenopus* embryos

When embryos reached the desired stages for study, a sample (approximately 30 embryos per treatment) was removed to a separate beaker. Heat-shock treatments were carried out in 0.1X MBS in beakers sealed with parafilm and immersed in a 33°C water bath for 1 h. Control embryos were maintained at 22°C. Following treatment the embryos destined for RNA isolation were transferred to microcentrifuge tubes, excess media was removed and tubes were immersed in liquid nitrogen and then stored at -80°C. Embryos destined for whole mount *in situ* hybridization were fixed in MEMPFA (0.1 M 3-morpholino propane sulfonic acid (MOPS; BioShop, Burlington, ON), pH 7.4, 2 mM ethylenediamine tetraacetic acid

(EDTA; BioShop), 1 mM $MgSO_4$, 4% paraformaldehyde (PFA; Bioshop)) for 2 h at room temperature on a nutating table, transferred to 100% methanol, and then frozen at -20°C.

2.2 Characterization of *Xenopus hsp110* cDNA

2.2.1 *Hsp110* cDNA

A partially sequenced expressed sequence tag clone encoding a putative HSP105-like protein (ID no. 4055839) was purchased from the Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) consortium of the ATCC. The *hsp105* clone was received in DH10 cells, in pCMV-SPORT6 plasmid, cloned into *Not1* and *Sal1* restriction endonuclease sites. The inserted region of this clone was sequenced (Mobix, Hamilton, ON), which required several sequencing reactions to obtain overlapping high quality sequence for this large gene. At each step of sequencing, gene specific primers were synthesized (Mobix) and another sequencing reaction performed until reliable sequence was obtained for the entire length of the inserted cDNA.

2.2.2 DNA sequencing

To obtain the nucleotide sequence of the plasmid insert, automated sequencing was contracted from MOBIX. Initially, standard T7 and SP6 primers were used to obtain sequence from each end of the plasmid insert. From this sequence information, custom internal primers were chosen to further elongate the sequence (Table 1). This process was repeated until the sequence overlapped in the middle of the insert. Computer analysis of

nucleotide sequences was done with Gene Tools Lite 1.0 (Bio Tools Incorporated, 2000). Eventually the DNA sequence was used to obtain a putative amino acid sequence using the Expasy proteomics server. Alignment of nucleotide and amino acid sequences was performed with ClustalW. The expasy proteomics server was also used to determine potential glycosylation sites also.

Table 1. PCR primers used for *hsp110* sequencing

Forward primers	Reverse primers
SP6 : GATTTAGGTGACACTATAG	T7 : TAATACGACTCACTATAGGG
105b : GGATGCTGCCCAGATTGT	105a : CCACCGGGTCCTGATCCA
105d : TTGCGTGCCGAAGATGTC	105c : GGGCTTCTTCGCTTCTGG

2.2.3 Plasmid isolation

Plasmids were isolated using a phenol chloroform method, modified from Sambrook and Russell, (2001). Cells containing the plasmid of interest in 5 mL LB broth (1% (w/v) tryptone (Difco, Lawrence, KS), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl (BioShop) supplemented with 100 µg/mL ampicillin (BioShop) propagated overnight at 37°C were centrifuged at 5 000 rpm for 5 min at 4°C in an Eppendorf Centrifuge 5810R (Brinkmann Instruments Ltd.) with a swinging bucket rotor. After the supernatant was removed the bacterial pellet was resuspended in 200 µL of ice-cold alkaline lysis solution I (50 mM glucose (Sigma-Aldrich), 25 mM Tris (BioShop) (pH 8.0), 1 mM EDTA (pH 8.0)) and transferred to a 1.5 mL microcentrifuge tube. To each suspension 200 µL of freshly made alkaline lysis solution II (0.2 N NaOH (BioShop), 1% (w/v) SDS (BioShop)) was added and

gently mixed by slowly inverting the tube several times and placed on ice. Next, 200 µL of ice-cold alkaline lysis solution III (3 M potassium acetate (Sigma-Aldrich), 5 M glacial acetic acid (Fisher Scientific, Pittsburgh, PA) was added and tubes were slowly inverted 3 - 5 times and then placed on ice for 5 min. The tubes were then centrifuged at 14 000 rpm at 4°C for 5 min and supernatant transferred to a fresh tube.

Residual RNA was eliminated with 20 µL RNase A (10 µg/mL; Roche Molecular Biochemicals, Laval, QC) and incubating at 37°C for 2 h. Following incubation, 600 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and vortexed. Tubes were then centrifuged for 3 min at 4°C and the upper aqueous layer transferred to a new tube and the previous step repeated. The top aqueous layer was once again transferred to a new tube and 600 µL of choloform:isoamyl alcohol (24:1) was added. Tubes were vortexed, centrifuged at the above settings and the upper aqueous layer was transferred to a fresh tube. Precipitation of plasmids was accomplished with 600 µL of isopropanol at room temperature (RT) for 2 min followed by centrifugation at 14 000 rpm in a microcentrifuge for 5 min. The supernatant was discarded and the tubes were inverted to allow all fluid to drain out, and the pellet to dry. To wash the pellet 1 mL of 70% ethanol was added and then the tubes were centrifuged at 14 000 rpm for 2 min. The supernatant was once again discarded and the tubes were once again inverted to dry the pellet. Upon drying the pellet the plasmids were dissolved in 50 µL of TE buffer (50 mM Tris, 2 mM EDTA, pH 8.0). At this point plasmids could be subjected to UV-spectrophotometry for quantification and agarose gel electrophoresis for determination of plasmid integrity.

2.2.4 Rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR)

In order to obtain the missing segment of the *Xenopus hsp110* gene, 5' RACE-PCR was required. 5' RACE-PCR was carried out using the SMART-RACE cDNA amplification system according to the manufacturer's instructions (BD Biosciences, Mississauga, ON). One μg of total RNA isolated from heat-shocked A6 cells was used for first strand cDNA synthesis. This reaction consisted of 1 μg total A6 RNA, 1 μL 5' CDS primer, 1μL BD SMART II A oligo, and water for 5 μL total volume. The components were gently mixed, pulse-spun and then incubated at 70°C for 2 min followed by 2 min on ice with a final pulse spin. At this point 2 μL 5X first strand buffer, 1 μL 20 mM dithiothreitol (DTT), 1 μL 10mM dNTP mix and I μL BD Powerscript reverse transcriptase were added to the initial components. The tube was then gently mixed again, pulse-spun, incubated at 42°C for 1.5 h, 100 μL Tricine-EDTA buffer was added, a final incubation at 72°C for 7 min and this first strand cDNA product was then stored at -20°C.

For 5' RACE, 3 μL of 5' cDNA was added to the reverse gene-specific primer (HSP110R - CCGGTTCTGTATTGGAGTGCC) and the universal primer (UPM - provided), in addition to the appropriate amount of prepared master mix (containing BD Advantage 2 PCR buffer, dNTP mix and BD Advantage 2 Polymerase Mix). The tube contents were gently mixed and pulse-spun to collect in the bottom of the tube. After 2 min at 94 °C, 30 cycles with the following parameters ensued: 30 s @ 94°C, 30 s @ 62°C and 3.5 min @ 72°C. All PCR products were electrophoresed on a 0.8% agarose gel (in 1X TAE; 40 mM Tris-acetate, 1 mM EDTA) and visualized with ethidium bromide. The 2.5 kb bands that resulted were

excised from the gel and purified using the NucleoTrap Band Purification kit (Clontech, Mountain View, CA).

The excised band was combined in a tube with buffer NT1 and nucleotrap suspension and then incubated at 50°C for 15 min. The contents were centrifuged at 10 000 g for 30 s at RT and the supernatant discarded. The pellet was combined with buffer NT2 and centrifuged under the same conditions, and the same was repeated with buffer NT3. The resulting DNA pellet was dried and solubilized in 20 μL Tris EDTA buffer (TE; 10 mM Tris-HCl, 1mM EDTA, pH 8.0). This solution was centrifuged at 10 000 g for 30 s at RT and the supernatant (DNA) was transferred to a clean tube. A 1 μL sample of the purified DNA was electrophoresed on a 0.8% agarose gel with a molecular mass marker to determine the approximate yield of DNA.

The 5'RACE products were then ligated into the T/A cloning vector, pGEM®-T Easy (Promega, Madison, WI) for sequencing. The ligation reaction contained approximately 30 ng of 5'RACE cDNA insert, 50 ng pGEM®-T Easy, DNA ligase, and 1X ligase buffer and was incubated overnight at 4°C. Competent JM109 cells (50 μL; Promega) were then transformed with the ligated hsp110-pGEM®-T Easy products via a 60 s 42°C 'heat shock' following a 20 min incubation on ice. The cells were allowed to recover on ice for 2 min and then 950 μL warm LB was added and the cells were incubated at 37°C for 1.5 h. At this point all cells were plated on LB-agar plates containing 100 μg/mL ampicillin (BioShop), with 40 μL 20 mg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; BioShop) and 4

μL isopropyl β-D-1-thiogalactopyranoside (1M IPTG; BioShop) and grown overnight at 37°C.

The next day, colonies were selected that had incorporated the hsp110-pGEM®-T Easy plasmid and grown overnight in LB broth containing 100 μg/mL ampicillin. Plasmid was isolated from broth cultures the following day and the insert was removed from the plasmid using *EcoRI.* This revealed that an insert of approximately the correct size was present in some of the isolated plasmid, and samples of the successfully ligated plasmid were sequenced yielding a full-length *hsp110* nucleotide sequence.

2.2.5 cDNA sequence analysis

Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). The tree was prepared using the neighbour-joining method with pairwise deletion, and Jukes and Cantor (1969) correction. A bootstrap value of 1 000 was employed. Putative amino acid sequences were inferred from nucleotide sequences (Expasy Proteomics Server online) and aligned using ClustalW (Thompson *et al.*, 1994). Generunner software was used to display aligned nucleotides and corresponding amino acids.

2.3 RNA isolation

2.3.1 RNA isolation from A6 cells

RNA was isolated from A6 cells for use as a template for first strand cDNA synthesis or for analysis with northern hybridization. Total RNA was isolated from *Xenopus* A6 cells

using the RNeasy mini RNA extraction kit (Qiagen, Mississauga, ON) following the manufacturer's instructions. Briefly, 600 μL of buffer RLT supplemented with 0.1% (v/v) β-mercaptoethanol (BioShop) was added to frozen cell pellets and this mixture was vortexed thoroughly. Next, the solution was passed through a 20-gauge needle attached to a 1 mL syringe 10 times to shear genomic DNA. At this point an equal volume of 70% cold ethanol was added to the lysate. The lysate was loaded onto an RNeasy mini column and centrifuged for 15 s at 14 000 rpm in an Eppendorf 5415D microcentrifuge (Brinkmann Instruments Ltd.) (this was repeated until all lysate was loaded onto the column). After all flow-through had been discarded 750 mL of buffer RPE was added to the column and centrifuged as previously described. Upon placement of the column in a fresh microcentrifuge tube 750 μL buffer RW1 was added. After centrifugation and discarding the flow-through this process was repeated. The column was then dried by centrifuging void of buffer for 2 min at 14 000 rpm. Finally, the columns were transferred to a fresh microcentrifuge tube, and 30 μL of RNase-free water was added to the column filter. After centrifugation, the eluant was collected in the bottom of the tube, and 30 μL more RNase-free water was added to the column to increase the RNA yield. Finally 60 μL of high yield total RNA was placed on ice prior to quantification.

2.3.2 RNA isolation from *Xenopus laevis* embryos

RNA was isolated from *Xenopus* embryos for analysis with northern hybridization. Total RNA was isolated from *Xenopus* embryos using a GIT/CsCl ultracentrifugation method originally described by Chirgwin *et al.* (1979), and modified by Ohan and Heikkila (1995).

As a precaution against RNA contamination all glassware used for RNA isolation was subjected to 140°C for 8 h. Plasticware was sprayed with RNase Away (VWR, Mississauga, ON) and rinsed with 0.1% diethyl pyrocarbonate-(DEPC; Sigma-Aldrich) treated water, or treated with 3% (v/v) hydrogen peroxide. Similarly, any solutions used were prepared in 0.1% DEPC, and subsequently autoclaved to inactivate the DEPC.

Approximately thirty embryos were homogenized in 9 mL GIT (4 M guanidine isothiocyanate (BioShop), 25 mM sodium acetate (BioShop), pH 6.0, 120 mM β-mercaptoethanol (Sigma-Aldrich) -in DEPC-treated water) using a PowerGen 125 homogenizer (Fisher). The homogenate was carefully layered onto 3.3 mL cesium chloride buffer (5.7 M cesium chloride (BioShop), 25 mM sodium acetate (BioShop) pH 6.0 in 0.1% DEPC-treated water) in ultra-clear centrifuge tubes (Beckman, Palo Alto, CA). Samples were centrifuged at 30 000 rpm for 23 h at 21°C (SW41 rotor, Beckman L-90K ultracentrifuge) to pellet RNA.

Following centrifugation, the supernatant was removed from the pellet and the pellet was allowed to dry. The pellets were then resuspended in 360 μL of TES buffer (0.01M Tris (BioShop), pH 7.4, 5 mM ethylenediamine tetraacetic acid (EDTA) (BioShop), pH 8.0, 1% (w/v) sodium dodecyl sulphate (SDS) (BioShop) on ice for 15 min and transferred to a microcentrifuge tube. To precipitate the RNA, 0.3M sodium acetate was added to the solution as well as 1 mL of 100% ice-cold ethanol, and was placed at -20°C for 30 min. Finally, the samples were centrifuged at 14 000 rpm at 4°C for 10 min. After removal of the supernatant, this set of precipitation steps was repeated. The pellet was dissolved in 50 μL of

DEPC-treated water. Purity, integrity and concentration of RNA were established by spectrophotometry and formaldehyde agarose gel electrophoresis.

2.3.3 RNA concentration and integrity

Total RNA isolated from both A6 cells and embryos was subjected to quantification and quality assessment. UV-spectrophotometry was used to assess the concentration of nucleic acids present. Of each sample, 5 μL was diluted to 1 000 μL with RNase-free water and the absorbance at 260 nm was measured using quartz cuvettes and a Varian Cary 50 Bio UV-Visible spectrophotometer (Varian Medical Systems, Palo Alto, CA). The absorbance at 260 nm was multiplied by 200 (dilution factor), and by 40 (RNA OD value), and finally divided by 1 000 (to move from the mL value provided by the machine, to μL) to arrive at the concentration of RNA in μg/μL units. Also, the ratio of absorbance at 260 nm: 280 nm was determined to estimate RNA purity. All RNA values used in this thesis were greater than 1.6.

Once the concentration was determined the samples were subjected to formaldehyde-agarose gel electrophoresis. Generally 1 μg total RNA was resuspended in denaturing loading buffer (1 μL 10X MOPS, pH 7.0, 1.6 μL formaldehyde (37%; BioShop), 5 μL formamide (BioShop), 2 μL loading dye (0.2% (w/v) bromophenol blue, 1mM EDTA, pH 8.0, 50% (v/v) glycerol (Sigma-Aldrich), 0.5 μL 10 mg/mL ethidium bromide). The RNA was mixed gently with the loading buffer and denatured at 68°C for 10 min and immediately placed on ice for 5 min. The samples were then electrophoresed in a formaldehyde-agarose gel (1.2% (w/v) agarose (BioShop), 10% (v/v) 10X MOPS, 16% (v/v) formaldehyde (37%))

for approximately 2 h in 1X MOPS running buffer. Visualization with UV light showed ribosomal RNA bands (18s and 28s) that could be used to determine if the RNA was degraded or was equally loaded into each lane.

2.4 Restriction enzmye (RE) digestion

Many RE digests were performed both for confirming plasmid insert sizes as well as for preparing linear templates for in vitro transcription reactions. RE digestion of recombinant plasmid DNA was performed as described in Sambrook and Russell (2001). The appropriate amount of DNA was used for each case: 1 μg for insert checking, or up to 10 μg for linear template preparation. In the case of template preparation10 μg of plasmid was added to a microcentrifuge tube (16 μL), along with 2 μL of 10X RE buffer (supplied with each RE by the manufacturer) and 2 μL of RE. The contents of the tube were gently mixed and incubated at 37°C for 2 h (unless the enzyme worked optimally at another temperature, for example *Sma1* @ 25°C). After the reaction was completed the digested plasmid DNA was run on a gel to confirm complete digestion and product size. In general a 1% agarose (w/v) gel in 1X TAE with 0.2 μg/mL ethidium bromide was used, and the entire 20 μL digest reaction volume was loaded with the appropriate amount of 6X gel-loading buffer (50% (v/v) glycerol, 0.1% bromophenol blue) into individual lanes.

2.5 Gel purification of DNA templates

The linearized templates produced by restriction enzyme digestion were purified out of the agarose gel for downstream *in vitro* transcription reactions as per the following. This

procedure provides a pure pool of DNA of the same size and prevents any carryover of indigested DNA. Individual bands of the correct size were excised from the agarose gel using a clean razor blade, taking as little agarose as possible, and placed into a gel nebulizer within an ultrafree-MC purification column from a Montage Ultrafree-DA kit (Millipore, Billerica, MA). This step had to be done using a hand-held UV lamp in order to see the band of interest. The tube(s) were centrifuged at 5 000 x g for 10 min. The flow-through contained the DNA in electrophoresis buffer separated from the agarose gel. To further purify the DNA it was precipitated using 0.1 volume of 3M sodium acetate (BioShop), pH 5.2, and 2.5 volumes of 100% ethanol. This solution was placed at -20°C for 30 min and then collected by centrifuging at 14 000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 30 μL of DEPC-treated water.

2.6 Riboprobe preparation

Digoxygenin (DIG)-labelled anti-sense riboprobes were employed for both northern hybridization and whole mount *in situ* hybridization. Probe synthesis was carried out according to manufacturer's instructions with minor modifications (Roche Molecular Biochemicals). *In vitro* transcription reactions were carried out similarly for each probe/gene except that each linearized template was unique and the RNA polymerase used was dictated by the orientation of the insert with respect to the polymerase site on the plasmid. Each reaction consisted of 5.5 μL linearized template, 4 μL NTP's (2.5 mM rGTP, 2.5 mM rATP, 2.5 mM rCTP, 1.65 mM rUTP and 0.875 mM DIG-11-UTP) (Roche Molecular Biochemicals), 4μL 100 mM dithiothreitol (DTT) (BioShop), 4 μL 5X transcription buffer

(Roche Molecular Biochemicals), 0.5 μL RNase inhibitor (Roche Molecular Biochemicals) and 2 μL RNA polymerase (MBI Fermentas, Burlington, ON). This solution was mixed gently and incubated at 37°C for 2 h. Following this, 2 μL RNase-free DNase 1 (Roche Molecular Biochemicals) was added to digest the original DNA template and left to incubate for 15 min at 37°C. The resulting transcripts were precipitated with 10 μL 3M sodium acetate (BioShop), pH 5.2, 220 μL 100% ethanol, 80 μL of 1% SDS (w/v) (BioShop) in Tris-EDTA buffer (TE) at -20°C for 30 min. The RNA was collected by centrifugation at 14 000 rpm at 4°C for 10 min. The transcripts were stored at -80°C until used for hybridization. The supernatant was removed and the pellet resuspended in 21 μL DEPC-treated water. A 1 μL aliquot of the riboprobe was run on a formaldehyde-agarose gel containing ethidium bromide and visualized with UV light to ensure a single band of the expected size.

2.6.1 Specific riboprobe conditions

The *hsp110* and *ef-1α* cDNA clones (Genbank accession no. BF428504 and BG160504 respectively) in pCMV-Sport6 vectors (Invitrogen Life Technologies, Frederick, MD) were obtained from the ATCC. Both *hsp110,* and *ef-1α,* anti-sense riboprobes were produced by linearizing the vector constructs with *Sma1*, and transcribing with T7 RNA polymerase, as described previously (Figure 3 and Figure 4). Sense *hsp110*-riboprobe was produced by linearizing the construct with *Xho1* and transcribing with SP6 RNA polymerase. The *hsp27* clone (Genbank accession no. DQ473544) in pCS107 plasmid was also obtained from the

ATCC. To synthesize anti-sense riboprobe, pCS107-*hsp27* was linearized with *BamHI*, and transcribed *in vitro* with T7 RNA polymerase (Figure 5).

Figure 3. Template for *hsp110 in vitro* transcription

A portion of the coding region of *hsp110* was cloned into pCMV-SPORT6 (Invitrogen) to generate pCMV-SPORT6-hsp110 (ATCC). To generate antisense riboprobe, pCMV-SPORT6-hsp110 was linearized with *SmaI* and transcribed *in vitro* with T7 RNA polymerase. To generate sense riboprobe, pCMV-SPORT6 was linearized with *XhoI* and transcribed with SP6 RNA polymerase.

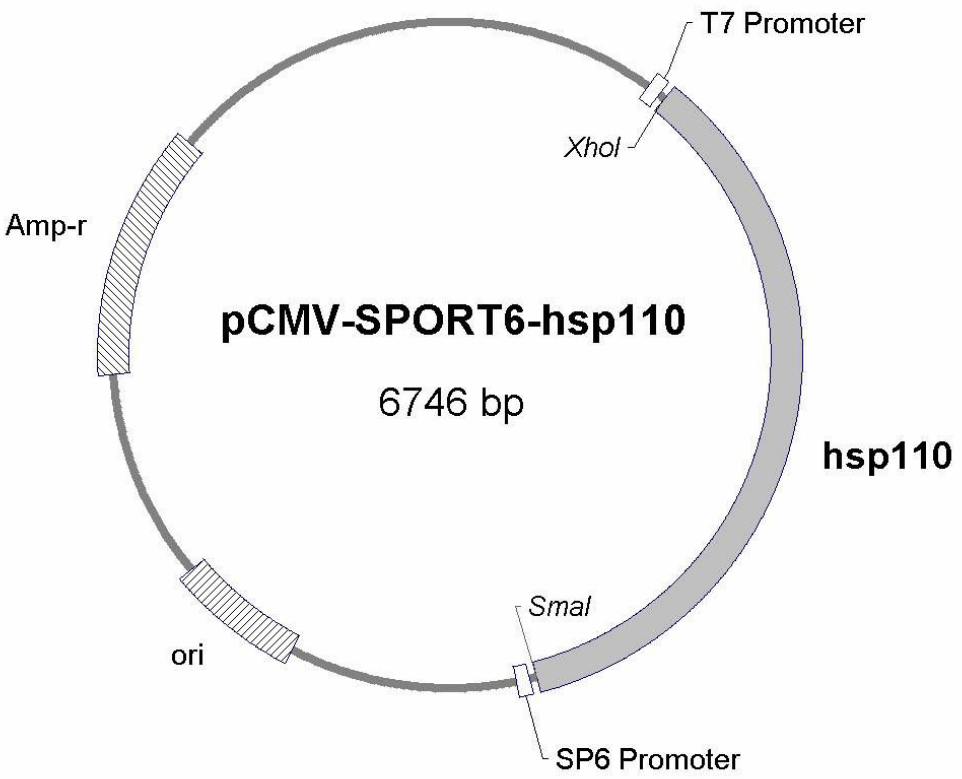

T7 Promoter
XhoI
Amp-r
pCMV-SPORT6-hsp110
6746 bp
hsp110
SmaI
ori
SP6 Promoter

Figure 4. Template for *ef-1α in vitro* transcription

The coding region of *ef-1α* was cloned into pCMV-SPORT6 (Invitrogen), generating pCMV-SPORT6-ef-1α (ATCC). To synthesize antisense riboprobe, pCMV-SPORT6-ef-1α was linearized with *SmaI* and transcribed *in vitro* with T7 polymerase.

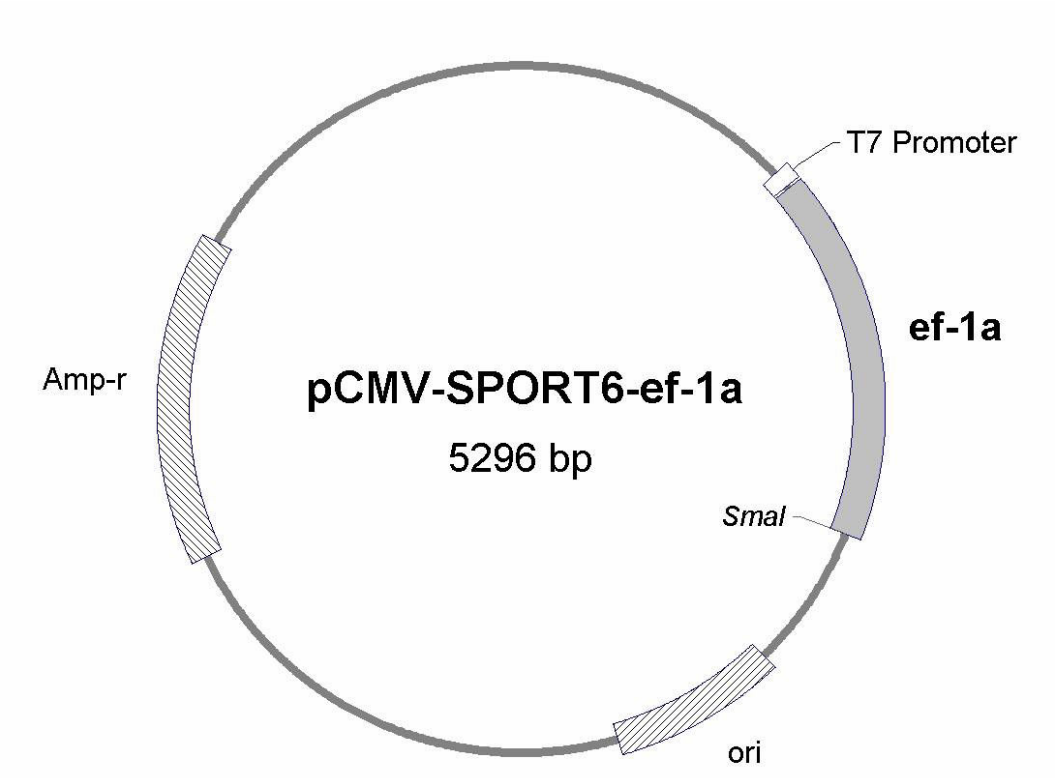
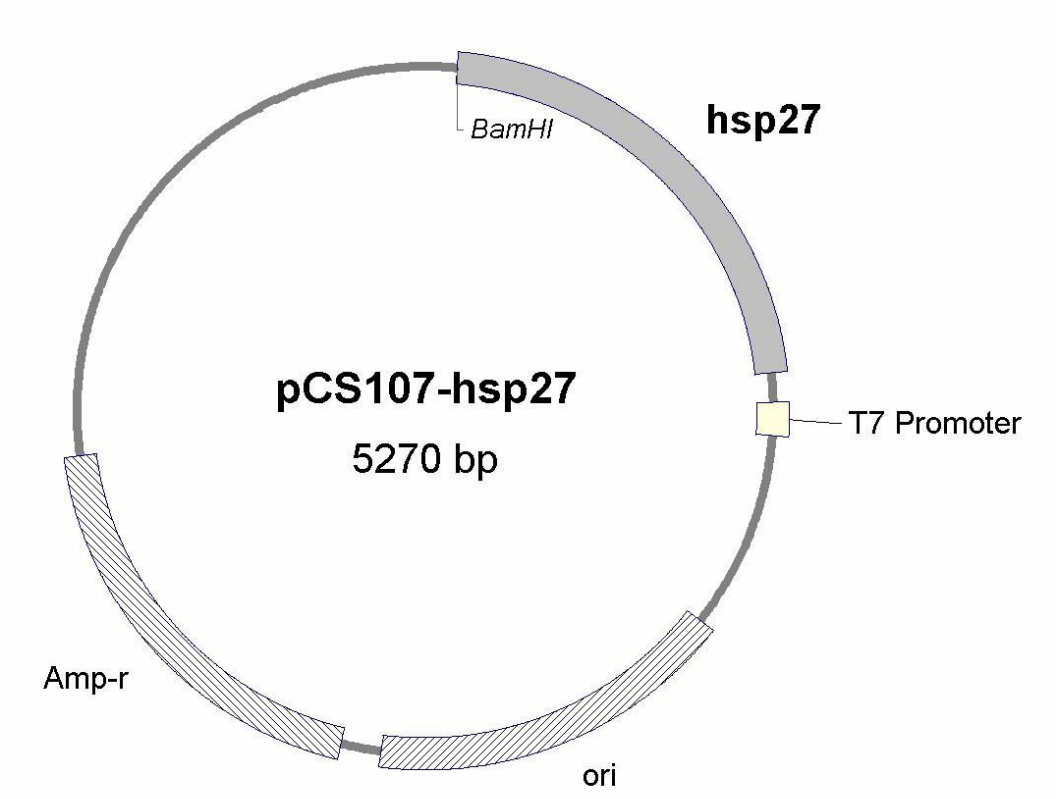

T7 Promoter
ef-1a
Amp-r
pCMV-SPORT6-ef-1a
5296 bp
SmaI
ori

Figure 5. Template for *hsp27 in vitro* transcription

The coding region of the *hsp27* gene was cloned into pCS107 (Invitrogen) to generate pCMV-SPORT6-hsp27 (ATCC). To synthesize antisense riboprobe, pCMVSPORT6-hsp27 was linearized with *BamHI* and transcribed *in vitro* with T7 RNA polymerase.


BamHI
hsp27
pCS107-hsp27
5270 bp
T7 Promoter
Amp-r
ori

The *actin* clone used here was the type 8 cytoskeletal actin cDNA from pXIcAI (Mohun *et al.,* 1983). The coding region of *actin* was previously cloned into the *HindIII* site of pBlueScript KS (Gibco/BRL Laboratories, Burlington, ON) generating pBlue-actin. To synthesize anti-sense riboprobe, pBlue-actin was linearized with *EcoRI* and transcribed *in vitro* with T3 polymerase (Figure 6). The coding region of hsp70 from pXL16P (Bienz, 1984) was excised with *Fsp1* and *Pst1*, and cloned into *SmaI* and *PstI* of pSP72 (Promega) generating pSP72-hsp70. To synthesize anti-sense riboprobe, pSP72-hsp70 was linearized with *MluNI* and transcribed *in vitro* with SP6 RNA polymerase (Figure 7). To synthesize sense riboprobe, pSP72-hsp70 was linearized with *XhoI* and transcribed *in vitro* with T7 RNA polymerase, as described previously (Lang *et al.,* 2000). All specific riboprobe generation information has been summarized in Table 2.

Table 2. Specific riboprobe generation conditions

Gene Insert	Plasmid	Restriction Enzyme	RNA Polymerase
Hsp110	pCMV-SPORT6	*Sma1*	T7
Hsp27	pCS107	*BamH1*	T7
Hsp70	pSP72	*MluN1*	SP6
Actin	pBluescript KS	*EcoR1*	T3
EF-1α	pCMV-SPORT6	*Sma1*	T7

Figure 6. Template for *actin in vitro* transcription

The coding region of the type 8 cytoskeletal *actin* gene contained in pXIcAI (Mohun *et al.*, 1983) was cloned into the *HindIII* site of pBluescript KS (Fermentas), generating pBlue-actin. To generate antisense riboprobe template, the plasmid was linearized with *EcoRI* and transcribed with T3 RNA polymerase.

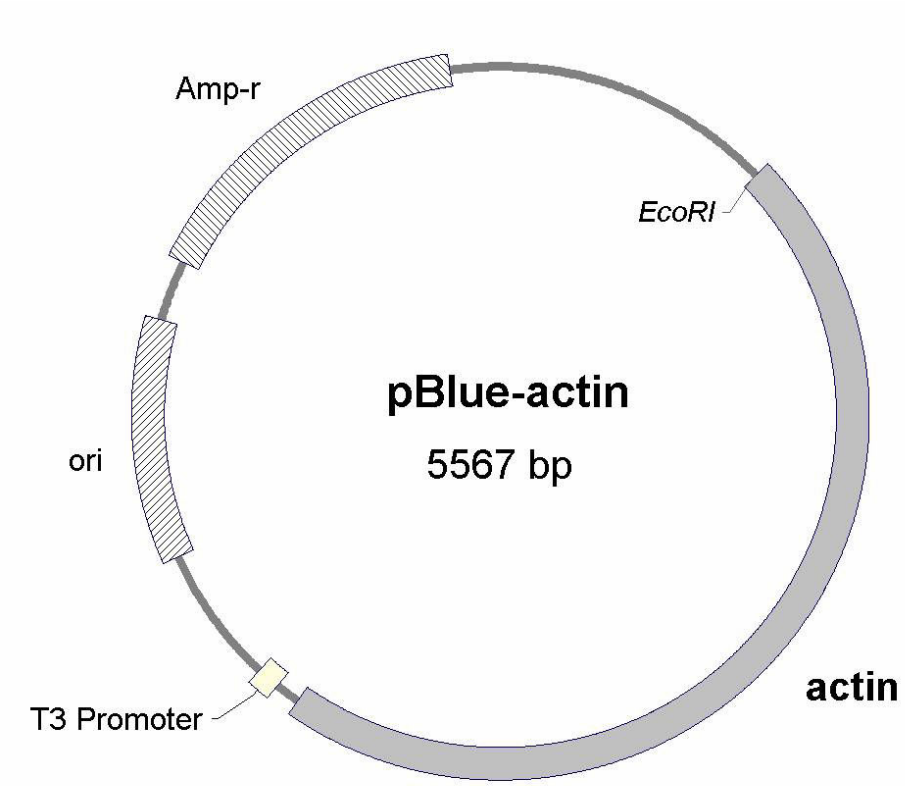
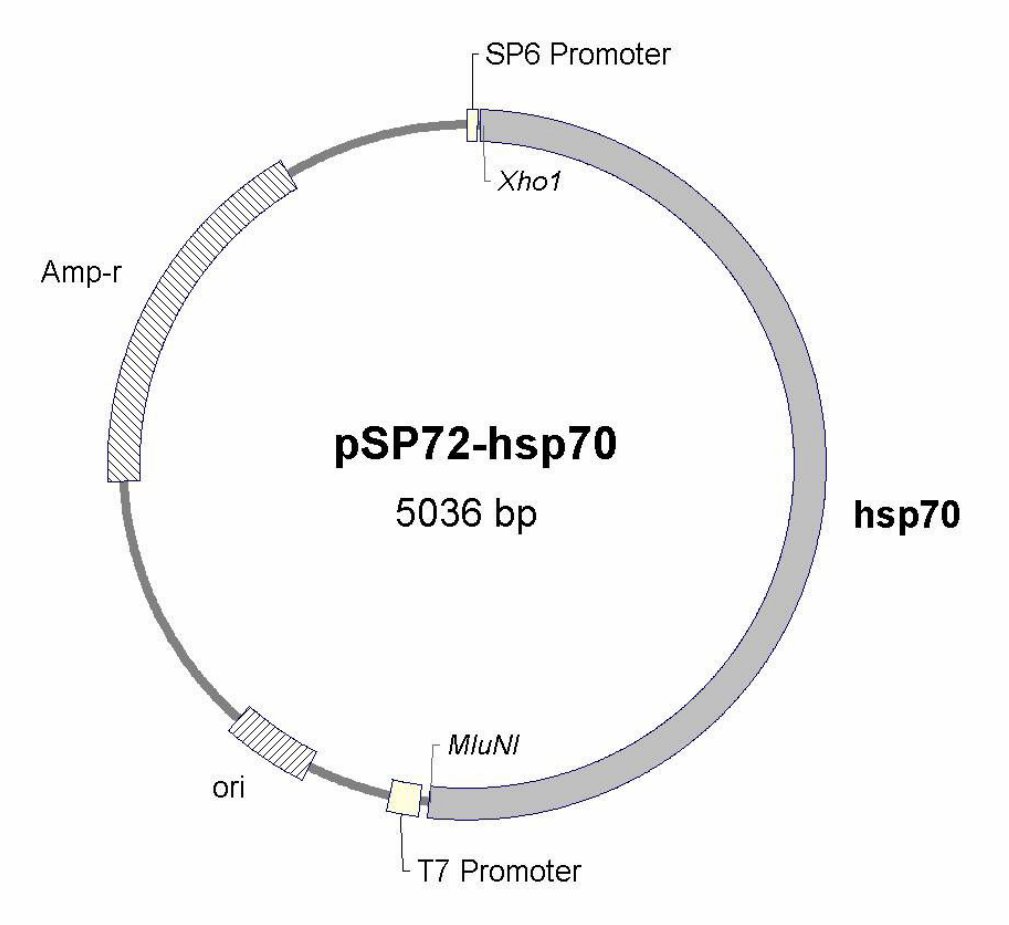

Amp-r
EcoRI
pBlue-actin
5567 bp
ori
T3 Promoter
actin

Figure 7. Template for *hsp70 in vitro* transcription

The coding region of the *hsp70* gene contained in pXL16P (Bienz, 1984b) was cloned into the *SmaI* and *Pst1* sites of pSP72 (Promega), generating pSP72-hsp70. To generate antisense riboprobe template, the plasmid was linearized with *MluNI* and transcribed with SP6 polymerase. To generate sense riboprobe template, the plasmid was linearized with *XhoI* and transcribed with T7 RNA polymerase.


SP6 Promoter
Xho1
Amp-r
pSP72-hsp70
5036 bp
hsp70
MluNI
ori
T7 Promoter

2.7 Northern hybridization

Northern hybridizations were performed, with modifications, as per Sambrook and Russell (2001), and manufacturer's directions (Roche Molecular Biochemicals). RNA samples were aliquotted in microcentrifuge tubes, and if necessary, concentrated using a speed vac (Savant, Ramsey, MN). RNA was electrophoresed as described previously, at 60V for approximately 4 h to ensure adequate resolution. To prepare the gel for transfer it was immersed for 20 min in 0.05N NaOH, followed by two 20 min soaks in 20X SSC (3M NaCl, 300 mM sodium citrate, pH 7.0, in DEPC-treated water).

At this point the RNA was transferred by capillary action to a positively charged nylon membrane (Roche Molecular Biochemicals). The gel was placed on a 3MM Whatman filter paper wick, inverted. This apparatus was positioned in a dish containing 20X SSC to saturate the wick and gel continuously. A positively charged nylon membrane was cut to the size of the gel and placed directly on it. Then two pieces of 3MM Whatman filter paper of the same size were placed on the membrane, followed by a stack (approximately 10 cm tall) of paper towels, also cut to the size of the gel. At each step, air bubbles were eliminated by gently rolling with a clean pipette. Parafilm was placed on all edges of the gel support to prevent any contact between the wick and any element of the transfer above the gel. Finally, a glass plate was placed on the paper towel stack with a weight of approximately 500 g to evenly distribute weight and facilitate overnight capillary transfer.

The next day the RNA was cross-linked using a UVC 515 Ultraviolet Multilinker (120 000 μJ/cm2; UltraLum Inc., Claremont, CA) to the membranes with UV light and stained with

reversible RNA blot stain (Sigma-Aldrich) to verify equal sample loading and transfer efficiency prior to hybridization. This was done by first immersing the membrane in 10% (v/v) acetic-acid (in DEPC-treated water) for 5 min, followed by 5 min in a 1:10 dilution of Blot Stain Blue blot staining solution (Sigma-Aldrich). The membrane was then rinsed with water to remove any non-specific background staining. The membrane was then scanned with a conventional computer scanner. Upon confirmation of a good transfer, the membrane was probed for the gene of interest.

Prior to adding the riboprobe the membrane was prepared for hybridization. The membrane was sealed in a KAPAK sealable pouch (VWR Canlabs, Mississauga, ON) with 50 mL prehybridization buffer (5X SSC, 50% formamide, 2% (w/v) blocking reagent (Roche Molecular Biochemicals), 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS) and gently mixed at 68°C for 4 h. Then the hybridization buffer was added, which is 50 mL of ‘prehybridization buffer with 20 μL of riboprobe prepared as previously described. The hybridization was left overnight.

The hybridization buffer was removed (and could be reused in the future) and the membrane was washed twice in 2X SSC, 0.1% (w/v) SDS at 68°C for 5 min, once in 0.5X SSC, 0.1% (w/v) SDS at 68°C for 15 min, and once in 0.1X SSC, 0.1% (w/v) SDS at room temperature (RT) for 15 min. The membrane was then briefly washed in washing buffer (100 mM maleic acid (BioShop), 150 mM NaCl, pH 7.5, 0.3% (v/v) Tween 20 (Sigma-Aldrich) for 1 min and then blocked in 2% blocking buffer (2% (w/v) blocking reagent, 100 mM maleic acid, 150 mM NaCl, pH 7.5) for 30 min with gentle shaking at RT. The anti-DIG

alkaline phosphatise FAB fragments antibody (Roche Molecular Biochemicals) was then added to the membrane in fresh 2% blocking buffer at 1:8 000 dilution for 30 min at RT. The membrane was then washed twice in washing buffer for 15 min at RT, and once in detection buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl) for 2 min at RT. Finally, the membrane was placed on a fresh sealable bag and the substrate CDP-Star (Roche Molecular Biochemicals) was added evenly to the membrane, avoiding air bubbles, for 10 min in the dark at RT. Chemiluminescence was detected using a FluorChem imager (Alpha Innotech, San Leandro, CA).

2.8 Whole mount *in situ* hybridization

Albino embryos were solely used for whole mount *in situ* hybridization, to avoid any confusion between natural pigmentation and colorimetric detection of the DIG-labelled riboprobe, using procedures modified from those previously described (Harland, 1991; Lang *et al.*, 1999; 2000). The riboprobes were prepared exactly as for a northern hybridization, as described above. Sense-riboprobes were used for *in situ* as a negative control in these experiments. For each experimental condition/stage, approximately 15 embryos were used, to ensure representative information.

Embryos previously treated and fixed, as described previously, were transferred to appropriately labelled 6 mL glass vials (VWR Canlabs) which had been previously treated with Sigmacote (Sigma-Aldrich) to prevent embryos from sticking to the glass. Since the embryos had been stored in 100% methanol, they were gradually rehydrated. This was done by washing them in decreasing concentrations of methanol (100% to 75% to 50% methanol

in water, to 25% methanol in TTw buffer (10 mM NaCl, 2.5 mM Tris, pH 7.4, 0.1% (v/v) Tween 20), and finally to straight TTw for 3 washes). After this the embryos were permeabilized for 20 min with 5 μg/mL proteinase K (Roche Molecular Biochemicals), and then immersed twice for 5 min in 0.1 M triethanolamine, pH 7. Next the embryos were treated with 5 μL/vial acetic anhydride for 5 min to prevent non-specific binding of the riboprobe. This step was repeated to refresh the acetic anhydride. The embryos were then washed twice for 5 min in TTw and then re-fixed in MEMPFA for 20 min. After this, the embryos were washed thoroughly 5 times to prepare for hybridization. Embryos were rinsed in prehybridization buffer (50% (v/v) formamide, 5X SSC, 1 mg/mL Torula RNA, 1X Denhardts solution, 0.1% (v/v) Tween 20, 5 mM EDTA, 100 μg/mL heparin) briefly. After this was removed, the embryos were immersed in fresh prehybridization buffer for 2 h at 65°C. Finally, the prehybridization buffer was removed, and replaced with the appropriate DIG-labelled riboprobes in the same buffer. The embryos were then allowed to hybridize overnight at 60°C.

The next day, the probe was removed (for reuse) and the embryos were gradually moved out of hybridization buffer. First they were rinsed in 100% hybridization buffer for 10 min at 60°C, followed by 50% hybridization buffer/50% 2X SSC for 10 min at 60°C, followed by 25% hybridization buffer/75% 2X SSC at 60°C, and finally 100% 2X SSC twice for 20 min at RT. At this time 1 μL/mL RNase A in 2X SSC was added to the embryos for 30 min at 37°C to degrade any remaining probe. This was followed by two high stringency washes in 0.2X SSC at 60°C for 1 h. At this point the embryos were prepared for the secondary

antibody incubation with a 10 min incubation in TTw at RT, followed by a 10 min incubation in TBT (20 mM NaCl, 2.5 mM Tris, pH 7.4, 2 mg/mL BSA, 0.1% (v/v) Triton X-100) at RT. In order to prevent non-specific binding, the embryos were blocked with TBT containing 20% lamb serum at RT for 1 h, and then the same solution was again added to the embryos, but this time containing a 1:3 000 dilution of alkaline phosphatase-conjugated anti-DIG antibody. The embryos were then left overnight at 4°C with gentle shaking.

The next day, the embryos were washed 12 times for 30 min in TBT at RT, to remove excess antibody. Then they were incubated twice for 15 min at RT with alkaline phosphatase (AP) buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 2 mM levamisol (Sigma-Aldrich), 0.1% Tween 20). They were then incubated overnight at 4°C with staining solution (0.85 μL/mL nitroblue tetrazolium chloride (NBT; Roche Molecular Biochemicals), 0.875 μL/mL bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Molecular Biochemicals) in AP buffer).

On the second last day of the procedure the hybridized message was visualized in the embryos. First the staining reaction was stopped by dehydrating the embryos in methanol gradually (0% to 25% to 50% to 75% to 100% methanol: water). The embryos were maintained in chilled 100% methanol until any background staining was sufficiently removed (no more stain was leaching out). When the results looked satisfactory the embryos were rehydrated by gradually decreasing the concentrations of methanol and finally counterstained with Bouin's Fixative (VWR Canlabs) overnight at RT.

On the last day of the procedure visualization of embryos was facilitated by clearing. They were once again dehydrated by subjecting them to increasing concentrations of methanol (as previously described). Finally, they were immersed in clearing buffer (benzyl alcohol:benzyl benzoate, 1:2; BABB) in the wells of a glass watchplate. Embryos were viewed using a Nikon SMZ 1500 dissecting microscope and photographed with a Nikon Coolpix 995 digital camera. After photographing the embryos were rinsed twice in 100% methanol and then stored in 100% methanol at -20°C.

2.9 A6 cell protein isolation

A6 cell pellets were resuspended in homogenization buffer (200 mM sucrose, 2mM EGTA, 1mM EDTA, 40 mM NaCl, 30 mM HEPES, 1% SDS, 100 μg/mL PMSF, 1 μg/mL aprotnin, 0.5 μg/mL leupeptin, pH 7.4) in a micro-centrifuge tube, homogenized with 10 passes of a Teflon pestle, and sonicated using 15 0.65-s pulses at 35 W (Branson model 240 Sonifier, Danbury, CT). Cellular debris was removed by centrifugation at 14 000 rpm in an Eppendorf Centrifuge 5810R (Brinkmann Instruments Ltd.) for 30 min at 4°C and the protein-containing supernatant was transferred to a new tube.

2.9.1 Protein quantification

In order to determine the protein concentration in each sample the bicinchoninic acid (BCA) method was used according to manufacturer's instructions (Pierce, Brockville, ON). Bovine serum albumin (BSA) was used as the standard and was diluted in 0.2 mg/mL increments from the original 2 mg/mL concentration to zero, to obtain a standard dilution

curve. A6 cell protein samples were diluted 1:10 in water prior to assaying to obtain values within the standard curve dimensions. For the assay, 10 μL of all samples, including A6 cell protein unknowns and BSA standard dilutions, was added to individual wells of a 96-well plate in triplicate. BCA reagent (reagent A: reagent B, 50:1) was mixed immediately prior to use, and 80 μL was added per well. The plate was incubated at 37°C for 30 min, and then at RT for 10 min following. The absorbance at 562 nm was measured using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, CA) and Soft Max pro software. The protein concentrations in each sample were determined using the absorbance values and interpolating concentrations from the standard curves generated from the BSA standards. The protein was then resolved on a Coomassie Brilliant Blue stained gel described in the next section) to assess reliability of protein concentrations and protein degradation.

2.10 Custom antibody production

Several commercially available antibodies for Hsp110 were tried in immunoblot analyses with *Xenopus laevis* protein, although they were not marketed to display cross-reactivity with *Xenopus*. I tried SPA1101D, SPA1103D (Stressgen, Victoria, BC) and H-7287(HD19) (Sigma-Aldrich), each of which are hsp110 antibodies with differing epitope regions. Unfortunately all antibodies produced extensive non-specific binding of the antibody to the membrane, rendering them ineffective for western blotting, and especially for immunohistochemical analysis.

Rabbit anti-(*Xenopus*) antibodies against Hsp110 and Hsp70 were custom produced for our laboratory (Abgent, San Diego, CA). Briefly, a C-terminal peptide was synthesized (Hsp110

– ACHPNEKSTIDMDFD, Hsp70 - AEKYKADDDAQRERVC), with a cysteine added to the N-terminal end of the peptide to enable keyhole limpet hemocyanin (KLH) conjugation, which ultimately increases antigenicity. The methodology employed by the company involved the collection of a serum sample prior to immunization of the rabbits (pre-immune serum). Rabbits were then immunized subcutaneously and intra-muscularly with 200 μg/mL conjugated peptide emulsified in Complete Freund's Adjuvant (CFA) (week 0). The rabbits were re-immunized 100 μg antigen 2 weeks later (week 2). Two weeks following that, they were immunized with 100 μg antigen in Incomplete Freund's Adjuvant (IFA) (week 4). From then until week 9 the rabbits received 100 μg antigen in PBS per week. Test bleeds were collected from rabbits on week 5, and enzyme-linked immunosorbent assay's (ELISA's) and western blots were carried out to ensure that antibody was being produced. The terminal bleed was carried out on week 10. The antibody was then affinity purified against the peptide and a final ELISA and western blot was done to ensure specificity and titre of the antibody.

2.11 Immunoblot analysis

Protein samples to be subjected to immunoblot analysis were thawed slowly and aliquotted into new microcentrifuge tubes based on the protein concentrations from the BCA assay. The amount of protein loaded on the gel was dependent upon the potency of the antibody to be used. The protein was mixed with an appropriate amount of water, to equalize volume between samples, and denaturing loading buffer (0.0625 M Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue).

Immediately prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were boiled for 10 min, cooled and briefly centrifuged to collect the sample in the bottom of the tube.

Discontinuous gel electrophoresis was carried out with a Mini Protean III (Bio-Rad, Mississauga, ON) and manufacturer's instructions were followed. The separating gel (10% polyacrylamide gels (10% (v/v) polyacrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS), 0.15% (v/v) n,n,n'n'-Tetramethylethylenediamine (TEMED)) was poured and fully polymerized (approximately 30 min) followed by the stacking gel (4% (v/v) polyacrylamide, 0.125 M Tris pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.2% (v/v) TEMED) (approximately 25 min). The gels were assembled in the rig, immersed in 1X TGS (25 mM Tris, 0.2 M glycine, 1 mM SDS) and then the protein samples were loaded into the wells and electrophoresed at 90V. When the loading dye front reached the bottom of the separating gel the voltage was stopped.

If the protein gel was destined for Coomassie staining (checking protein quality, as mentioned earlier), the gel was removed from the apparatus, rinsed in water, and immersed in Coomassie Staining Solution (50% methanol, 10% glacial acetic acid, 0.25% Coomassie Brilliant Blue R-250 (Sigma-Aldrich)). The gel was gently shaken in this solution for 1 h at RT, and then rinsed overnight in destaining solution (50% methanol, 10% glacial acetic acid) to remove background staining.

Gels destined for immunoblot analysis were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol) for 15 min, while the membranes they were to be

transferred to were soaked for 30 min. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) at 20V for 20 min using a Trans Blot Semi-dry Transfer Cell (Bio-Rad). Following transfer, membranes were stained immediately in Ponceau-S (Sigma-Aldrich; 0.19% (w/v) Ponceau-S, 5% (v/v) acetic acid)) for 10 min, to verify equal loading of protein and transfer efficiency. The membranes were scanned with a Hewlett Packard ScanJet 3300C. The membranes were then blocked with blocking solution (5% (w/v) skim milk powder in wash buffer (TBS-T; 2mM Tris, pH 7.5, 30 mM NaCl, 0.1% (v/v) Tween 20)) for 1 h at RT with gentle shaking.

The membranes were then incubated in the appropriate polyclonal primary antibody diluted in blocking solution and gently shaken at RT for 1 h (Table 3). After this, the membranes were washed in TBS-T three times for 10 min.

Table 3. Polyclonal antibodies employed for this study

Antibody:	Animal raised in:	Dilution used:	Manufacturer:
Hsp110-commercial	Rabbit	1:2000	StressGen, Victoria, BC
Hsp110-custom	Rabbit	1:850	Abgent, San Diego, CA
Hsp70-custom	Rabbit	1:200	Abgent, San Diego, CA
Actin-commercial	Mouse	1:200	Sigma-Aldrich

Membranes incubated with the commercial hamster Hsp110 antibody (StressGen, cat. #SPA1101), were incubated with horseradish peroxidise (HRP)–conjugated goat anti-rabbit IgG secondary antibody (Cedarlane, Hornby, ON) diluted 1:3 000 in blocking solution for 1 h. The membranes were again washed three times in TBS-T for 10 min. Finally they were detected using ECL-plus chemiluminescent substrate (Amersham Biosciences, Brockville,

ON), and imaged using the FluorChem Chemiluminescent imager (Alpha Innotech Corp., San Leandro, CA). Actin blots were incubated with AP-conjugated secondary IgG (Bio-Rad), equilibrated in AP detection buffer, with NBT chloride and BCIP.

Membranes that were incubated with all other primary antibodies were treated in the same way, but the secondary antibody used was AP-conjugated goat anti-rabbit IgG at 1:3 000 (Bio-Rad). For these membranes, the washing steps were the same, but for detection they were immersed in AP detection buffer (50 mM Tris, 50 mM NaCl, 25 mM $MgCl_2$, pH 9.5) with 0.33% NBT and 0.17% BCIP, and allowed to develop until the band intensity was satisfactory.

2.12 Laser scanning confocal microscopy (LSCM)

Cells were prepared for imaging by LSCM on glass coverslips in sterile Petri dishes. It was necessary before growing A6 cells on the coverslips to thoroughly clean the coverslips so that cells could properly attach. Glass coverslips (VWR) were placed in small Coplin jars to ensure full contact with the cleaning solution (49.5% (v/v) ethanol, 0.22M NaOH) for 30 min with periodic shaking at RT. The coverslips were then rinsed under running deionized water for 3 h and dried on Whatman paper. Finally the coverslips were flamed in the laminar flow hood and stored in sterile Petri dishes until used.

To prepare A6 cells for an experiment, coverslips were placed in new sterile Petri dishes and A6 cell suspension was added to the dish, and allowed to attach to the coverslips at 22°C for 24-48 h. A6 cells were then treated directly in the Petri dishes either in the 22°C incubator, for chemical treatments, or parafilm-sealed and immersed in a heated water bath

for heat shocking. Following treatment, the cells were allowed to recover at 22°C for 2 h before preparing for imaging.

After treatment, the L-15 media the cells were grown in was removed and the cells were washed twice in phosphate-buffered saline (PBS; 1.37 M NaCl, 67 mM Na_2HPO_4, 26 mM KCl, 14.7 mM KH_2PO_4, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) and the coverslips were transferred to new small Petri dishes (1 coverslip/dish). The cells were fixed (3.7% (w/v) PFA, pH 7.4 in PBS) for 10 min and then washed 3 times in PBS for 5 min. Next, the cells were permeabilized (0.1% Triton X-100 (Sigma-Aldrich) in PBS) for 10 min and then washed 3 times in PBS for 5 min. Finally, the cells were blocked (5% (w/v) BSA Fraction V (Sigma-Aldrich), filter-sterilized using a 0.45 μm filter (Pall Filtration Corp.) in PBS) overnight at 4°C.

The next day the cells were incubated in primary antibody (custom HSP110 at 1:250 in blocking solution) for 1 h. After three, 5-min washes in PBS, the cells were indirectly labeled by incubation in a fluorescent-conjugated secondary antibody (goat-anti rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) at 1:2 000 in blocking solution) for 30 min, in the dark, to avoid any chance of photo-bleaching of the fluorescent signal. After this, the cells were immediately probed for actin (rhodamine-tetramethylrhodamine-5-isothiocyanate phalloiden (TRITC (Molecular Probes), 1:60 in PBS) for 20 min, in the dark. The cells were then washed three times for 5 min in PBS. The coverslips were then mounted (cell side down) in one drop of VectaShield (Vector Laboratories Inc, Burlingame, CA) on a glass slide and sealed with clear nail polish. Once dried, the slides were visualized according to

manufacturer's instructions with a Zeiss Axiovert 200 microscope and LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, ON).

Chapter 3: Results

3.1 *Hsp110* cDNA sequence

A putative *Xenopus hsp105* cDNA clone was obtained from the ATCC. This expressed sequence tag (EST) clone was unverified by the distributor, and sold as an "*hsp105*-like" sequence. The insert was cloned into *NotI* and *SalI* RE sites in the pCMV-SPORT6 plasmid. After complete sequencing of the insert, it was determined that the 5' portion of the nucleotide sequence was missing, including the initiation codon. A complete cDNA sequence of the *hsp110* open reading frame (Figure 8) was generated after the missing coding sequence, and part of the 5' untranslated region (UTR), was obtained by 5'RACE-PCR (Figure 9).

The amino acid sequence was predicted using the Expasy proteomics server, and aligned, using ClustalW, with several species to confirm its identity. The predicted *Xenopus* HSP110 amino acid sequence resembled other organisms in length (854 amino acids), molecular mass (96,112 kDa) and pI (5.63). In comparison to hamster HSP110, the *Xenopus* HSP110 amino acid sequence displayed 73% identity with 4 additions and 178 substitutions, 114 of which were conserved (Figure 10). There was one NLS (KKPK) identical to hamster, starting at amino acid 586. Also, there were seven potential N-glycosylation sites, the first 3 of which were identical to hamster (at amino acid 45, 61, 280, 517, 700, 731, and 831, Figure 10). The *Xenopus laevis hsp110* sequence also shared 74% identity with human and mouse *hsp105,* 49% identity with sea urchin egg receptor for sperm and 41% identity with yeast *pss1* (Table 4).

Figure 8. Nucleotide sequence and predicted amino acid sequence of the complete *Xenopus laevis hsp110* coding region

Nucleotide sequence and predicted amino acid sequence of the complete *Xenopus laevis hsp110* coding region (accession number DQ458776). The top line shows the 854 predicted amino acids, and the bottom line shows the nucleotide sequence of the cDNA (2 562 nucleotide bases). The start (ATG, nucleotide position 1) and stop (TAA, nucleotide position 2 559) codons are underlined and shown in bold. The NLS (KKPK) is indicated in bold and underlined, at amino acid position 581. The seven potential N-glycosylation sites (amino acid positions 45, 61, 280, 517, 700, 731, and 831) are also underlined and indicated in bold.

```
     M  S  V  V  G  F  D  L  G  F  Q  N  C  H  V  A  I  A  R  A  G  G  I  E  T  V  A  N  E  F  S  D  R
   1 ATGTCGGTTG TCGGGTTCGA CTTGGGTTTT CAGAACTGTC ACGTTGCTAT AGCCCGAGCC GGCGGCATCG AGACCGTTGC CAATGAATTT AGCGACCGCT

     C  T  P  A  V  V  S  F  G  L  K  N  R  T  I  G  I  A  A  K  N  Q  L  I  T  N  A  N  N  T  V  S  S  F
 101 GCACCCCCGC TGTAGTATCG TTTGGATTGA AAAACAGGAC AATTGGCATT GCTGCCAAAA ACCAGCTAAT CACAAATGCC AACAACACTG TATCCAGCTT

     K  K  F  H  G  R  A  F  N  D  A  F  V  Q  K  E  K  N  N  L  P  Y  K  L  V  Q  T  N  N  G  G  V  G
 201 TAAGAAATTC CATGGCCGTG CATTTAATGA TGCATTTGTG CAGAAAGAGA AGAATAATTT GCCCTACAAG CTAGTACAAA CGAATAACGG TGGTGTTGGT

     V  K  V  N  Y  L  E  E  E  H  V  F  S  I  A  Q  I  T  A  M  L  L  T  K  L  K  E  T  A  E  N  N  L
 301 GTTAAGGTCA ATTATCTGGA AGAAGAGCAT GTATTTAGCA TTGCACAAAT AACTGCCATG CTGTTAACTA AGCTAAAGGA GACTGCAGAG AACAACCTCA

     K  K  P  V  T  D  C  V  I  S  V  P  F  F  F  T  D  A  E  R  R  S  V  L  D  A  A  Q  I  V  G  L  N  C
 401 AGAAGCCGGT CACTGACTGT GTCATATCTG TACCATTCTT CTTCACAGAT GCAGAGAGGA GATCTGTTTT AGATGCTGCC CAGATTGTTG GCCTTAACTG

     L  K  L  M  N  D  M  T  A  V  A  L  N  Y  G  I  Y  K  Q  D  L  P  A  P  E  E  K  P  K  I  V  V  F
 501 CCTGAAGTTA ATGAATGACA TGACAGCAGT TGCTTTGAAC TATGGAATAT ATAAGCAAGA CCTTCCAGCT CCAGAGGAGA AACCTAAAAT TGTAGTTTTT

     A  D  M  G  H  S  S  F  Q  L  S  A  C  A  F  N  K  G  K  L  K  V  L  G  T  A  F  D  P  Y  F  G  G
 601 GCTGACATGG GACACTCCTC TTTCCAATTA TCTGCCTGTG CCTTCAATAA AGGAAAGCTA AAGGTTCTTG GCACAGCCTT CGACCCATAC TTCGGAGGAA

     R  N  F  D  E  K  L  V  E  H  F  C  V  E  F  K  T  K  Y  R  L  D  V  K  S  K  I  R  A  V  L  R  L  Y
 701 GAAATTTTGA TGAAAAGCTC GTTGAACACT TTTGTGTAGA ATTCAAAACA AAATACAGGC TGGATGTGAA ATCCAAAATC AGAGCTGTTC TGCGCCTCTA

     Q  E  C  E  K  L  K  K  L  M  S  S  N  S  T  D  L  P  L  N  I  E  C  F  M  N  D  L  D  V  S  G  R
 801 TCAAGAATGC GAAAAGCTGA AGAAACTAAT GAGCAGCAAT AGCACCGATC TGCCGCTAAA TATTGAATGC TTTATGAATG ACCTTGATGT ATCAGGGCGT

     M  N  R  A  G  F  E  D  L  C  S  D  L  L  Q  R  I  E  D  P  L  R  S  L  M  E  Q  T  Q  L  R  A  E
 901 ATGAACAGGG CTGGATTTGA AGATTTATGT TCTGACCTCT TGCAAAGAAT AGAAGATCCT CTTCGCTCAT TAATGGAACA GACGCAGTTG CGTGCCGAAG

     D  V  S  A  V  E  V  I  G  G  A  T  R  I  P  A  V  K  E  R  I  A  K  F  F  G  K  D  V  S  T  T  L  N
1001 ATGTCTCTGC AGTGGAGGTC ATTGGGGGCG CCACTCGAAT TCCAGCTGTG AAGGAGAGGA TTGCTAAGTT CTTTGGGAAG GATGTCAGCA CGACATTAAA

     A  D  E  A  V  A  R  G  C  A  L  Q  C  A  I  L  S  P  A  F  K  V  R  E  F  S  V  T  D  V  V  P  Y
1101 CGCAGATGAA GCTGTTGCAA GAGGCTGTGC TCTGCAGTGT GCGATCCTTT CTCCAGCGTT CAAAGTGAGG GAGTTCTCCG TCACAGATGT TGTTCCTTAC

     P  I  S  L  K  W  S  T  E  A  D  E  T  E  G  V  H  E  V  F  S  K  N  H  A  A  P  F  S  K  V  L  T
1201 CCCATCTCTC TGAAATGGAG CACTGAAGCA GATGAGACTG AGGGAGTCCA TGAAGTTTTC AGCAAAAACC ACGCTGCTCC TTTTTCTAAA GTTCTCACCT

     F  Y  R  K  N  A  F  Q  I  E  A  F  Y  S  D  P  S  A  V  P  Y  P  E  S  K  I  G  Q  F  V  V  Q  N  I
1301 TCTACCGAAA GAATGCTTTC CAGATTGAAG CTTTTTACTC TGATCCTTCT GCTGTCCCCT ATCCAGAATC AAAAATAGGT CAATTTGTTG TGCAGAATAT

     C  A  A  K  D  G  E  K  S  K  V  K  V  K  V  R  V  N  T  H  G  I  F  S  V  S  T  A  S  M  V  E  R
1401 TTGTGCAGCT AAAGATGGCG AGAAGTCGAA GGTGAAAGTG AAGGTACGCG TGAACACGCA TGGCATTTTC AGTGTCTCCA CTGCTTCCAT GGTAGAGCGA

     M  E  V  D  E  S  E  E  Q  T  G  E  M  V  G  D  N  Q  S  A  T  E  T  L  E  T  D  K  G  V  Q  Q  D
1501 ATGGAAGTGG ATGAGTCTGA GGAGCAGACT GGGGAAATGG TTGGAGATAA CCAGAGCGCT ACTGAGACCC TGGAAACCGA TAAAGGTGTC CAGCAAGACA

     S  S  D  A  G  T  Q  P  Q  V  Q  T  D  G  Q  H  C  P  P  S  P  E  T  S  S  G  D  H  K  M  T  E  T  D
1601 GCAGTGATGC TGGAACACAG CCCCAGGTAC AAACTGATGG GCAGCACTGT CCCCCTTCAC CCGAGACATC CAGTGGGGAT CATAAAATGA CTGAAACTGA

     K  G  N  E  K  K  T  D  Q  P  P  E  A  K  K  P  K  I  K  V  K  N  I  E  L  P  I  E  T  N  L  I  W
1701 CAAAGGCAAT GAAAAGAAAA CGGATCAGCC TCCAGAAGCG AAGAAGCCCA AGATAAAGGT GAAGAACATA GAGCTGCCCA TTGAAACAAA TCTTATATGG

     Q  L  S  K  D  L  L  N  M  Y  I  E  N  E  G  K  M  I  L  Q  D  K  L  E  K  E  R  N  D  A  K  N  A
1801 CAGCTGAGCA AGGACCTTCT CAATATGTAC ATTGAGAACG AGGGCAAAAT GATCCTTCAA GATAAACTGG AGAAAGAGAG GAACGACGCC AAGAATGCTG

     V  E  E  Y  V  Y  E  F  R  D  K  L  S  G  P  Y  E  K  F  V  S  D  K  D  Q  S  R  F  L  E  L  L  T  E
1901 TGGAGGAGTA CGTCTACGAG TTCAGAGACA AACTCTCTGG GCCATACGAA AAATTTGTCA GTGATAAGGA CCAAAGCAGA TTTCTAGAGC TTCTAACAGA

     T  E  N  W  L  Y  E  D  G  E  D  Q  R  K  Q  V  Y  I  E  K  L  E  E  L  K  K  R  G  T  P  I  Q  N
2001 AACGGAGAAC TGGCTGTATG AGGATGGGGA AGACCAACGC AAGCAGGTGT ATATTGAGAA GCTGGAAGAG CTGAAGAAAC GTGGCACTCC AATACAGAAC

     R  S  R  E  A  E  E  R  P  R  K  F  E  E  L  G  Q  R  L  Q  H  Y  A  K  I  V  E  E  Y  R  N  K  S
2101 CGGTCTAGAG AGGCAGAAGA GCGTCCAAGA AAGTTTGAAG AGCTCGGACA GAGACTTCAA CACTATGCCA AAATTGTAGA AGAATACAGG AATAAGAGTG

     E  A  Y  Q  H  I  S  S  T  D  M  E  K  V  E  K  C  V  G  E  T  M  E  W  M  N  N  V  M  N  A  Q  A  K
2201 AAGCCTATCA ACACATCAGC AGCACTGACA TGGAAAAGGT GGAAAAATGT GTCGGAGAGA CCATGGAGTG GATGAATAAT GTGATGAATG CACAGGCAAA

     Q  R  L  D  Q  D  P  V  V  K  V  H  E  I  K  A  K  C  T  S  L  D  S  S  C  H  G  I  V  S  Q  P  K
2301 GCAGAGACTG GATCAGGACC CGGTGGTAAA AGTGCACGAA ATCAAAGCAA AATGCACATC TTTGGACAGC AGCTGTCATG GAATAGTATC ACAACCAAAA

     P  K  V  E  S  P  K  E  E  K  S  V  N  G  V  N  E  N  H  K  D  G  T  N  S  E  K  H  T  N  N  P  T
2401 CCCAAGGTTG AATCTCCAAA AGAAGAAAAA TCTGTAAATG GCGTCAATGA AAACCACAAA GACGGCACTA ACAGCGAAAA GCACACGAAT AACCCGACCC

     Q  P  Q  Q  N  G  A  C  H  P  N  E  K  S  T  I  D  M  D  F  D  *
2501 AACCGCAGCA GAACGGAGCA TGTCACCCTA ATGAAAAGAG CACCATTGAC ATGGACTTTG ATTAA
```

Figure 9. Additional *hsp110* sequence obtained using 5' RACE-PCR

The displayed nucleotide sequence was absent from the initially obtained EST clone (ATCC; IMAGE Clone ID no. 4055839). This additional sequence, which completed the open reading frame and added to the 5' UTR (underlined), was generated using 5' RACE-PCR. The start codon is indicated in bold.

TTACCCAGACTGCGGTTCGAACCGAGGACTTTGCACTGATTACCCAGAGTTCCGCCGCTGCTGCAGGT
TGTTATC**ATG**TCGGTTGTCGGGTTCGACTTGGGTTTTCAGAACTGTCACGTTGCTATAGCCCGAGCCG
GCGGCATCGAGACCGTTGCCAATGAATTTAGCGACCGCTGCACCCCCGCTGTAGTATCGTTTGGATTG
AAAAACAGGACAATTGGCATTGCTGCCAAAAACCAGCTAATCACAAATGCC

Figure 10. Amino acid sequence comparison of *Xenopus laevis* HSP110 predicted amino acid sequence and hamster HSP110

Amino acid sequence comparison of *Xenopus laevis* HSP110 predicted amino acid sequence and hamster HSP110 (accession number CAA87768). Asterisks (*) denote complete amino acid identity between sequences, colons (:) denote conserved substitutions and periods (.) denote semi-conserved substitutions.

```
Xenopus         MSVVGFDLGFQNCHVAIARAGGIETVANEFSDRCTPAVVSFGLKNRTIGIAAKNQLITNA 60
Hamster         MSVVGLDVGSQSCYIAVARAGGIETIANEFSDRCTPSVISFGPKNRTIGVAAKNQQITHA 60
                *****:*:* *.*::*:********:**********:*:*** ******:***** **:*

Xenopus         NNTVSSFKKFHGRAFNDAFVQKEKNNLPFKLVQMKNGGVGVKVNYLEEDHVFSIAQITAM 120
Hamster         NNTVSSFKRFHGRAFSDPFIQKEKESLSYDLVPMKNGGVGIKVMYMDEEHLFSVEQITAM 120
                ********:******.*.*:****:.*.:.** *******:** *::*:*:**: *****

Xenopus         LLTKLKETAENNLKKPVTDCVISVPSFFTDAERRSVLDAAQIVGLNCLKLMNDMTAVALN 180
Hamster         LLTKLKETAENNLKKPVTDCVISVPSFFTDAERRSVLDAAQIVGLNCLRLMNDMTAVALN 180
                **************************************************:**********

Xenopus         YGIYKQDLPAPEEKPKIVVFADMGHSSFQLSACAFNKGKLKVLGTAFDPYFGGRNFDEKL 240
Hamster         YGIYKQDLPNADEKPQGSGVCGHGPSSFQVSACAFNKGKLKVLGTAFDPFLGGKNFDEKL 240
                ********* .:***:   ... * ****:*******************::**:******

Xenopus         VEHFCVEFKTKYRLDVKSKIRAVLRLYQECEKLKKLMSSNSTDLPLNIECFMNDLDVSGR 300
Hamster         VEHFCAEFKTKYKLDAKSKIRALLRLHQECEKLKKLMSSNSTDLPLNIECFMNDKDVSAK 300
                *****.******:**.******:***:************************** ***.:

Xenopus         MNRAGFEDLCSDLLQRIEDPLRSLMEQTQLRAEDVSAVEVIGGATRIPAVKERIAKFFGK 360
Hamster         MNRSQFEELCAELLQKIEVPLHSLMEQTHLKTEDVSAIEIVGGATRIPAVKERIAKFFGK 360
                ***: **:**::***:** **:******:*::*****:*::******************

Xenopus         DVSTTLNADEAVARGCALQCAILSPAFKVREFSVTDVVPYPISLKWSTEADETEGVHEVF 420
Hamster         DVSTTLNADEAVARGCALQCAILSPAFKVREFSVTDAVPFPISLVWNHDSEETEGVHEVF 420
                ************************************.**:**** *. :::**********

Xenopus         SKNHAAPFSKVLTFYRKNAFQIEAFYSDPSAVPYPESKIGQFVVQNICAAKDGEKSKVKV 480
Hamster         SRNHAAPFSKVLTFLRRGPFELEAFYSDPQGVPYPEAKIGRFVVQNVSAQKDGEKSKVKV 480
                *:************ *:..*::*******..*****:***:*****:.* **********

Xenopus         KVRVNTHGIFSVSTASMVERMEVDESEEQ--TGEMVGDNQSATETLETDKGVQQDSSDAG 538
Hamster         KVRVNTHGIFTISTASMVEKVPTEEDDGSSVEADMECPNQKPAESSDVDKNSQQDNSEAG 540
                **********::*******:: .:*.: .   .:*   **..:*: :.**. ***.*:**

Xenopus         TQPQVQTDGQ---HCPPSPETSSGDHKMTETDKGNEKKTDQPPEAKKPKIKVKNIELPIE 595
Hamster         TQPQVQTDGQQTSQSPPSPELPSEENKIPDADKANEKKVDQPPEAKKPKIKVVNVELPVE 600
                **********   :.***** .* ::*:.::**.****.************* *:***:*

Xenopus         TNLIWQLSKDLLNMYIENEGKMILQDKLEKERNDAKNAVEEYVYEFRDKLSGPYEKFVSD 655
Hamster         ANLVWQLGRDLLNMYIETEGKMIMQDKLEKERNDAKNAVEECVYEFRDKLCGPYEKFICQ 660
                :**:***.:********.*****:***************** ********.******:.:

Xenopus         KDQSRFLELLTETENWLYEDGEDQRKQVYIEKLEELKKRGTPIQNRSREAEERPRKFEEL 715
Hamster         QEHEKFLRLLTETEDWLYEEGEDQAKQAYIDKLEELMKMGNPVKVRFQEAEERPKVLEEL 720
                :::.:**.******:****:**** **.**:***** * *.*:: * :******: :***

Xenopus         GQRLQHYAKIVEEYRNKSEAYQHISSTDMEKVEKCVGETMEWMNNVMNAQAKQRLDQDPV 775
Hamster         GQRLQHYAKIAADFRSKDEKYNHIDESEMKKVEKSVNEVMEWMNNVMNAQAKRSLDQDPV 780
                **********. ::*.*.* *:**..::*:****.*.*.************: ******

Xenopus         VKVHEIKAKCTSLDSSCHGIVSQPKPKVESPKEEKSVNGVNENHKDGTNSEKHTNNPTQP 835
Hamster         VRTHEIRAKVKELNNVCEPVVNQPKPKIESPKLERTPNGPNLDKKEDLEG-KDNFGAEAP 839
                *:.***:** ..*:. *. :*.*****:**** *:: ** * ::*:. :..*.. ..  *

Xenopus         QQNGACHPNEKSTIDMDFD 854
Hamster         HQNGECHPNEKGSVNMDLD 858
                :*** ******.:::**:*
```

In a preliminary phylogenetic tree, *Xenopus* groups outside of mammals, in its own subgroup. It is more closely related to the mammals than it is to sea urchin, mold or yeast (Figure 11). The majority of the identity was among the residues in the amino terminus, which has been designated as the ATPase domain based on sequence (Lee-Yoon *et al.*, 1995).

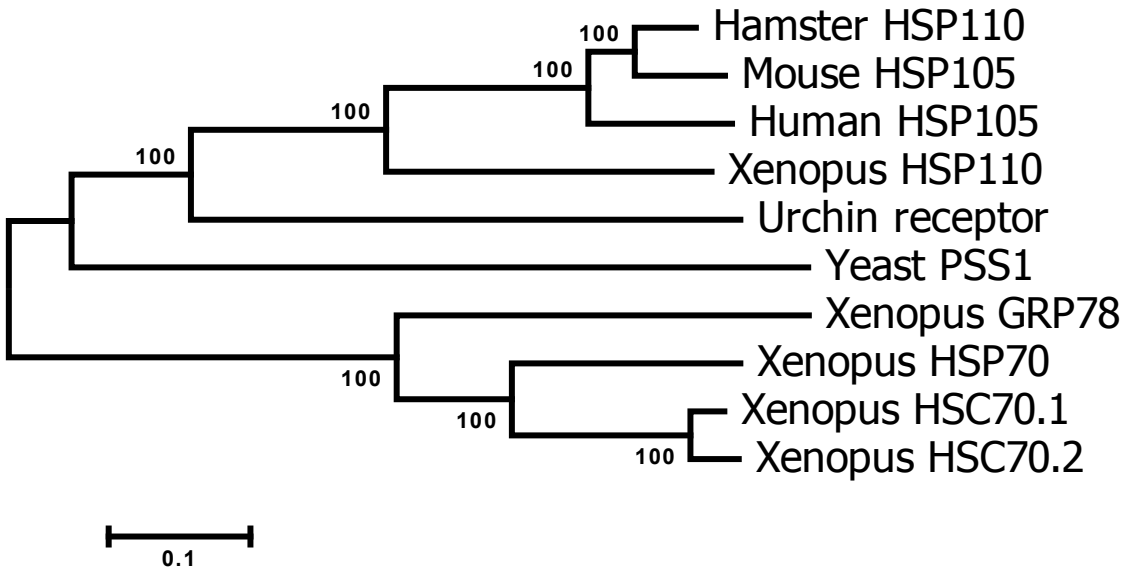
Table 4. Comparison of the amino acid sequence of *Xenopus* HSP110 with members of the HSP70/DnaK superfamily

Organism	Protein	Accession #	% Identity with *Xenopus* HSP110
Mouse	HSP105	NP_038587	74
Human	HSP105	Q92598	74
Hamster	HSP110	CAA87768	72
Sea Urchin	Egg receptor	NP_999695	49
Yeast	PSS1	O59838	41
Xenopus	HSP70	CAA25576	29
Xenopus	HSC70.1	AAB97092	27
Xenopus	HSC70.2	AAB41583	28
Xenopus	BiP	AAB41582	28

The percent identity of *Xenopus* HSP110 with selected member of the HSP70/DnaK superfamily was determined using ClustalW. Genbank accession numbers are shown.

Figure 11. Preliminary phylogenetic analysis of HSP110

Sequences were aligned using ClustalW and a phylogenetic tree was generated using the program MEGA 3.1. The tree was prepared using a neighbor-joining method with pairwise deletion, a bootstrap value of 1000 and Jukes and Cantor (1969) correction.


Hamster HSP110
Mouse HSP105
Human HSP105
Xenopus HSP110
Urchin receptor
Yeast PSS1
Xenopus GRP78
Xenopus HSP70
Xenopus HSC70.1
Xenopus HSC70.2
100
100
100
100
100
100
100
0.1

3.2 *hsp110* mRNA accumulation in A6 cells

Hsp110 mRNA was present constitutively at relatively low levels in A6 cells, as determined by northern blot analysis. Temperature-induced elevation of the 3.4 kb *hsp110* message was detected at 30°C, peaked at 33-35°C and was markedly reduced at 37°C (Figure 12). In comparison, *hsp70* was not detected constitutively at 22°C, and was strictly stress inducible. An enhanced accumulation of *hsp70* mRNA was detected at 30°C with peak levels at 35°C and a slight reduction at 37°C.

Temporal characterization of *hsp110* mRNA accumulation in A6 cells was carried out at 33°C (Figure 13). A slight *hsp110* mRNA accumulation was detected initially at 0.5 h with maximal accumulation at 2 h, and then a gradual decrease over time up to 6 h. In comparison, *hsp70* mRNA accumulation was easily detectable after only 0.5 h. Maximal induction of *hsp70* was detected at 1 h, and then declined to undetectable levels after 4 h of heat shock. A temporal characterization of *hsp110* and *hsp70* mRNA accumulation was also carried out at 35°C in A6 cells (Figure 14). A similar pattern was seen at 35°C for *hsp110.* The peak accumulation of *hsp70* message at 35°C was seen at 2 h, and the message was detectable for the full 6 h treatment. *Actin* mRNA levels were relatively unaffected over the 6 h heat shock period at both temperatures.

Figure 12. The effect of temperature on *hsp110* and *hsp70* mRNA accumulation in A6 cells

A6 cells were incubated at 22, 30, 33, 35, or 37°C for 1 h. Total RNA was isolated and the relative levels of *hsp110* and *hsp70* mRNA were analyzed by northern hybridization using DIG-labeled antisense riboprobes as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity was demonstrated by staining the membrane prior to northern hybridization with RNA blot stain (bottom panel). Arrows indicate the position of the transcripts.

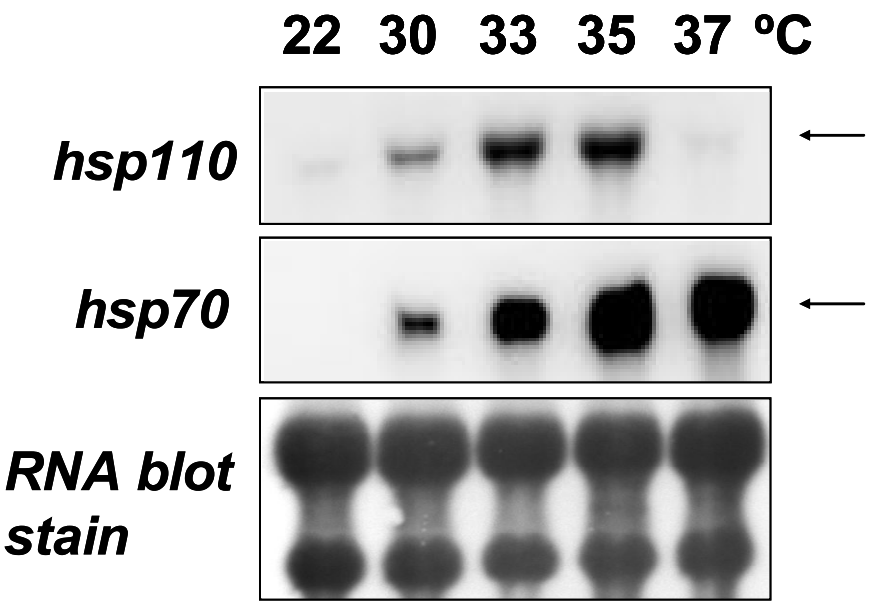

22 30 33 35 37 ºC
hsp110
hsp70
RNA blot stain

Figure 13. The temporal pattern of *hsp110, hsp70* and *actin* mRNA accumulation in A6 cells subjected to continuous heat shock

A6 cells were incubated at 33°C for 0.5, 1, 2, 4, or 6 h, or maintained at 22°C (C). Total RNA was isolated and the relative levels of *hsp110, hsp70* and *actin* mRNA were analyzed by northern hybridization, using DIG-labeled antisense riboprobes, as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity was demonstrated by staining the membrane prior to northern hybridization with RNA blot stain (bottom panel). Arrows indicate the position of the transcripts.

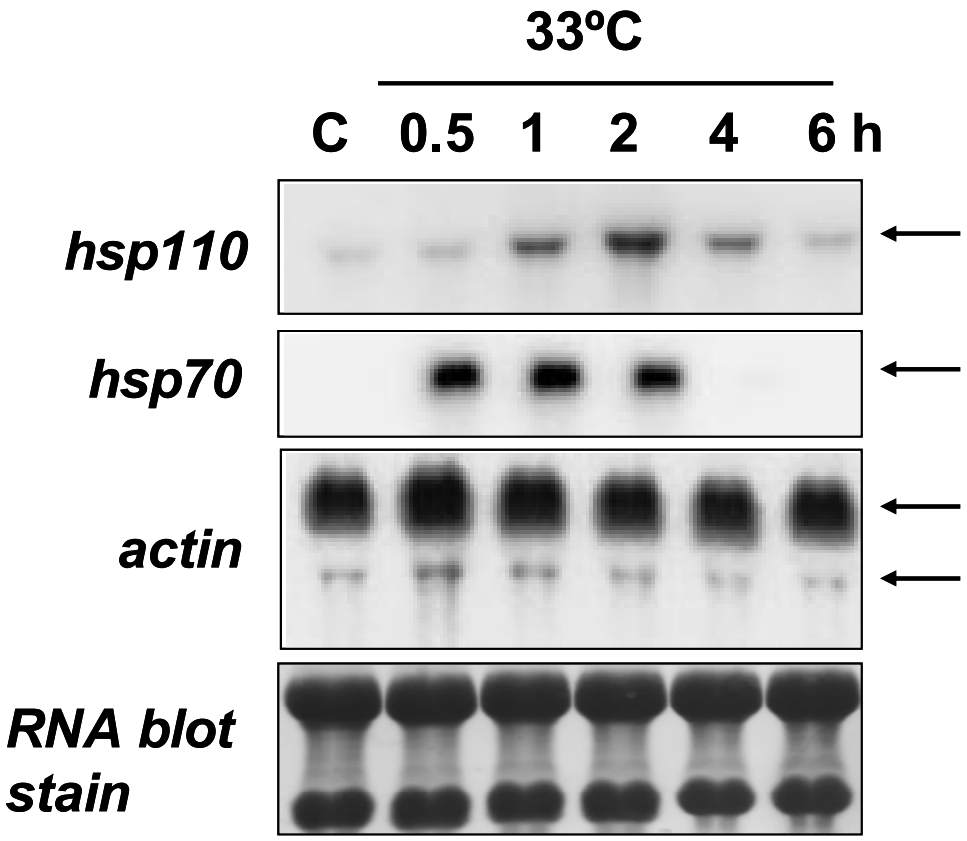
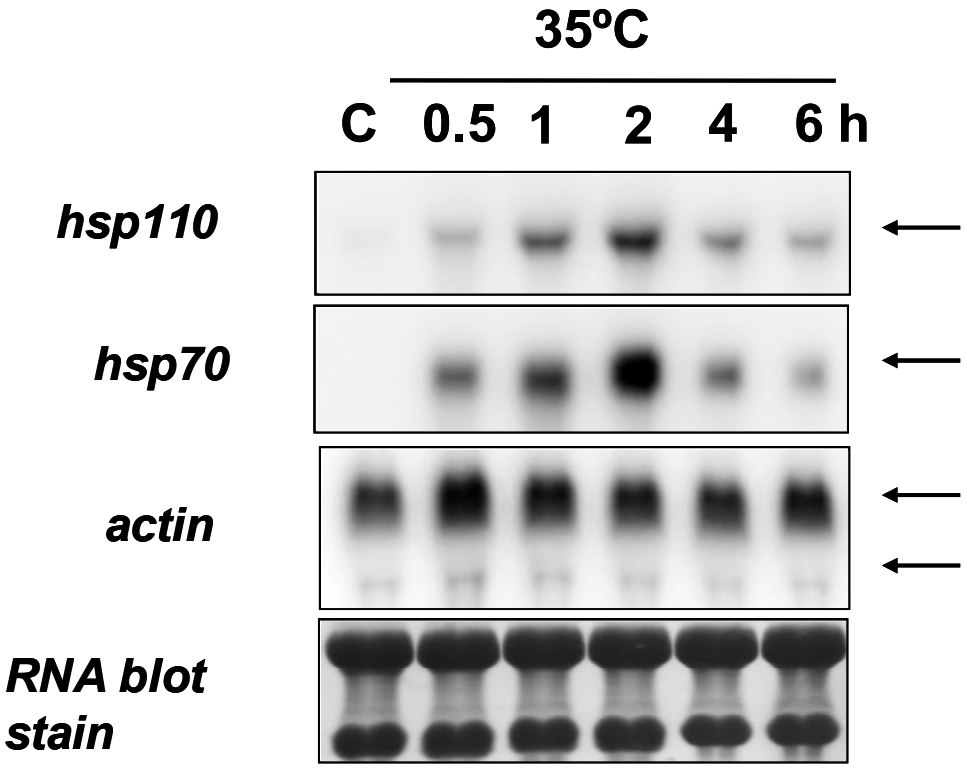

33ºC
C 0.5 1 2 4 6 h
hsp110
hsp70
actin
RNA blot stain

Figure 14. The temporal pattern of *hsp110, hsp70* and *actin* mRNA accumulation in A6 cells subjected to continuous heat shock

A6 cells were incubated at 35°C for 0.5, 1, 2, 4, or 6 h, or maintained at 22°C (C). Total RNA was isolated and the relative levels of *hsp110, hsp70* and *actin* mRNA were analyzed by northern hybridization, using DIG-labeled antisense riboprobes, as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity was demonstrated by staining the membrane prior to northern hybridization with RNA blot stain (bottom panel). Arrows indicate the position of the transcripts.


35ºC
C 0.5 1 2 4 6 h
hsp110
hsp70
actin
RNA blot stain

After establishing that *hsp110* mRNA accumulated in response to heat shock, A6 cells were exposed to other stressors to further characterize the stress response of *Xenopus hsp110* (Figure 15). After a 5 h exposure of A6 cells to 6% ethanol, *hsp110* mRNA levels were not altered when compared to control cells, whereas elevated levels of *hsp70* mRNA were detected. Neither *hsp110*, nor *hsp70* mRNA accumulated upon exposure to 200 μM copper sulphate for 5 h. However, treatment with 200 μM cadmium chloride for 5 h resulted in enhanced accumulation of *hsp110* and *hsp70* mRNA. Sodium arsenite exposure for 5 h clearly resulted in a markedly enhanced accumulation of both *hsp110* and *hsp70* mRNA compared to the control.

It was evident that sodium arsenite treatment of A6 cells elicited a strong accumulation of *hsp110* mRNA, so this was explored further. A6 cells were exposed to 50 μM sodium arsenite for 1, 2, 3, 4, or 6 h, and the total RNA isolated was analyzed by northern hybridization (Figure 16). An increase in the relative amount of *hsp110* mRNA was detectable after 2 h, and reached maximal levels after 6 h of exposure. A similar trend was noted with *hsp70* message, although enhanced accumulation in response to sodium arsenite treatment occurred after only 1 h. *Actin* mRNA levels were relatively unaffected by continuous sodium arsenite exposure. In other studies elevated levels of *hsp110* mRNA were detected at 12 h also, and detected albeit reduced after 24 h (data not shown).

Figure 15. The effect of selected non-heat shock stressors on *hsp110* and *hsp70* mRNA accumulation in A6 cells

A6 cells were incubated at 22°C (C), in the absence or presence of 6% ethanol (Et), 200 μM copper sulfate (Cu), 200 μM cadmium chloride (Cd) or 100 μM sodium arsenite (Na) for 5 h. Total RNA was isolated and the relative levels of *hsp110* and *hsp70* mRNA were analyzed by northern hybridization, using DIG-labeled antisense riboprobes, as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity was demonstrated by staining the membrane prior to northern hybridization with RNA blot stain (bottom panel). Arrows indicate the position of the transcripts.

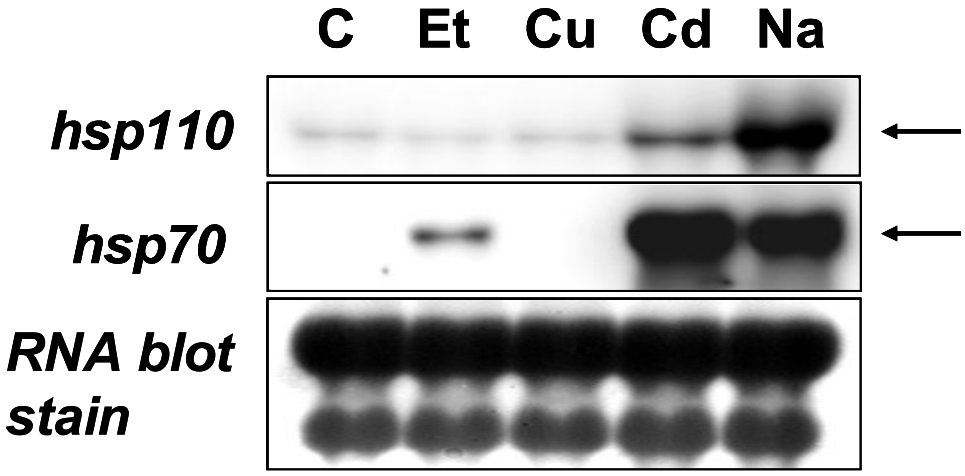
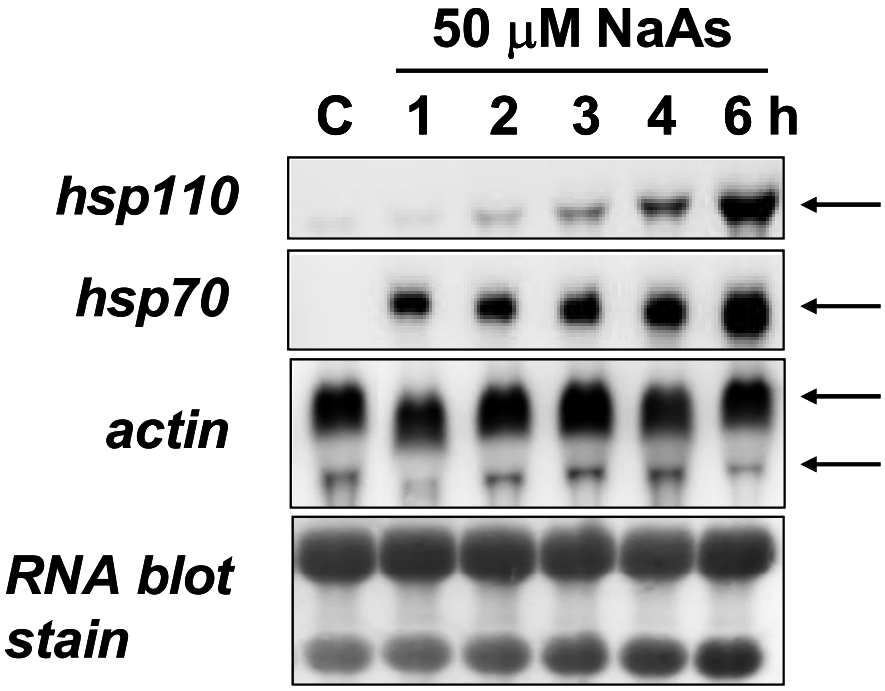

C
Et
Cu
Cd
Na
hsp110
hsp70
RNA blot stain

Figure 16. The effect of sodium arsenite on *hsp110, hsp70* and *actin* mRNA accumulation in A6 cells

A6 cells were incubated at 22°C in the absence (C), or presence of 50 μM sodium arsenite (NaAs) for 1, 2, 3, 4 or 6 h. Total RNA was isolated and the relative levels of *hsp110, hsp70* and *actin* mRNA were analyzed by northern hybridization, using DIG-labeled antisense riboprobes, as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity was demonstrated by staining the membrane prior to northern hybridization with RNA blot stain (bottom panel). Arrows indicate the position of the transcripts.


50 μM NaAs
C 1 2 3 4 6 h
hsp110
hsp70
actin
RNA blot stain

3.3 *Hsp110* mRNA accumulation in *Xenopus* embryos

Northern hybridization analysis revealed that *hsp110* message was present in unfertilized eggs, and considered a maternal message (Figure 17). The *hsp110* message, unlike *hsp70*, was present constitutively throughout development, from unfertilized egg to tadpole (Figure 18). Although always present, the relative levels of constitutive *hsp110* mRNA differed between developmental stages. *Hsp110* mRNA was detected in late blastula embryos, but then declined to very low levels in gastrula, neurula and early tailbud embryos. However, the relative amount of constitutive *hsp110* mRNA increased by midtailbud, and then remained elevated until at least tadpole stage (data not shown). Heat shock treatment of embryos resulted in enhanced *hsp110* and *hsp70* mRNA accumulation at all stages following MBT. The relative amounts of heat-induced *hsp110* mRNA accumulation increased with developmental stage from late blastula to midtailbud. Unlike the larger *hsps, hsp27* mRNA accumulation was earliest observed in heat shocked early tailbud stage embryos. *Hsp27* message was observed in control embryos and was also heat inducible at mid and late tailbud stages. In contrast to *hsp110, hsp70 and hsp27* mRNA, the relative levels of *ef-1α* mRNA did increase slightly throughout development, but the message was unaffected by heat shock.

Figure 17. *hsp110* mRNA is present as a maternal message

At various stages of development, *Xenopus laevis* eggs and embryos were maintained at either 22°C (C) or exposed to a 33°C heat shock (H) for 1 h. Unfertilized eggs (U), fertilized eggs (F), and cleaving embryos (Cl) were harvested and total RNA was isolated (panel A). In comparison, A6 cells were maintained at either 22°C (C) or exposed to a 33°C heat shock (H) for 2 h (panel B). Relative levels of *hsp110* mRNA was analyzed by northern hybridization, using DIG-labeled antisense riboprobes as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity are demonstrated by staining the membrane prior to northern hybridization with Blot Stain Blue (bottom panel). Arrows indicate the position of the transcripts.

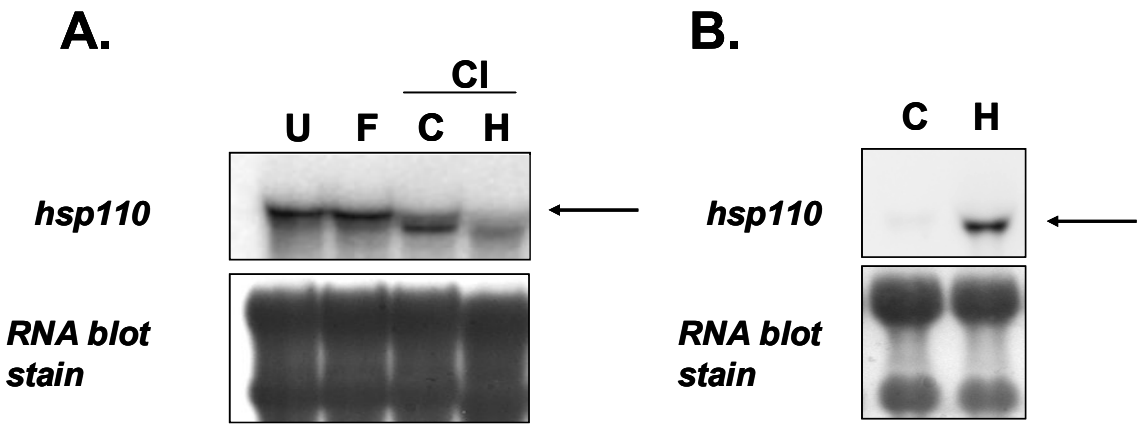
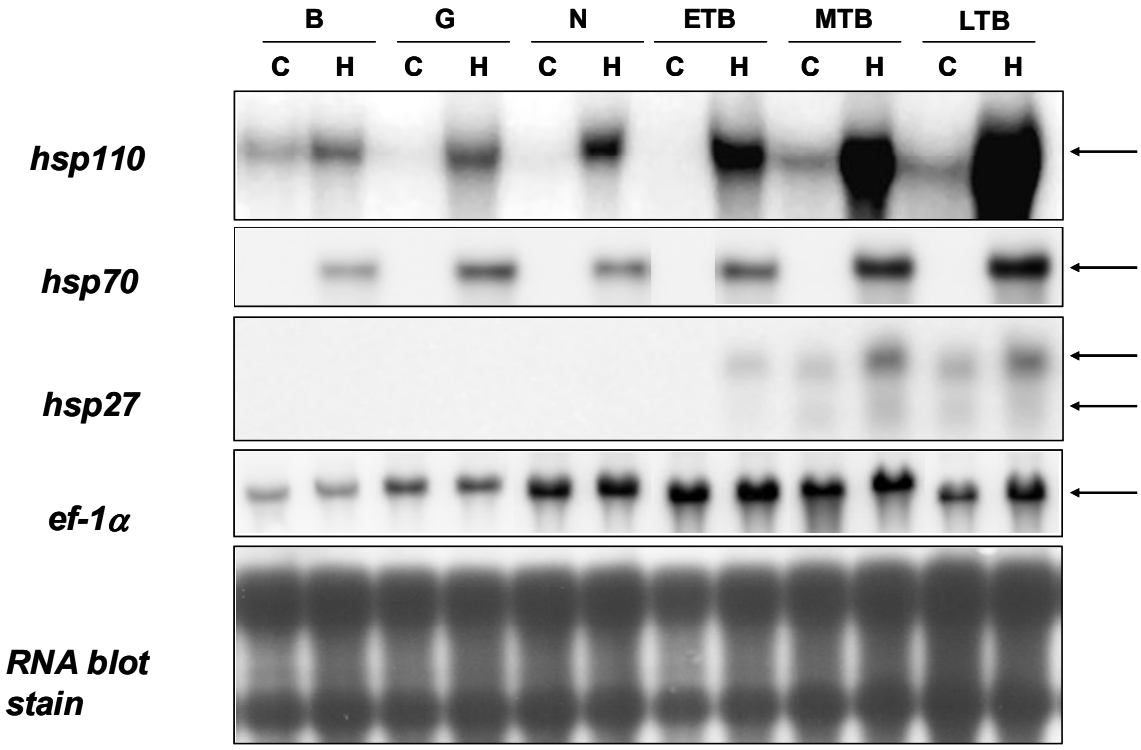

A.
Cl
U
F
C
H
hsp110
RNA blot stain
B.
C
H
hsp110
RNA blot stain

Figure 18. *hsp* mRNA accumulation during early development

At various stages of development, *Xenopus laevis* embryos were maintained at either 22°C (C) or exposed to a 33°C heat shock (H) for 1 h. Late blastula (B), gastrula (G), neurula (N), early tailbud (etb), mid tailbud (mtb), and late tailbud (ltb) embryos were harvested and total RNA was isolated. Relative levels of *hsp110*, *hsp70, hsp27* and *elongation factor-1α (ef-1α)* mRNA were analyzed by northern hybridization, using DIG-labeled antisense riboprobes as described in Materials and Methods. Equal loading, transfer efficiency and RNA integrity are demonstrated by staining the membrane prior to northern hybridization with Blot Stain Blue (bottom panel).


B
G
N
ETB
MTB
LTB
C H C H C H C H C H C H
hsp110
hsp70
hsp27
ef-1α
RNA blot stain

The spatial distribution of *hsp110, hsp70* and *hsp27* mRNA in early *Xenopus laevis* embryos was investigated using whole mount *in situ* hybridization. Constitutive levels of *hsp110* mRNA were detected globally in gastrula embryos (Figure 19). Heat shock treatment enhanced this pattern of message accumulation. In heat shocked neurula embryos, there was an accumulation of *hsp110* mRNA along the neural fold. Early tailbud embryos displayed constitutive *hsp110* mRNA accumulation in the lens vesicle and head region. Following heat shock, enhanced *hsp110* mRNA accumulation was seen additionally in the notochord, somites and brain region. Control mid and late tailbud stage embryos showed *hsp110* mRNA accumulation in the lens vesicle, head and heart region. Embryos that were heat shocked showed an enhancement of this pattern of *hsp110* mRNA accumulation, plus enhanced *hsp110* mRNA levels in the brain region, notochord, heart region and somites. In contrast to the above results, *in situ* hybridization of embryos with a sense *hsp110* riboprobe did not produce a detectable DIG signal.

Hsp70 mRNA accumulated in embryos only in response to heat shock (Figure 20). There was no detectable *hsp70* mRNA accumulation in control embryos as detected by either northern, or *in situ,* hybridization. *Hsp70* mRNA did accumulate in a similar pattern to that of *hsp110* in embryos following heat shock, and in general was more widespread. In neurula embryos, *hsp70* accumulated along the neural fold. Early tailbud embryos showed accumulation of *hsp70* mRNA in the proctodeum, notochord, somites, brain region, heart region and lens vesicle. In mid and late tailbud embryos the same pattern prevailed, but *hsp70* mRNA accumulated in the cement gland as well.

Figure 19. Pattern of *hsp110* mRNA accumulation during development

Albino *Xenopus laevis* embryos were incubated at 22°C (control) and 33°C (heat shock) for 1 h at various stages of development (gastrula (st. 11), neurula (st. 14), early tailbud (st. 25), mid tailbud (st. 27), and late tailbud (st. 38)). Whole mount *in situ* hybridization analysis was carried out with *hsp110* DIG-labeled antisense riboprobes as described in Materials and Methods. NF, neural fold; LV, lens vesicle; BR, brain region; NC, notochord; S, somites; H, heart region.

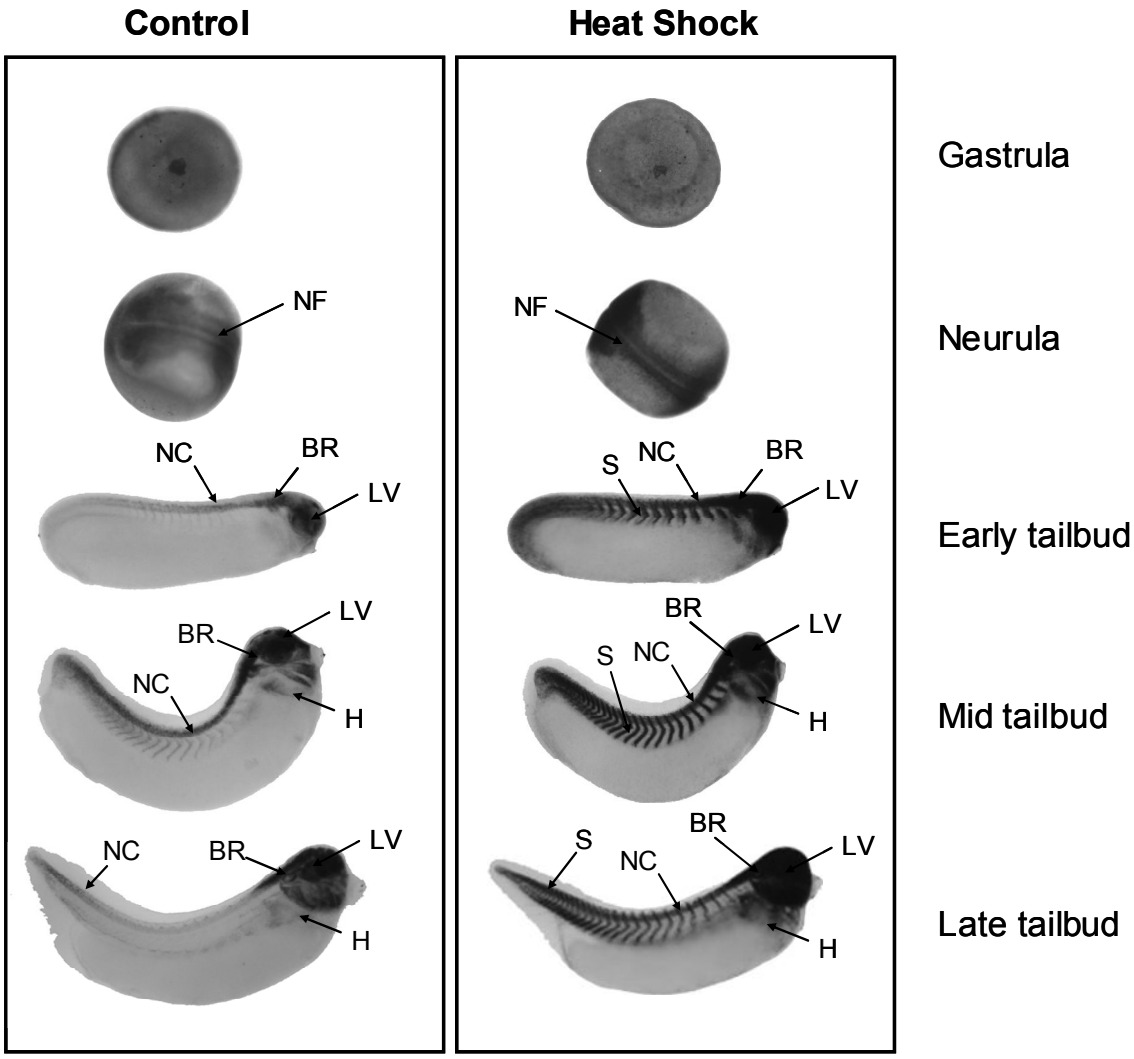
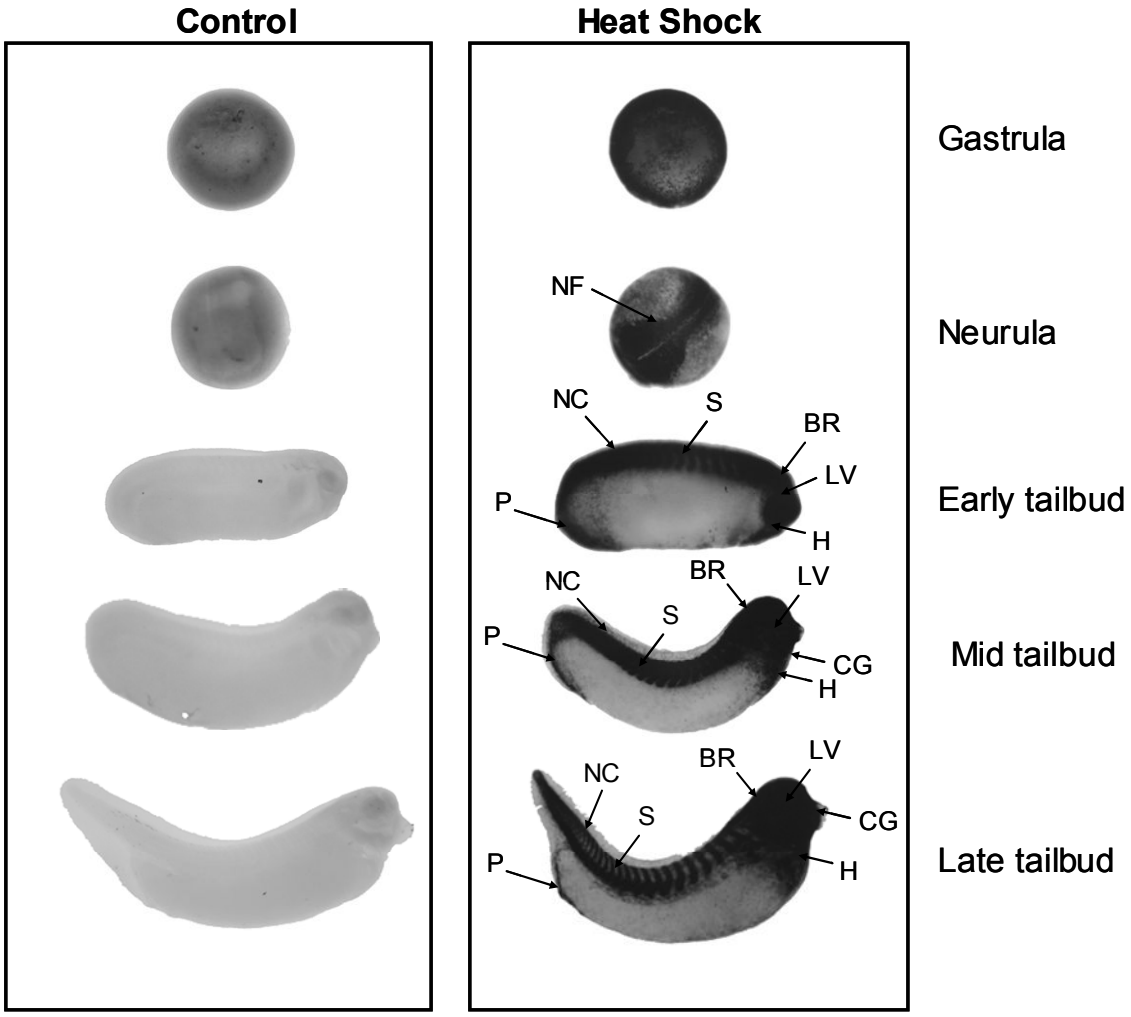

Control
Heat Shock
NF
NF
NC
BR
LV
S
NC
BR
LV
LV
BR
NC
H
BR
S
NC
LV
H
NC
BR
LV
H
S
NC
BR
LV
H
Gastrula
Neurula
Early tailbud
Mid tailbud
Late tailbud

Figure 20. Spatial pattern of *hsp70* mRNA accumulation during development

Albino *Xenopus laevis* embryos were incubated at 22°C (control) and 33°C (heat shock) for 1 h at various stages of development (gastrula (st. 11), neurula (st. 14), early tailbud (st. 25), mid tailbud (st. 27), and late tailbud (st. 38)). Whole mount *in situ* hybridization analysis was carried out with *hsp70* DIG-labeled antisense riboprobes as described in Materials and Methods. NF, neural fold; LV, lens vesicle; BR, brain region; NC, notochord; S, somites; H, heart region; P, proctodeum; CG, cement gland.


Control
Heat Shock
Gastrula
NF
Neurula
NC
S
BR
LV
P
H
Early tailbud
NC
BR
LV
S
P
CG
H
Mid tailbud
BR
LV
NC
S
CG
P
H
Late tailbud

Hsp27 mRNA accumulated in embryos under both control and heat shock conditions (Figure 21). Control early tailbud embryos displayed an accumulation of *hsp27* mRNA in the somites and lens vesicle, and this was enhanced with heat shock. An accumulation of *hsp27* mRNA was observed in the somites, lens vesicle and heart of both mid and late tailbud control embryos, and this pattern was enhanced with heat shock.

3.4 Production of anti-*Xenopus* HSP70 and HSP110 custom polyclonal antibodies

An initial examination of the relative level of HSP110 protein by western blot analysis employed a rabbit polyclonal antibody raised against hamster HSP110 since a homologous anti-*Xenopus* HSP110 antibody was unavailable. This antibody recognized two proteins (under all conditions) of approximately 107 and 110 kDa in size from A6 cells (Figure 22). The relative levels of these putative HSP110 proteins were elevated when cells were incubated at 35°C for 2-6 h. Exposure of A6 cells to sodium arsenite for 12 and 24 h resulted in an elevation of HSP110 protein compared to untreated cells. Actin protein levels remained relatively constant. Due to the detection of several non-specific bands with the commercial antibody, a custom anti-*Xenopus* HSP110 antibody was obtained. In addition, an antibody specific to HSP70 that did not bind to HSC70, had not been previously available, and a custom antibody was simultaneously produced for HSP70.

Figure 21. Spatial pattern of *hsp27* mRNA accumulation during development

Albino *Xenopus laevis* embryos were incubated at 22°C (control) and 33°C (heat shock) for 1 h at various stages of development (early tailbud (st. 23), mid tailbud (st. 27), and late tailbud (st. 38)). Whole mount *in situ* hybridization analysis was carried out with *hsp27* DIG-labeled antisense riboprobes as described in Materials and Methods. LV, lens vesicle; S, somites; H, heart region.

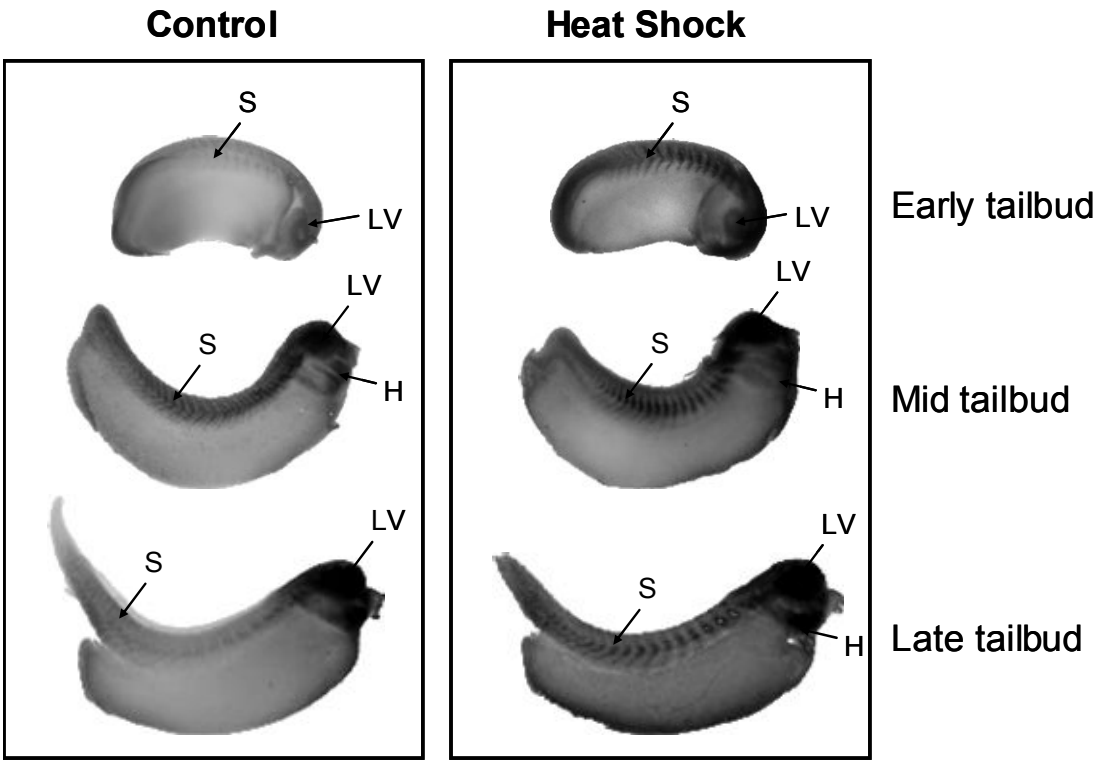
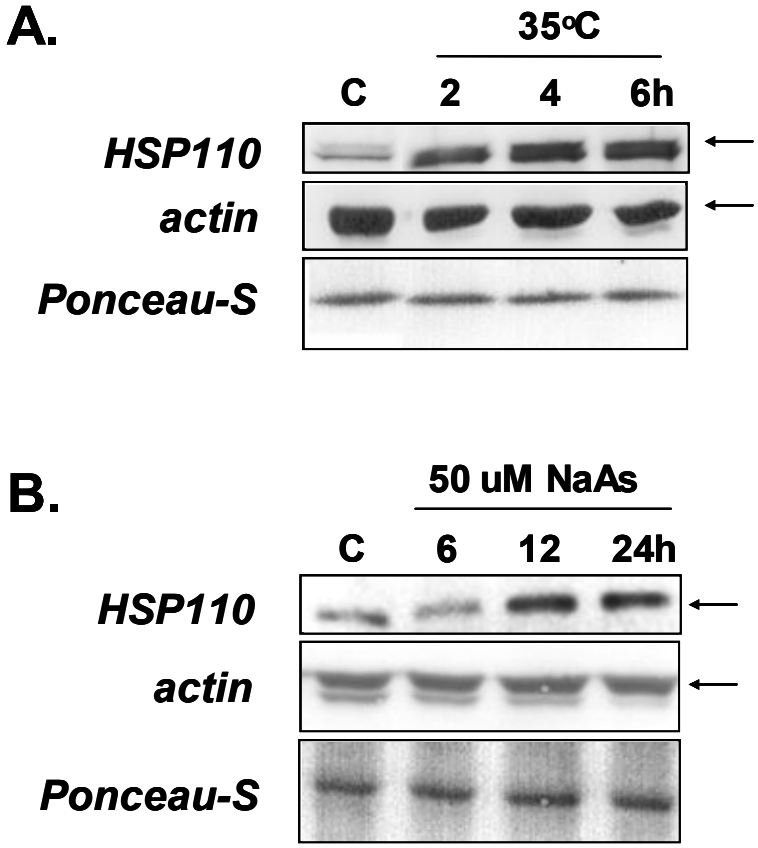

Control
Heat Shock
S
LV
S
LV
H
S
LV
Early tailbud
Mid tailbud
Late tailbud

Figure 22. HSP110 protein accumulation in A6 cells measured with a commercially available antibody

In panel A, A6 cells were maintained at 22°C (C), or heat-shocked at 35°C for 2, 4, or 6 h, and allowed to recover at 22°C for 2 h. In panel B, A6 cells were exposed to 50 μM sodium arsenite (NaAs) for 6, 12, or 24 h, while being maintained at 22°C (C). Total protein was isolated and resolved on an 8% SDS-polyacrylamide gel. Protein was transferred to a PVDF membrane and probed with either a hamster HSP110 polyclonal antibody, or an anti-actin polyclonal antibody as described in Materials and Methods. Ponceau S stained membranes are included to show efficient transfer and equal loading of protein.


A.
35°C
C
2
4
6h
HSP110
actin
Ponceau-S
B.
50 uM NaAs
C
6
12
24h
HSP110
actin
Ponceau-S

The custom designed polyclonal antibodies were raised in rabbits and the antigens were *Xenopus laevis* C-terminal peptides coupled to KLH, as described in Materials and Methods. For HSP110 this resulted in a specific antibody that recognized only one protein under most conditions, at approximately 110 kDa (Figure 23). The protein was present constitutively and also accumulated with elevated temperature or elevated concentrations of sodium arsenite. At 35°C two bands were detected, one at 110 kDa and an additional band at approximately 97 kDa, which was similar to results found in other species, and will be discussed later. For HSP70 a specific antibody that recognized only one protein, at approximately 70 kDa was produced (Figure 24). HSP70 protein was not present under control conditions but did accumulate in response to elevated temperatures of 33, 35 and 37°C, as well as 12 and 24 h of exposure to 50 μM sodium arsenite.

3.5 HSP110 protein accumulation in *Xenopus* A6 cells

A comparative examination of the relative level of selected heat shock proteins induced by various temperatures was completed by western blot analysis (Figure 25). HSP110 protein was present under control conditions (22°C), whereas HSP30 and HSP70 were not, and were strictly stress-inducible. Upon temperature increase, HSP110, HSP70 and HSP30 protein accumulated, reaching a maximum amount of protein at 35°C and declined slightly at 37°C.

Figure 23. HSP110 protein accumulation in A6 cells

A6 cells were maintained at 22°C (C) or heat shocked for 2 h at 30, 33, 35 or 37°C, and allowed to recover at 22°C for 2 h. Alternatively, A6 cells were exposed to 50 μM sodium arsenite (NaAs) for 6, 12, or 24 h at 22°C (C). Total protein was isolated and resolved on a 10% SDS-polyacrylamide gel. Protein was transferred to a PVDF membrane and probed with a custom produced anti-*Xenopus* HSP110 polyclonal antibody as described in Materials and Methods. Ponceau S stained membranes were included to show efficient transfer and equal loading of protein. Arrows indicate the position of the proteins.

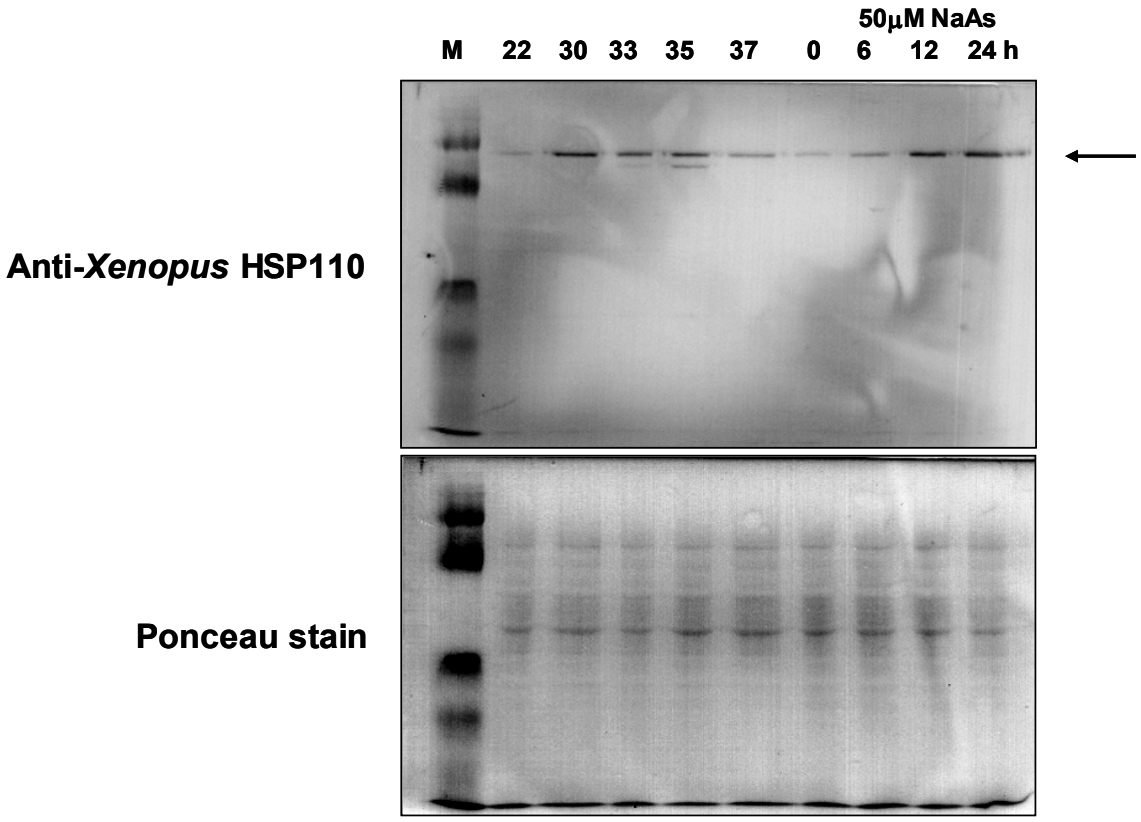

50μM NaAs
M 22 30 33 35 37 0 6 12 24 h
Anti-*Xenopus* HSP110
Ponceau stain

Figure 24. HSP70 protein accumulation in A6 cells

A6 cells were maintained at 22°C (C) or heat shocked for 2 h at 30, 33, 35 or 37°C, and allowed to recover at 22°C for 2 h. Alternatively, A6 cells were exposed to 50 μM sodium arsenite (NaAs) for 6, 12, or 24 h at 22°C (C). Total protein was isolated and resolved on a 10% SDS-polyacrylamide gel. Protein was transferred to a PVDF membrane and probed with a custom produced anti-*Xenopus* HSP70 polyclonal antibody as described in Materials and Methods. Ponceau S stained membranes were included to show efficient transfer and equal loading of protein. Arrows indicate the position of the proteins.

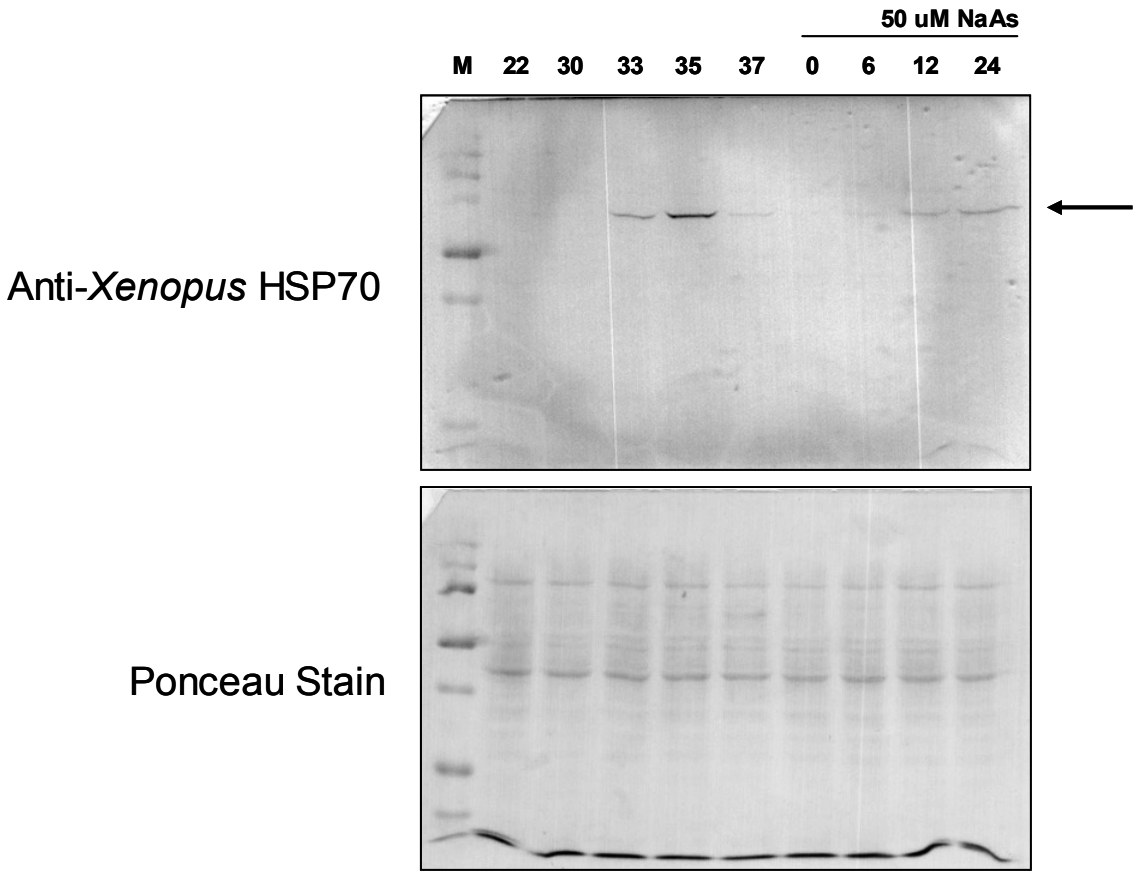
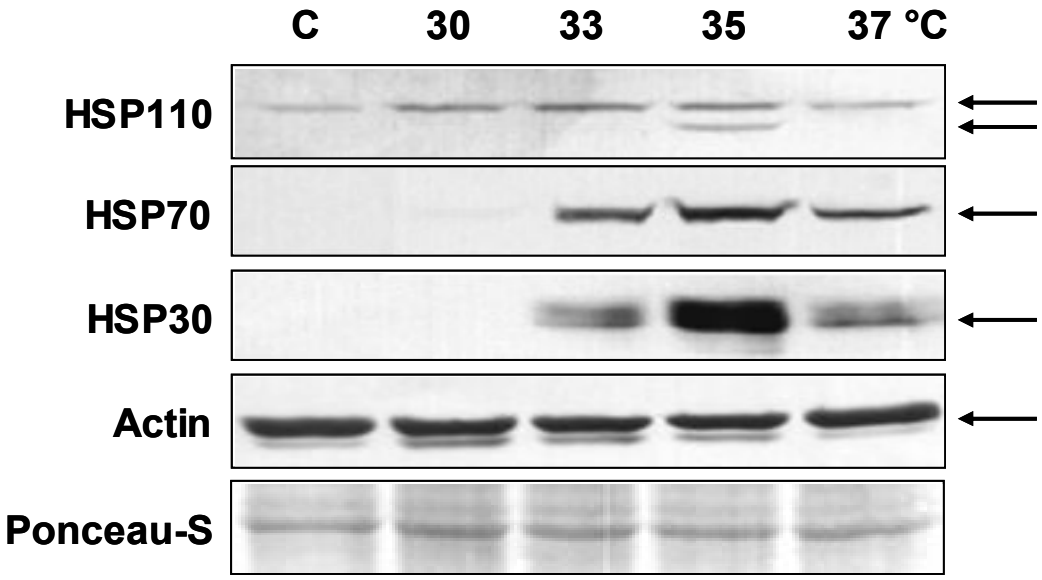

50 uM NaAs
M 22 30 33 35 37 0 6 12 24
Anti-*Xenopus* HSP70
Ponceau Stain

Figure 25. Protein accumulation in response to elevated temperature in A6 cells

A6 cells were maintained at 22°C (C), or heat shocked at 30, 33, 35 or 37°C for 2 h, and allowed to recover at 22°C for 2 h. Total protein was isolated and resolved on 10% SDS-polyacrylamide gels. Protein was transferred to a PVDF membrane and probed with (anti-*Xenopus*) HSP110, HSP70, HSP30 or anti-actin polyclonal antibodies as described in Materials and Methods. Ponceau S stained membranes are included to show efficient transfer and equal loading of protein. Arrows indicate the position of the protein.


C
30
33
35
37 °C
HSP110
HSP70
HSP30
Actin
Ponceau-S

At 35°C a band of approximately 97 kDa was detected with the HSP110 antibody, in addition to the 110 kDa band. This second band was only detected with a 35°C heat shock, and under no other conditions. Similarly, HSP30 protein was detected as multiple bands on a western, but this was regardless of treatment. Actin protein levels remained relatively constant.

A temporal investigation of the effects of heat induced stress was carried out on A6 cell extracts using HSP110, HSP70, HSP30 and actin antibodies (Figure 26). Again, HSP110 was present under control conditions, while HSP70 and HSP30 were detected only upon heat shock. Exposure to a constant 33°C heat shock resulted in protein accumulation of all three HSPs. Both HSP110 and HSP70 protein were present at elevated levels at 33°C and 35°C and remained elevated in both cases for up to 6 h. HSP30 was also elevated by a 33°C and 35°C heat shock, although this protein appeared to be present at the highest levels after 4 h of 33°C and then declined. After 2 h of 35°C, HSP30 protein accumulated, increasing further after 4 h, and remaining elevated for at least 6 h. Actin protein levels remained relatively constant.

Sodium arsenite affected the amount of protein present in A6 cells for all HSPs studied (Figure 27). As expected, HSP110 protein was present even in the absence of sodium arsenite treatment, but was elevated with increased concentration reaching a maximum at 50 μM (this elevation remains at 100 μM also, data not shown). HSP70 protein accumulated with sodium arsenite exposure reaching a maximum at 25 μM, and declined at 50 μM. A similar situation was

Figure 26. Temporal response of heat shock proteins to elevated temperatures in A6 cells

A6 cells were maintained at 22°C (C), 33°C or 35°C for 2, 4, or 6 h. Total protein was isolated and resolved on 10% SDS-polyacrylamide gels. Protein was transferred to a PVDF membrane and probed with (anti-*Xenopus*) HSP110, HSP70, HSP30 or anti-actin polyclonal antibodies as described in Materials and Methods. Ponceau S stained membranes are included to show efficient transfer and equal loading of protein. Arrows indicate the position of the protein.

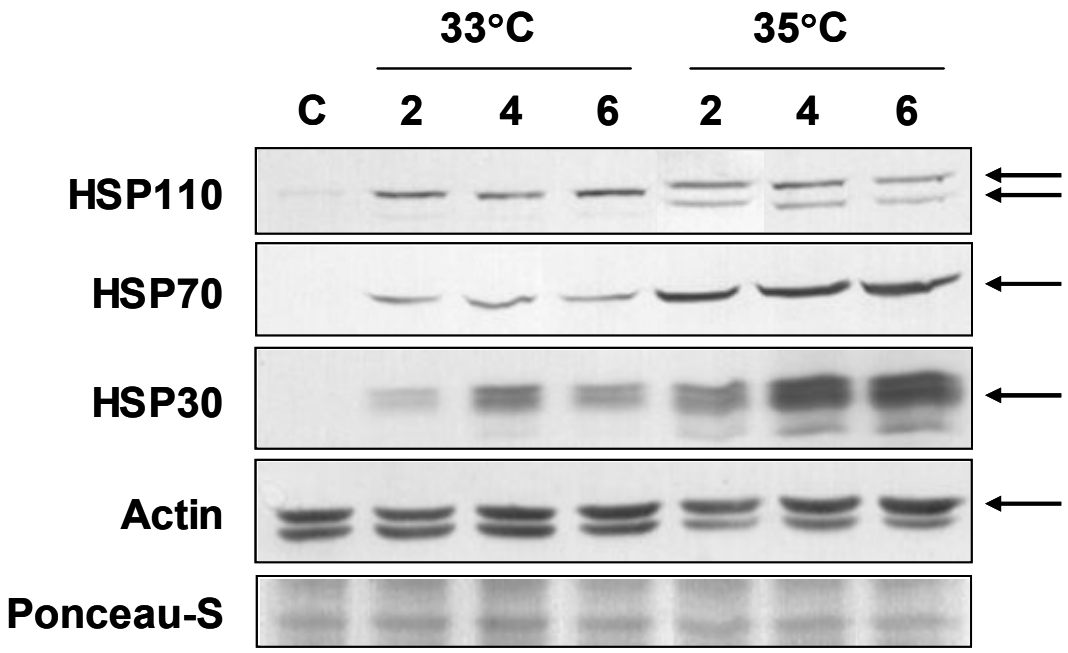
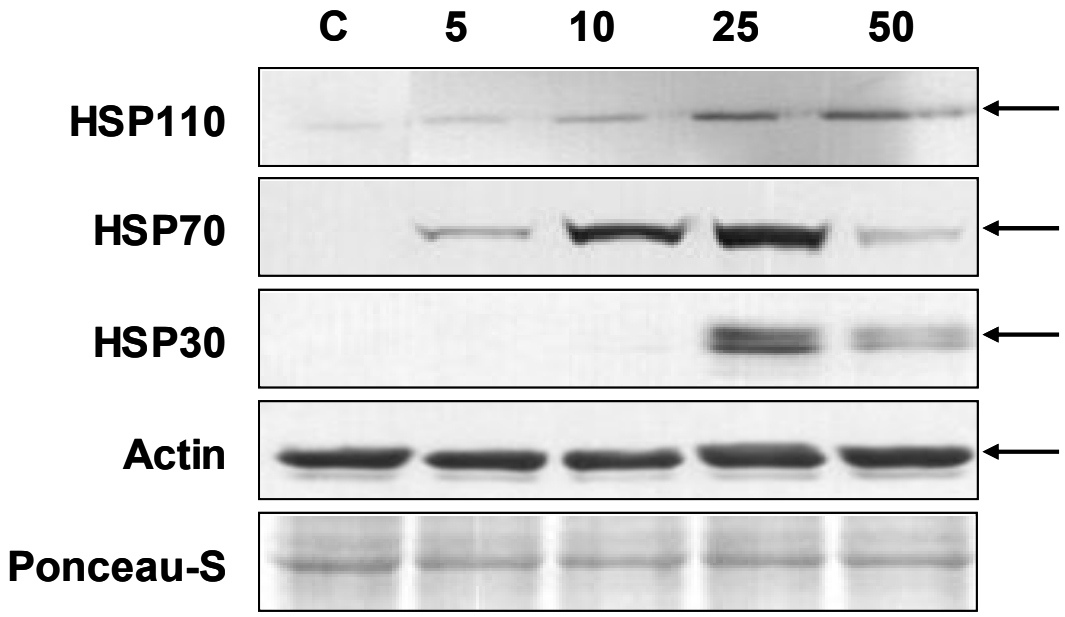

33°C
35°C
C
2
4
6
2
4
6
HSP110
HSP70
HSP30
Actin
Ponceau-S

Figure 27. Protein accumulation in response to selected sodium arsenite concentrations in A6 cells

A6 cells were maintained at 22°C in the absence of sodium arsenite (C), or with 5, 10, 25 or 50 μM sodium arsenite for 12 h. Total protein was isolated and resolved on 10% SDS-polyacrylamide gels. Protein was transferred to a PVDF membrane and probed with (anti-*Xenopus*) HSP110, HSP70, HSP30 or anti-actin polyclonal antibodies as described in Materials and Methods. Ponceau S stained membranes are included to show efficient transfer and equal loading of protein. Arrows indicate the position of the protein.


C
5
10
25
50
HSP110
HSP70
HSP30
Actin
Ponceau-S

seen with HSP30 protein, which was present maximally at 25 μM. Actin protein levels remained relatively constant.

3.6 Intracellular accumulation of HSP110 and HSP30 in *Xenopus* A6 cells

3.6.1 Intracellular accumulation in response to temperature

Immunofluorescence using confocal laser scanning microscopy (CLSM) revealed that HSP110 in cells maintained at 22°C was localized to both the cytoplasm and nucleus (Figure 28). In contrast, HSP30 was not detectable in A6 cells maintained at 22°C. In these control preparations, the phalloidin-stained actin cytoskeleton was clearly visible as red fibers. Heat treatment at 30°C elicited a low relative amount of HSP30, whereas HSP110 protein levels were increased compared to control cells. At 33, 35 and 37°C enhanced levels of both HSPs were clearly detectable. However, the pattern of their intracellular accumulation was very different. For example, HSP110 protein accumulated in very distinct, punctate areas within the cell, especially surrounding the nucleus, whereas HSP30 protein was homogeneously expressed in the cytoplasm. Also, while temperature stress resulted in HSP110 protein increases in the cytoplasm and nucleus, HSP30 was primarily enriched in the cytoplasm.

A6 cells continuously incubated at 33°C displayed an accumulation of HSP110 primarily in the cytoplasm (Figure 29). HSP110 was routinely observed surrounding the nucleus in long, spindle-shaped structures at the cell periphery. HSP110 protein accumulation compared to the control did not increase substantially from 2 to 6 h at 33°C.

Figure 28. Cellular localization of HSP110 and HSP30 proteins in A6 cells in response to elevated temperatures

A6 cells were maintained at 22°C (control; A1-A4), or heat-treated for 2 h at 30°C (B1-B4), 33°C (C1-C4), 35°C (D1-D4) or 37°C (E1-E4), followed by a 2 h recovery period at 22°C. Cells were then labeled for HSP110 (columns 1-2), HSP30 (columns 3-4) and F-actin (columns 2 and 4) expression using Alexa Fluor 488 (green; for HSPs), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.

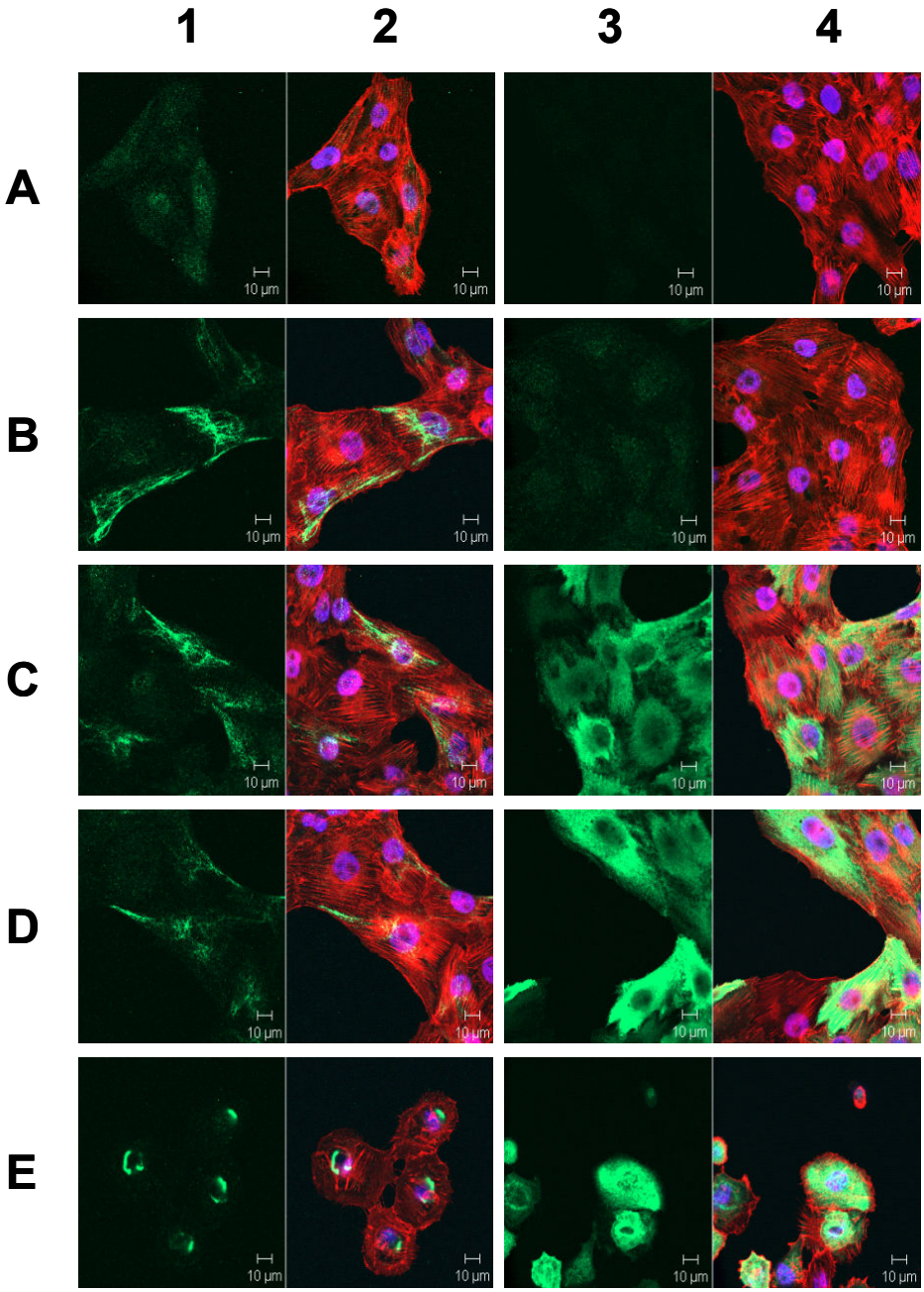
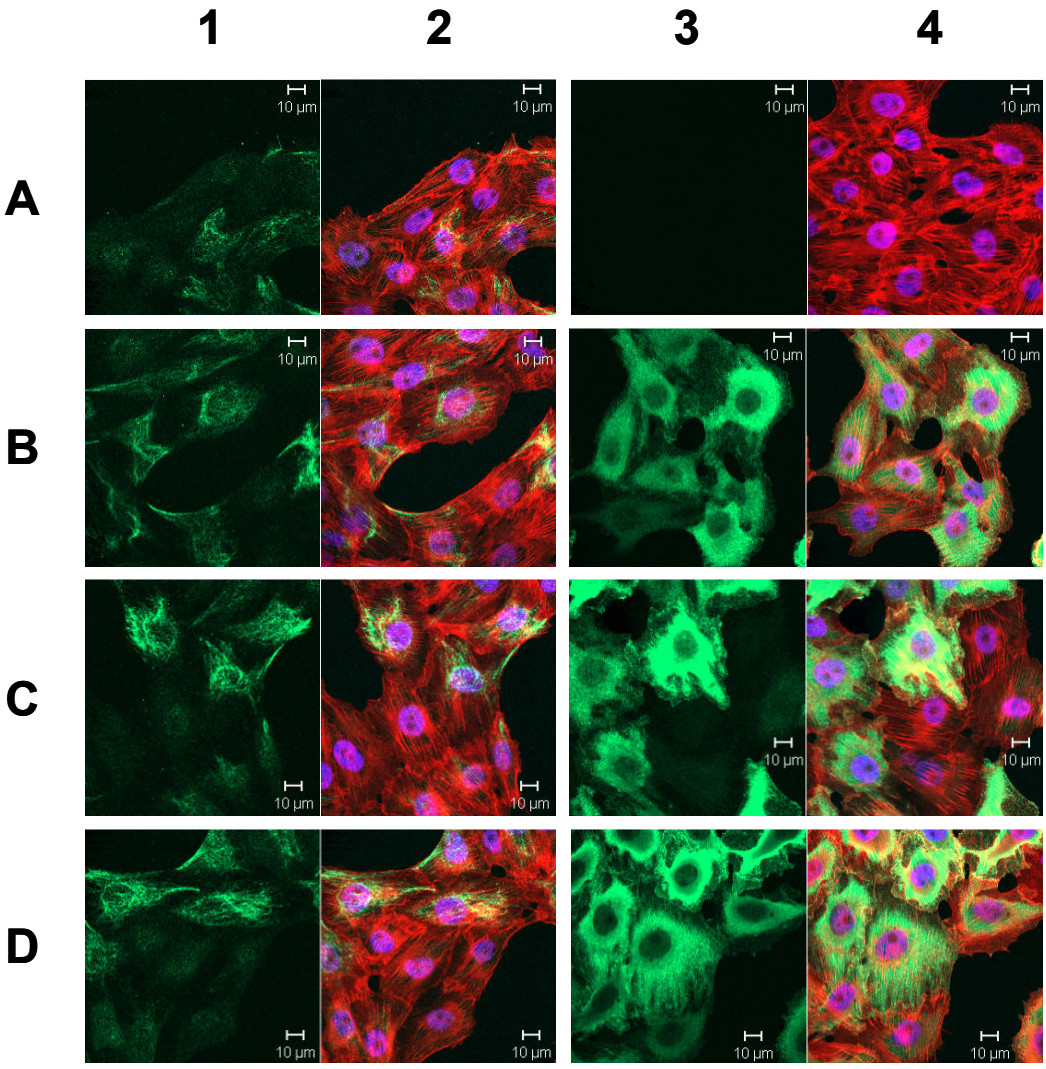

1
2
3
4
A
B
C
D
E
10 µm

Figure 29. Cellular localization of HSP110 and HSP30 protein in A6 cells in response to extended temperature elevation at 33°C

A6 cells were maintained at 22°C (control; A1-A4), or exposed to 33°C for 2 h (B1-B4), 4 h (C1-C4) or 6 h (D1-D4). Cells were then labeled for HSP110 (columns 1-2), HSP30 (columns 3-4) and F-actin (columns 2 and 4) expression using Alexa Fluor 488 (green; for HSPs), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.


1
2
3
4
A
B
C
D
10 µm

However, relative levels of HSP30 increased markedly at 33°C (2 to 6 h) compared to control. In A6 cells continuously incubated at 35°C, HSP110 accumulation was observed in the cytoplasm with enrichment around the nucleus (Figure 30). After 6 h at 35°C, there was less HSP110 accumulation than observed at 4 h. In comparison, the relative levels of HSP30 were markedly increased with time at 35°C showing maximal levels from 4 to 6 h. After 2 h at 35°C HSP30 was localized diffusely in the cytoplasm. By 4 h, HSP30 was focused on the edges of the cytoplasm, and by 6 h, relatively high levels of HSP30 were homogeneously localized throughout the cytoplasm. Interestingly, in some cells, this treatment caused an increased accumulation of HSP30 protein at the periphery of the nucleus.

3.6.2 Intracellular accumulation in response to sodium arsenite

The effect of varying concentrations of sodium arsenite on A6 cell HSP110 and HSP30 localization is shown in Figure 31. Exposure of A6 cells to 10 μM was the minimum concentration resulting in differences in HSP110 or HSP30 accumulation compared to control cells. At this concentration, HSP110 accumulation was visualized as scattered cytoplasmic spindle-shaped structures whereas HSP30 accumulated in a diffuse pattern in the cytoplasm. Exposure of A6 cells to 25 μM sodium arsenite resulted in the localization of HSP110 protein at opposite ends of several nuclei, while HSP30 remained diffuse throughout the cytoplasm. A similar pattern of HSP110 and HSP30 accumulation and localization was observed at 50 μM sodium arsenite. Also exposure of A6 cells to 10 μM or 50 μM sodium arsenite for extended periods of time resulted in few differences in HSP110 accumulation (Figure 32 and Figure 33). Finally, co-localization of HSP110 or HSP30 with actin or tubulin

Figure 30. Cellular localization of HSP110 and HSP30 protein in A6 cells in response to extended temperature elevation at 35°C

A6 cells were maintained at 22°C (control; A1-A4), or exposed to 35°C for 2 h (B1-B4), 4 h (C1-C4) or 6 h (D1-D4). Cells were then labeled for HSP110 (columns 1-2), HSP30 (columns 3-4) and F-actin (columns 2 and 4) expression using Alexa Fluor 488 (green; for HSPs), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.

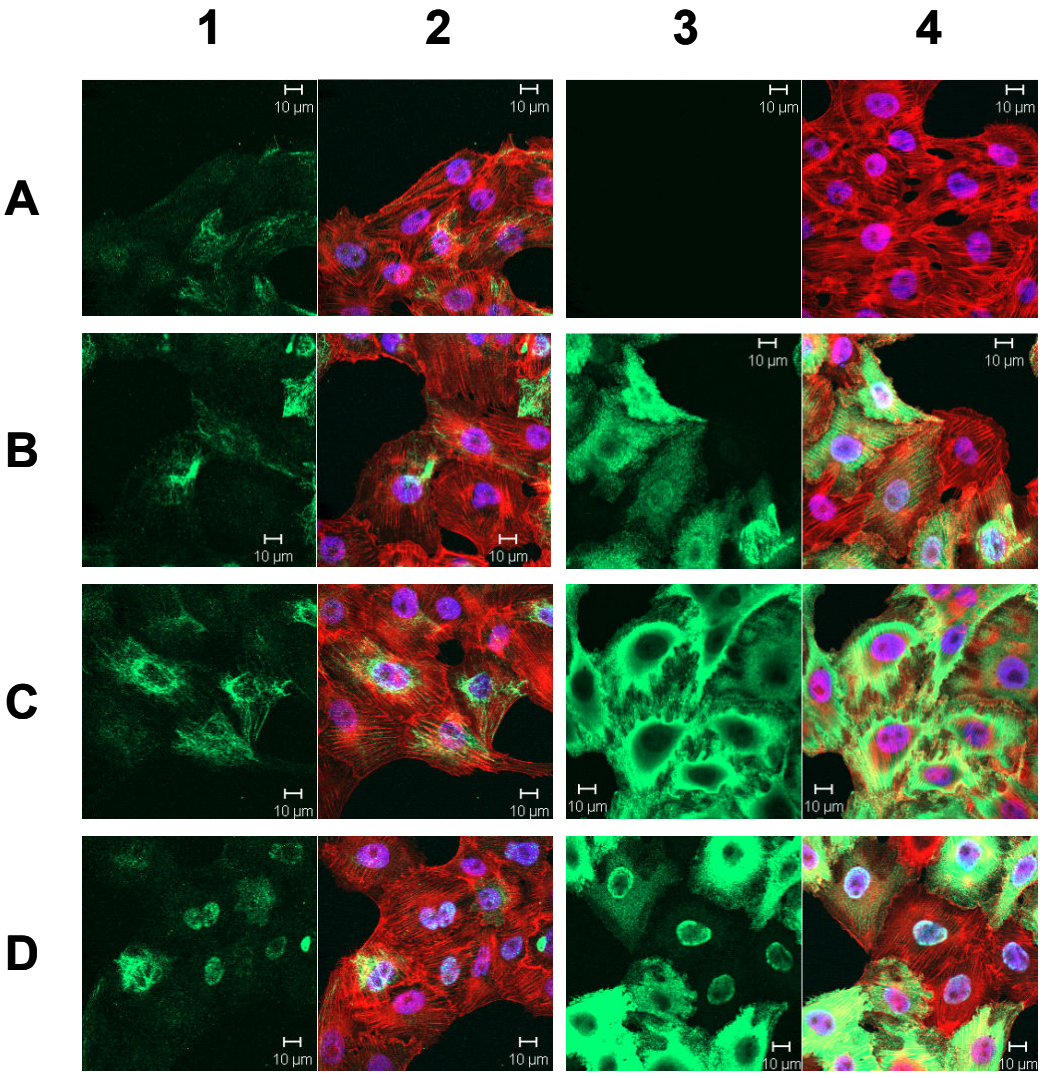

1
2
3
4
A
B
C
D
10 µm

Figure 31. Cellular localization of HSP110 and HSP30 proteins in A6 cells in response to sodium arsenite

A6 cells were maintained at 22°C (control; A1-A4), or exposed to sodium arsenite for 12 h at 5 μM (B1-B4), 10 μM (C1-C4), 25 μM (D1-D4) or 50 μM (E1-E4). Cells were then labeled for HSP110 (columns 1-2), HSP30 (columns 3-4) and F-actin (columns 2 and 4) expression using Alexa Fluor 488 (green; for HSPs), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.

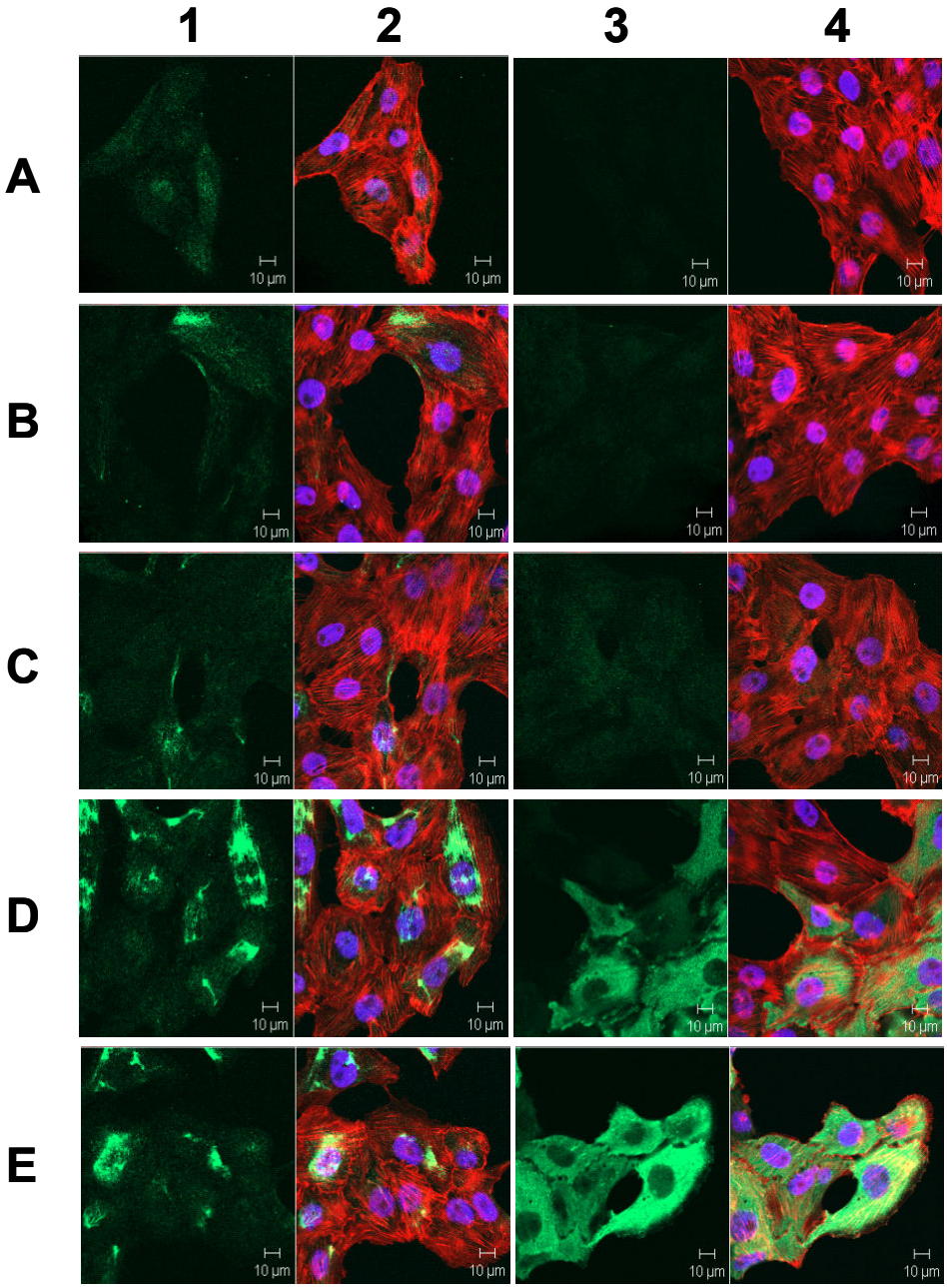

1
2
3
4
A
B
C
D
E
10 µm

Figure 32. Cellular localization of HSP110 protein in A6 cells in response to extended sodium arsenite exposure at 10 μM

A6 cells were maintained at 22°C (control; A1-A2), or exposed to 10 μM sodium arsenite for 5 h (B1-B2), 12 h (C1-C2) or 24 h (D1-D2). Cells were then labeled for HSP110 and F-actin expression using Alexa Fluor 488 (green; for HSP110), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.

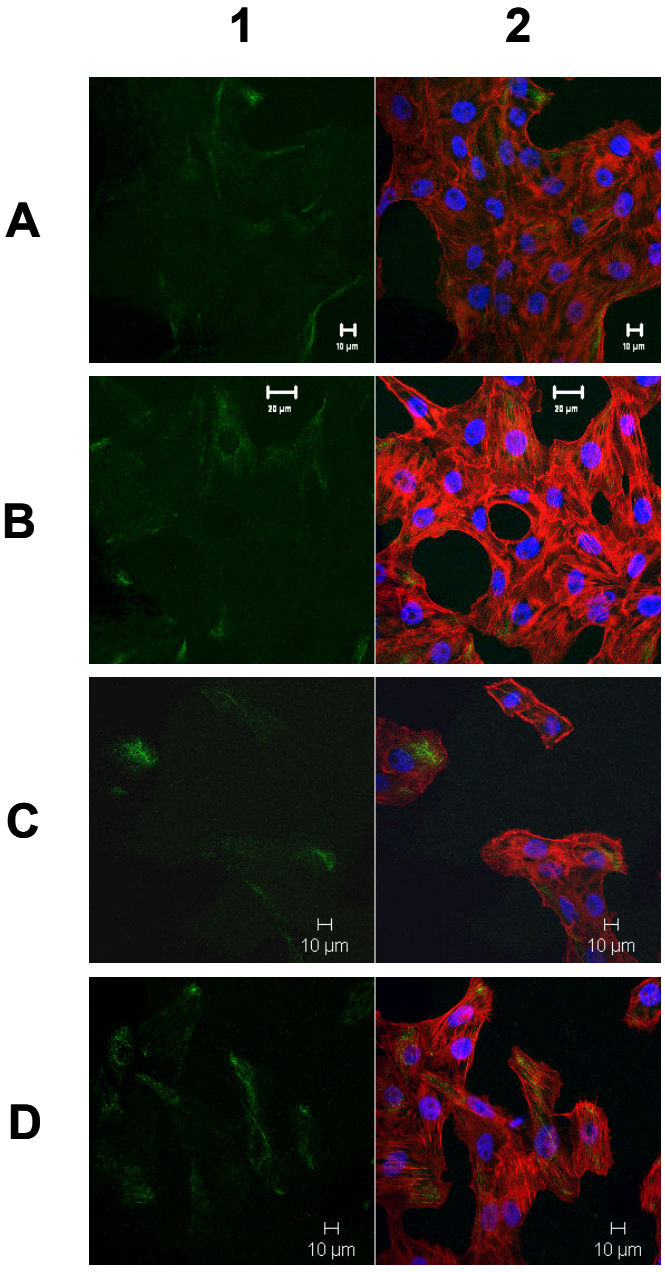
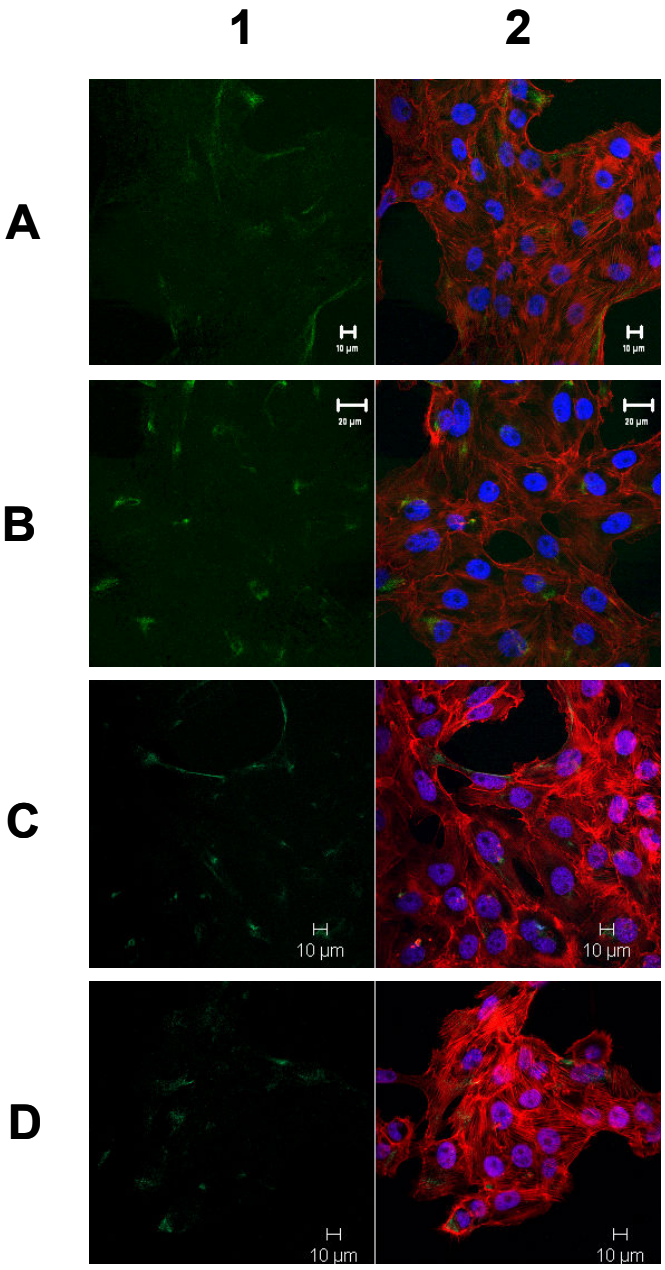

1
2
A
B
C
D
10 µm

Figure 33. Cellular localization of HSP110 protein in A6 cells in response to extended sodium arsenite exposure at 50 μM

A6 cells were maintained at 22°C (control; A1-A2), or exposed to 50 μM sodium arsenite for 5 h (B1-B2), 12 h (C1-C2) or 24 h (D1-D2). Cells were then labeled for HSP110 and F-actin expression using Alexa Fluor 488 (green; for HSP110), TRITC (red; for F-actin) and DAPI (blue; for the nucleus) as outlined in Materials and Methods. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.


1
2
A
B
C
D
10 µm
20 µm

was not directly observed in any of the aforementioned treatments. This would have been indicated by a yellow merged signal. Although in some images a yellow signal is visible, suggesting colocalization of HSP30 and actin, this may be due to a coincidental overlap of protein, rather than direct protein interaction. Further experiments would be necessary to confirm such interactions.

Chapter 4: Discussion

In this study, we characterized for the first time, a *Xenopus hsp110* cDNA sequence and the expression of this gene in *Xenopus* cultured cells and embryos. *Xenopus* HSP110 shared identity with HSP110-like proteins from other organisms including mouse, human, and sea urchin (Hatayama *et al.*, 1994; Ishihara *et al.*, 1999; Foltz *et al.*, 1993). For example, amino acid sequence comparison of the coding region of *Xenopus* HSP110 with HSP110/105 proteins from mammalian species revealed an identity of 72-74% while members of the *Xenopus hsp70* family (*hsp70, hsc70.1, hsc70.2* and *BiP*) only shared an identity of 27-29%. Most of the identity within the superfamily resided in the ATP-binding domain in the N-terminus. The *Xenopus* HSP110 NLS (KKPK) was identical in sequence and position to hamster as were three of seven potential N-glycosylation sites. Phylogenetic analysis indicated that *Xenopus* HSP110 grouped outside of mammals. A comprehensive phylogenetic analysis of the entire HSP70/DnaK superfamily has been presented previously (Lee-Yoon *et al.*, 1995; Easton *et al.*, 2000).

Analysis of *hsp110* mRNA levels in *Xenopus* A6 kidney epithelial cells revealed that this message was constitutive as reported previously for mammalian cultured cells (Yasuda *et al.*, 1995; Ishihara *et al.*, 1999; Hatayama *et al.*, 2001; Yamagishi *et al.*, 2002). The pattern of heat shock-induced *hsp110* mRNA accumulation in A6 cells incubated at different temperatures and during continuous treatment at 33°C and 35°C was similar to the pattern observed with *hsp70* mRNA. Heat shock treatment also enhanced the relative level of HSP110 protein as determined by immunoblot analysis. While the *Xenopus hsp110* gene has

not been isolated it is likely that heat shock-induced expression was the result of HSF-HSE interaction as determined for other *hsp* genes in *Xenopus* and other organisms (Heikkila, 2004; Morimoto, 1998). Genomic cloning of murine *hsp105* revealed 2 HSEs in the 5' UTR (Yasuda, *et al.*, 1999). In contrast to *Xenopus hsp70* mRNA, the relative levels of *hsp110* mRNA were quite low when A6 cells were incubated at 37°C. If the *Xenopus hsp110* gene has introns, as determined for mammalian *hsp110* (Feige *et al.*, 1996; Yasuda *et al.*, 1999), then it is possible that the low level of *Xenopus hsp110* mRNA accumulation at 37°C was due to an inhibition of RNA splicing as found with *hsp90* primary transcripts (Yost and Lindquist, 1986; Kay *et al.*, 1987; Bond, 1988). This is a viable possibility since introns are present in the *hsp110* gene of *Xenopus tropicalis* as revealed by the genome project for that organism (U.S. Department of Energy Joint Genome Institute; http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Since *Xenopus laevis* is tetraploid it is possible that multiple copies of the *hsp110* gene exist. Therefore, the results presented with A6 cells and embryos in the present study may reflect the overall response of the different *hsp110* genes to the various stressors.

In contrast to heat shock, treatment of A6 cells with sodium arsenite produced a gradual increase in *hsp110* and *hsp70* mRNA accumulation for at least 12-24 h. A gradual increase in *hsp105* mRNA levels after sodium arsenite exposure was reported in both HeLa cells and FM3A cells (Ishihara *et al.*, 1999; Yasuda *et al.*, 1995). Cadmium chloride also enhanced *Xenopus hsp110* and *hsp70* mRNA accumulation in A6 cells while treatment with copper sulfate had no detectable effect on either message. However, treatment of A6 cells with ethanol resulted in an accumulation of *hsp70* mRNA but with no accompanying increase in

hsp110 mRNA levels compared to control. This latter finding suggests the presence of regulatory mechanisms that lead to a stressor-specific accumulation of different *hsp* mRNAs. In support of this possibility, stressor-specific activation of *hsp* genes has been observed in rat hepatoma cells (Wiegant *et al.*, 1994; Ovelgonne *et al.*, 1995).

This study investigated *hsp110* mRNA accumulation in developing *Xenopus* embryos. The detection of constitutive levels of *hsp110* mRNA in unfertilized eggs, cleavage and early blastula stage embryos suggested that these transcripts were maternally inherited since the zygotic genome was not activated until after the midblastula stage (Kimmelman *et al.*, 1987; Hair *et al.*, 1998). The relative levels of constitutive *hsp110* message declined in gastrula, neurula and early tailbud embryos and then increased in midtailbud and later stage embryos. The reasons for this decline are not known although it is possible that this phenomenon reflects the decay in maternal *hsp110* mRNA followed by enhanced zygotic constitutive *hsp110* mRNA accumulation in midtailbud. Similar findings were observed with maternal transcripts encoding insulin receptor and S-adenosylmethionine decarboxylase during *Xenopus* early development (Groigno *et al.* 1999; Shinga *et al.*, 1996). In comparison to *hsp110* mRNA, *hsp70* was detected only in response to stress and the magnitude of the response increased with development. Unlike *hsp70* and *hsp110* messages, which accumulated in blastula embryos, *hsp27* message was differentially expressed in a heat-inducible fashion beginning in early tailbud embryos, similar to *Xenopus hsp30* (Krone and Heikkila, 1988).

In the present study, whole mount *in situ* hybridization revealed an enhanced accumulation of *hsp110* mRNA in lens vesicle, notochord, brain, and heart region of *Xenopus* late tailbud

embryos. We found a similar result for *hsp70* mRNA, *hsp27* mRNA and previously for the mRNA encoding collagen-binding *hsp47* (Hamilton and Heikkila, 2006). This pattern of mRNA enrichment was similar to that of fish, avian and mammalian embryos for *hsp27* (Mao and Shelden, 2006; Kawazoe *et al.*, 1999; Armstrong *et al.*, 2001), mouse embryos for *hsp105* (Hatayama *et al.*, 1997), and fish and *Drosophila* embryos for *hsp70* (Lele *et al.*, 1997; Wang and Lindquist, 1998). Tissue-specific enrichment of mouse *hsp105* mRNA was also reported in brain, lung, liver, heart, intestine and limb at gestational days 11 and 14 (Hatayama *et al.*, 1997; Satoh *et al.*, 1998). While the mechanism for tissue-specific enrichment of *hsp110* mRNA is not known, the presence of this mRNA in selected tissues of early *Xenopus* embryos suggests that *hsp110* may have a role during organogenesis. A possible role in apoptosis was suggested for mouse HSP105 during early development since this protein was observed specifically in cells undergoing programmed cell death in the interdigital regions of the limb tissue (Hatayama *et al.*, 1997). This proposed apoptotic role was supported by recent data linking HSP110 expression to caspase-3 expression during mouse development (Evrard *et al.,* 1999; 2000; Gashegu *et al.*, 2007).

Whole mount *in situ* hybridization analysis revealed that heat shock enhanced *hsp110* mRNA accumulation in selected tissues including the notochord, somites, lens vesicle, heart region and brain region of late tailbud embryos. The mechanism for preferential enrichment of *hsp110* message in selected tissues of normal and heat shocked *Xenopus* embryos is not known. There are a number of possibilities for this finding including preferential mRNA stability or tissue-specific transcription. Unfortunately, very little is known about either possibility. In fact, the mouse *hsp105* gene promoter sequence has been the only one

analyzed for its constitutive and heat-inducible expression in transfected cultured cells (Yasuda *et al.*, 1999). While heat shock-induced activation of *hsp* genes by HSF-HSE interaction is well documented, the mechanism responsible for preferential accumulation of *hsp110* mRNA in selected tissues is unclear. Previous studies with heat shocked *Xenopus* embryos have suggested that a reduced temperature set point for HSF activation in selected tissues may be a possible mechanism for the tissue-specific enrichment of *hsp30* and *hsp70* mRNA (Lang *et al.*, 1999; 2000). Also HSF activation at a reduced temperature compared to other cells or tissues has been reported in mouse pachytene spermatocytes and adult *Xenopus laevis* heart (Sarge, 1995; Ali *et al.*, 1997). Given these findings, it is possible that under heat shock conditions the interaction between HSF and HSE of the *hsp110* promoter may be superimposed on the mechanisms associated with tissue-specific expression. Future analysis of the *Xenopus hsp110* gene may aid in elucidating the mechanisms associated with the pattern of *hsp110* gene expression during early embryogenesis. Finally, it is possible that the preferential accumulation of *hsp110* mRNA in selected tissues during heat shock may indicate a protective role for HSP110. Previous experiments have documented the ability of HSP110 to confer heat resistance and/or act as a molecular chaperone (Oh *et al.*, 1997; 1999; Hatayama *et al.*, 2001).

In order to investigate HSP110 protein accumulation, a polyclonal rabbit-anti hamster HSP110 commercial antibody was initially employed. While this antibody detected HSP110, it also detected several non-specific proteins, which prevented us from examining cellular localization using CLSM. A custom polyclonal rabbit-anti *Xenopus* HSP110 antibody was prepared using a KLH-coupled C-terminal peptide. This preparation provided much greater

specificity and enabled us to investigate both protein accumulation via western blot analysis, and cellular localization of HSP110 with confidence. This antibody produced a single band of approximately 110 kDa via western blot analysis, and detected HSP110 protein under both control and stressed conditions, which was expected based on the preliminary results obtained from the commercial preparation. Also, the production of an antibody specific to *Xenopus* HSP70 was particularly helpful. Previously, an antibody that would detect HSP70 exclusively, and not HSC70, was not available.

Western blot analyses indicated HSP110 protein accumulation in response to both elevations in temperature and increasing sodium arsenite concentrations. Similar patterns of protein accumulation were found for HSP70 and HSP30, although these two proteins were only detected in response to stress. Interestingly, HSP70 and HSP30 protein levels declined in response to 50 μM sodium arsenite, while HSP110 protein remained elevated. It is possible that HSP110 protein, or *hsp110* mRNA have longer half-lives than HSP70, HSP30 or their messages. This may be feasible given that *hsp70* and *hsp30* gene expression is primarily stress-inducible whereas *hsp110* is a constitutively expressed gene.

Two HSP110 protein bands were detected at 33-35°C with western blot analysis. One possibility that may account for this finding is alternative splicing. A similar finding was also reported in mouse and human, which express two HSP105 isoforms α and β, the latter alternatively spliced from the former (Yasuda *et al.*, 1995; Ishihara *et al.*, 1999). Mammalian HSP105β protein, a 90 kDa band, was detected only at heat shock temperatures of 42°C and not under any other conditions. In the present study, the 97 kDa *Xenopus* HSP110 band was

detected only at 33-35°C, and not under the other heat shock or sodium arsenite treatments employed. Given the similarity between the patterns of mouse and *Xenopus* HSP110 accumulation, it is possible that alternative splicing may be the mechanism for the appearance of the 97 kDa HSP110 band. However, since a genomic analysis of *Xenopus* HSP110 has not been completed we are unable to confirm this hypothesis. It is also possible that the additional *Xenopus* HSP110 band may be the result of post-translational modifications, especially given the number of putative glycosylation sites in the HSP110 protein. Finally, multiple *hsp110* genes are also a possibility since *Xenopus laevis* is tetraploid. Alterations in the coding regions of *hsp110* genes during evolution could have given rise to HSP110 isoforms with different sizes.

In this study, *Xenopus* HSP110 localized primarily in the cytoplasm, showing enrichment near, and in some cases was present in, the nucleus. Immunofluorescent detection showed that *Xenopus* HSP110 was present under control conditions and accumulated in response to heat shock and sodium arsenite, which was in agreement with western blot analysis results. This general pattern of HSP110/HSP105 localization was shared with mouse (Hatayama *et al.*, 1994; Yasuda *et al.*, 1995) and human cultured cells (Ishihara *et al.*, 1999). Hamster cells also shared this pattern of intracellular distribution, but additionally accumulated in the nucleolus (Subjeck *et al.*, 1983; Lee-Yoon *et al.*, 1995). Under control conditions, *Xenopus* HSP110 demonstrated diffuse staining in the cytoplasm and in the nucleus in some cases, but was never documented in the nucleolus. This is similar to what has been reported in mouse cultured cells (Hatayama *et al.*, 1994). In A6 cells, long spindle-shaped HSP110 structures close to the cell periphery were frequently noted. Increases in temperature (30, 33 and 35°C),

as well as prolonged high temperature exposure resulted in a general increase in HSP110 cytoplasmic staining, and also an increase in the frequency of the spindle-shaped structures, which have not thus far been reported in the literature. It is possible, based on the morphology, that perhaps this localization is related to microtubules or the cytoskeleton. This possibility is supported well by findings in other organisms. For example, HSP105, which was shown to co-localize with α-tubulin in HeLa and COS-7 cells, was suggested as having a stabilizing role in microtubular structure (Saito *et al.*, 2003). It was suggested that this relationship between HSP105α and α-tubulin would be protective for the cell, through binding to denaturing microtubules and preventing their aggregation and disorganization, and also possibly preventing apoptosis. In the present study, a monoclonal mouse anti-α-tubulin antibody was employed with *Xenopus* A6 cells to investigate a possible co-localization of HSP110 with α-tubulin. While no co-localization of these two proteins was observed (data not shown) it was possible that this phenomenon could be explained by co-localization of HSP110 with a different cytoskeletal protein other than F-actin or α-tubulin. Additionally, HSP110 immunofluorescence was observed not just as thread or spindle-shaped fibers (which may parallel stress fibers), but also in the nucleus, as aggregates at the periphery of the nucleus, and amorphously in the cytoplasm. It is possible given its role as a molecular chaperone that HSP110 may function in different subcellular locations with selective client proteins during stress.

In contrast to HSP110 localization, HSP30 was strictly stress-inducible and displayed a granular, or punctate, cytoplasmic localization, that was ameliorated in response to increasing temperature and sodium arsenite exposure. This pattern has been suggested to be due to the

formation of super-aggregated HSP30 structures (Gellalchew and Heikkila, 2005; Ohan *et al.*, 1998). Also, upon exposure to higher temperatures, F-actin is prone to cytoplasmic disorganization, and HSP30 was observed to co-localize to these areas. It is possible that HSP30 was involved in cytoskeletal protection since it has been shown to bind actin by co-immunoprecipitation (Fernando *et al.*, 2003) and it co-localized with actin in areas of disorganization (Gellalchew and Heikkila, 2005). Unlike HSP110, very little nuclear or perinuclear localization of HSP30 was observed in this study, but did increase slightly at high temperatures. HSP30 does not possess a NLS, but has been reported in the nucleus at very low levels in response to high temperature exposure (Gellalchew and Heikkila, 2005; Manwell and Heikkila, 2007). Additionally, in response to prolonged exposure to high temperatures, HSP30 was initially enriched at the cell periphery, and longer treatments resulted in enrichment at the nuclear periphery. Although, few similarities were noted between the localization of HSP30 and HSP110, this is not surprising given these two HSPs belong to different HSP families entirely.

HSP110 was also routinely observed surrounding the nucleus following stress, especially concentrated at opposite ends of the nucleus. This localization pattern of HSP110 was found in response to both elevated temperature and sodium arsenite treatment. Although treatment of A6 cells at 37°C resulted in detachment of cells from the substrate, and the death of many cells, in cells that did survive this stress, HSP110 localized in distinct intensely stained areas at opposite ends of the nucleus. It is unclear what caused this distinctive pattern of HSP110 localization. Furthermore, in A6 cells subjected to prolonged heat shock, HSP110 was detected in the nucleus. Nuclear localization in response to prolonged heat shock was

previously observed with HSP30 in *Rana catesbeiana* FT cells, human HSP27, mouse HSP25 (Mulligan-Tuttle and Heikkila, 2007; Van de Klundert *et al.*, 1998; Adhikari *et al.*, 2004).

Recently Saito *et al.* (2007) produced antibodies that were able to distinguish between mouse HSP105α and HSP105β. This study revealed that HSP105α and HSP105β localized in different cellular compartments, with HSP105β specifically in the nucleus of mammalian COS-7 cells. While the antibody prepared in the present study was not able to distinguish between putative HSP110α and HSP110β, it is tempting to speculate that *Xenopus* HSP110 would behave in a similar manner. Although the antibody employed is unable to differentiate HSP110α from HSP110β, one would expect to observe HSP110 in the nucleus at 35°C to a greater extent than other treatments. However, the *Xenopus* HSP110 intracellular localization data presented in this study do not support this hypothesis. An increased amount of HSP110 protein was observed in the nucleus, but only when A6 cells were treated at 35°C for 6 h. While it is clear that western blot analysis supports the presence of a putative HSP110β, more extensive localization investigation would be required to conclude the presence of a similarly functioning HSP110α and HSP110β in *Xenopus laevis*.

The presence of HSP110 in control A6 cells and embryos suggests that HSP110 may play a role in unstressed cells. Oh *et al.* (1997) implied that HSP110 likely serves an important function under control conditions simply due to the levels that are present constitutively, which have been reported to be 0.7% of total cell protein in the mouse brain (Subjeck *et al.*, 1982c). The developmental regulation of *hsp110* mRNA accumulation in embryos also

suggests a role for this protein during embryogenesis. A pattern of mRNA accumulation similar to *Xenopus* was observed in mouse embryos (Hatayama *et al.*, 1997), and those authors proposed that HSP105 played a role in organogenesis. Moreover, the localization of HSP105 to apoptotic bodies during development may indicate additional roles in metamorphosis, neural tube closure, parietal plate closure, or mesonephros degeneration. It remains to be determined whether *Xenopus* HSP110 is directly involved in any of these processes, and the possibility should not be ruled out. In mouse FM3A cells, HSP105 was shown to interact with HSP70 family proteins, forming large complexes (of heterodimers and heterotetramers) of HSP105 and HSC70/HSP70 under unstressed conditions (Hatayama *et al.*, 1998). The authors proposed that either HSP105 was regulating the function of HSC70, or that HSC70 was regulating the function of HSP105, or that HSP105 was functioning cooperatively with HSC70/HSP70. A new role as a "nucleic acid chaperone" was put forward, in which the hamster N-terminal ATP-binding domain of HSP110 was shown to bind directly to RNA under control conditions, possibly for the unwinding of complex secondary RNA structures to expose *cis*-acting sequences critical for recognition and binding by other protein factors (Henics *et al.*, 1999). In summary, a role for HSP110 under normal cellular conditions has been well-documented in several animal systems. Although HSP110 mRNA and protein are clearly present constitutively, a role in *Xenopus* is yet to be determined.

This study has presented for the first time a characterization of *Xenopus laevis* HSP110, including the full-length sequence, mRNA accumulation data, spatial patterning of mRNA, the production of polyclonal antibody, characterization of protein accumulation and cellular

localization data. This work has opened the door to many potential studies regarding *Xenopus* HSP110. For example, a thorough high magnification analysis, possibly including immunoelectron microscopy, would shed light on the interesting and unique localization patterns exhibited by *Xenopus* HSP110. Similarly, a localization study using CLSM in embryos might yield interesting results and would nicely complement the whole mount *in situ* hybridization study presented here. Furthermore, a determination of potential client proteins of HSP110 might help to elucidate its function. This could be accomplished with immunoprecipitation studies, or a yeast two hybrid analysis. Additionally, given that HSP110 displays documented chaperone activity in hamster (Oh *et al.*, 1999), it would be interesting to determine whether *Xenopus* HSP110 functions as a chaperone. Another function that has been well-documented for HSP105 is an involvement in apoptosis (Hatayama *et al.*, 1997; Hatayama *et al.*, 2001; Yamagishi *et al.*, 2002; Yamagishi *et al.*, 2006). The *Xenopus laevis* system would lend itself well to such a project given the availability of the A6 and XTC cell lines, as well as an excellent developmental system. A last approach to function of this protein could be inhibitory in nature. Antisense morpholino oligonucleotides have enjoyed much success in the *Xenopus laevis* embryo system, allowing a simple way to 'knockdown' a gene, in order to determine a function in its absence (Heasman, 2002).

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