Characterization of the expression and function of *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27

by

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

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Abstract

Exposure of cells to environmental or chemical stressors will initiate the heat shock response, which is mediated by heat shock proteins. Heat shock proteins are molecular chaperones which are classified by size into six main families: HSP100, HSP90, HSP70, HSP60, HSP40 and the small heat shock proteins (sHsps). The sHsp family members bind to denatured proteins and maintain them in a folding competent state such that they may be refolded by other molecular chaperones.

The present study examined the expression and function of two amphibian sHsps, namely, *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27. Initially, an antisense riboprobe was produced to study the mRNA accumulation of *Rana hsp30* in cultured tongue fibroblast (FT) cells. Results showed that *Rana hsp30* mRNA was optimally induced when maintained at 35^{o}C for 2 h. An antibody to the recombinant *Rana* HSP30 protein was also produced in order to study HSP30 protein accumulation in *Rana* FT cells. Analysis showed that *Rana* HSP30 was heat-inducible and accumulated maximally at 4 h when maintained at 35^{o}C and then allowed to recover at 22^{o}C for 2 h. Immunocytochemical analysis indicated that *Rana* HSP30 protein was present primarily in the nucleus, with diffuse localization in the cytoplasm. Additional immunocytochemical analysis showed that *Rana* HSP30 remained in the nucleus following heat stress and extended periods of recovery.

The molecular chaperone function of *Rana* HSP30 was also studied. Recombinant *Rana* HSP30 was found to inhibit the heat induced aggregation of various target proteins including citrate synthase, luciferase and malate dehydrogenase. Also, no major difference

was detected between *Rana* HSP30 and *Xenopus* HSP30C in the inhibition of heat-induced aggregation of target proteins.

This study also examined the expression and function of *Xenopus laevis* HSP27. Analysis of the putative amino acid sequence of the *Xenopus hsp27* cDNA revealed that it had an identity of 71% with chicken, 65% with zebrafish, 63% with human and 53% with topminnow. Most of the identity was located within the α-crystallin domain of the protein. Interestingly, *Xenopus* HSP27 shared only a 19% identity with 2 other *Xenopus* sHsps, HSP30C and HSP30D.

Western blot analysis using an anti-*Xenopus* HSP27 antibody revealed that HSP27 was not detectable in cultured kidney epithelial cells. However, examination of early *Xenopus* embryos revealed that HSP27 was first detected in tadpole embryos (stage 44). Heat-inducible HSP27 was also first detected at this stage. The accumulation pattern of *Xenopus* HSP27 protein was distinct from *Xenopus* HSP30, which was heat-inducible at midtailbud stage 26, approximately two and a half days earlier in development.

Analysis of recombinant HSP27 by native pore exclusion limit electrophoresis showed that it formed high molecular weight, multimeric complexes. The molecular chaperone function of HSP27 was assessed by means of thermal aggregation assays employing citrate synthase, luciferase and malate dehydrogenase. *Xenopus* HSP27 inhibited the heat-induced aggregation of all of these target proteins. This study has revealed that *Xenopus* HSP27 is a member of the HSP27 subfamily of small heat shock proteins in *Xenopus* and distinct from the HSP30 family. The accumulation of HSP27 under constitutive and stress-inducible conditions is developmentally regulated. Finally, this sHsp appears to function as a molecular chaperone.

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1 Introduction

All life on earth is governed by ambient temperatures, and as such, it follows that all physiological processes are temperature-dependent. Although the systematic mechanisms of adaptation to changes in an organism's environment are well-known (Katschinski, 2004), the molecular and genetic mechanisms are less so. Organisms are known to react to changes in temperature by either increasing or decreasing protein production. This is mediated by a common set of proteins known as heat shock proteins (Hsps) whose expression pattern is conserved almost universally (Katschinski, 2004). However, despite decades of research, the functional significance of heat shock proteins remains to be entirely elucidated.

1.1 The heat shock response

The heat shock response is a rapid and transient response to stress that is mediated by heat shock proteins. It is essentially the activation and mediation of heat shock protein (Hsp) genes. It has been extensively studied and characterized. Heat shock protein genes are transcribed upon the activation of a heat shock factor (HSF). Activation of HSF can occur as a result of environmental (anoxia, heavy metals, heat shock and ethanol) or physiological (fever, inflammation, oxidative stress, growth and development and differentiation) stresses (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Ehrnsperger *et al*., 1997). HSFI is the stress-responsive member of the HSF family and is responsible for the activation of the heat shock response in higher organisms (Morimoto, *et al*., 1994). The structure of HSFI is highly conserved, consisting of 100 amino acids in a helix-turn-helix DNA binding motif, as well as a transcriptional transactivation domain, a

C-terminal hydrophobic region and an oligomerization domain instrumental in trimer formation. HSFI, which exists as an inactive monomer in the cytosol, is bound by HSP70 and HSP90. HSFI is converted to an active homotrimer in the nucleus upon heat shock, following its release from HSP70 and HSP90 (Heikkila, 2003; Katschinski, 2004). Once active, HSFI interacts with the 5' upstream heat shock element (HSE) on the heat shock protein gene, which mediates transcription (Heikkila, 2003), initiating the RNA polymerase II transcription of the hsp genes. A diagrammatic representation of this process is outlined in Figure 1. Once these proteins are in circulation within the cell, they seek out and bind to unfolded or denatured proteins, maintaining them in a folding competent state until the stress is no longer a threat to survival. Following the removal of the stressor, the cell stops producing excess heat shock proteins and levels return to pre-stress levels following a recovery period (Heikkila, 2003).

1.2 Heat shock proteins

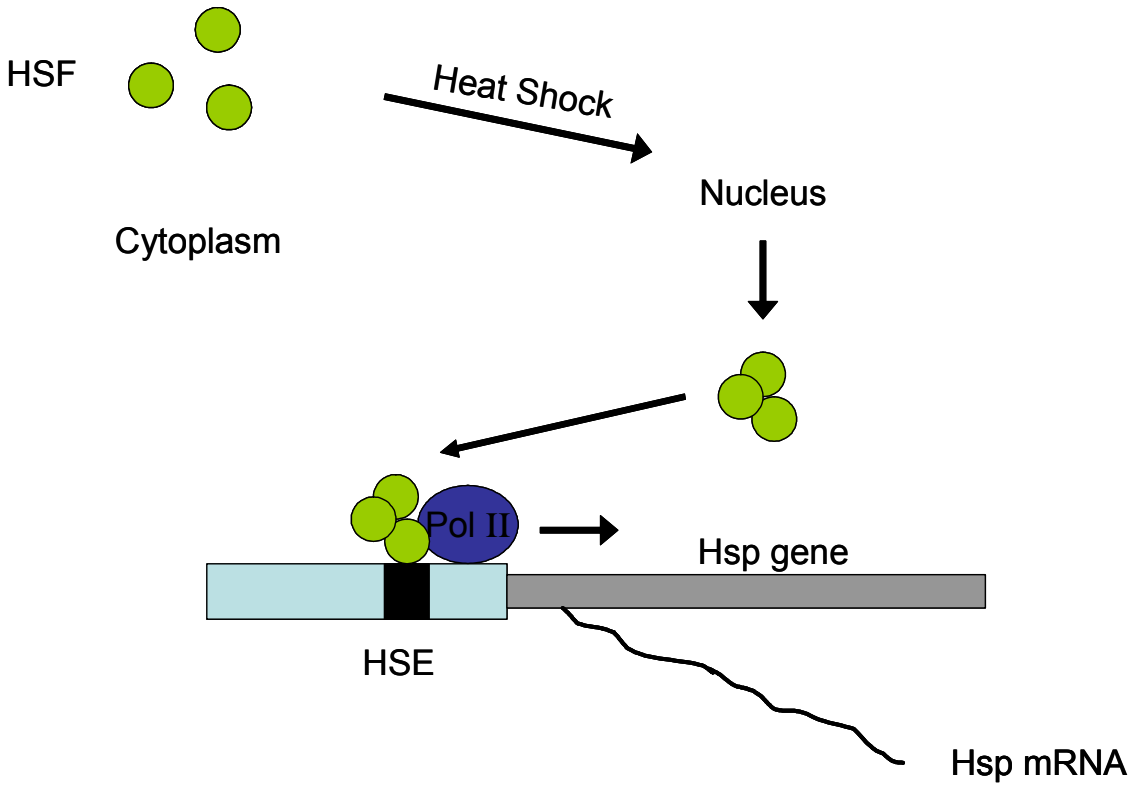
Heat shock proteins were first discovered in the salivary glands of *Drosophila* by Ritossa in 1962. Continued research into both the heat shock response and its key players, heat shock proteins, has revealed a plethora of information in a number of model systems that point to a complex cascade of events surrounding the stress response. Heat shock proteins are cellular components that have been found in all prokaryotic and eukaryotic organisms studied to date (Heikkila *et al*., 1997b; Robert, 2003; Stromer *et al.*, 2003). Although some Hsps are expressed constitutively within the cell under normal physiological conditions, aiding in vital cellular processes such as protein folding, assembly and transport, others are only expressed upon heat shock or under other stress conditions (Currie *et al*., 2000; Höhfeld, 2001; Azzoni *et al*., 2004; Katschinski, 2004;

Wataba *et al*., 2004). Those proteins expressed under normal physiological conditions in the cell are differentially expressed or hormonally regulated during the cell cycle as well as during different stages of development (Wataba *et al*., 2004) and are mediated at the level of mRNA synthesis, mRNA stability and translational efficiency as well as through post-translational modifications (Katschinski, 2004). It is widely accepted that these proteins act as molecular chaperones, maintaining other proteins in a folding competent state and preventing the aggregation of denatured or damaged proteins (Agashe and Hartl, 2000), while remaining separate from the final functional structures (Heikkila *et al*., 1997b; Azzoni *et al*., 2004). The heat shock response, as this process is known, is a cellular survival mechanism that ensures cells survive the onslaught of environmental stresses in the course of an organism's lifespan. Once the cell returns to normal unstressed conditions, those heat shock proteins acting as molecular chaperones deliver their substrate proteins to other heat shock proteins that can refold them in the presence of ATP (Agashe and Hartl, 2000; Wang and Spector, 2001).

Heat shock proteins are generally named and grouped according to their molecular weight. There are six main families of heat shock proteins: HSP100, HSP90, HSP70, HSP60, HSP40 and the small heat shock proteins (sHsp) (Katschinski, 2002). Many Hsps require the formation of large polymeric complexes ranging in size from 100 kDa to 10 MDa (Fernando *et al*., 2003; Azzoni *et al*., 2004) for functionality; while others, like HSP40, work as co-chaperones, regulating and modulating the activity and substrate specificity of other Hsps (Tam and Heikkila, 1995). Heat shock proteins are key players in the following processes: DNA replication, RNA translation, protein folding and translocation through the

Figure 1: A model of heat shock factor function *in vivo*.

Heat shock factor (HSF) exists as an inactive monomer in the cytoplasm of the cell. Upon heat shock, or other stressor, HSF localizes to the nucleus. Here it trimerizes (thus becoming active) and binds to the heat shock element (HSE) located in the 5' untranslated region (UTR) of heat shock protein genes allowing for polymerase II to bind and transcribe the gene into mRNA.


HSF
Heat Shock
Nucleus
Cytoplasm
Pol II
Hsp gene
HSE
Hsp mRNA

endoplasmic reticulum and mitochondrial membranes, cell signalling and protection at higher temperatures (Tam and Heikkila, 1995; Heikkila *et al*., 1997b; Phang *et al*., 1999; Katschinski, 2004).

1.3 Small heat shock proteins

The small heat shock protein family is comprised of those Hsps with molecular weights between 16 and 42 kDa (Buchner *et al*., 1998; Heikkila, 2003; Stromer *et al*., 2003). The sHsp family differs from other Hsp families in its lack of conservation. The number, size and sequence of the sHsp family members vary from organism to organism; with intra-species variation being relatively small compared to the variation seen between organisms of different species (Arrigo and Landry, 1994; Stromer *et al*., 2003). They all consist of an amino terminal end and a carboxy terminal end with a variably-sized linker region (Azzoni *et al*., 2004). The only conserved region is a C-terminal domain composed of 80 to 100 amino acids, known as the α-crystallin domain (Lindner *et al.*, 1998; van den Ijssel *et al*., 1999; Heikkila, 2003). The domain name derives itself from the lens protein α-crystallin, to which sHsps are evolutionarily related, which is found in abundance in the vertebrate eye. The α-crystallin domain is thought to play a more significant role in the formation of the functional oligomeric complexes that sHsps are known to form than it does in their function as molecular chaperones (Quinlan and van den Ijssel, 1999; Wieske *et al*., 2001; Young *et al*., 2003). The flexible C-terminal allows interaction with cellular proteins, playing a key role in maintenance of target protein solubility (Fernando *et al*., 2003) and is required for sHsp function (Abdulle *et al*., 2002) in *Xenopus*, as well as in murine HSP25 and *Drosophila* HSP27 (Abdulle *et al*., 2002), as a molecular chaperone. Research has found that in many organisms, with the notable exception of the nematode,

the N-terminal end has little effect on chaperone function whereas deletion of amino acids from the C-terminal end of the protein results in a dramatic reduction in chaperone activity (Fernando and Heikkila, 2000; Abdulle *et al*., 2002). C-terminal truncation mutants of αA-crystallin lost the ability to prevent heat-induced aggregation (Takemoto *et al.*, 1993; Andley *et al.*, 1996). Similar results were evidenced with *Caenorhabditis elegans* HSP12.2 and HSP12.3 that lacked the extension (Kokke *et al.*, 1998). However, the N-terminal is thought to play a major role in the formation of oligomeric complexes (Merck *et al.*, 1992; Leroux *et al.*, 1997).

The number of members within the sHsp family varies depending on the species; one has been found in yeast, four in *Drosophila*, three in mammals, sixteen members have been detected in *Xenopus laevis*, and more than thirty have been identified in plants (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Arrigo and Landry, 1994; Tam and Heikkila, 1995; Ohan and Heikkila, 1995; Helbing *et al*., 1996; Heikkila *et al*., 1997b).

sHsps, not unlike α-crystallin, are not limited to any particular organ, tissue or cell type. Although this is generally the case, there are some sHsps that demonstrate constitutive tissue specific expression. Among these, human HSP27 was found in the epithelial cell lining of target organs for estrogen, including the placenta, connective and nervous tissue of the female reproductive tract (Ciocca *et al.*, 1993). Interestingly, in rats, HSP27 was found to accumulate in the lens, heart, stomach, colon, lung, bladder, anterior pituitary and all muscle types.

sHsps appear to be responsible for acquired thermotolerance as well as aiding in resistance against apoptosis (Romano *et al*., 2004) and other functions common to other Hsp families. In order to function within the cell, sHsps aggregate into large, oligomeric

complexes that remain separate from the final structures they help to form (Freeman and Yamamoto, 2002; Azzoni *et al*., 2004). Synthesis or mutation of small heat shock proteins have been associated with a variety of diseases including cancer, muscle myopathy, cataracts, multiple sclerosis, Alzheimer's disease and a number of other neuropathologies (Quinlan and van den Ijssel, 1999; Heikkila, 2003). Recent genetic evidence points to mutations in two human homologues of *Methanococcus jannaschii*, αA-crystallin and αB-crystallin, which are responsible for autosomal dominant congenital cataract and desmin-related myopathy, respectively (Quinlan and van den Ijssel, 1999; van den Ijssel *et al*., 1999).

As molecular chaperones, sHsps are efficient at interacting with non-native substrate proteins. They show no substrate specificity in binding to and preventing the aggregation of these proteins (Heikkila, 2003; Stromer *et al*., 2003). Although sHsps do not show substrate specificity, their morphology when binding substrates is predictable. sHsps can form both homo- and heterocomplexes but it has been shown that the morphology these complexes take depends on the first substrate bound each time and is independent of the sHsp itself (Stromer *et al*., 2003). The non-native proteins remain surface-exposed, allowing for continued interaction with antibodies while bound to the sHsp; however, these proteins are in a soluble but inactive state and are not refolded by sHsps. Once the stress has been removed, the sHsps are responsible for bringing their substrate protein to ATP-dependent Hsps, like HSP70, which will refold the protein into its native state in the presence of ATP. The sHsps are removed from the complex through phosphorylation, which alters the secondary structure and inhibits oligomerization (Fernando *et al*., 2003). It has been shown that all sHsps form large oligomeric complexes

of between 9 and 30 subunits in order to function (Ohan *et al*., 1998b; Heikkila, 2003; Stromer *et al*., 2003). This trend is conserved in a number of organisms including yeast, crustaceans, insects, nematodes, mammals and amphibians.

In addition to their thermoprotective function, sHsps have been implicated in cellular differentiation, the modulation of redox parameters and actin capping and decapping (Heikkila, 2003). Like other heat shock proteins, the small heat shock protein family has both constitutive and inducible members in many animal development systems including *Drosophila*, mouse, rat, brine shrimp and nematode (Heikkila, 2003). Many sHsps interact with the p38 MAP kinase pathway. Both mammalian HSP27 and HSP25 are phosphorylated by the MAPKAPK-2 stress-activated signaling pathway. It has also been determined that MAPKAPK-2 has a role in phosphorylating HSP30C from *Xenopus laevis* following both heat and chemical stress (Fernando *et al*., 2003). In the case of HSP30C, phosphorylation inactivates it as a molecular chaperone, altering its secondary structure and inhibiting oligomerization. This causes the release of HSP30C from its substrate (Fernando *et al*., 2003), which allows for other Hsps, including HSP70 to bind and refold the protein.

1.3.1 Oligomerization of sHsps

sHsps form high molecular weight aggregates with a molecular mass of approximately 100 kDa to 10 MDa under conditions of cellular stress (Arrigo and Landry, 1994; Fernando *et al*., 2003; Azzoni *et al*., 2004). Multimerization, as this process is known, is the result of the interaction of sHsp subunits and is believed to be crucial for chaperone activity (Ehrnsperger *et al.*, 1997; Leroux *et al.*, 1997). Several studies have linked aggregate size with the molecular chaperone activity of sHsps. The formation of

large oligomeric structures by sHsps is essential for their interaction with unfolded or partially unfolded proteins (Ehrnsperger *et al.*, 1997). While disagreements exist with respect to which amino acids are responsible for aggregate formation, both the conserved α-crystallin domain and the variable N-terminal region have been implicated. Studies involving the N-terminal and C-terminal domains of αA-crystallin showed the formation of large complexes with the N-terminus while the C-terminus formed smaller dimers and tetramers (Merck *et al.*, 1992).

1.3.2 Functional domains of sHsps

Small Hsps are evolutionarily related to the lens protein α-crystallin, whose gene contains a 5' HSE that can be induced by various stressors. Although there is limited sequence similarity within the sHsp family, there exist certain regions that display structural conservation among and between species (Lindquist, 1986). All sHsps contain two major domains, each consisting of two similar motifs, a moderately conserved carboxy-terminal region that corresponds to exon 1 of α-crystallin, and the highly conserved α-crystallin domain (de Jong *et al.*, 1993). The degree of correlation in the sequence of the α-crystallin domain ranges from ~20% between distantly related members to more than 60% between mammalian species (de Jong *et al.*, 1993; Caspers *et al.*, 1995; de Jong *et al.*, 1998).

Although the function of the flexible variable C-terminal extension is not clear, it has been suggested that its primary role is to maintain the solubility of the hydrophobic protein (Fernando and Heikkila, 2000; Abdulle *et al*., 2002; Fernando *et al*., 2003) and to help form the highly polymeric structures these proteins are known to form with target

substrates (Lindner *et al.*, 2000; Fernando *et al*., 2002). In the case of most C-terminal extensions, they consist mainly of polar amino acid residues (Ehrnsperger *et al.*, 1997; Fernando *et al*., 2002) and the replacement of these residues with hydrophobic ones resulted in the reduction of flexibility, heat-stability, solubility and chaperone activity (Smulders *et al.*, 1996). Conversely, the function of the highly conserved α-crystallin domain, found within the C-terminus, is well-known. Its function is to form defined oligomeric complexes (Ehrnsperger *et al.*, 1997). Arguably the most important function of the α-crystallin domain is its involvement in the molecular chaperone activity of the protein. The importance of the α-crystallin domain as a regulator of sHsp molecular chaperone activity has been demonstrated by the deletion of the last 42 amino acids in *Drosophila* HSP27, which resulted in the loss of its ability to protect cells against thermal stress (Jakob and Buchner, 1994). Murine HSP25 and αB-crystallin have been shown to inhibit actin polymerization although the stepwise deletion of the first three amino acids of the N-terminus of peptide 11, which corresponds to residues 92 to 106 of the full-length protein, reduced activity by ~25%, ~5% and ~0%, respectively. This finding provides direct evidence of an interaction of actin with defined sequence segments of either murine HSP25 or αB-crystallin, thus implying a function as an inhibitor of actin polymerization (Wieske *et al*., 2001).

1.4 The HSP30 Family

The HSP30s are a family of stress-inducible small heat shock proteins that act as molecular chaperones. HSP30 genes have only been isolated in fish, frog and avian species, indicating that these genes may be oviparous animal-specific. As these animals develop in an egg where temperatures are not constant, the probability for temperature

stress during development is high which would account for the need of an additional family of Hsps for survival (Katoh *et al*., 2004). Their function has been studied in chicken, fish and amphibians; however, much is left to be elucidated. The number of genes within the family varies depending on the species; three members have been identified in chicken (Panasenko *et al*., 2003), at least five members have been seen in *Xenopus laevis* (Ohan and Heikkila, 1995; Tam and Heikkila, 1995), up to 18 isoforms have been identified in Poeciliid fishes (Norris *et al*., 1995) and as many as ten have been found in *Rana catesbeiana* (Helbing *et al*., 1996).

1.4.1 Expression of HSP30

HSP30, like other sHsps, including those from *Drosophila*, brine shrimp, mouse, rat and nematode (Fernando and Heikkila, 2000), is differentially expressed during development (Heikkila *et al*., 1997b). Four complete genes in two gene clusters have been isolated from the HSP30 family in *Xenopus laevis*, two of which give rise to functional proteins (Ohan and Heikkila, 1995; Tam and Heikkila, 1995). The first cluster includes *hsp30A* and *hsp30B*, neither of which are representative of the HSP30 gene family, as *hsp30A* contains a 21 base pair insertion in the coding region and *hsp30B* has been identified as a pseudogene (Bienz, 1984a; Bienz, 1984b). The second cluster contains *hsp30C*, *hsp30D* and a portion of *hsp30E*. All are intronless and share a conserved α-crystallin domain and *hsp30C* and *hsp30D* both encode for 24kDa proteins (Heikkila, 2003). *Hsp30C*, the most extensively studied *hsp30* gene in *Xenopus*, contains 2 TATA boxes, 3 HSEs and a CCAAT box within its 5' regulatory region while the 3' UTR is AT-rich and contains both a polyadenylation element and an mRNA instability sequence (Heikkila, 2003).

Studies with early embryos show that none of the *hsp30* genes are constitutively expressed, nor are they heat inducible until at least the late neurula/early tailbud stage of development (Lang *et al*., 1999; Heikkila, 2003). Expression patterns indicate that there is a preferential accumulation of *hsp30* mRNA and protein in the cement gland, lens placode, somites and proctodeum of midtailbud embryos (Lang *et al*., 1999). Although studies using histone deacetylase inhibitor (HDI) treatments, which enhance the acetylation of lysine residues, resulting in a looser chromatin conformation, resulted in *hsp30* mRNA accumulation as early as gastrula stage embryos (Ovakim and Heikkila, 2003). HDI treatment only alters the temporal expression pattern; it did not in any way alter its spatial pattern of heat-induced *hsp30* mRNA accumulation in neurula and tailbud embryos. Similar results were obtained in *Drosophila hsp26* gene expression studies (Nightingale *et al*., 1998). The mechanism responsible for this phenomenon is as yet undiscovered.

There appears to be constitutive accumulation of *hsp30* mRNA in the cement gland of early and midtailbud embryos, although its presence there is transient. The transcripts found within the cement gland, however, do not coincide with any of the known mRNA of *hsp30* family members, leading to the belief that this is a thus far undiscovered member of the HSP30 family in *Xenopus laevis* (Lang *et al*., 1999). *Rana catesbeiana* HSP30 has been looked at briefly and its functions have not yet been thoroughly assessed although it has been shown to be present constitutively during metamorphosis in the liver, which may imply a protective role in developing embryos (Helbing *et al*., 1996).

In adult *Xenopus laevis*, heart tissue was found to have a lower HSF activation temperature than does the liver (Ali *et al*., 1997). Similar results were obtained with mouse pachytene spermatocytes (Sarge, 1995). Adult *Xenopus* A6 kidney epithelial cells have

been used for comparative studies with early embryos. These cells have been used to determine the effect of various stressors including heat shock, sodium arsenite, herbimycin A, and ethanol. All these stressors have been seen to induce *hsp30* gene expression and protein synthesis in *Xenopus* A6 cells in the cytoplasm (Heikkila, 2003; Gellalchew and Heikkila, 2005). Exposure causes transient but very different temporal patterns of *hsp30* mRNA accumulation and protein synthesis.

Studies of the Atlantic salmon, *Salmo salar*, have found that it possesses a homolog of the sHsp family, HSP30. Similar in identity to *Xenopus laevis* HSP30, *Poeciliopsis* HSP30, and to chicken HSP25, salmonid HSP30 also displays an inducible expression pattern. Juvenile Atlantic salmon exposed to common forms of hatchery stress (including a 26^{o}C heat stress) were found to have elevated levels of *hsp30* mRNA after 30 minutes (Zarate and Bradley, 2003). Although this indicates a stress response of the animal, the amount of time required to see an increase in *hsp30* levels makes it a poor indicator of stress in this regard. However, in both field and laboratory studies with Miramichi Atlantic salmon, *hsp30* mRNA, but not the protein, was significantly induced between 22^{o}C and 25^{o}C; 23^{o}C being the threshold for mRNA accumulation (Lund *et al*., 2002). Interestingly, *hsp30* mRNA followed the same pattern as *hsp70* mRNA. *Hsp30* expression patterns appear to be the least conserved among tissues in salmonids as yet another phenomenon was seen when looking at the relationship between Hsp levels and the hormonal stress response (Ackerman *et al*., 2000). *Hsp30* levels were compared with those of plasma cortisol, glucose and ion concentrations in response to various stressors associated with handling procedures. It was determined that handling procedures do not increase levels of *hsp30* and in many cases, including the netting of the fish prior to heat shock, actually

cause a significant decrease in *hsp30* levels in gill tissue compared to heat shock alone (Ackerman *et al*., 2000). Hightower *et al*. (1999) determined that there was a threshold for *hsp30* induction, a temperature that was rarely, if ever, selected for in the wild. Similarly, Currie *et al*. (2000) found that *hsp30* upregulation occurred at a set point that was unaffected by acclimation to different environmental temperatures.

The desert topminnow, *Poeciliopsis*, shows a high degree of variability in the HSP30 gene family. To date, 18 isoforms have been identified with only two or three being synthesized in more than one species (White *et al*., 1994; Norris *et al*., 1995). These genes show a high degree of biochemical variability, even among closely related species and this diversity appears to be geographically linked (Norris *et al*., 1995). Evidence shows that post-translational phosphorylation does not contribute to the diversity of HSP30 isoforms in *Poeciliopsis*, making it likely that they represent the products of individual genes (White *et al*., 1994). The number of isoforms is similar to that seen in both *Drosophila* and *Xenopus*. Like its counterparts in salmon and *Xenopus*, minnow *hsp30* is not expressed constitutively, but is heat-inducible and transient in its expression (Norris *et al*., 1997). *Hsp30* mRNA is induced after 2 h at heat shock temperatures, decreasing in intensity after 9 h of heat shock while the protein is not seen until approximately 6 h at heat shock temperatures. Interestingly, its synthesis declines in parallel with *hsp70* following heat shock. This phenomenon is also seen in *Xenopus* (Darasch *et al*., 1988; Tam and Heikkila, 1995).

Expression of sHsps in many vertebrate species is restricted to the muscle tissues and the eye lens. However, chicken HSP25, a member of the HSP30 family, is the first member to be expressed ubiquitously in tissues exposed to external stresses (Kawazoe *et*

al., 1999). Several studies have shown that the expression of HSP25 is not constitutive (Voellmy and Bromley, 1982; Wolfe and Zatz, 1994; Yahav *et al*., 1996; Kawazoe *et al*., 1999; Katoh *et al*., 2004), which coincides with similar findings in *Xenopus laevis* (Heikkila, 2003), but is inducible under several conditions of stress including lactacystin (Katoh *et al*., 2004), heat shock (Voellmy and Bromley, 1982; Wolfe and Zatz, 1994; Yahav *et al*., 1996; Kawazoe *et al*., 1999), and sodium arsenite (Wang *et al*., 1981). HSP25 is seen to accumulate in a perinuclear fashion, co-localizing with γ-tubulin (Wieske *et al*., 2001; Katoh *et al*., 2004) and requires the presence of both HSF1 and HSF3 for induction (Katoh *et al*., 2004). The expression profile of HSP25 being similar to that of the minnow (Norris *et al*., 1997) and *Xenopus laevis* (Lang *et al*., 1999). HSP25 is synthesized in fibroblasts, pineal cells (Wolfe and Zatz, 1994) and in a number of organs including the heart, liver, brain and lung; the greatest expression occurring in the liver (Voellmy and Bromley, 1982), and is expressed in all tissues of the developing embryo under conditions of hyperthermia (Kawazoe *et al*., 1999).

1.5 HSP27

HSP27 has been extensively studied in a variety of mammals, especially human. It is constitutively expressed at a basal level, being elevated upon heat shock or other stresses and is thought to play a role in determining the viability and function of a cell (Wataba *et al*., 2004). Its functions include anti-apoptotic protection through interaction with both pro-apoptotic and anti-apoptotic genes, including Bcl-2 and AKT (Efthymiou *et al*., 2004; Rocchi *et al*., 2004), cytoprotection by increasing thermal tolerance to heat shock and from multiple drugs and chemotherapeutic reagents (Rocchi *et al*., 2004), and as a molecular chaperone working to maintain the cell in a homeostatic condition (Efthymiou *et al*., 2004;

Ohnishi *et al*., 2004; Rocchi *et al*., 2004). Interestingly, overexpression of HSP27 in murine cells can increase heat tolerance (Ohnishi *et al*., 2004) and protect against myocardial infarction by decreasing the size of the infarct (Efthymiou *et al*., 2004), while in *Drosophila*, overexpression of the *hsp27* gene can increase longevity (Wang *et al*., 2004). It has been proposed that there is a paralogous relationship between *Xenopus* HSP30 and the HSP27 lineage (de Jong *et al*., 1993). Recent findings with *Poeciliopsis lucida*, a desert topminnow, lend credence to this hypothesis, pointing to a gene duplication event early in the evolution of vertebrates prior to the divergence of fish and tetrapods (Norris *et al*., 1997).

1.5.1 Expression of HSP27

Hsp27 is developmentally regulated, its expression varying depending on the tissue-type and developmental stage (Ciocca *et al*., 1993). For example, although in adult humans *hsp27* is constitutively expressed in breast, uterine, cervical, placental and skin tissues as well as in platelets, it is not detected in fetal organs (Ciocca *et al*., 1993). Conversely in rodents, *hsp27* is expressed in both fetal and adult lens, heart, stomach, colon, lung and bladder (Ciocca *et al*., 1993). Overexpression of murine *hsp27* in the heart has been linked with potent protection against lethal ischaemia/reperfusion injury by reducing the size of infarct (Rocchi *et al*., 2004). In *Drosophila*, the *hsp27* gene is activated along with the hormone, β-ecdysone (Ciocca *et al*., 1993) and may function to regulate some stages of *Drosophila* development. 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 is expressed as homopolymers that

range in size from 2- to 24-mers, depending entirely on the state of phosphorylation (Thériault *et al*., 2004). In the unstressed state, HSP27 exists in its unphosphorylated form and is found as a 24mer; however, under conditions of stress, the p38 pathway is activated and HSP27 is phosphorylated and dimers are the predominant form present (Thériault *et al*., 2004). *Poeciliopsis lucida*, the desert topminnow, has three isoforms of the HSP27 protein, each being a differently phosphorylated form of a single peptide (Norris *et al*., 1997). These phosphorylation sites are conserved and, as in mammals and avians, may be involved in the signal transduction to the actin cytoskeleton (Norris *et al*., 1997) mediated by changes in the phosphorylation state of HSP27 in response to stress, mitogens, and several inflammatory mediators (Arrigo and Landry, 1994). Finally, *hsp27* is induced by a number of naturally occurring components of the body including retinoic acid (in rodents; Ciocca *et al*., 1993), estrogen, tumor necrosis factor α (TNFα), interleukin 1, and thrombin (in humans; Ciocca *et al*., 1993).

1.6 Function of HSP30 and HSP27

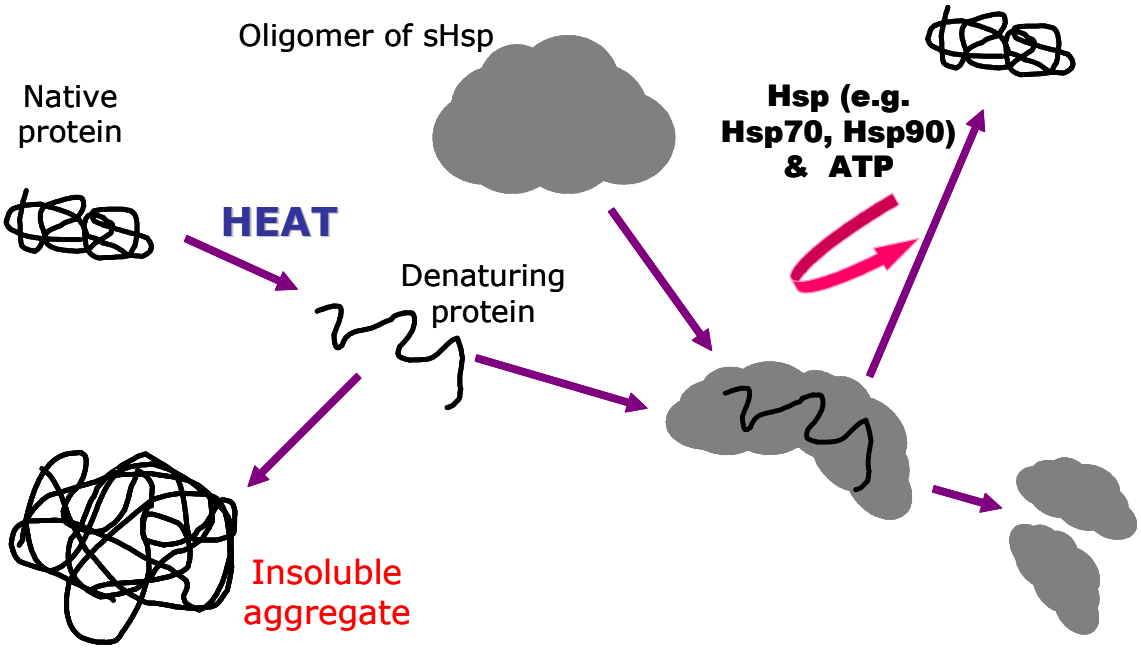
As with all other sHsps, HSP30 and HSP27 have been shown to function as molecular chaperones. A molecular chaperone is a protein which non-covalently (Robert, 2003) and transiently binds to exposed hydrophobic surfaces of non-native proteins and stabilizes these proteins through regulated binding and release, facilitating their correct fate *in vivo* (Agashe and Hartl, 2000; Höhfeld *et al*., 2001). Molecular chaperones function in a number of ways including folding following *de novo* synthesis, intramembranous transport, oligomeric assembly, switching between active and inactive conformations, intracellular transport and proteolytic degradation, either alone or with the help of co-factors (van den Ijssel *et al*., 1999; Höhfeld *et al*., 2001).

Following transcription, small heat shock proteins exist as large multimers in cells. Upon exposure to heat shock, a series of structural changes occur that result in a dissociated form of sHsp multimer whose hydrophobic binding surfaces are exposed, allowing for the binding of other proteins. At the same time, native proteins are denaturing as a result of the heat and either aggregate, in the absence of heat shock proteins, or complex with sHsps through interaction with their exposed hydrophobic residues. The sHsps prevent the further aggregation and denaturation of native proteins and maintain them in a state such that upon return to physiological conditions, they can be refolded in the presence of ATP by co-chaperones such as HSP70 and HSP90. In the absence of such co-chaperones, sHsp/substrate complexes often become large and insoluble aggregates that eventually precipitate out of solution (Figure 2).

Both HSP30 and HSP27 function in the protection of non-native proteins against their unfolding and aggregation in the presence of stressors. Although these sHsps are instrumental in maintaining their substrate proteins in a folding-competent state (Heikkila *et al*., 1997b; Abdulle *et al*., 2002; Fernando *et al*., 2003; Heikkila, 2003), they are incapable of reversing the heat-induced inactivation of enzyme activity (Fernando and Heikkila, 2000). This phenomenon has also been documented in mouse and pea (Lee *et al*., 1995; Ehrnsperger *et al*., 1997). Chicken HSP25 has been shown to preferentially bind to target proteins that are partially unfolded (Katoh *et al*., 2004). Similar results have been obtained with *Xenopus* HSP30C and HSP30D with luciferase (LUC) and citrate synthase (CS) (Fernando *et al*., 2003; Heikkila, 2003), HSP30C being capable of maintaining LUC in a folding-competent state such that under non-stressed conditions, co-chaperones were able to refold it and reactivate LUC to between 80 and 100% activity (Fernando *et al*.,

Figure 2: sHsp chaperone activity: a model.

Native proteins exist in the cell and upon heat or other stress, can become denatured. In the absence of sHsps, the denatured protein becomes an insoluble aggregate and can lead to cell death. However, in the presence of sHsps, the denaturing protein is maintained in a partially unfolded state until the stress is removed and physiological conditions recommence, at which time sHsps are phosphorylated, allowing for the release of the substrate protein. Other hsps, including HSP70, can then, in the presence of ATP, refold the protein. Adapted from van Montfort *et al*., 2002.


Oligomer of sHsp
Native protein
HEAT
Denaturing protein
Hsp (e.g. Hsp70, Hsp90) & ATP
Insoluble aggregate

2003). *Rana* HSP30 was also seen to prevent the heat-induced aggregation of CS and of DNA restriction endonuclease, *PstI* (Kaldis *et al*., 2004). Mutation analysis showed that the C-terminus is essential to function as a molecular chaperone (Fernando and Heikkila, 2000; Abdulle *et al*., 2002; Fernando *et al*., 2002); the removal of the last 25 amino acids of the C-terminus of HSP30C inhibited chaperone activity and precipitated out of solution at high concentrations (Fernando and Heikkila, 2000). The His-tag of recombinant protein, however, does not alter the function of recombinant protein (Friedrich *et al*., 2004).

In addition to preventing stress-induced aggregation of non-native proteins, both HSP30 and HSP27 have been implicated in the signal transduction to the actin cytoskeleton. In *Xenopus*, immunocytochemical analysis has shown that HSP30C co-localizes with F-actin and is enriched in the perinuclear region (Gellalchew and Heikkila, 2005). Similarly, in both chicken and murine cells, overexpression of HSP25 and HSP27 have been shown to stabilize microfilaments. HSP27 in humans, as well as in *P. lucida*, has been implicated in signal transduction to the actin cytoskeleton through the conserved phosphorylation sites (Arrigo and Landry, 1994; Norris *et al*., 1997). HSP27 in particular, has a role in the capping/decapping of actin. In its phosphorylated state, HSP27 decaps actin, allowing for the growth of the microfilaments. Conversely, when unphosphorylated, actin is capped and cannot elongate (Miron *et al*., 1991).

In addition to the functions that HSP27 shares with HSP30, this sHsp may also play a role in the modulation of redox parameters. Evidence shows that HSP27 plays a direct role in the interference of caspase activation points to its role in the modulation of oxidative stress (Rocchi *et al*., 2004). In *Drosophila*, *hsp27* has been shown to be a longevity gene, not only increasing the mean lifespan of the organism by up to 30% (Wang

et al., 2004), but it has also been shown to increase glucose-6-phosphate dehydrogenase activity, which maintains the cellular level of glutathione, thereby protecting cells from reactive oxygen species. In patients suffering from *Helicobacter pylori*-induced ulcers, treatment with an antioxidant, α-tocopherol, increased levels of HSP27 and reduced the resultant oxidative stress caused by the ulcers (Oh *et al*., 2005). A role of HSP27 in the signal transduction pathways of cell regulators seems to be undisputed. A variety of organisms and organ systems have been found where HSP27 is a part of individual signalling pathways including that of rat Leydig cells, human breast cancer cell growth, interleukin 1, TNFα, leukemia inhibitory factor and thrombin (Ciocca *et al*., 1993). It also binds cytochrome c from the mitochondria, protecting the cells against apoptosis (Wang *et al*., 2004). Also, work with human cells has identified HSP27 as the p29 estrogen receptor-associated protein (Ciocca *et al*., 1993); this finding coincides with the presence of HSP27 in human estrogen target organs.

Finally, HSP27 has been implicated in the anti-apoptotic pathways of mammals. In murine cells, it interacts with AKT, an anti-apoptotic protein, and maintains it in a biologically active form (Efthymiou *et al*., 2004). Research on various forms of cancer has revealed that HSP27 can have a negative effect on hormonal and drug treatments that signal apoptosis of proliferating cells. HSP27 appears to blocks apoptotic signals in response to these hormones, leading to poor prognoses for patients testing positive for HSP27 expression in both prostate (Rocchi *et al*., 2004) and breast cancers (Ciocca *et al*., 1993). In endometrial carcinomas, the presence of HSP27 correlated with the degree of tumour progression and differentiation as well as the presence of estrogen and

progesterone receptors (Ciocca *et al*., 1993) and has been correlated with a negative outcome in patients with gastric cancer.

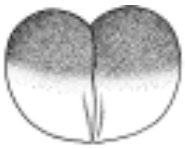
1.7 *Xenopus laevis* and *Rana catesbeiana* as model systems

The majority of Hsp work with amphibians has been done with *Xenopus laevis,* the African clawed frog. *Xenopus* has been well studied and its life cycle and much of its cellular and molecular biology has been characterized and extensively examined (Bienz 1984a, 1984b; Krone *et al*., 1992; Ali *et al*., 1996a, 1996b; Miskovic *et al*., 1997). *Xenopus laevis* has been used extensively as a model system for vertebrate development. The comparative ease of maintaining adult frogs in a laboratory setting makes the use of *Xenopus* ideal. Adult *Xenopus* females can be induced to produce thousands of eggs, which can be fertilized *in vitro* and the resulting embryos are easy to maintain and manipulate. The large size of *Xenopus* eggs and embryos provide an abundant source of RNA, DNA and protein, which was exploited in this study. Also, the characterization of *Xenopus* oogenesis and embryogenesis has been extensively documented at the cellular and molecular levels, providing researchers with a reliable, practical and predictable model of the various stages of embryological development (Figure 3). Further study of amphibians would be beneficial, as they experience a broad range of environmental conditions, and their sHsp response to these changes would expand the current knowledge base on amphibian stress response. It was for this reason that *Rana catesbeiana,* the North American bullfrog, was also studied here. *Rana* is indigenous to the temperate regions of northern United States and southern Canada, and as such its response to heat and other stressors is ecologically relevant. These frogs are exposed to hypoxic conditions while underwater or in burrows of leaf litter, where they spend the cold winter months (Tattersall

Figure 3: A pictorial view of the early developmental stages of *Xenopus laevis* from fertilized egg to tadpole.

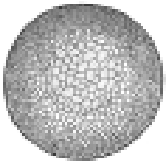
Adapted from Nieuwkoop and Faber, 1967.

Fertilized egg



Early cleavage (Stage 2)

Late cleavage (Stage 7)



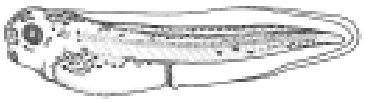
Blastula (Stage 9)

Gastrula (Stage 11)

Neurula (Stage 16)

Early tailbud (Stage 23)

Mid-tailbud (Stage 31)



Late tailbud (Stage 40)

Tadpole (Stage 46)

and Boutilier, 1997) and are capable of living at higher temperatures than most other North American frogs, due to their higher critical thermal maximum (Duellman and Trueb, 1986). Furthermore, *hsp30* and *hsp70* have been cloned and sequenced from *Rana* and their properties are similar to Hsps found in *Xenopus* (Helbing *et al*., 1996; Kaldis *et al*., 2004) and a cell line from tongue fibroblasts of *Rana* was available from ATCC, allowing for *in vitro* cell stressor studies. *Rana* HSP30 has not been characterized to the extent that the *Xenopus* HSP30 family has, providing a good research starting point.

1.8 Objectives

Most of our knowledge of HSP30 has come from research carried out with *Xenopus laevis*, the desert topminnow, salmon and recently chicken. Little is known, however, about the expression and function of HSP30 in *Rana catesbeiana*. Therefore, the main objective of this Master's thesis is to characterize the expression and function of the HSP30 protein in the North American bullfrog, *Rana catesbeiana*. During this research I also participated in a study that examined the expression and function of another small heat shock protein, *Xenopus laevis* HSP27. The main objectives of this study are listed below:

1. Subclone the cDNA encoding the open reading frame of *Rana catesbeiana hsp30* into an expression vector and generate an antisense riboprobe from it.
2. Northern blot analysis of *hsp30* gene expression in *Rana catesbeiana* FT cells exposed to heat shock or sodium arsenite.
3. Production and purification of recombinant forms of *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27.
4. Production and purification of rabbit polyclonal antibodies raised against *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27 recombinant proteins.
5. Western blot analysis of *Rana* HSP30 and *Xenopus* HSP27 protein levels in cells and/or embryos exposed to heat shock or sodium arsenite.
6. Immunocytochemical analysis of HSP30 in heat shocked *Rana catesbeiana* FT cells using confocal laser scanning microscopy (CLSM).
7. Determination of the molecular chaperone properties of *Rana catesbeiana* HSP30 and of *Xenopus laevis* HSP27 recombinant proteins in thermal aggregation assays.

2 Experimental Procedures

2.1 Maintenance of *Rana catesbeiana* FT cells and *Xenopus laevis* A6 kidney epithelial cells and embryos

2.1.1 Culturing of *Rana catesbeiana* FT and *Xenopus laevis* A6 kidney epithelial cells

The *Rana catesbeiana* FT tongue epithelial cell line was obtained from the American Type Culture Collection (ATCC) and cultured in 10% fetal bovine serum (FBS; Sigma, Oakville, Ontario) and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; Sigma) supplemented with 90% Leibovitz (L)-15 media (Sigma) at 22^{o}C in T75 cm^{2} flasks. Cell treatments were performed 1-2 weeks post-splitting to allow for 100% cell confluency, following the same protocol used for *Xenopus* A6 cell cultivation (Miskovic *et al*., 1997). Following treatment, cells were harvested and immediately stored at -80^{o}C.

The *Xenopus laevis* A6 kidney epithelial cell line was obtained from ATCC and cultured in 55% Leibovitz (L)-15 media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) at 22^{o}C in T75 cm^{2} flasks. Cell treatments were performed 2-3 days post-splitting to allow for 90-100% cell confluency, following the same protocol used by Miskovic *et al*. (1997). Following treatment, cells were harvested and immediately stored at -80^{o}C.

2.1.2 Treatment and harvesting of *Rana* FT cells

The expression of the *Rana* HSP30 protein was determined using heat shock as a cell stressor. Cells were initially exposed to temperatures of 22, 30, 33, 35 and 37^{o}C to determine the optimal temperature at which to conduct time course studies. Flasks

containing FT cells were immersed in water baths for 2 h at the above temperatures (with the exception of 22^{o}C controls) and then allowed to recover at 22^{o}C.

Following the 2 h recovery period, cells were immediately harvested. The media was aspirated off and 2 mL of 65% Hank's balanced salt solution (HBSS; Sigma) was added to rinse the cells. 1 mL of 100% HBSS was then added and the cells were scraped from the bottom of the flask and transferred to a 1.5 mL microcentrifuge tube. Cells were immediately pelleted by centrifugation in an Eppendorf Centrifuge 5415D microcentrifuge (Brinkman Instruments Ltd., Mississauga, Ontario) for 1 min at 13,200 rpm. The supernatant was discarded and the cells were stored at -80^{o}C.

2.1.3 *In vitro* fertilization and maintenance of *Xenopus* eggs and embryos

Xenopus laevis eggs were collected and fertilized according to the procedure outlined in Sive *et al*. (2000). Female *Xenopus* frogs (Boreal, St. Catharines, Ontario) were injected with 50 units (U) of human chorionic gonadotropin (hCG; Sigma) resuspended in sterile 0.65% (w/v) NaCl by injection into the dorsal lymph sac using a 26 gauge needle five days prior to induction. Approximately 9 to 10 h prior to egg collection, frogs were induced to hyper-ovulate with 1000 U of hCG. Eggs were then collected by applying gentle but firm pressure to the abdomen. Eggs were squeezed out into 55 x 15 mm petri dishes containing 1X Modified Barth's Saline (MBS; 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES-KOH, 2.5 mM $NaHCO_3$, pH 7.8).

Following egg collection, a male was sacrificed; the testes were excised, and placed in a petri dish containing 1X MBS. All MBS was removed from the petri dishes containing the eggs and, using a segment of testes, eggs were fertilized by touching a cut end of the testes to each egg. The eggs were then covered with 0.1X MBS and placed on an orbital

shaker for 20 minutes at room temperature to allow for sperm penetration. Evidence of penetration could be seen by the cortical rotation of fertilized eggs so that the animal pole faced upward. After fertilization, eggs were dejellied using 2% (w/v) L-cysteine (Sigma) in 0.1X MBS, pH 8.0. The embryos were subsequently washed 5 to 8 times in 0.1X MBS to remove residual L-cysteine. The embryos were then allowed to develop at 22^{o}C. Any dead or deformed embryos were immediately removed and 0.1X MBS was periodically replaced during the course of development. Developmental staging was determined by observing morphological changes, as outlined in Nieuwkoop and Faber (1967).

2.1.4 Treatment of *Xenopus* embryos

Embryos were collected at desired stages for control and heat shock treatments. Embryos to be heat shocked were placed in beakers containing 0.1X MBS, covered with parafilm and subsequently placed in water baths at either 33 or 35^{o}C for 2 h. Following heat shock, embryos were placed at 22^{o}C for a 2 h recovery period. Control embryos were maintained at 22^{o}C during the course of the heat shock treatment. Embryos were then staged and transferred to 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen and stored at -80^{o}C.

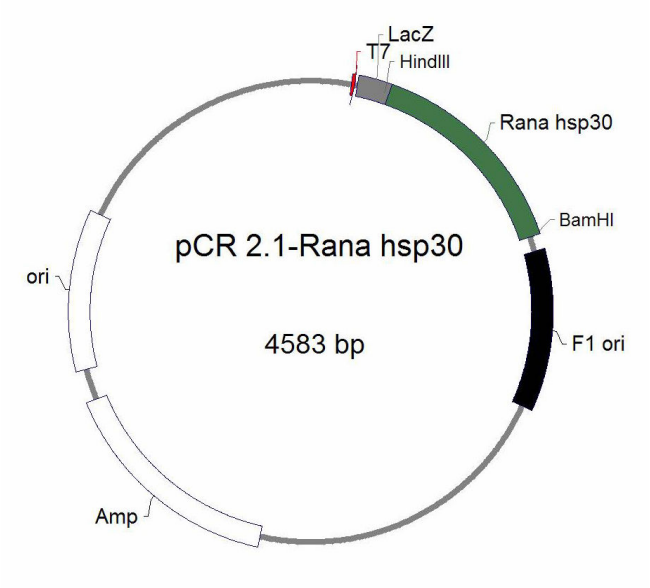
2.2 Characterization of *Rana hsp30* cDNA

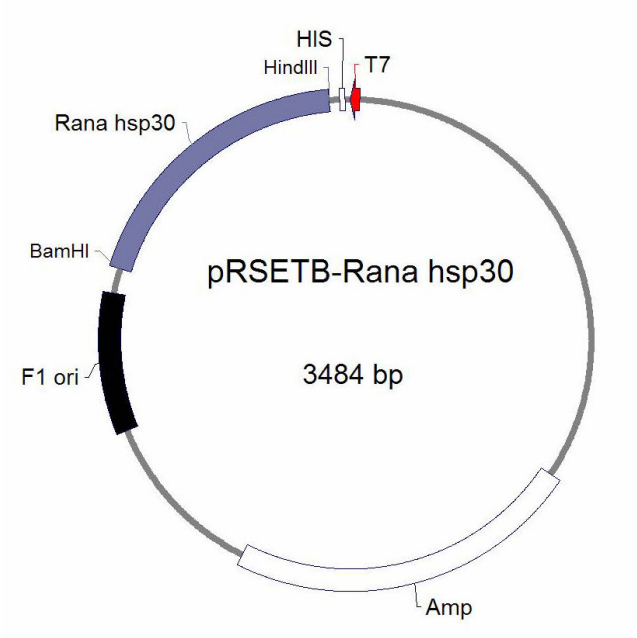
2.2.1 *Hsp30* cDNA

The *Rana catesbeiana hsp30* cDNA clone was obtained from Dr. Burr Atkinson (University of Western Ontario). The entire open reading frame (ORF) was contained within the pCR2.1 plasmid vector (Figure 4). The ORF was previously amplified by

Figure 4: Full-length *Rana catesbeiana hsp30* cDNA in the expression vector pCR2.1.

A cDNA encoding the open reading frame of *Rana catesbeiana hsp30* was a gift from Dr. Burr Atkinson (University of Western Ontario). The open reading frame was amplified by Angelo Kaldis and cloned into the expression vector pRSETB such that a *BamHI* site and a *HindIII* site were created as flanking regions of the open reading frame.


LacZ
T7
HindIII
Rana hsp30
BamHI
pCR 2.1-Rana hsp30
4583 bp
ori
F1 ori
Amp


HIS
HindIII
T7
Rana hsp30
BamHI
pRSETB-Rana hsp30
3484 bp
F1 ori
Amp

polymerase chain reaction (PCR; Angelo Kaldis, 2004), such that a *BamHI* restriction endonuclease (RE) site was created immediately upstream of the start codon and a *HindIII* RE site was created immediately downstream, of the translational stop codon and inserted into the pRSETB expression vector (Invitrogen, Carlsbad, California), as outlined in Sambrook and Russell. (2001). Plasmid vectors containing the *hsp30* insert were then transformed into *Escherichia coli* DH5α cells.

2.2.2 Isolation of plasmid DNA by phenol chloroform

Plasmids were isolated using the phenol chloroform method, as outlined in Sambrook and Russell, (2001). In order to accommodate use of a microcentrifuge, modifications were made to the volumes of the reagents. DH5α cells containing the plasmid of interest in 5 mL LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) supplemented with 100 µg/mL ampicillin (BioShop, Burlington, Ontario) that had been grown overnight at 37^{o}C, were pelleted by centrifugation at 5,000 rpm for 10 min at 4^{o}C in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) using a swinging bucket rotor. The supernatant was removed. The bacterial pellet was resuspended in 200 µL of ice-cold alkaline lysis solution I (50 mM glucose, 25 mM Tris (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, 1% (w/v) SDS) was added and gently mixed by slowly inverting the tube rapidly 5 times (tubes were stored on ice from this step onwards). Next, 200 µL of ice-cold alkaline lysis solution III (3 M potassium acetate, 5 M glacial acetic acid) was added and tubes were slowly inverted 3 to 5 times to disperse throughout suspension and then placed on ice for 5 min. Tubes were then centrifuged at 14,000 rpm for 5 min at 4^{o}C and the supernatant was transferred to a new

tube. RNA present within each sample was eliminated by adding 20 µL of RNase A (10 µg/mL; BioShop) and incubating at 37°C for 2 h. Following incubation, 600 µL of phenol:chloroform (1:1) was added to each tube and vortexed to mix. Tubes were centrifuged for 3 min at 4°C and the upper aqueous layer was transferred to a new tube and the phenol:chloroform step was repeated. The top aqueous layer was once again transferred to a new tube and 600 µL of isoamyl:chloroform (1:24) was added. Tubes were vortexed, centrifuged at the above settings and the upper aqueous layer was transferred to a new tube. Nucleic acids were precipitated using 600 µL of isopropanol. Samples were vortexed and allowed to stand at room temperature (RT) for 2 min followed by centrifugation at 13,200 rpm in an Eppendorf Centrifuge 5415 D microcentrifuge (Brinkman Instruments Ltd.) for 5 min at RT. The supernatant was removed and tubes were inverted to allow all fluid to drain out. Finally, 1 mL of 70% ethanol was added to the pellet and DNA was recovered by centrifugation at 13,200 rpm for 2 min at RT. The supernatant was removed and tubes were once again inverted to allow fluid to drain out. Once tubes were dry, the nucleic acids were dissolved in 50 µL of TE buffer (50 mM Tris, 2 mM EDTA; pH 8.0).

2.2.3 Isolation of plasmid DNA using the QIAgen QIAprep Miniprep Kit

To produce recombinant protein, cells must be grown in which to express the protein. To begin, one LB agar (1% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bacto-agar, pH 7.5) plate supplemented with ampicillin (100µg/mL) was streaked with the DH5α cells which contained the insert of interest and incubated at 37°C overnight. A broth culture of LB media (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) supplemented with ampicillin (100µg/mL) was inoculated with a single colony from the plate and grown at 37°C overnight with shaking in a

Gyrotory® Water Bath Shaker (New Brunswick Scientific, Edison, New Jersey). Using the Qiagen Kit, QIAprep Spin Miniprep Kit (250), and the buffers supplied within (Buffers P1, P2, N3 and PR), plasmids were isolated as outlined on pp.22-23 of the booklet contained within and outlined below. Cells were pelleted by centrifugation at 5,000 rpm for 10 min at 4°C in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) using a swinging bucket rotor. The supernatant was removed. The bacterial pellet was resuspended in 250 μL of Buffer P1 containing RNase A. The suspension was transferred to a microcentrifuge tube and an equal volume of Buffer P2 was added to each tube. Each tube was mixed gently by inverting tubes 4-6 times. Next, 350 μL of Buffer N3 was added to each lysis reaction and tubes were inverted as above and centrifuged for 10 min at 13,200 rpm in an Eppendorf Centrifuge 5415 D microcentrifuge (Brinkman Instruments Ltd.) at RT. The supernatants were the applied to the QIAprep column and centrifuged at above settings for 1 min. The flow-through was discarded and 750 μL of Buffer PR was added. Columns were centrifuged as above for 1 min, the flow-through discarded and centrifuged for an additional minute. The flow-through was discarded. The QIAprep column was then placed in a 1.5 mL microcentrifuge tube and DNA was eluted by adding 50 μL of Buffer EB (10 mM Tris-Cl, pH 8.5) to the column. The columns were allowed to stand for 1 min at RT prior to centrifugation for 1 min at the above settings. Eluted plasmids were stored at -20°C.

2.3 Characterization of *Xenopus* HSP27

2.3.1 *Hsp27* cDNA

The *Xenopus laevis hsp27* cDNA clone was obtained from ATCC. The ORF was sequenced by Mobix (McMaster University, Hamilton, Ontario). Previously in our laboratory, Norman Chan designed primers such that a *BamHI* RE site was created immediately upstream of the translational start site, and a *HindIII* RE site was created immediately downstream of the translational stop codon. The resulting fragment was cloned into the pRSETB expression vector (Invitrogen) and transformed into DH5α cells.

2.4 Preparation of Digoxygenin-labelled riboprobes

2.4.1 Subcloning of *Rana hsp30* cDNA and cloning of L8, actin and EF1α cDNAs

The ORF of the *Rana catesbeiana hsp30* cDNA, which was previously cloned into the pRSETB vector by Angelo Kaldis, was excised from this expression vector using the flanking *BamHI* and *HindIII* sites and incubating at 37^{o}C for 2 h (Figure 4). This was done because this expression vector provided for the creation of a sense riboprobe, not an antisense riboprobe, as there exists only the T7 promoter at the 3' end. The resulting fragment was run on a 1% (w/v) agarose gel and cut out using a razor blade. The gel fragment was then placed in a DNA extraction tube (Millipore Corp., Bedford, Massachusetts) and spun at 5,000 rpm for 10 min in an Eppendorf Centrifuge 5415D microcentrifuge (Brinkman Instruments Ltd.). DNA was precipitated once the filter was removed by adding 2x volume of 100% ice-cold ethanol and a 1/10x volume of 3 M sodium acetate (pH 5.2) and placing tubes at -80^{o}C overnight. Samples were then spun at

4°C for 10 min in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) and the supernatant was discarded. The pellet was rinsed with 120 µL of 70% ice-cold ethanol and spun at the above settings and the supernatant was discarded. Samples were air dried and resuspended in 20 µL of sterile water. The expression vector pBlueScript II KS (+) was likewise digested with *BamHI* and *HindIII* and electrophoretically separated under the same conditions. Both fragments were then ligated using a 1:1 ratio of plasmid: insert in a solution containing equal volumes of both 2X rapid ligation buffer (Promega, Madison, Wisconsin) and T4 DNA ligase (Promega) and being incubated at 22°C for 3 h (Figure 5). Plasmids were then transformed into either JM109, BL21 (DE3), or DH5α cells in 1:1 and 1:1.5 ratios cells: plasmids and streaked on LB agar plates supplemented with ampicillin (100 µg/mL), x-gal (20 mg/mL), and 1 M IPTG and incubated overnight at 37°C. Transformants were selected based on the colour of colony. White colonies indicated a successful transformation. One colony from the JM109 plate and one colony from the DH5α plate were aseptically transferred to 5 mL LB broth supplemented with ampicillin (100µg/mL) and incubated at 37°C overnight in a Gyrotory® Water Bath Shaker (New Brunswick Scientific). The following morning, cultures were used to isolate plasmids for production of antisense riboprobe, as outlined in section 2.2.2. Isolated plasmids were then singly digested with either *BamHI* or *HindIII* at 37°C for 2 h and run on a 1% (w/v) agarose gel, cut out and precipitated prior to being used for *in vitro* transcription reactions for the production of an *hsp30* antisense riboprobe.

The coding region of L8 was cloned into pBlueScript (present from Dr. Yun-Bo Shi from NIH) to generate pBlue-L8 (Figure 6). To synthesize antisense riboprobe, pBlue-

Figure 5: *Hsp30* template for *in vitro* transcription.

To generate *hsp30* riboprobes, the coding region of the *hsp30* gene was cloned into the *HindIII* and *BamHI* sites of pBlueScript (present from Dr. Brian Dixon). To synthesize antisense riboprobe, the vector was linearized with *BamHI* and transcribed *in vitro* with T7 polymerase.

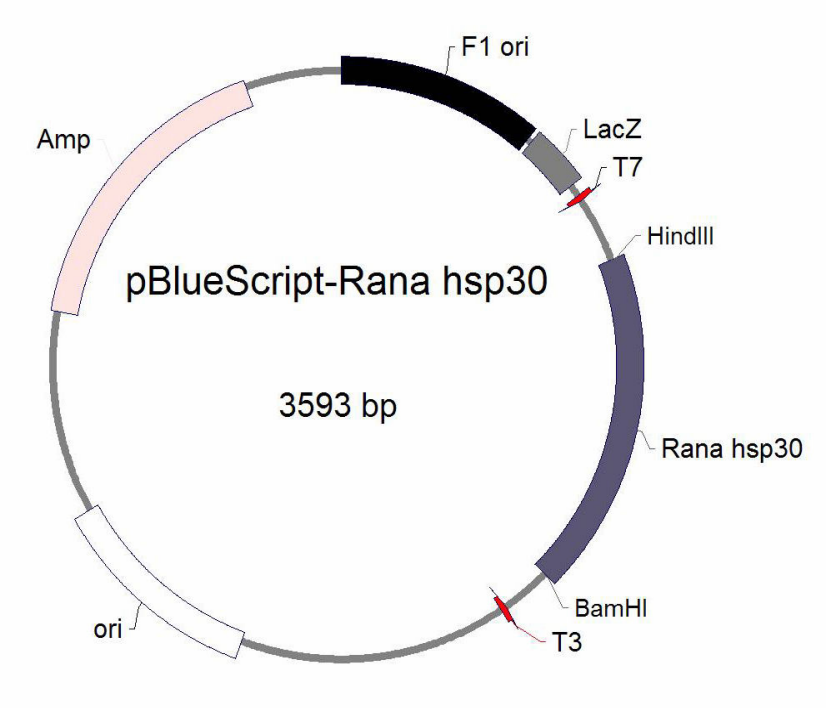
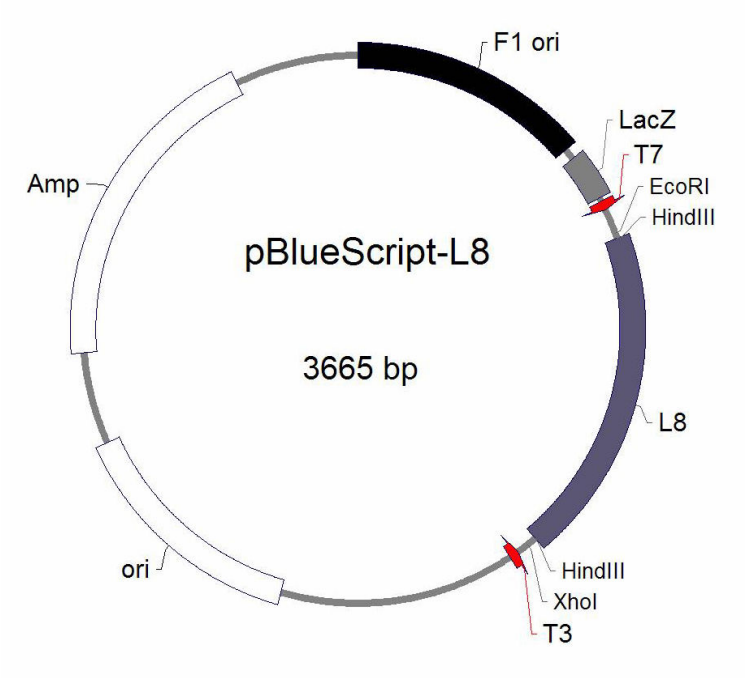
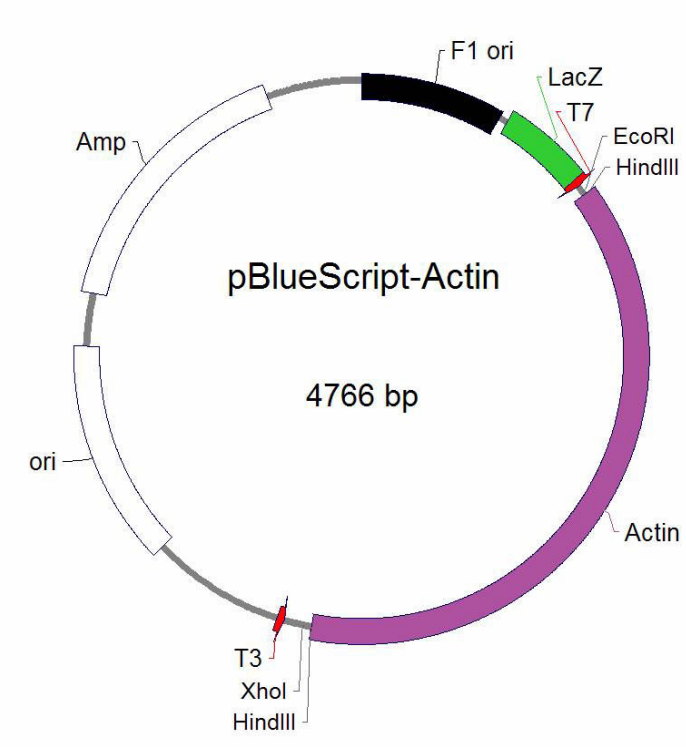

F1 ori
LacZ
T7
HindIII
pBlueScript-Rana hsp30
3593 bp
Rana hsp30
BamHI
T3
ori
Amp

Figure 6: Templates for L8 and Actin for *in vitro* transcription.

The coding region of L8 was cloned into pBlueScript (present from Dr. Yun-Bo Shi) to generate pBlue-L8. To synthesize antisense riboprobe, pBlue-L8 was linearized with *XhoI* and transcribed *in vitro* with T3 polymerase. The coding region of actin was cloned into pBlueScript to generate pBlue-actin. To synthesize antisense riboprobe, pBlue-actin was linearized with *EcoRI* and transcribed *in vitro* with T3 polymerase.


F1 ori
LacZ
T7
EcoRI
HindIII
Amp
pBlueScript-L8
3665 bp
L8
ori
HindIII
XhoI
T3


F1 ori
LacZ
T7
EcoRI
HindIII
Amp
pBlueScript-Actin
4766 bp
ori
Actin
T3
XhoI
HindIII

L8 was linearized with *XhoI* and transcribed *in vitro* with T3 polymerase (Figure 8, lanes 9 and 10).

The coding region of actin was cloned into pBlueScript (Gibco/BRL Laboratories, Burlington, Ontario) to generate pBlue-actin (Figure 6). To synthesize antisense riboprobe, pBlue-actin was linearized with *EcoRI* and transcribed *in vitro* with T3 polymerase (Figure 8, lanes 3 and 4).

The coding region of EF1α was cloned into pCMV-SPORT6 to generate pCMV-SPORT6- EF1α (ATCC, Figure 7). To synthesize antisense riboprobe, pCMV-SPORT6-EF1α was linearized with *SmaI* and transcribed *in vitro* with T7 polymerase (Figure 8, lanes 6 and 7).

2.4.2 *In vitro* transcription

Using the linearized vectors described previously, Digoxygenin (DIG)-labelled riboprobes were generated using *in vitro* transcription reactions. For each transcription reaction, 4 μL of linearized DNA template, 4 μL of rNTP mix (2.5 mM rATP, 2.5 mM rCTP, 2.5 mM rGTP, 1.625 mM rUTP (Promega), 0.875 mM DIG-11-UTP (Roche Molecular Biochemicals)), 1.5 μL DEPC-treated water 4 μL of 100 mM DTT (final concentration of 20 mM; Promega), 4 μL of 5x transcription buffer (final concentration of 1x; MBI Fermentas, Burlington, Ontario), 0.5 μL RNase inhibitor (MBI Fermentas) and 40 U of the appropriate RNA polymerase (T3 RNA polymerase (MBI Fermentas), T7 RNA polymerase (MBI Fermentas) or SP6 RNA polymerase (Roche Molecular Biochemicals)) were combined in a 1.5 mL microcentrifuge tube. The reaction was allowed to take place at 37^{o}C for 1 h, at which time an additional 40 U of the appropriate RNA polymerase was

Figure 7: EF1α template for *in vitro* transcription.

The coding region of EF1α was cloned into pCMV-SPORT6 to generate pCMV-SPORT6-EF1α (ATCC). To synthesize antisense riboprobe, pCMV-SPORT6- EF1α, was linearized with *SmaI* and transcribed *in vitro* with T7 polymerase.

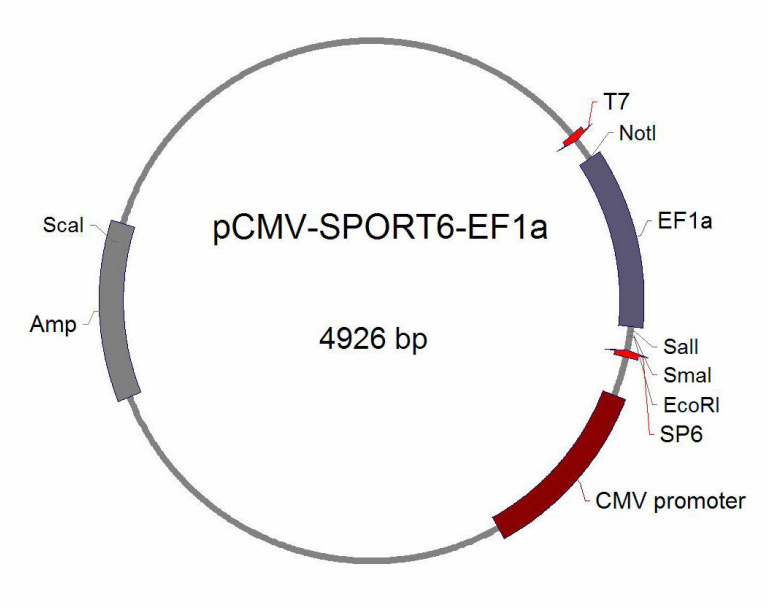
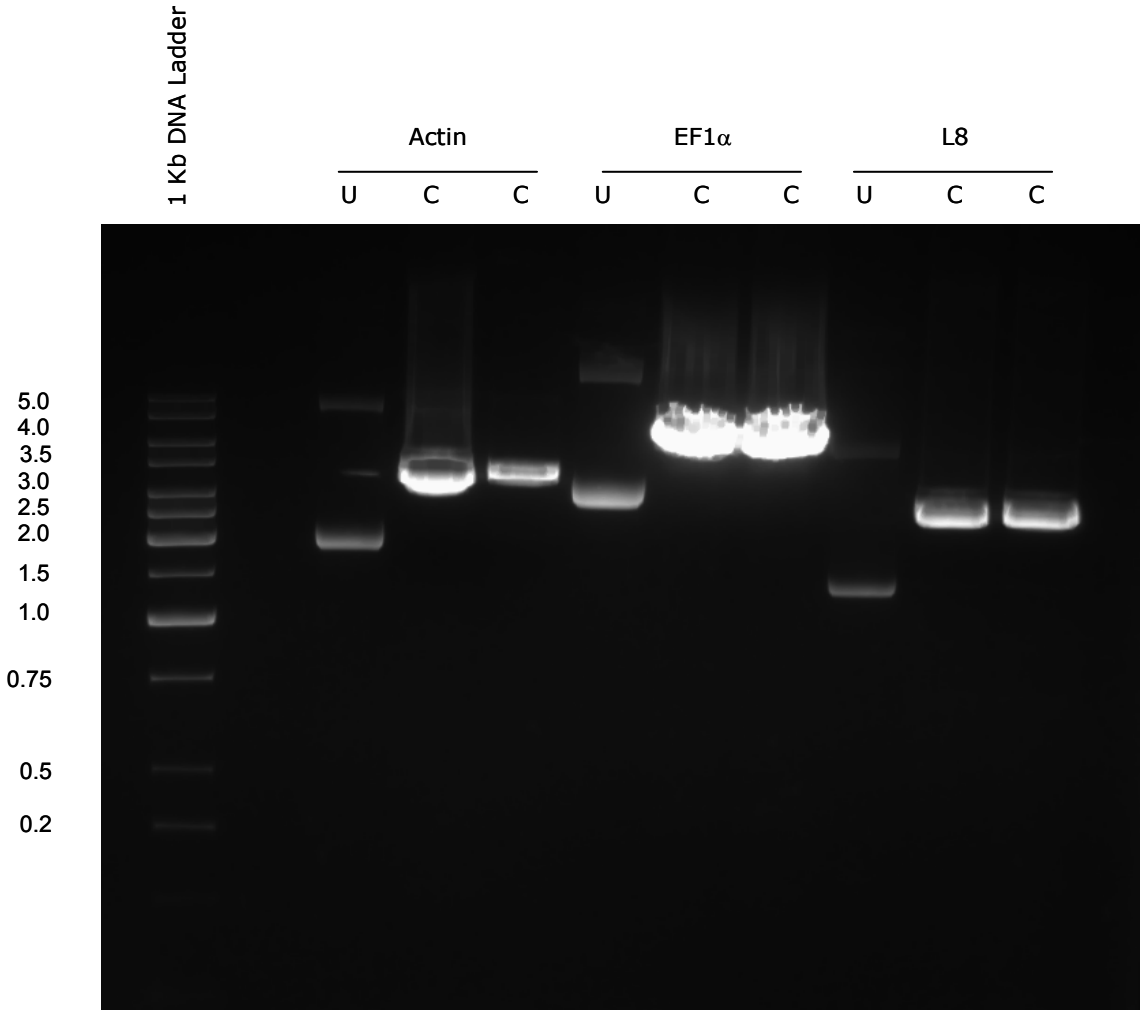

pCMV-SPORT6-EF1a
4926 bp
T7
NotI
EF1a
SalI
SmaI
EcoRI
SP6
CMV promoter
Amp
ScaI

Figure 8: Vectors involved in the production of riboprobes.

The vectors containing actin, EF1α and L8 cDNAs were linearized using *EcoRI, SmaI* and *XhoI*, respectively. Lanes 2, 5 and 8 show uncut (U) vectors containing their respective cDNA, while lanes 3, 4, 6, 7, 9 and 10 contain cut fragments (C) which were excised from the gel using a new razor blade for each band and precipitated out of the gel. The DNA was resuspended and used as a template for production of DIG-labelled antisense riboprobes. The molecular weight standard sizes are shown to the left of the gel in kilobases.


1 Kb DNA Ladder
Actin
EF1α
L8
U
C
C
U
C
C
U
C
C
5.0
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.75
0.5
0.2

added and incubated for an additional 1 h. The DNA template was then digested out of the reaction by adding 2 μL of RNase-free DNase I (Roche Molecular Biochemicals) for 10 min at 37^{o}C. To each tube, 10 μL of 3 M sodium acetate (pH 5.2), 80 μL of TES (10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 1% (w/v) SDS) and 220 μL of ice-cold 100% ethanol was added and incubated at -80^{o}C for 30 min. The precipitate was then pelleted by centrifugation for 15 min at 14,000 rpm at 4^{o}C in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) followed by the removal of the supernatant. The pellet was air dried and resuspended in 21 μL of DEPC-treated water, of which 1 μL was removed for electrophoretic analysis (Figure 9). The remainder was stored at -80^{o}C until use in northern blot analysis.

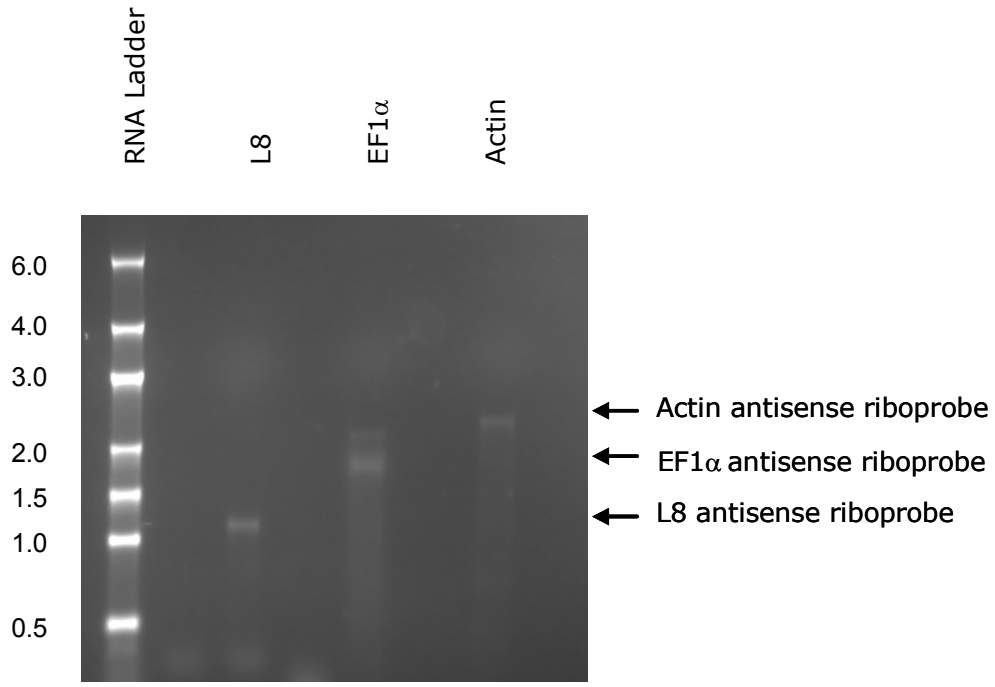
2.5 RNA Isolation and Northern Hybridization

2.5.1 Isolation and Quantification of RNA

RNA was isolated from FT cell treatments using the QIAgen RNeasy Mini Kit as detailed in RNeasy Mini Handbook Animal Cell Protocol (2001). RNA samples were then quantified by adding 5 μL of RNA in DEPC-treated water to 995 μL of sterile water and using UV-spectrophotometry at 260 nm in a Cary 50 Bio UV-visible spectrophotometer (Varian, Mississauga, Ontario). RNA integrity was checked prior to use in northern blot analysis using 1 μg of each sample on a 1.2% (w/v) formaldehyde agarose gel (1.2% (w/v) agarose, 10% (v/v) 10x MOPS (pH 7.0; 0.2 M 3-propane sulphonic acid, 50 mM anhydrous sodium acetate, 10 mM EDTA, pH 8.0) and 16% (v/v) formaldehyde). Prior to

Figure 9: DIG-labelled antisense riboprobes.

DIG-labeled antisense riboprobes, used in northern blot analysis, were made from cDNA templates for L8, EF1α and actin mRNA as outlined in Experimental Procedures. Probes were run on a 1.2% formaldehyde-agarose gel with ethidium bromide to ensure that they were transcribed. Northern blot analysis was then conducted using these probes as controls. The molecular weight standard sizes are shown to the left of the gel in kilobases.


RNA Ladder
L8
EF1α
Actin
6.0
4.0
3.0
2.0
1.5
1.0
0.5
Actin antisense riboprobe
EF1α antisense riboprobe
L8 antisense riboprobe

loading, 10 μL loading buffer (1 μL 10x MOPS, 1.6 μL formaldehyde, 2 μL RNA loading dye (0.2% (w/v) bromophenol blue, 1 mM EDTA (pH 8.0), 50% (v/v) glycerol), 5 μL formamide and 0.5 μg/mL ethidium bromide) was added to each sample. Samples were heat-denatured for 10 min at 68^{o}C, cooled on ice for 5 min and loaded into the gel and run for 45 min to 2 h at 90 V. Distinct 18S and 28S rRNA banding indicated intact RNA.

2.5.2 Northern Blot analysis

For Northern hybridization of *hsp30*, 5 μg of total RNA was electrophoresed in a 1.2% formaldehyde agarose gel as described above with the following exceptions. The loading buffer contained all components except ethidium bromide and the gel was electrophoresed for 2 to 3.5 h at 65 V. Following electrophoresis, the RNA in the gel was denatured by soaking the gel in 0.05 N NaOH for 20 min then rinsed in DEPC-treated water and soaked twice for 20 min each time in fresh 20x SSC buffer (3 M sodium chloride, 300 mM sodium citrate (pH 7.0)). The RNA was then transferred overnight to a positively charged nylon membrane (Roche Molecular Biochemicals) by capillary action as outlined here. A piece of blotting paper, which served as a wick, was pre-soaked in 20x SSC and placed on a plexiglass support over a Pyrex® dish containing 500 mL of 20x SSC. Onto this wick was placed the inverted gel, followed by a piece of nylon membrane slightly larger in dimensions than the gel itself. This was then covered by two pieces of blotting paper cut to the same size as the gel (also pre-soaked in 20x SSC), and paper towels were then stacked about 10 cm high on top of the blotting paper. A glass support and a weight of approximately 500 g were placed on top of the apparatus to aid transfer.

The following morning, the apparatus was disassembled and lanes were marked on the membrane using a lead pencil. RNA was UV-crosslinked twice to the membrane using

a UVC-515 Ultraviolet Multilinker (120,000 μJ/cm^2; UltraLum Inc., Claremont, California). The quality of the transfer was checked using 1x reversible blot stain (Sigma). The stained blot was photographed and then incubated in 50 mL of pre-heated prehybridization buffer (50% (v/v) formamide, 5x SSC, 0.02% SDS, 0.01% N-lauryl sarcosine, 2% blocking reagent) in a hybridization bag (SealPAK pouches, VWR, Mississauga, Ontario) at the appropriate hybridization temperature (60^{o}C for L8 and EF1a, 65^{o}C for actin and 68^{o}C for *Rana hsp30*) for 4 h in a Shake N' Bake Hybridization Oven (Boekel Scientific, Feasterville, Pennsylvania). After prehybridization, the buffer was replaced with hybridization buffer (same components as prehybridization buffer) containing the appropriate DIG-labeled antisense riboprobe and the membrane was returned to the hybridization oven overnight.

The membrane was then washed to remove any unbound probe. The membrane was first washed twice in 2x SSC and 0.1% (w/v) SDS at RT, then once each in 0.5x SSC and 0.1% (w/v) SDS and 0.1x SSC and 0.1% (w/v) SDS, both at hybridization temperature, for 15 min. The blot was then equilibrated for 1 min at RT in washing buffer (100 mM maleic acid buffer, 0.3% (v/v) Tween 20) and blocked using blocking solution (2% (w/v) blocking reagent, 10% (v/v) maleic acid buffer (pH 7.5)) for 1 h at RT. The blot was then incubated in blocking solution containing 1:8000 alkaline phosphatase-conjugated anti-DIG-Fab fragments (Roche Molecular Biochemicals) antibody for 30 min at RT. The membrane was then washed twice in washing buffer for 20 min each and then equilibrated in detection buffer (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl) for 2 min at RT prior to detection using CDP-star, a chemiluminescent reagent, which was applied to the membrane and allowed to develop in a hybridization bag for 10 min. The membrane was

then visualized on a Fluorchem 8000 Chemiluminescence and Visible Imaging System (filter position 1; Alpha Innotech Corp., San Leandro, California) for up to 30 min depending on the strength of the signal.

2.6 Cloning and expression of recombinant *Rana* HSP30 and *Xenopus* HSP30C and HSP27 proteins

The open reading frame of the *Rana hsp30* cDNA was cloned into the pRSETB vector downstream from the T7 DNA promoter and the histidine tag (Kaldis *et al*, 2004). *Escherichia coli* DH5α cells are good hosts for the pRSETB plasmid but for the production of recombinant protein, are not very good at actually producing the recombinant protein itself (Kroll *et al*, 1993). Therefore, the plasmid was isolated from these cells and transformed into *E. coli* BL21 (DE3) cells, which are much more adept at the production of recombinant protein.

Transformed DH5α cells were cultured in LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl), supplemented with 100 μg/mL ampicillin, overnight at 37^{o}C and then their plasmids were isolated using either the QIAgen Kit, QIAprep Miniprep Kit (250) or the phenol-chloroform method (Sambrook and Russell, 2001). Plasmids were then digested using appropriate DNA restriction endonucleases (all restriction endonucleases were obtained from Roche Molecular Biochemicals, Laval, Quebec) and then electrophoresed along with uncut plasmid samples in a 1% agarose gel to ensure that the plasmid insert was isolated successfully (Figures 10 and 11). BL21 (DE3) cells were propagated and transformed with the plasmids, cultured and then induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Figure 10: Isolation and verification of plasmids containing *Xenopus hsp30C* or *Rana hsp30* from DH5α cells.

Plasmids containing either *Xenopus hsp30C* or *Rana hsp30* cDNA were isolated from DH5α cells, as outlined in Experimental Procedures. Samples were electrophoresed on a 1% agarose gel to determine the approximate size of the insert. The sizes of the molecular weight are shown in base pairs.

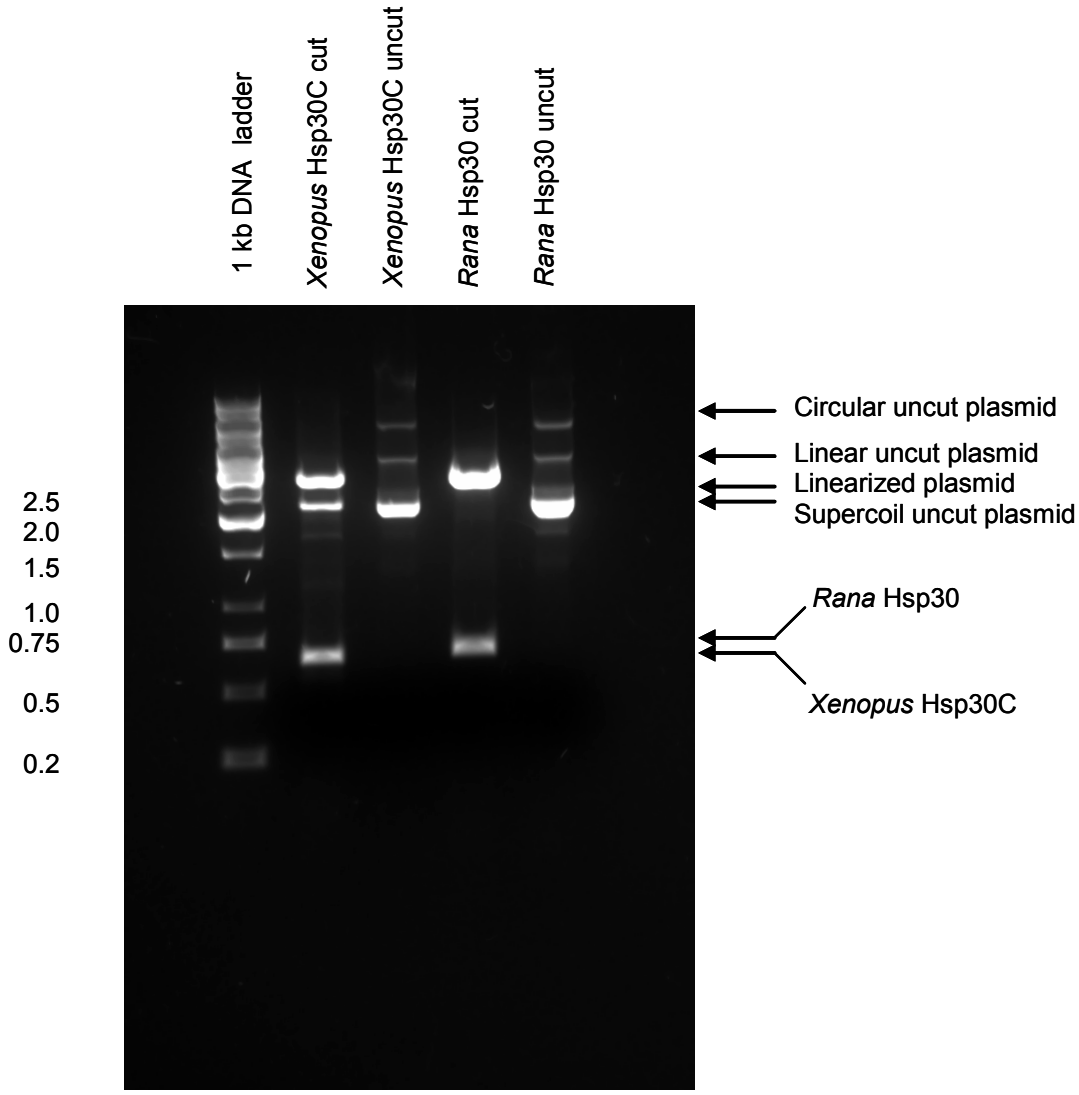
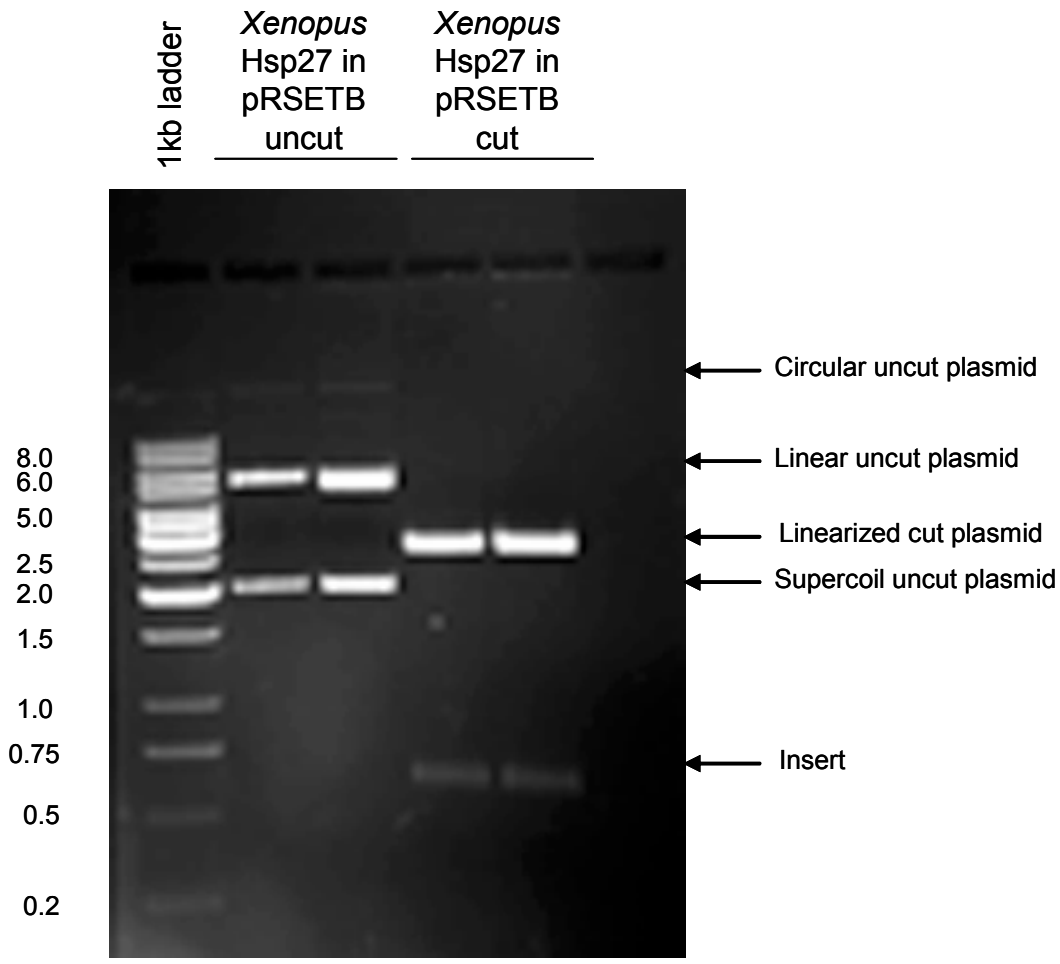

1 kb DNA ladder
Xenopus Hsp30C cut
Xenopus Hsp30C uncut
Rana Hsp30 cut
Rana Hsp30 uncut
2.5
2.0
1.5
1.0
0.75
0.5
0.2
Circular uncut plasmid
Linear uncut plasmid
Linearized plasmid
Supercoil uncut plasmid
Rana Hsp30
Xenopus Hsp30C

Figure 11: Isolation and verification of plasmids containing *Xenopus hsp27* from DH5ą cells.

Plasmids containing *Xenopus hsp27* cDNA were isolated from DH5α cells, as outlined in Experimental Procedures. Samples were electrophoresed on a 1% agarose gel to determine the approximate size of the insert. The desired insert is 741 base pairs. Sizes are shown in kDa.


1kb ladder
Xenopus Hsp27 in pRSETB uncut
Xenopus Hsp27 in pRSETB cut
8.0
6.0
5.0
2.5
2.0
1.5
1.0
0.75
0.5
0.2
Circular uncut plasmid
Linear uncut plasmid
Linearized cut plasmid
Supercoil uncut plasmid
Insert

Cells were homogenized in guanidium lysis buffer (6.0 M guanidine hydrochloride, 20 mM Na_2HPO_4, 500 mM NaCl, pH 7.8). Cells in guanidium lysis buffer were then sonicated using a Branson sonifier (Branson Sonic Power Co.) for 20 bursts and 3 repetitions (70% duty cycle and 5 output), then centrifuged at 3000 x g in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) for 25 min at 4^{o}C to remove cellular debris. The protein, which contained a 6-histidine residue tag at the N-terminal end, was purified under denaturing conditions on a nickel affinity column, which binds his-tagged proteins and allows others to pass through the column. Purified protein was then eluted from the column with Denaturing Buffer (pH 4.0; 8.0 M urea, 20 mM NaPO4, 0.5 M NaCl) and dialyzed in TEN buffer (50 mM Tris, 25 mM NaCl, 0.2 mM EDTA, pH 8.0), and concentrated using a MicroSep 3K concentrator column (Pall Filtration Corp., Northborough, Massachusetts) and spun at 3000 x g in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) and stored at -20^{o}C (Fernando and Heikkila, 2000).

2.7 Purification of and His-tag removal from recombinant protein

2.7.1 Purification of recombinant protein

A Poly Prep chromatography column (BioRad) was prepared by adding 2 mL of ProBond resin (Invitrogen) containing nickel and centrifuging at 800 x g for 2 min to remove the storage buffer. The column was washed twice with 5 mL of distilled water, followed by two washes with 5 mL of denaturing buffer (8.0 M urea, 20 mM $NaPO_4$, 0.5 M NaCl, pH 7.8). The bacterial lysate containing the recombinant protein was pH adjusted to 7.8 and applied to the nickel-coated column and bound for 30 min at RT. The recombinant protein was then purified under denaturing conditions according to

manufacturer's instructions with a few modifications. The column was washed with denaturing buffer three times at pH 6.0, five times at pH 5.3 and three times at pH 5.0. The protein was then eluted off the column by gravity with the addition 10 mL of denaturing buffer at pH 4.0. The eluted protein was then dialyzed against TEN buffer (50 mM Tris, 25 mM NaCl, 0.2 mM EDTA, pH 8.0) for 15 h at 4^{o}C. Following dialysis, the purified protein was concentrated using a MicroSep 3K Concentrator Column (Pall Filtration Corp.) by centrifugation at 3,000 x g at 4^{o}C.

2.7.2 His-tag removal

Purified recombinant protein destined to have its His-tag removed was dialysed for 15 h against 1X EnterokinaseMax™ (EKMax™) reaction buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, 0.1% (v/v) Tween-20, pH 8.0) at 4^{o}C. The protein was then concentrated as outlined in Section 2.7.1. EKMax™ (Invitrogen) was added, undiluted to fusion protein such that it was in a 1:5 ratio of EKMax™:fusion protein. A 1X concentration of EKMax™ buffer (Invitrogen) was added to the reaction mixture and incubated at 37^{o}C for 16 hours to allow for cleavage of the His-tag from the recombinant protein. Following incubation, the reaction mixture (pH 7.8) was bound to a Poly Prep chromatography column (BioRad) with ProBond resin (Invitrogen) containing nickel for 1 h at RT to remove the EKMax™-His-tag complex. The eluted protein was collected, quantified as outlined in Section 2.11.2 and stored at -20^{o}C until use.

2.8 Protein analysis by SDS-PAGE

Protein samples were prepared by resuspending the pelleted cells in TEN buffer (50 mM Tris-Cl, 2.5 mM NaCl, 0.2 mM EDTA, pH 8.0) followed by sonication (output 5,

70% duty cycle for 20 bursts) using a Branson sonifier (Branson Sonic Power Co.) and centrifugation at 5,000 x g in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) at 4^{o}C for 25 min. The supernatant was transferred to a new tube and the pellet was resuspended in 20 µL of TEN buffer and mixed with 1x loading buffer (0.0625 M Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.00125% bromophenol blue). The samples were then boiled for 10 min and separated from the other cellular content by SDS-PAGE. SDS-PAGE gels were made up of a separating gel (12% acrylamide, 0.32% n'n'-bis methylene acrylamide, 0.375 M Tris (pH 8.8), 1% SDS, 0.2% ammonium persulfate (APS) and 0.14% tetramethylethylenediamine (TEMED), which, upon pouring, were layered with 100% ethanol to ensure a smooth surface. Once the separating gels solidified, the ethanol was removed and stacking gels (4% acrylamide, 0.11% n'n'-bis methylene acrylamide, 0.125 M Tris (pH 6.8), 1% SDS, 0.4% APS and 0.21% TEMED) were cast on top of the separating gel and combs were inserted in the gel until solidified, at which time they were removed. Gels were cast in a Mini Protean II gel apparatus (BioRad) and the apparatus was placed in a tank with 800 mL of 1x electrophoresis buffer (25 mM Tris, 0.2 M glycine, 1 mM SDS). Samples were electrophoresed at 90 V through the stacking gel and at 150 V until the dye front had reached the bottom of the gel. Gels were stained using Coomassie Brilliant Blue R-250 (40% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue R-250) for 1 h at RT and then destained in destaining solution (10% acetic acid, 5% methanol) overnight followed by transillumination exposure using a Fluorchem 8000 Chemiluminescence and Visible Imaging System (filter position 2; Alpha Innotech Corp.) (Fernando and Heikkila, 2000).

2.9 Polyclonal antibody production

Recombinant protein of interest was diluted to 1.0 μg/μL in sterile 0.85% saline solution followed by the addition of an equal volume of Freund's Complete Adjuvant (Sigma). The mixture was emulsified using an emulsification needle for 30 min and then expelled into a sterile 1 mL syringe with an 18-gauge needle on the end. Prior to injection, 10 mL of blood was collected from the rabbit from the marginal ear vein for use as pre-immune serum. Once collected, the blood samples were allowed to clot overnight at 4^{o}C and then centrifuged at 14,000 rpm at 4^{o}C for 60 min in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) to separate the serum from the rest of the blood, the serum was then aliquoted and stored at -20^{o}C. The rabbit was given three additional protein injections at 3-week intervals with the Freund's Incomplete Adjuvant (Sigma) being substituted for Freund's Complete Adjuvant. At the end of the twelve week period, the rabbit was euthanised.

A 1.5 mL nickel affinity column containing either bound *Rana* HSP30 or *Xenopus* HSP27 recombinant protein (via the six-histidine residues at the N-terminus) was used for the affinity purification of their respective antibodies from the rabbit anti-sera. The column was initially equilibrated in equilibration buffer (50 mM Tris, 2 mM NaCl, pH 7.4), followed by the addition of 2 mL of crude anti-serum diluted 4 times in TBST (25 mM Tris, 150 mM NaCl, 2.5 mM KCl, 0.1% Tween-20), which was allowed to filter through the column by gravity at 4^{o}C. The column was subsequently washed 5 times with 5 mL of equilibration buffer followed by 5 washes of 5 mL of wash buffer (50 mM Tris, 150 mM NaCl, pH 7.4). Elution of the antibody from the column was achieved by soaking the column in 4 M $MgCl_2$ for 15 min at 4^{o}C. This step was repeated and eluants were pooled

and dialyzed against TEN buffer for 15 h at 4°C and then concentrated in a MicroSep 3K Concentrator column (Pall Filtration Corp.).

2.10 Antibody titre by ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed on sera samples beginning with the pre-immune and primary injection sera to determine if an immune response was elicited and to obtain a relative comparison of the titre of the antibody. Recombinant HSP30 or HSP27 and bovine serum albumin (BSA) (experimental and control antigens, respectively) were first diluted to 10 μg/mL in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) and 100 μL was pipetted into the appropriate wells of a 96-well plate. Coating buffer alone was added in 100 μL aliquots to the remaining wells and the plate was covered in parafilm and incubated at 4°C overnight. The wells were then washed 3 times with TBS-Tw (25 mM Tris, 150 mM NaCl, 2.5 mM KCl, 0.05% Tween-20, pH 8.0). All wells were then blocked for 2 h at RT with 300 μL of blocking solution (5% skim milk powder (w/v) in TBS-Tw). The blocking solution was then removed and serial dilutions of the anti-sera (1:100, 1:2,000) in blocking solution were put into the wells and incubated at 37°C for 2 h. The wells were then washed 3 times with TBS-Tw as above. Alkaline phosphatase-conjugated goat anti-rabbit IgG (100 μL; BioRad, Toronto, Ontario) secondary antibody was added at a dilution of 1:1,000 in blocking solution to the respective wells and incubated at 37°C for 2 h. The Sigma *Fast*™ p-NPP substrate solution (Sigma) was prepared according to manufacturer's instructions. The secondary antibody was removed and the plate was washed 3 times in TBS-Tw as above prior to the addition of 100 μL of the substrate solution. The colour reaction was

allowed to develop in the dark for 30 min prior to the addition of 100 μL of 0.03 M NaOH, which stopped the reaction. The optical density of each sample was then read at 405 nm on a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, California).

2.11 Protein isolation and Western blot analysis

2.11.1 Protein isolation from FT and A6 cells

Rana FT or *Xenopus* A6 cells were homogenized using a Teflon pestle and homogenization buffer (200 mM sucrose, 2 mM EGTA, 1mM EDTA, 40 mM NaCl, 30 mM HEPES) and then sonicated (output 4.5, 65% duty cycle for 15 bursts) using a Branson sonifier (Branson Sonic Power Co.). Samples were then centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5810R (Brinkman Instruments Ltd.) for 25 min at 4^{o}C to pellet cellular debris. The supernatant was transferred to a new tube, quantified using a BCA protein assay kit (Pierce, Rockford, Illinois) and stored at -20^{o}C.

2.11.2 Protein quantification

Recombinant protein was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce) according to manufacturer's instructions. A 2 mg/mL stock of BSA was diluted to make a series of dilutions from 0 mg/mL to 2 mg/mL in 0.2 mg/mL increments. A blank using 50 μL of TEN buffer was also prepared. A series of dilutions of the recombinant protein was prepared in sterile water. Into the wells of a 96 well assay plate, 10 μL of each of the BSA standard dilutions and the unknown protein concentrations was loaded in triplicate. BCA reagents A and B were combined in a ratio of 50:1 and 80 μL was loaded into each well and pipetted 3 times to mix thoroughly. The plate was incubated

at 37°C for 30 min followed by a 10 min cooling at RT. The plate was then read at 562 nm using a Versamax Tunable microplate reader (Molecular Devices) and the Soft Max pro program. The BSA standards were then used to generate a standard curve, from which the concentrations of each protein sample was determined.

2.11.3 Native pore exclusion limit electrophoresis

The ability of *Xenopus* HSP27 to form oligomeric complexes was examined by native pore exclusion limit electrophoresis. Polyacrylamide gels with a 4-20% linear concentration gradient were obtained from BioRad. The samples were prepared for electrophoresis by adding a 5x native loading buffer (0.0625 M Tris (pH 6.8), 10% glycerol, 0.00125% bromophenol blue) to a final concentration of 1x. *Xenopus* HSP27 recombinant protein and the protein standards (bovine serum albumin, carbonic anhydrase and urease) were electrophoresed (15 μg each) at 100 V for 40 hours at 4°C to allow for maximal separation in 1x running buffer (25 mM Tris, 0.2 M glycine). The proteins were then visualized by Coomassie staining and photographed as described for SDS-PAGE.

2.11.4 Western blot analysis

Protein was first separated by SDS-PAGE and gels were incubated in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 min. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Nepean, Ontario) using a BioRad Trans-Blot Semi-Dry Transfer Cell (BioRad) at 25 V for 40 min. Ponceau S staining was then performed to determine the effectiveness of the transfer. The membrane was then rinsed with sterile water and incubated in blocking solution (5% skim milk powder (w/v) in 2 mM Tris (pH 7.5), 30 mM NaCl, 0.1% Tween-20 (TBS-T)) for 1 h

followed by incubation with the primary antibody, rabbit anti-*Rana catesbeiana* HSP30 antibody or rabbit anti-*Xenopus laevis* HSP27 antibody, at a 1:3000 dilution in blocking solution for 1 h. The blot was subsequently washed with TBS-T and then incubated with the secondary antibody, horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (BioRad), at a 1:3000 dilution in blocking solution for 1 h. Blots were then washed 3 times in TBS-T for 10 min and exposed to ECL+ chemiluminescent detection reagent (Amersham Biosciences, Piscataway, New Jersey) for 5 min and then detected using a Fluorchem 8000 Chemiluminescence and Visible Imaging System (filter position 1; Alpha Innotech Corp., San Leandro, California) for up to 15 min depending on the strength of the signal.

2.12 Thermal aggregation assays

Molecular chaperone studies were conducted to determine the ability of *Rana catesbeiana* HSP30 and *Xenopus laevis* HSP27 to prevent target protein aggregation. Citrate synthase (CS), luciferase (LUC) and malate dehydrogenase (MD) were used as substrate proteins in the aggregation assays. Each target protein was exposed to elevated temperatures known to cause aggregation for the specific protein (42^{o}C for both CS and LUC and 45^{o}C for MD), either alone at a 150 nM concentration in a 50mM HEPES-KOH (pH 7.5) or in varying molar ratios with the sHsp being studied in a final volume of 1 mL. Light scattering was determined by measuring the absorbance with a Cary 50 Bio UV-visible spectrophotometer (Varian) at 320 nm every 10 min over a 60-120 min time period. An increase in absorbance was indicative of protein aggregation (Fernando and Heikkila, 2000; Abdulle, *et al*., 2002).

2.13 Immunocytochemistry and Confocal Microscopy

Glass cover slips were acid washed in 2:1 nitric acid: hydrochloric acid, followed by rinsing in distilled water until the pH of the solution was between 6.7 and 7.0 as per the protocol outlined in Spector *et al*. (1998). Cells were plated on glass cover slips 24-48 h prior to fixing to ensure adhesion. Cells were then heat treated for 2-8 h at 35^{o}C followed by a 2 h recovery at 22^{o}C. Controls were maintained at 22^{o}C until time of fixing. Overlying media was aspirated and cover slips were washed twice with phosphate buffered saline (PBS; 8% NaCl, 0.2% KCl, 0.2% KH_2PO_4, 2.1% $Na_2HPO_4 \cdot 10H_2O$, 1 mM $MgCl_2$, 1 mM $CaCl_2$) solution for 3 min each at RT. Cover slips were then transferred to Parafilm-lined Petri dishes and fixed in paraformaldehyde (3.7% (w/v) paraformaldehyde, PBS) for 30 min and rinsed three times for 5 min each in PBS. Cells were then permeabilized in Triton X-100 (Sigma; 0.3% (v/v) Triton X-100, PBS) for 10 min, washed three times for 2 min each in PBS and blocked in BSA fraction V (Sigma; 3.7% (w/v) BSA, PBS, filter sterilized using a 0.4 μm filter (Pall Filtration Corp.)) for 1 – 16 h.

The following procedure was carried out in the dark. For indirect labelling using Fluorescein-5-isothiocyanate (FITC), cells were incubated with 1:500 rabbit anti-*Rana* HSP30 polyclonal antibody (in 3.7% BSA) for 1 h. The primary antibody was recovered and cells were washed three times for 2 min each wash with PBS then incubated with secondary mouse anti-rabbit IgG (whole molecule) FITC conjugate (Sigma) antibody at 1:320 in 3.7% BSA for 30 min. After incubation with FITC, cells were washed three times for 3 min each in PBS. Cells were then incubated with rhodamine-tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, Oregon) conjugate for 30 min then washed as above. Cover slips were then air dried and mounted in one drop of VectaShield

(Vector Laboratories Inc., Burlingame, California) on a glass slide and sealed with clear nail polish. Slides were stored in the dark at 4^{o}C overnight until visualization with a Zeiss Axiovert 200 microscope and LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, Ontario) as per manufacturer's instructions.

3 Results

3.1 *Rana catesbeiana hsp30* gene expression

3.1.1 Cloning of *Rana catesbeiana* cDNA into an expression vector and generation of an antisense riboprobe

Examination of the *Rana* small heat shock protein, *hsp30*, gene expression required the preparation of an antisense riboprobe from the cDNA clone. The open reading frame (ORF) was excised from the pRSETB vector using *BamHI* and *HindIII* restriction enzymes and successfully cloned into the pBlueScript expression vector. Restriction endonuclease digestion of the construct with *BamHI* and *HindIII* yielded the expected fragment of approximately 700 base pairs (Figure 12). Following the isolation of the pBlueScript-*Rana hsp30* construct from JM109 and DH5α cells, plasmids were digested with *BamHI* and transcribed with T7 RNA polymerase to produce an antisense riboprobe against *Rana hsp30* mRNA.

3.1.2 *Rana hsp30* mRNA accumulates in response to heat shock

Experiments on the accumulation of *Rana hsp30* were carried out using fibroblast tongue cells from *Rana catesbeiana* (Figure 13). Initial experiments examined the effect of different temperatures on the accumulation of *hsp30* mRNA in *Rana* FT cells (Figure 14). *Hsp30* mRNA occurred as a doublet in Northern blot analysis. *Rana hsp30* mRNA levels were slightly elevated at 33^{o}C, peaked at 35^{o}C and not detectable at 37^{o}C. In subsequent experiments, both 33^{o}C and 35^{o}C were used as FT cell treatment temperatures for *hsp30* mRNA accumulation studies.

Figure 12: Agarose gel electrophoresis of the restriction enzyme-digested pRSETB-*Rana hsp30* cDNA construct.

The pBlueScript vector DNA was digested with *BamHI*, while pRSETB-*Rana hsp*30 was digested with *BamHI* and *HindIII* as described in Experimental Procedures. The digestion products were then electrophoresed in 1% agarose gels and stained with ethidium bromide. The linearized pBlueScript vector is shown in lane 2 and the digested pRSETB vector and *Rana hsp30* fragment is shown in lane 3. Asterisks indicate the pBlueScript vector (linearized) and the *Rana hsp30* fragment that were excised from the gel and used for the production of the antisense riboprobe. The molecular weight markers (in kDa) are shown in the left column.

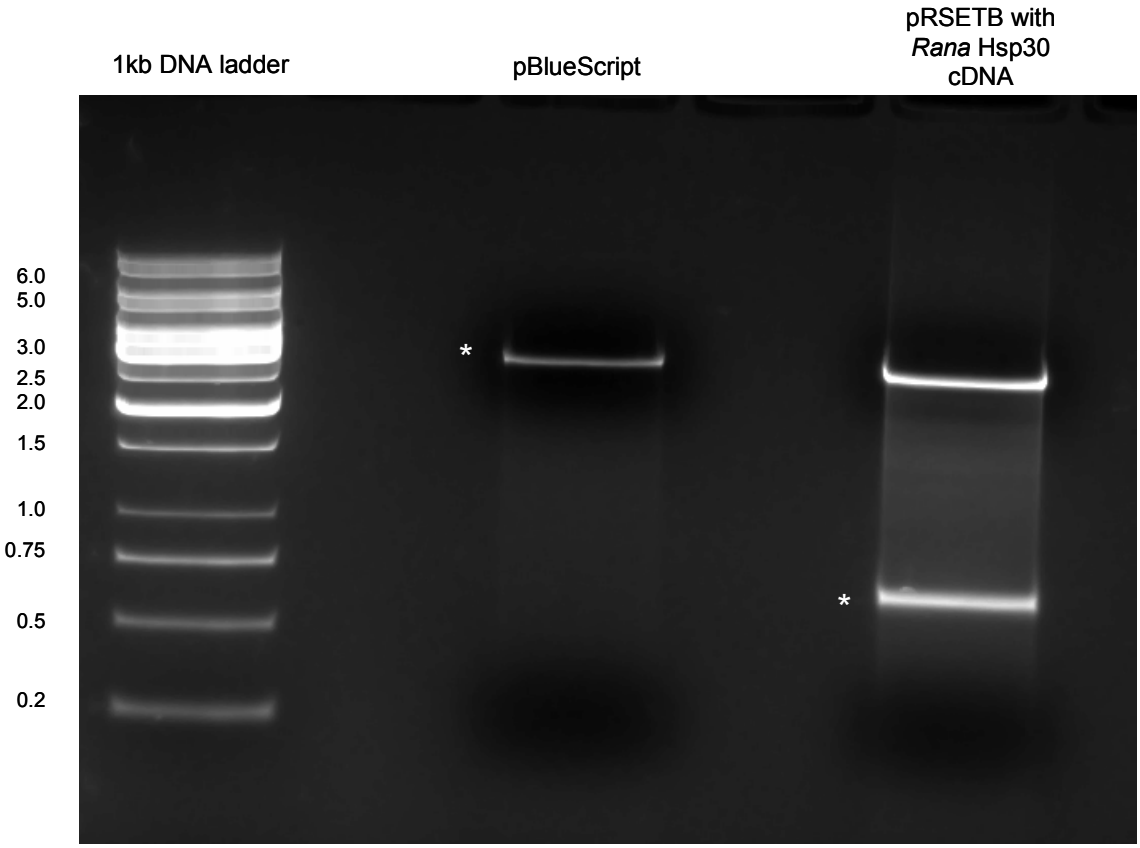

1kb DNA ladder
pBlueScript
pRSETB with
Rana Hsp30
cDNA
6.0
5.0
3.0
2.5
2.0
1.5
1.0
0.75
0.5
0.2
*
*

Figure 13: *Rana catesbeiana* fibroblast tongue cells.

A *Rana catesbeiana* fibroblast tongue (FT) cell line was obtained from ATCC and cultured for use in experiments for examining the accumulation of both *hsp30* mRNA and protein under control and heat shock conditions. The fibroblasts exhibited a characteristic elongated spindle fibre shape.

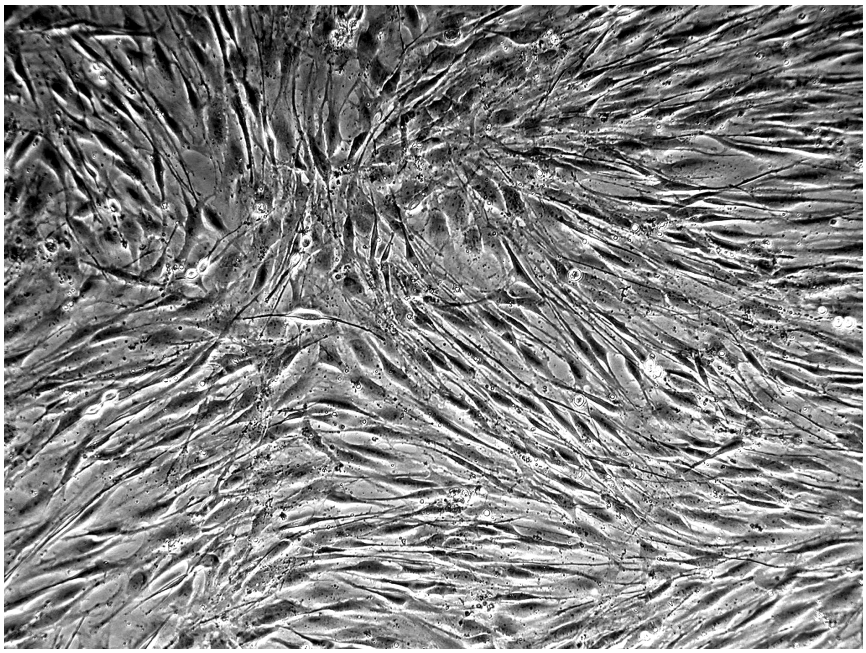
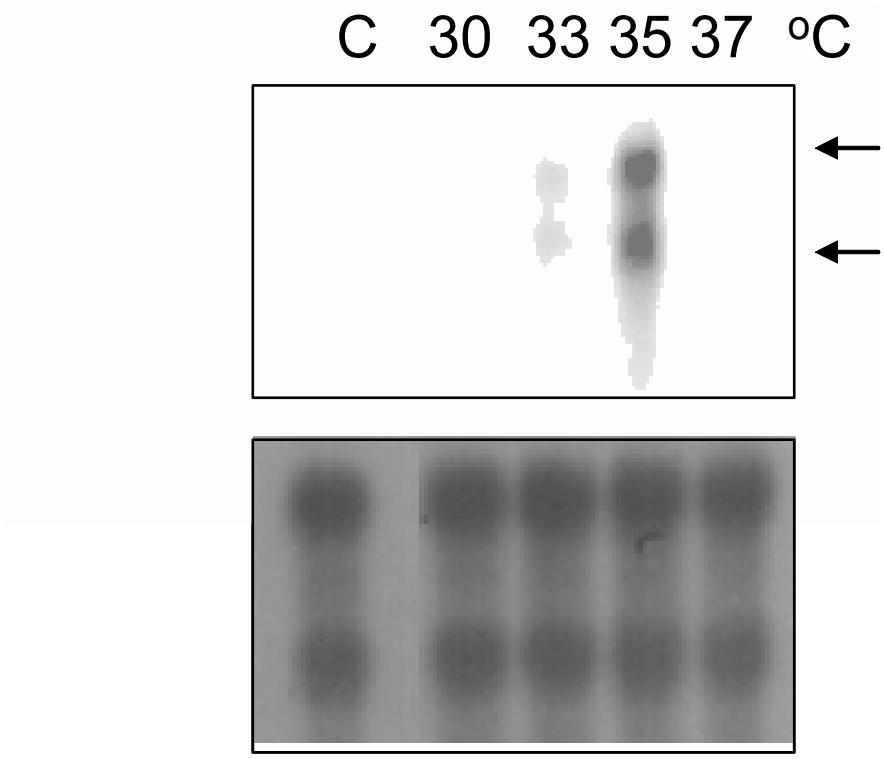


Figure 14: *Hsp30* mRNA accumulation in response to elevated temperatures in FT cells.

Rana FT cells were heat shocked at 30, 33, 35 or 37^{o}C for 2 h. Control cells were maintained at 22^{o}C until harvested. Total mRNA was isolated and quantified as outlined in Experimental Procedures. Northern blot analysis was performed using DIG-labelled antisense riboprobes to examine the relative levels of *hsp30* (using 5 μg total RNA) mRNA. Arrows indicate the position of the *hsp30* transcripts. The bottom panel is a representative RNA blot stain.


C 30 33 35 37 °C

Continuous exposure of *Rana* FT cells to a heat shock temperature of 33^{o}C indicated that *hsp30* mRNA was first detectable at 0.5 h, peaked at 2 h and was diminished at 6 h (Figure 15). A greater response was observed when cells were maintained continuously at 35^{o}C. As with 33^{o}C heat shock, FT cells incubated at 35^{o}C did not produce detectable *hsp30* mRNA until 0.5 h but the intensity of induction was much greater at 35^{o}C than at 33^{o}C. Levels of *hsp30* message peaked at 2 h and decreased thereafter.

3.1.3 Production of recombinant *Rana catesbeiana* HSP30 protein

Detection of *Rana* small heat shock protein, HSP30, protein in FT cells required anti-*Rana* HSP30 antibody. To this end, recombinant *Rana* HSP30 protein was produced. Following the transformation of the pRSETB-*Rana hsp30* construct into *E. coli* BL21 (DE3) cells, IPTG was added to induce the expression of *Rana* HSP30 recombinant protein. Bacterial lysates were then analysed by one-dimensional SDS-PAGE for analysis of recombinant protein presence and the time required for optimal accumulation after IPTG induction. An IPTG-induced 35 kDa protein band, which was not detected in samples lacking the pRSETB-*Rana hsp30* construct accumulated maximally after 8 h (Figure 16, compare lanes 2 and 3). *Rana* HSP30 was then purified by means of nickel affinity column chromatography. The eluted purified recombinant *Rana* HSP30 protein is shown in lane 4 of Figure 16.

3.1.4 Production of anti-*Rana* HSP30 polyclonal antibody

The recombinant *Rana* HSP30 protein was used to generate a polyclonal antibody in a New Zealand white rabbit as outlined in Section 2.9. Serum from the rabbit contained the polyclonal antibody against *Rana* HSP30. This was revealed in an enzyme-linked

Figure 15: *Hsp30* mRNA accumulates over time at 33°C and 35°C in FT cells.

FT cells were heat shocked at either 33°C or 35°C for 0.5, 1, 2, 4 or 6 h. Cells were harvested immediately and total RNA was isolated as outlined in Experimental Procedures. Northern blot analysis was performed using DIG-labelled antisense *hsp30* riboprobes to examine the relative levels of *hsp30* (using 5 μg total RNA) mRNA. The arrow indicates the position of the *hsp30* transcript. The bottom panel is a representative RNA blot stain.

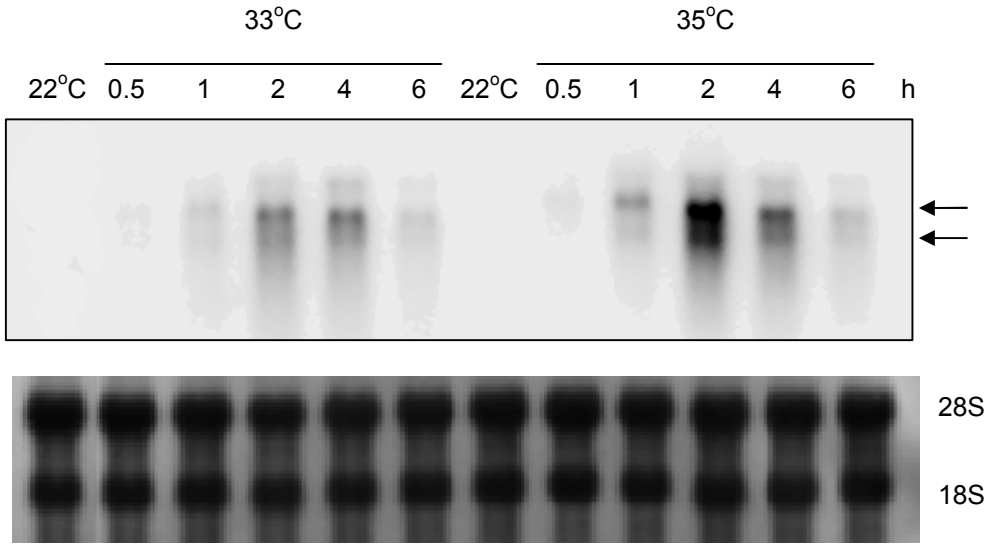
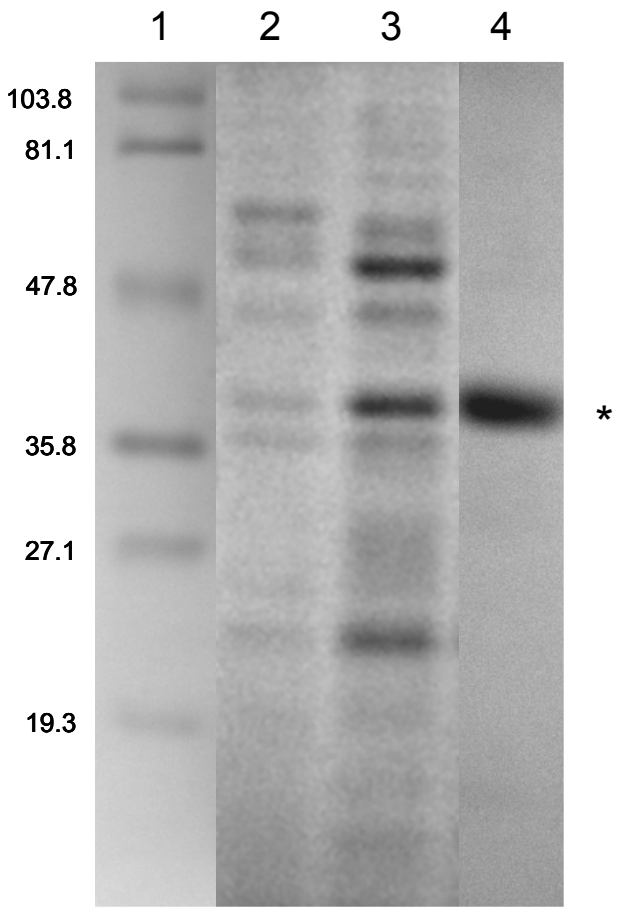

33°C
35°C
22°C 0.5 1 2 4 6 22°C 0.5 1 2 4 6 h
28S
18S

Figure 16: Purification of the *Rana* HSP30 recombinant protein.

Rana HSP30 was overexpressed in *E. coli* by induction with IPTG. The lysates from these cells were then passed over a nickel affinity column. The recombinant protein, which contains a six histidine tag, was bound to the column and was then purified as outlined in the Experimental Procedures. Purified protein was analysed by SDS-PAGE and visualised by Coomassie Brilliant Blue R-250 staining. Lane 1 contains molecular weight markers, masses shown in kDa. Bacterial lysates, not passed through a nickel affinity column, are shown in lanes 2 and 3: uninduced (lane 2) and induced (8h, lane 3) bacterial lysates, both containing the recombinant pRSETB-*Rana hsp30* construct. Lane 4 shows the purified recombinant *Rana* HSP30 protein. The asterisk indicates the position of the purified recombinant HSP30 protein.


1
2
3
4
103.8
81.1
47.8
35.8
27.1
19.3
*

immunosorbent assay (ELISA) with increasing titres of anti-*Rana* HSP30 antibody after three subsequent injections (Figure 17). The serum did not show any cross-reactivity with BSA, nor did the pre-immune serum react with recombinant *Rana* HSP30 (data not shown). The anti-*Rana* HSP30 antibody was purified on a nickel affinity column bound with the recombinant *Rana* HSP30 antigen. The antibody was tested with protein extracts from FT cells as well as with its recombinant protein as a positive control.

3.1.5 *Rana* HSP30 protein accumulated in response to heat shock

Enhanced *Rana* HSP30 protein accumulation in FT cells was not detectable at temperatures below 35^{o}C (Figure 18). However, since continuous exposure of temperatures above 35^{o}C were detrimental to the viability of FT cells, subsequent experiments were conducted at 35^{o}C. As evidenced in Figure 19, protein accumulation was not detectable by Western blot analysis until after 2 h of heat shock at 35^{o}C, with maximal levels at 4 h and a decrease at 8 h. As shown in Figure 20, the *Rana* HSP30 antibody also cross-reacted weakly with HSP30 in heat shocked *Xenopus* A6 cells.

3.1.6 Effect of sodium arsenite treatment on *Rana* HSP30 protein accumulation

Western blot analysis revealed that *Rana* FT cells were not susceptible to sodium arsenite treatment at 10 μM (Figure 21). A time course of treatment for up to 24 h failed to elicit any detectable accumulation of HSP30 in these cells. Further analyses with varying concentrations of NaAs, including 20, 50 and 100 μM, were also unsuccessful at inducing accumulation of *Rana* HSP30 in response to this stressor (data not shown).

Figure 17: Antibody titre using an enzyme-linked immunosorbent assay.

Serum from the rabbit used to generate an anti-*Rana* HSP30 antibody was tested for reactivity to *Rana* HSP30 before (Pre-immune) and after a number of re-immunization injections. The relative antibody titre was gauged by the absorbance at 405 nm in an ELISA. The levels of antibody present in the serum plateaued after the second boost. The serum did not cross-react with BSA when used as the antigen in the ELISA (data not shown).

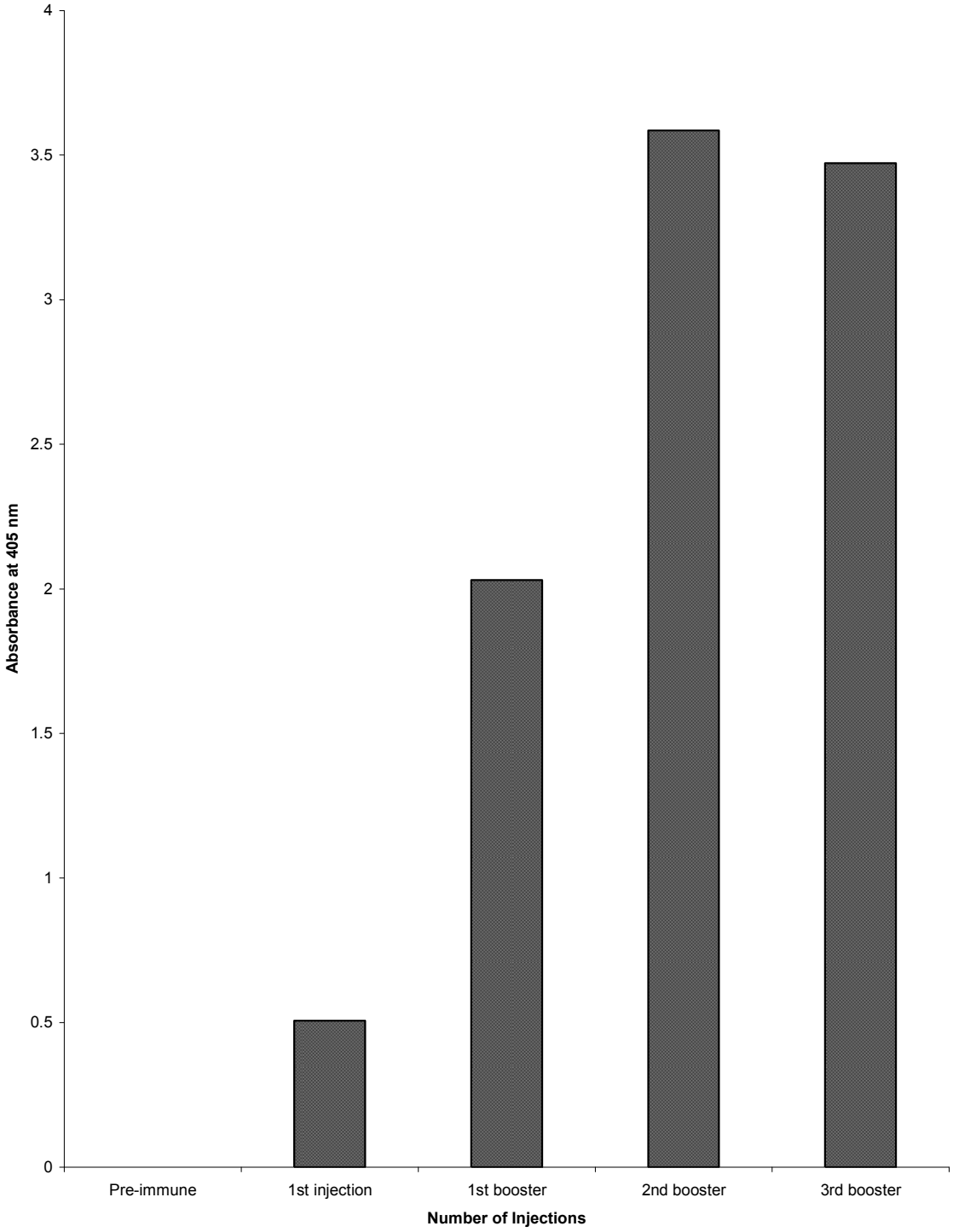

4
3.5
3
2.5
2
1.5
1
0.5
0
Absorbance at 405 nm
Pre-immune
1st injection
1st booster
2nd booster
3rd booster
Number of Injections

Figure 18: HSP30 protein accumulation in response to elevated temperatures in FT cells.

Rana FT cells were heat shocked for 2 h at 30, 33, 35 or 37^{o}C, followed by a 2 h recovery period at 22^{o}C. Control cells were maintained at 22^{o}C until harvested. Total protein was isolated and quantified as outlined in Experimental Procedures. Western blot analysis was performed using rabbit anti-*Rana* HSP30 antibody to detect the relative levels of HSP30 (using 10 μg of total protein). The arrow indicates the position of the HSP30 protein. The bottom panel is a representative Ponceau S stain of a portion of a stained blot.

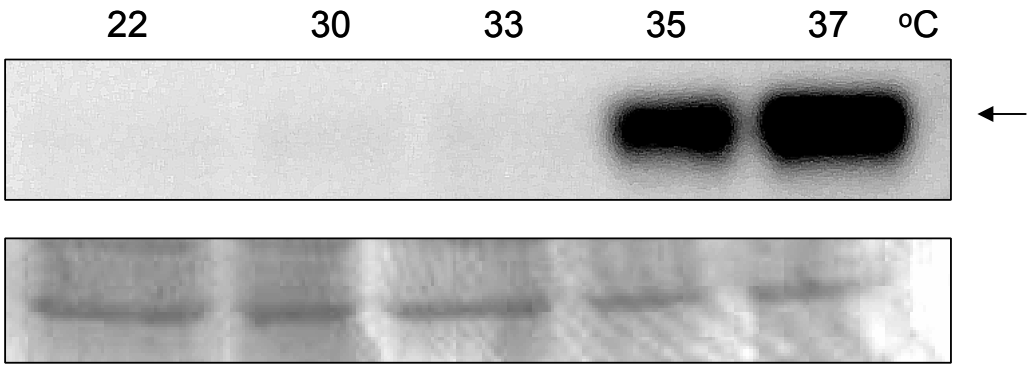

22
30
33
35
37
°C

Figure 19: HSP30 protein accumulation over time at 35^{o}C in FT cells.

Rana FT cells were heat shocked for 2 h at 35^{o}C, followed by a 2 h recovery period at 22^{o}C. Control cells were maintained at 22^{o}C until harvested. Total protein was isolated and quantified as outlined in Experimental Procedures. Western blot analysis was performed using rabbit anti-*Rana* HSP30 antibody to detect the relative levels of HSP30 (using 10 μg of total protein). The arrow indicates the position of the HSP30 protein. The bottom panel is a representative Ponceau S stain of a portion of a stained blot.

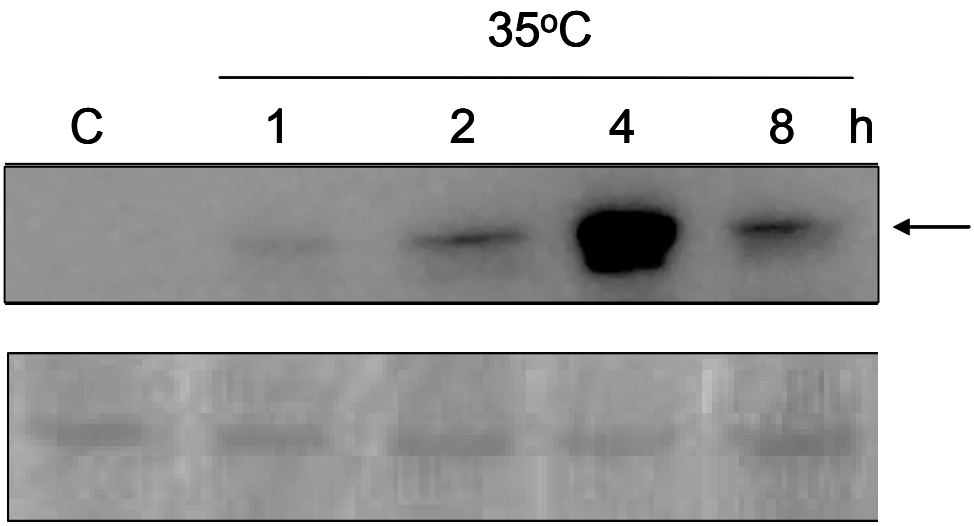

35°C
C
1
2
4
8
h

Figure 20: Anti-*Rana* HSP30 antibody cross reacts with *Xenopus* HSP30 protein.

Xenopus A6 cells were heat shocked at 33^{o}C for 1, 2, 4 or 8 h, followed by a 2 h recovery period at 22^{o}C. Control cells were maintained at 22^{o}C until harvested. Total protein was isolated as outlined in Experimental Procedures. Western blot analysis was performed using rabbit anti-*Rana* HSP30 antibody to determine the relative levels of HSP30C (using 10 μg total protein). The arrow indicates the position of the HSP30C protein. The bottom panel is a representative Ponceau S stain of a portion of a stained blot.

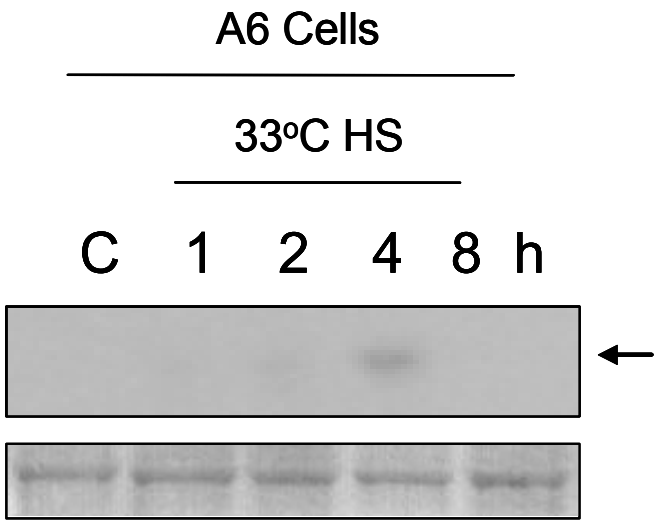
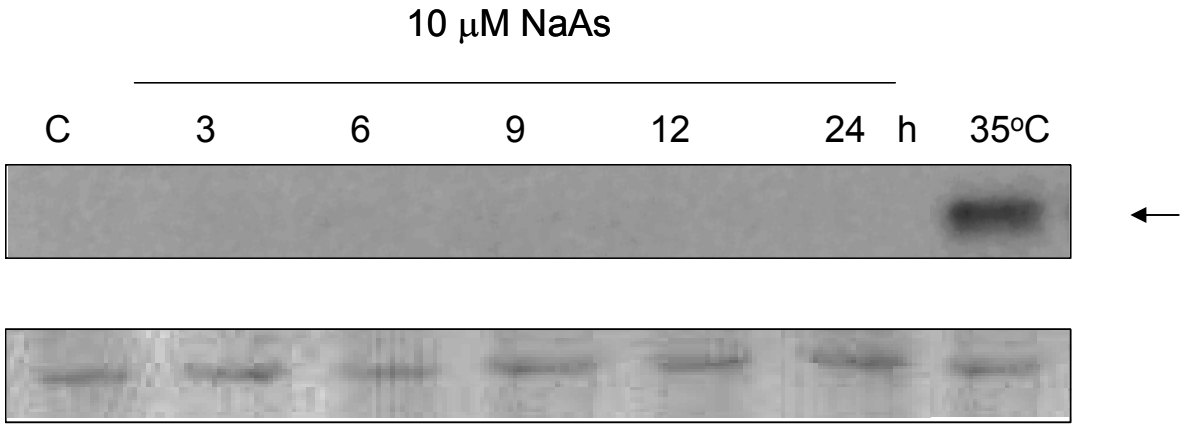

A6 Cells
33°C HS
C 1 2 4 8 h

Figure 21: HSP30 protein accumulation over time in 10 μM sodium arsenite.

Rana FT cells were treated for 3, 6, 9, 12, 24 or 36 h in 10 μM sodium arsenite in 90% L-15 (Leibovitz) media supplemented with 10% FBS (fetal bovine serum). Control cells were maintained in 90% L-15 media supplemented with 10% FBS. Some cells were also maintained at 35^{o}C for 2 h followed by a 2 h recovery period at 22^{o}C. Cells were immediately harvested and total protein was isolated and quantified as outlined in Experimental Procedures. Western blot analysis was conducted using 20 μg of total protein to determine the relative effects of sodium arsenite treatment on HSP30 protein accumulation in FT cells over time. The arrow indicates the position of the HSP30 protein. The bottom panel is a representative Ponceau S stain of a portion of a stained blot.


10 μM NaAs
C
3
6
9
12
24 h
35°C

3.1.7 Intracellular accumulation of HSP30 in *Rana* FT cells

Immunofluorescence using confocal laser scanning microscopy (CLSM) revealed that HSP30 was undetectable in cells maintained at 22^{o}C while the phalloidin stained actin cytoskeleton is clearly visible as red fibres (Figure 22, panels A1-A3). Similarly, cells incubated at 30 or 33^{o}C for 2 h also failed to show HSP30 accumulation (Figure 22, panels B1-C3). However, FT cells incubated at 35^{o}C resulted in accumulation of HSP30 primarily in the nucleus, with diffuse immunostaining in the cytoplasm (Figure 22, panel D1). Little obvious co-localization of HSP30 with actin was observed in these cells as indicated by yellow staining (Figure 22, D3). CLSM was used to examine the effects of continuous incubation at 35^{o}C on accumulation patterns of HSP30. HSP30 accumulation was detected in both the cytoplasm and the nucleus of FT cells only after 2 h at 35^{o}C (Figure 23, C1-C3). However, exposure of FT cells to 4 h or more at 35^{o}C resulted in a marked accumulation of HSP30 in the nucleus and a lessening of HSP30 in the cytoplasm (Figure 23, D1-F3). Actin structures did not show any detectable changes compared to control throughout the experiments. Further analysis using CLSM revealed that a 4 h heat shock at 35^{o}C followed by varying recovery times at 22^{o}C did not alter the localization of HSP30 (Figure 24). HSP30 remained detectable throughout the cytoplasm and nucleus for the entire 6 hr recovery period, gradually becoming more concentrated in the nucleus as recovery time increased (Figure 24).

Figure 22: *Rana* HSP30 protein accumulates under heat shock conditions in FT cells.

Rana catesbeiana FT cells were maintained at 22^{o}C (A1-A3), or heat-treated for 2 h at 30^{o}C (B1-B3), 33^{o}C (C1-C3) or 35^{o}C (D1-D3), followed by a 2 h recovery period at 22^{o}C. Cells were then labelled for F-actin and HSP30 expression using TRITC (red) and FITC (green), respectively, as outlined in Experimental Procedures. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The apparent dense actin staining is due to cell clumping and enhanced contrast during printing.

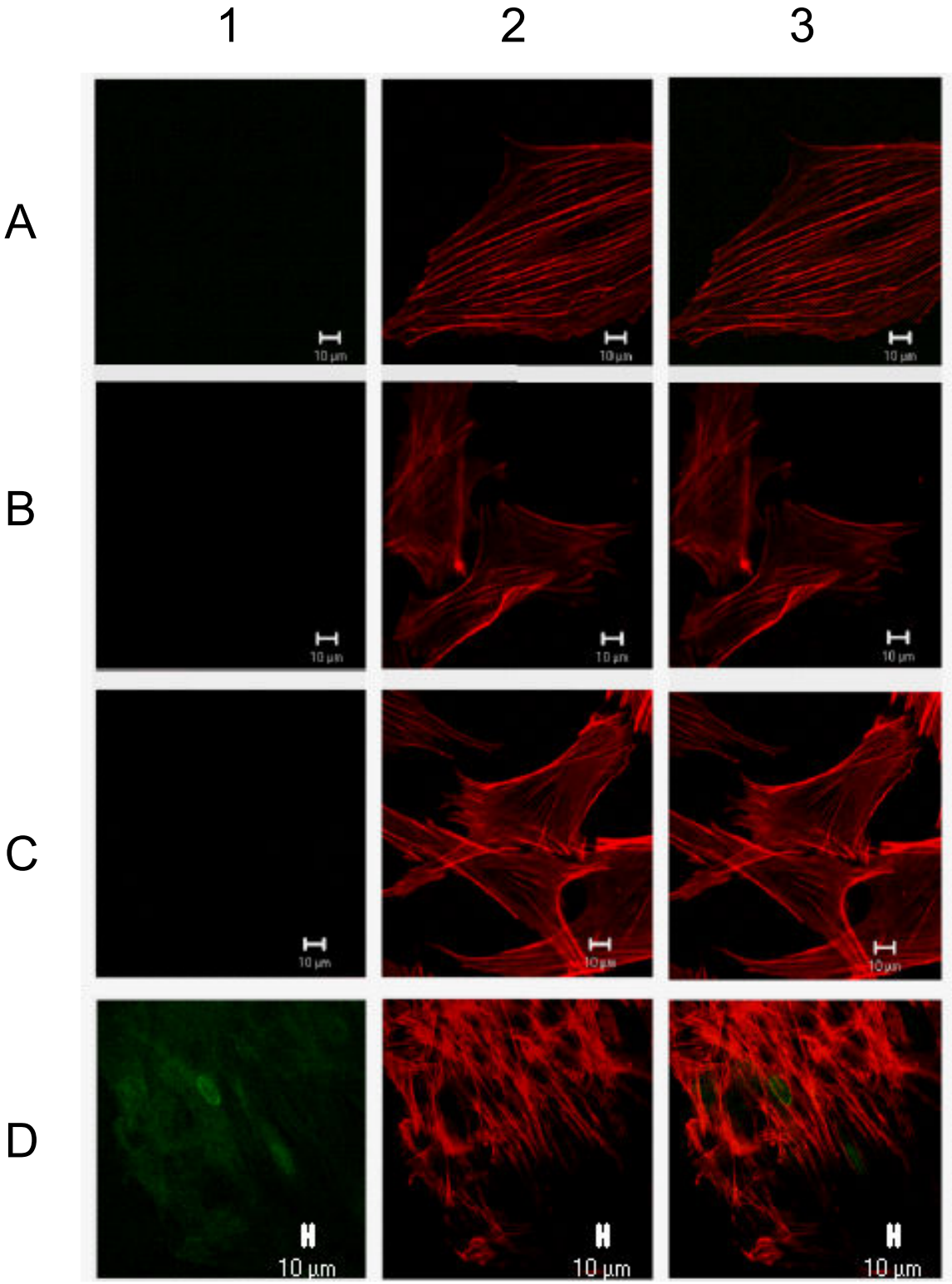
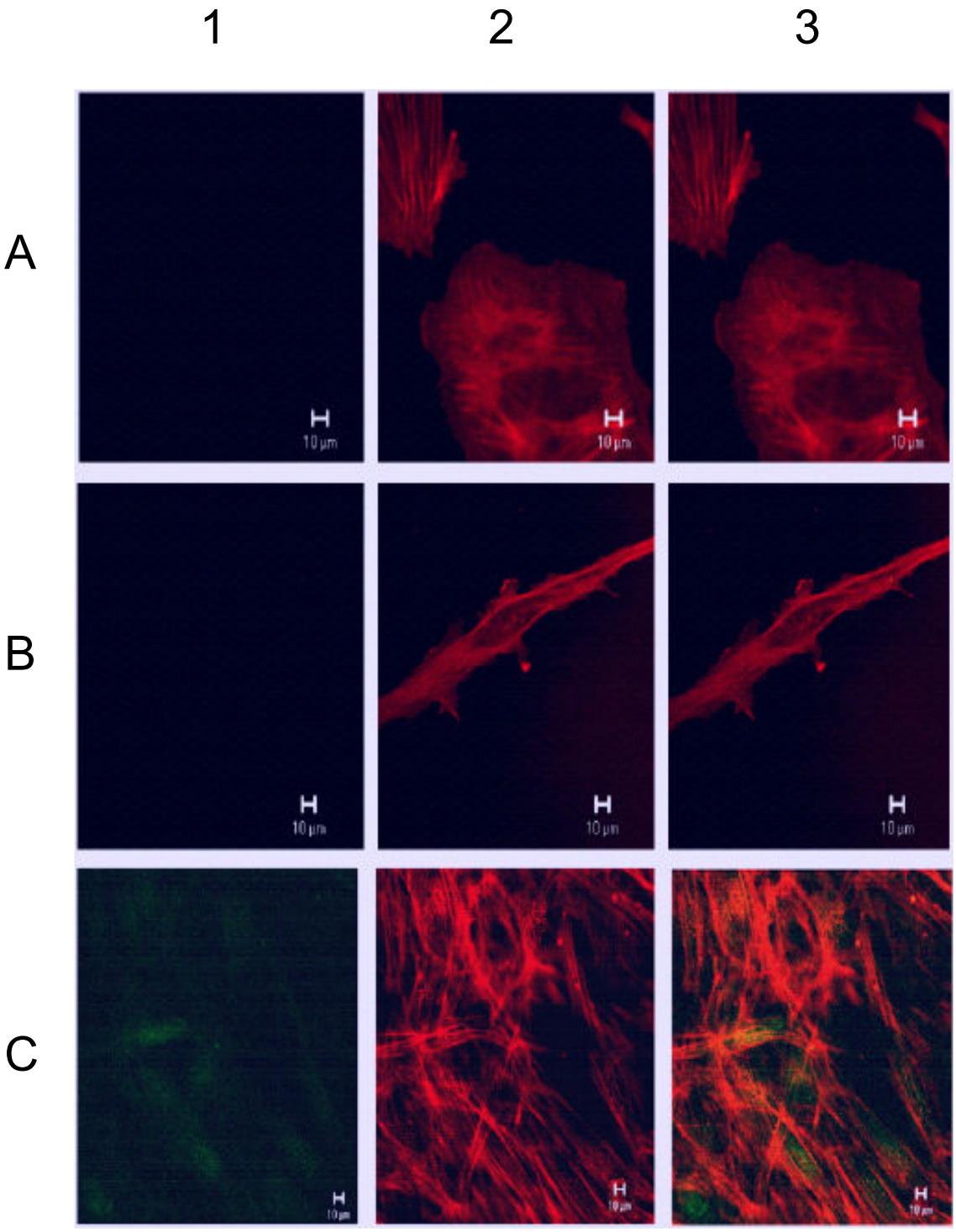

1
2
3
A
B
C
D
10 µm

Figure 23: *Rana* HSP30 protein is expressed under heat shock conditions in FT cells.

Rana catesbeiana FT cells were heat-treated for 0.5 (A1-A3), 1 (B1-B3), 2 (C1-C3), 4 (D1-D3), 6 (E1-E3) or 8 (F1-F3) h at 35^{o}C, followed by a 2 h recovery period at 22^{o}C. Cells were then labelled for F-actin and HSP30 expression using TRITC (red) and FITC (green), respectively, as outlined in Experimental Procedures. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The apparent dense actin staining is due to cell clumping and enhanced contrast during printing.


1
2
3
A
B
C
10 µm

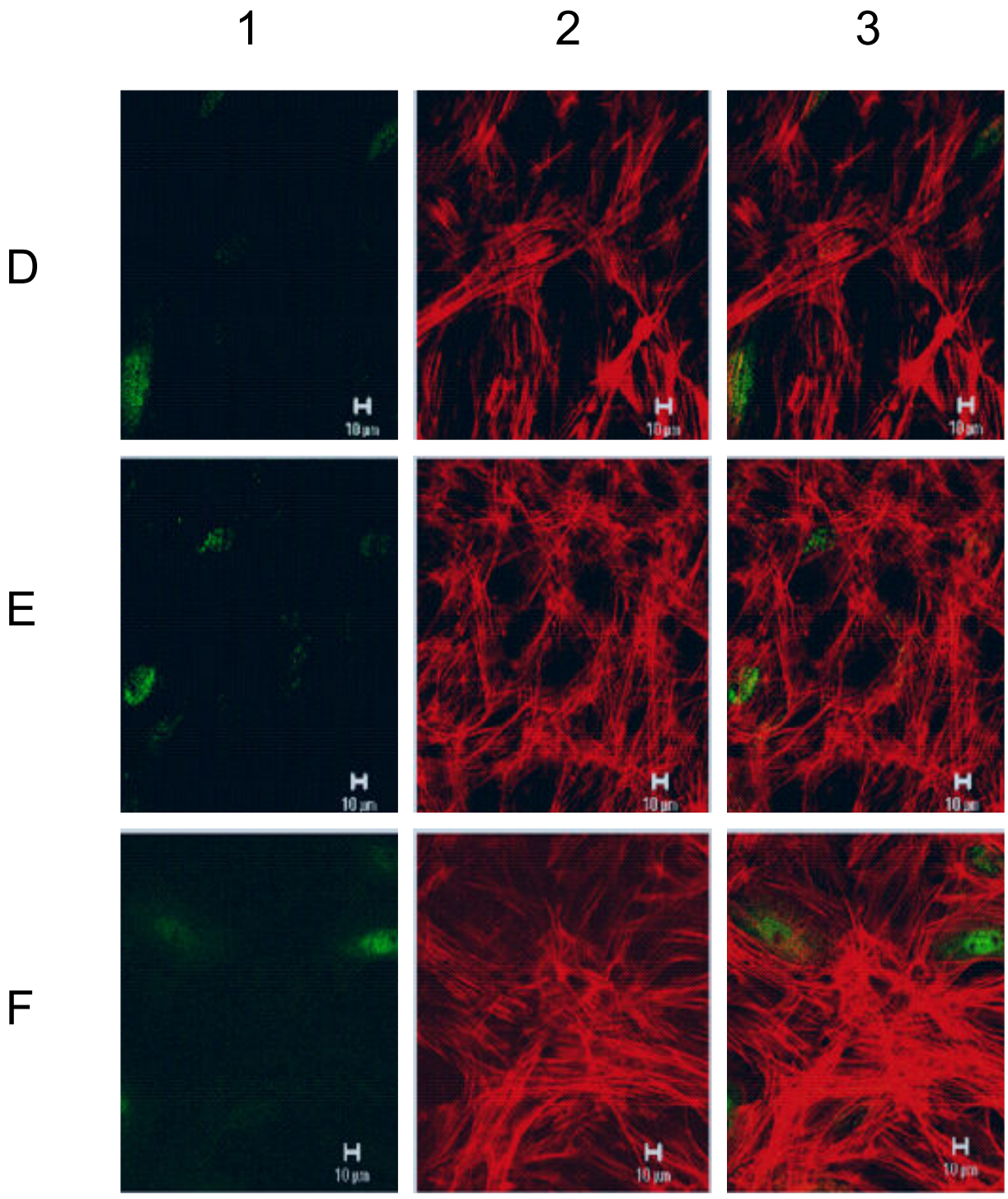
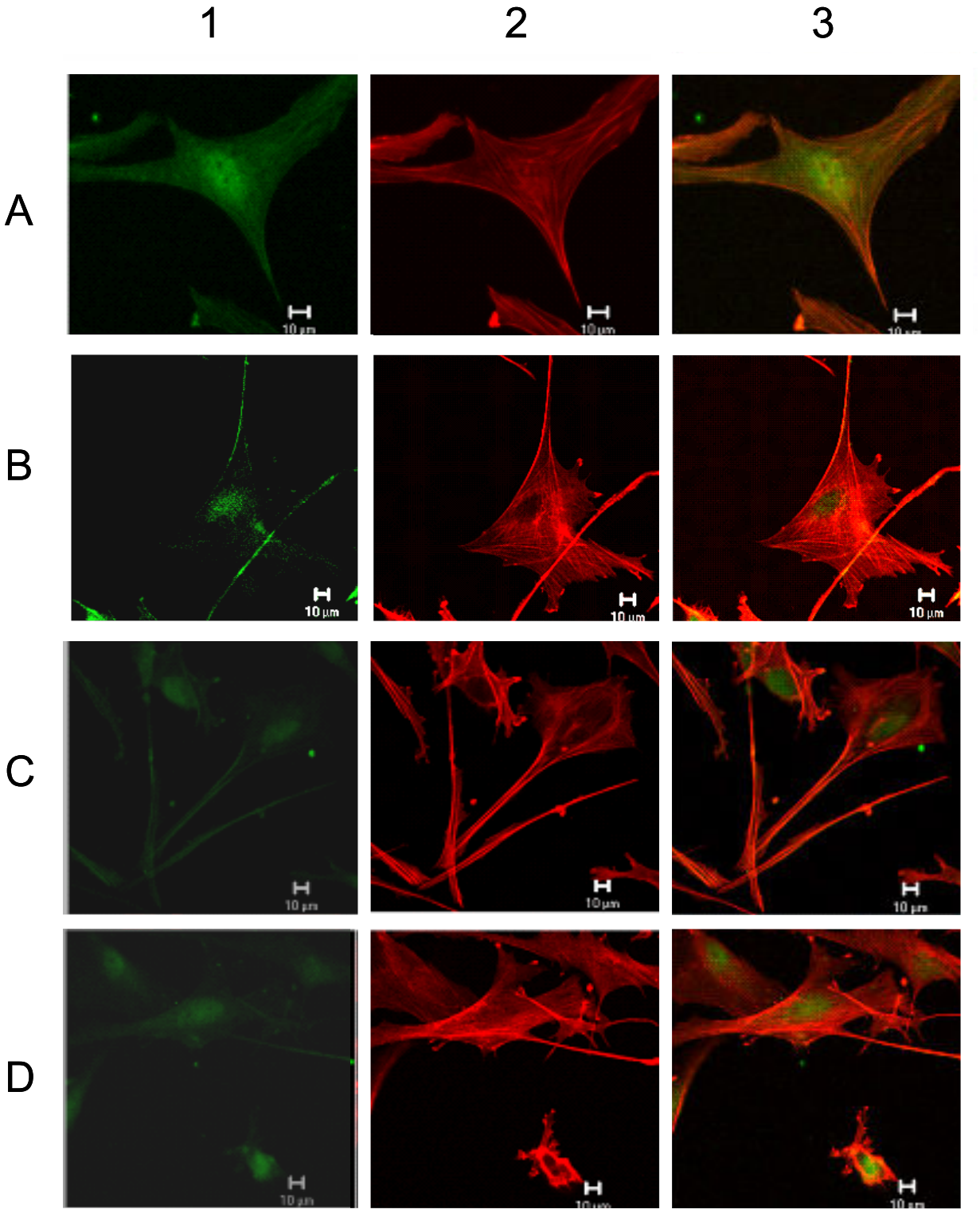

1
2
3
D
E
F

Figure 24: *Rana* HSP30 protein accumulates and remains in the nucleus upon extended recovery at 22^{o}C.

Rana catesbeiana FT cells were heat-treated for 4 h at 35^{o}C, followed by varying recovery periods at 22^{o}C; 0 h recovery (A1-A3), 2 h recovery (B1-B3), 4 h recovery (C1-C3) or 6 h recovery (D1-D3). Cells were then labelled for F-actin and HSP30 expression using TRITC (red) and FITC (green), respectively, as outlined in Experimental Procedures. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope.


1
2
3
A
B
C
D

3.2 Characterization of *Rana* HSP30 protein function

3.2.1 *Rana* HSP30 amino acid sequence comparison

Previous work done by Angelo Kaldis *et al*. (2004) revealed that HSP30 was a largely α-helical and β-sheet-like protein, similar to findings by Fernando *et al*. (2002, 2003) for *Xenopus* HSP30C and HSP30D. Analysis of the *Rana* HSP30 sequence revealed a putative 84 amino acid α-crystallin domain beginning at E90. This was consistent with the general length of the α-crystallin domain of 80 to 100 amino acids in small heat shock proteins (Takeuchi, 2006). The *Rana catesbeiana* HSP30 amino acid sequence was aligned with that of *Xenopus laevis* HSP30C. The *Rana* HSP30 amino acid sequence displayed 52% identity with 4 additions and 69 substitutions, 46 of which were conserved (Figure 25). The amino acid sequence comparison also revealed that *Rana* HSP30 has a more polar C-terminal extension than *Xenopus* HSP30C.

3.2.2 Recombinant *Rana* HSP30 inhibits the thermal aggregation of target proteins

Analysis of the function of *Rana* HSP30 involved the determination of its ability to inhibit the heat-induced thermal aggregation of citrate synthase (CS) *in vitro*. The amount of heat-induced aggregation was determined by measuring the increase in the amount of light scattering at 320 nm at 42^{o}C (Figure 26). Incubation of varying molar ratios of *Rana* HSP30 with CS showed its ability to prevent heat-induced aggregation. A 0.1:1 (HSP:CS) ratio resulted in a 30% reduction in aggregation. An additional 20% reduction was detected when a ratio of 0.25:1 was used. Increasing the molar ratio to 1:1 resulted in a 90% reduction in aggregation and higher ratios (HSP:CS) completely prevented aggregation of

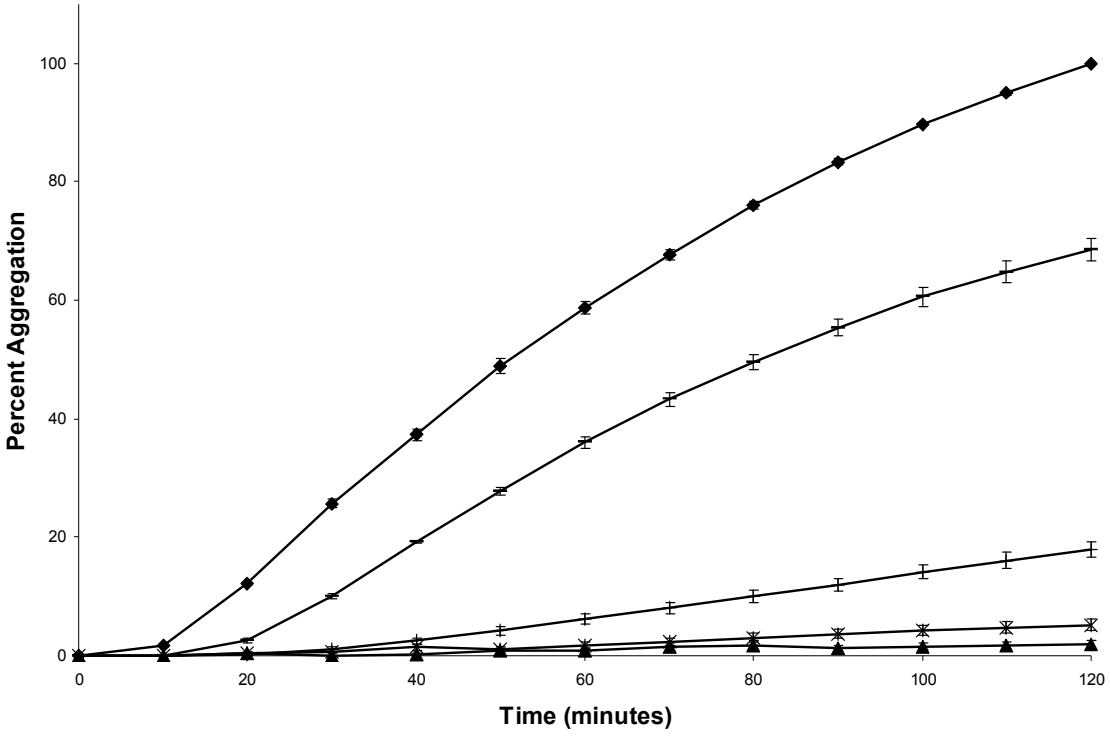
Figure 25: Comparison of the deduced amino acid sequences of *Rana* HSP30 and *Xenopus* HSP30C.

Comparison of the deduced amino acid sequences of *Rana* HSP30 and *Xenopus* HSP30C revealed a 52% identity between the two species. Asterisks under the sequences represent identical amino acids. Dots represent conservative amino acid substitutions and deletions are indicated by dashes. The α-crystallin domain is underlined. The C-terminal extension is shown in bold.

```
Rana hsp30          MFPLSLLQSSHSPVYVYHQPELPHWTTTHLILRQLEDDMLSVTSDMERRMQRLHQTYHLL
Xenopus hsp30C      MFPLSLVQPSHSPLCPCSQPALTLWPATRLIFGHLEDDILSMRNDMERRMQRVNEACRLL
                    ******:*.****:    ** *. *.:*:**: :****:**: .********:::: :**

Rana hsp30          ANDPDMRRGRGAQSRHPTAPQDKSPTEGKDEKENFELSLDVSPFSPEELTVRTEGRRLIV
Xenopus hsp30C      SKDTEMRR-ITDQNRQSRESEGTSPNSGKDGKDHFELTLNVRDFSPHELTVKTQGRRVIV
                    ::*.:***    *.*:.  .:..**..*** *::***:*:*  ***.****:*:***:**

Rana hsp30          AGKSDKKRETENGGFFQEYREWRREAELPEDVNPEDVLCSMSKNGRLHFWAPRLALPAAQ
Xenopus hsp30C      TGKHERKSDTEDGNYFHEYREWKREAELPESVNPEQVVCSLSKNGHLHIQAPRLALPPAP
                    :** ::* :**:*.:*:*****:*******.****:*:**:****:**: *******.*

Rana hsp30          QRSITITN EQSPGEGQESNPENQNHRGQQEQDLPQNSS
Xenopus hsp30C      ETPIPISMDTAPRDAQELPPDAQTSNAEGDQKVD----
                    : .*.*: : :* :.**  *: *. ..: :*.:
```

Figure 26: Inhibition of heat-induced aggregation of citrate synthase by *Rana* HSP30.

The effect of various molar amounts of recombinant *Rana* HSP30 on the heat-induced aggregation of citrate synthase (CS) was determined by measuring the amount of light scattered at 320 nm during incubation at 42^{o}C, as outlined in Experimental Procedures. Citrate synthase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 (HSP:CS) at 3:1 (▲), 1:1 (*), 0.5:1 (ı), and 0.1:1 (—). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of CS after 120 minutes and were expressed as the mean ± S.E.


Percent Aggregation
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (minutes)

CS. Further analysis of *Rana* HSP30's function as a molecular chaperone was carried out using luciferase (LUC) as the target protein. As with CS, the amount of light scattering at 320 nm was used as an indicator of heat-induced aggregation at 45^{o}C (Figure 27). Similarly, incubation of LUC with varying molar ratios of *Rana* HSP30 confirmed its ability to prevent heat-induced aggregation. A molar ratio of 0.1:1 (HSP:LUC) resulted in a 52% reduction in aggregation. Molar ratios in excess of 1:1 saw a 90% or greater reduction in aggregation. Additionally, malate dehydrogenase (MD) was used as a target protein to test further *Rana* HSP30's ability to inhibit heat-induced aggregation. A ratio of 0.1:1 (HSP:MD) resulted in a 57% reduction in aggregation, while a 0.5:1 ratio resulted in 94% reduction. Higher molar ratios completely inhibited heat-induced aggregation over the course of the experiment (Figure 28). Comparison with *Xenopus* HSP30C required the production of recombinant *Xenopus* HSP30C protein. Production was achieved in the same manner as with *Rana* HSP30 as outlined in Section 3.1.3. *Xenopus* HSP30C accumulated maximally after 8 h of induction with IPTG (Figure 29, lanes 2 and 3), with a molecular weight of approximately 35 kDa. Comparison between *Rana* HSP30 and *Xenopus* HSP30C showed no major difference between the two proteins as chaperones (Figures 30-32). In separate experiments, incubation of target proteins with varying molar ratios of BSA did not inhibit aggregation (data not shown) as shown previously in our laboratory (Fernando and Heikkila, 2000; Kaldis *et al*., 2004).

Figure 27: Inhibition of heat-induced aggregation of luciferase by *Rana* HSP30.

The effect of various molar quantities of *Rana* HSP30 on the heat-induced aggregation of luciferase (LUC) was determined by measuring the amount of light scattered at 320 nm while being incubated at 45^{o}C, as outlined in Experimental Procedures. Luciferase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 (HSP:LUC) at 3:1 (▲), 1:1 (|), and 0.1:1 (■). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of LUC after 60 minutes and were expressed as the mean ± S.E.

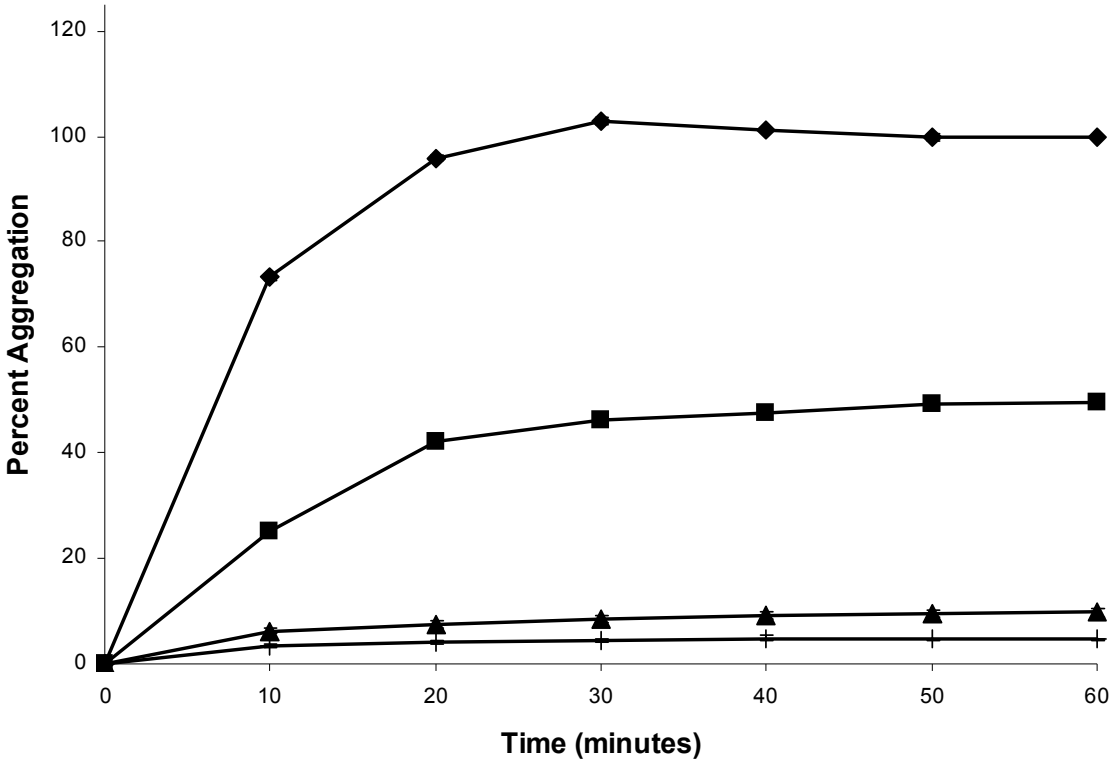

120
100
80
60
40
20
0
Percent Aggregation
0
10
20
30
40
50
60
Time (minutes)

Figure 28: Inhibition of heat-induced aggregation of malate dehydrogenase by *Rana* HSP30.

The effect of various molar quantities of recombinant *Rana* HSP30 on the heat-induced aggregation of malate dehydrogenase (MD) at 45^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Malate dehydrogenase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 (HSP:MD) at 3:1 (▲), 1:1 (|), and 0.1:1 (■). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of MD after 60 minutes and were expressed as the mean ± S.E.

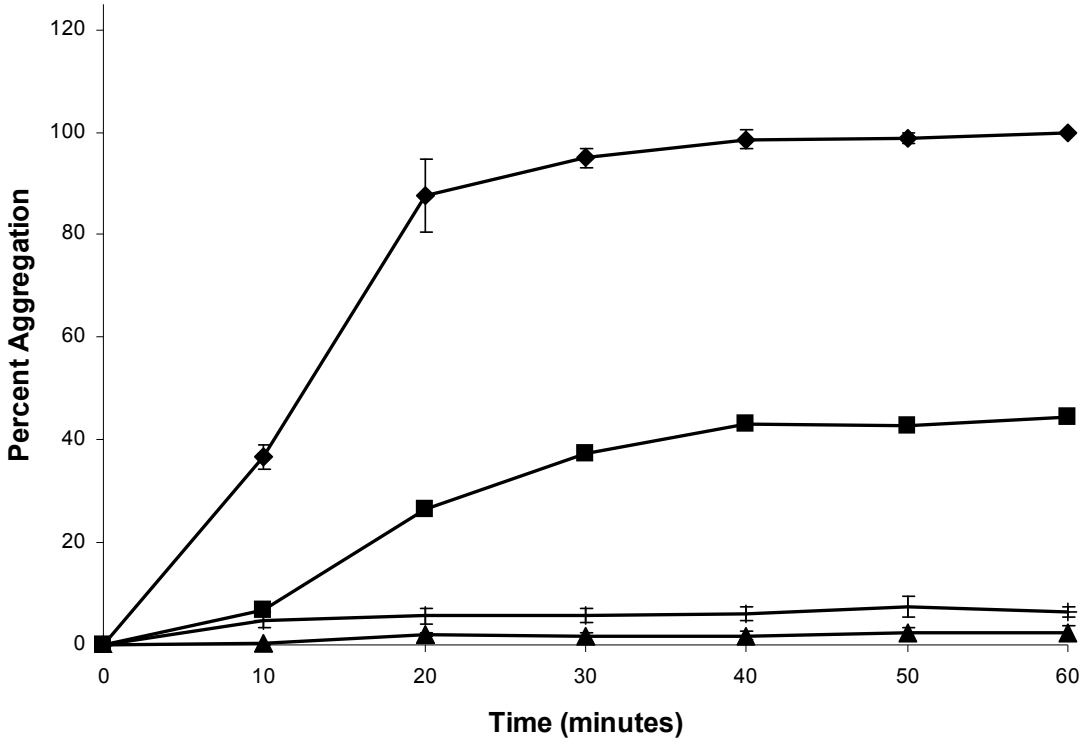

120
100
80
60
40
20
0
Percent Aggregation
0
10
20
30
40
50
60
Time (minutes)

Figure 29: Purification of the *Xenopus* HSP30C recombinant protein.

Xenopus HSP30C was overexpressed in *E. coli* by induction with IPTG. The lysates from these cells were then passed over a nickel affinity column. The recombinant protein, which contains a six histidine tag, was bound to the column and was then purified as outlined in the Experimental Procedures. Purified protein was analysed by SDS-PAGE and visualised by Coomassie Brilliant Blue R-250 staining. Lane 1 contains molecular weight markers, masses shown in kDa. Bacterial lysates, not passed through a nickel affinity column, are shown in lanes 2 and 3: uninduced (lane 2) and induced (8h, lane 3) bacterial lysates, both containing the recombinant pRSETB-*Xenopus hsp30C* construct. Lane 4 shows the purified recombinant *Xenopus* HSP30 protein. The asterisk indicates the position of the purified recombinant HSP30C protein.

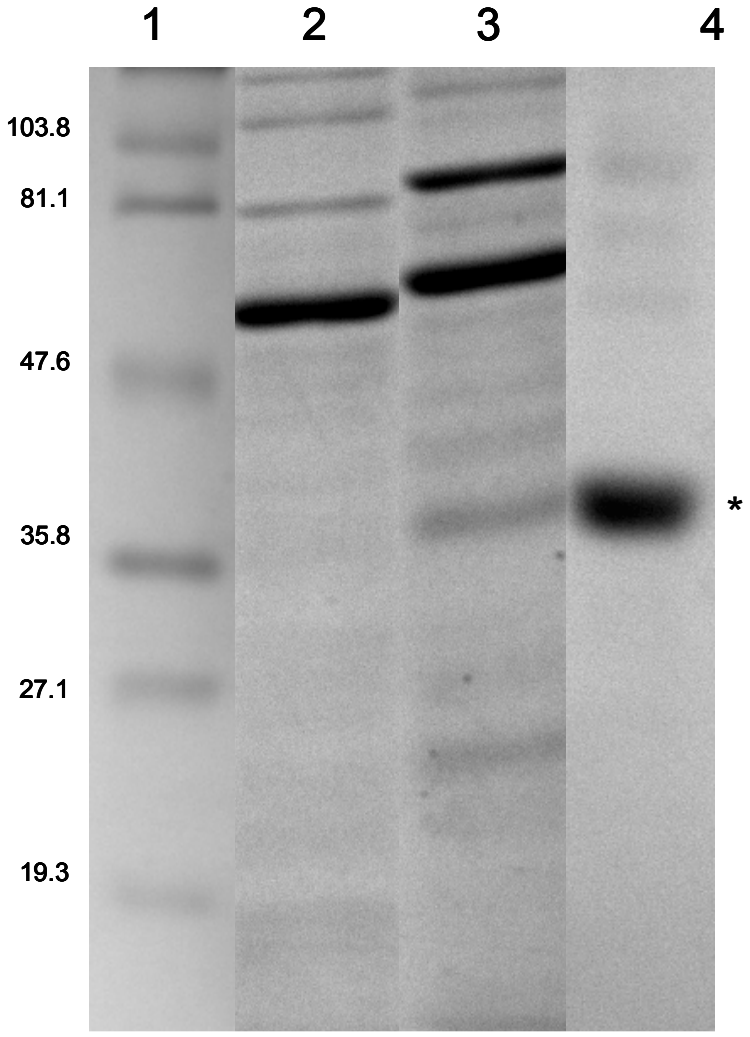

1
2
3
4
103.8
81.1
47.6
35.8
27.1
19.3
*

Figure 30: Inhibition of heat-induced aggregation of citrate synthase by *Rana* HSP30 or *Xenopus* HSP30C.

The effect of various molar quantities of *Rana* HSP30 or *Xenopus* HSP30C on the heat-induced aggregation of citrate synthase (CS) at 42^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Citrate synthase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 or *Xenopus* HSP30C (HSP:CS) at 2:1 (*Rana* ■, *Xenopus* ▲), 1:1 (*Rana* X, *Xenopus* *), 0.5:1 (*Rana* ●, *Xenopus* ׀), and 0.1:1 (*Rana* –, *Xenopus* —). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of CS after 120 minutes and were expressed as the mean ± S.E.

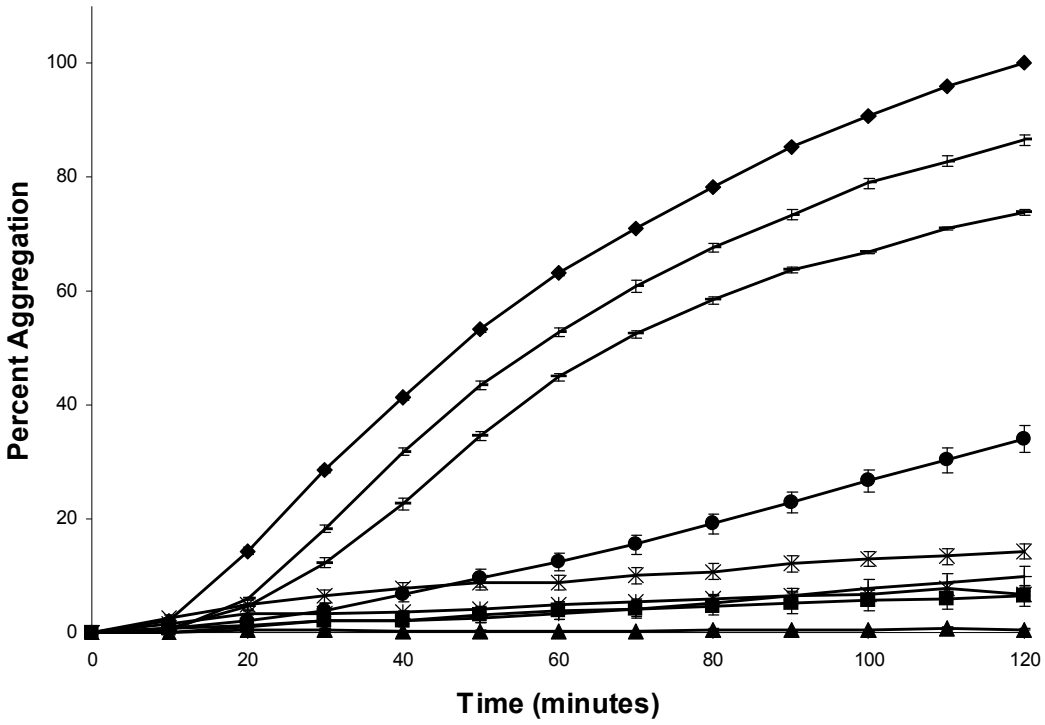

Percent Aggregation
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (minutes)

Figure 31: Inhibition of heat-induced aggregation of luciferase by *Rana* HSP30 or *Xenopus* HSP30C.

The effect of various molar quantities of *Rana* HSP30 or *Xenopus* HSP30C on the heat-induced aggregation of luciferase (LUC) at 45^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Luciferase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 or *Xenopus* HSP30C (HSP:LUC) at 2:1 (*Rana* ■, *Xenopus* ▲), 1:1 (*Rana* X, *Xenopus* *), 0.5:1 (*Rana* ●, *Xenopus* ׀), and 0.1:1 (*Rana* –, *Xenopus* —). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of LUC after 60 minutes and were expressed as the mean ± S.E.

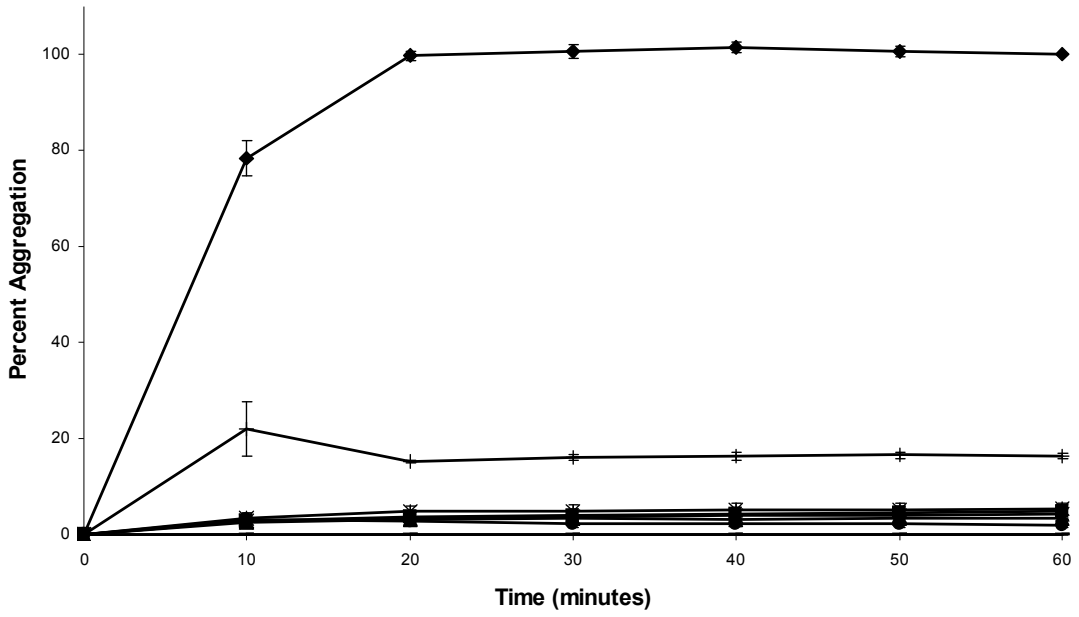
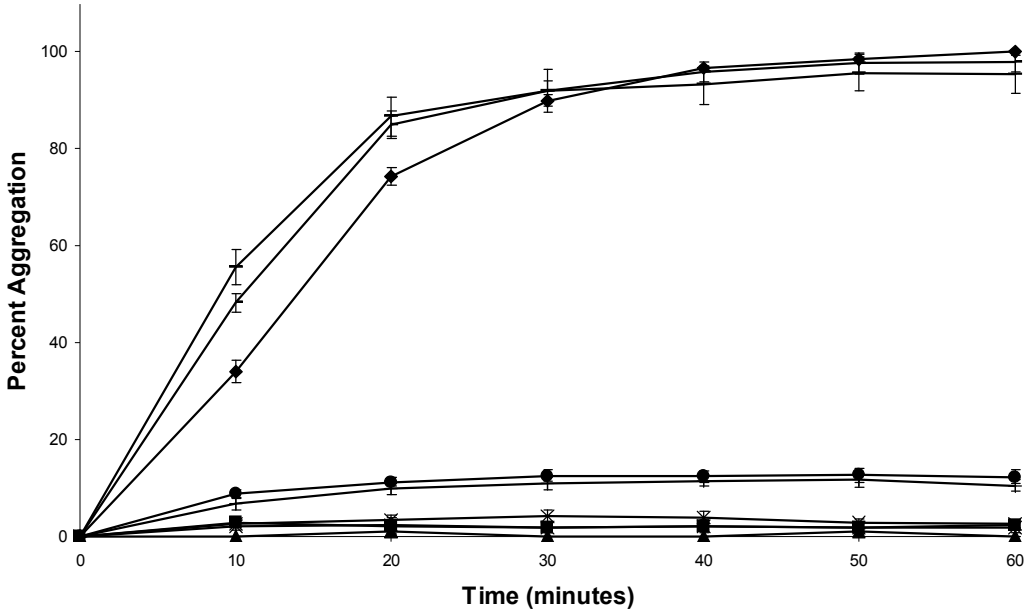

Percent Aggregation
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Figure 32: Inhibition of heat-induced aggregation of malate dehydrogenase by *Rana* HSP30 or *Xenopus* HSP30C.

The effect of various molar quantities of *Rana* HSP30 or *Xenopus* HSP30C on the heat-induced aggregation of malate dehydrogenase (MD) at 45^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Malate dehydrogenase was heated alone (♦) or combined in different molar ratios with *Rana* HSP30 or *Xenopus* HSP30C (HSP:LUC) at 2:1 (*Rana* ■, *Xenopus* ▲), 1:1 (*Rana* X, *Xenopus* *), 0.5:1 (*Rana* ●, *Xenopus* ׀), and 0.1:1 (*Rana* –, *Xenopus* —). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of MD after 60 minutes and were expressed as the mean ± S.E.


Percent Aggregation
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

3.3 *Xenopus laevis hsp27* gene expression

3.3.1 Analysis of the *Xenopus hsp27* amino acid sequence

The *Xenopus laevis hsp27* clone was obtained from the American Type Culture Collection (ATCC) and sequenced in our laboratory by Norman Chan. The *Xenopus laevis* HSP27 amino acid sequence derived from the cDNA sequence was aligned with that of chicken HSP27, human HSP27, zebrafish HSP27 and topminnow HSP27. The *Xenopus laevis* HSP27 amino acid sequence displayed 71% identity with chicken HSP27, while only a 65% identity with zebrafish, 63% identity with that of human, and a 53% identity with topminnow (Figure 33 and Table 1). *Xenopus laevis* HSP27 had an α-crystallin domain and a C-terminal extension, as indicated in Figure 34. Interestingly, *Xenopus* HSP27 only shared a 19% identity with either *Xenopus* HSP30C or 30D. In a preliminary phylogenetic tree (Figure 34), *Xenopus* grouped with chicken HSP27, just outside the mammals. It was more closely related to the mammals and avian HSP27s than other small heat shock proteins within *Xenopus* itself, namely HSP30C and HSP30D.

3.3.2 Production of *Xenopus* HSP27 recombinant protein and polyclonal antibody

Xenopus HSP27 recombinant protein was also produced using the same expression vector system and IPTG induction as *Rana* HSP30 recombinant protein as outlined in Section 3.1.3. *Xenopus* HSP27 accumulated maximally after only 6 h of induction with IPTG and was approximately 30 kDa in size (Figure 35, lanes 2 and 3). Following production of the recombinant protein, a polyclonal antibody against HSP27 was produced in a New Zealand white rabbit, as described in Section 2.9. Serum collected from the rabbit

Figure 33: Comparison of the deduced amino acid sequence of *Xenopus* HSP27.

Comparison of the deduced amino acid sequence derived from the cloned full-length *Xenopus laevis hsp27* cDNA to the deduced amino acid sequences of chicken, human, zebrafish and topminnow. Asterisks under the sequences indicate identical amino acids between all 5 species. Dots represent conservative amino acid substitutions while deletions are indicated by dashes. The underlined amino acids indicate the location of the α-crystallin domain while the C-terminal extension is shown in bold.

```
Xenopus hsp27       MSERRIPFTFLRTPSWDPFRDWYQGSSRLFDQSFGMPRIPEDWHQWPS-TSWPGYVR-LF
Chicken hsp27       MAERRVPFTFLTSPSWEPFRDWYHG-SRLFDQSFGMPHIPEDWYKWPSGSAWPGYFR-LL
Human hsp27         MTERRVPFSLLRGPSWDPFRDWYPH-SRLFDQAFGLPRLPEEWSQWLGGSSWPGYVR-PL
Zebrafish hsp27     MAERRIPFSFMRTPSWDPFRDWYQG-SRLLDQSFGMPALSEEMLTFPS-THWPGYMR-PF
Topminnow hsp27     MAERRIPFTLQRTPSWDVP--DLHQTSRIFDQAFGLPPVFEDFSGFPT-THWPGYMRPSL
                    *:***:**::   ***:         **::**:**:* : *:   :   : ****.*  :

Xenopus hsp27       PSQSMDVVPPTTPAPAGAPAPDFSRALSRQLSSGISEIRQTSDQWKISLDVNHFAPEELV
Chicken hsp27       PSESALLPAPGSPYGR----------ALSELSSGISEIRQSADSWKVTLDVNHFAPEELV
Human hsp27         PPAAIESPAVAAPAYS--------RALSRQLSSGVSEIRHTADRWRVSLDVNHFAPDELT
Zebrafish hsp27     GHPDFAALMQGPPVMPPMMTPSYGRALSRQLSSGMSEVKQTGDSWKISLDVNHFSPEELN
Topminnow hsp27     MTPDIMIPQSPMMYHPGHMMAHQARALSRQMSSGMSEIKQTQDNWKISLDVPHFSPEELV
                                              ::***:**:::: * *:::*** **:*:**

Xenopus hsp27       VKTKDGIVEITGKHEEKQDEHGFISRCFTRKYTLPPGVAINAVASSLSPDGILTVEAPLP
Chicken hsp27       VKTKDNIVEITGKHEEKQDEHGFISRCFTRKYTLPPGVEATAVRSSLSPDGMLTVEAPLP
Human hsp27         VKTKDGVVEITGKHEERQDEHGYISRCFTRKYTLPPGVDPTQVSSSLSPEGTLTVEAPMP
Zebrafish hsp27     VKTKDGVLEITGKHEERKDEHGFISRCFTRKYTLPPGVDSEKISSCLSPEGVLTVEAPLP
Topminnow hsp27     VKTKDGVLEISGKHEERKDEHGFVSRSFTRKYTLPPTANIEKVTSSLSPEGVLTVEAPIN
                    *****.::**:*****::****::**.********* .    : *.***:* ******:

Xenopus hsp27       KPAIQSAEISIPITF QSRAEIGTSEAKKGEEAAKK
Chicken hsp27       KPAIQSSEITIPVTVEAKKEEPAKK----------
Human hsp27         KLATQSNEITIPVTFESRAQLGGPEAAKSDETAAK
Zebrafish hsp27     KPAIQAPEVNIPVNKTTVSTTK-------------
Topminnow hsp27     KPALEYSETTIPVNVESSGAVAKK-----------
                    * * :  * .**:.  :
```

Table 1. ***A comparison of HSP27 and HSP30 amino acid sequences****

Percent identity with *Xenopus* HSP27	
Chicken HSP24 (Q00649)	71%
Zebrafish HSP27 (NP_001008615)	65%
Rat HSP27 (AAB29536)	64%
Human HSP27 (P04792)	63%
Mouse HSP25 (P14602)	62%
Topminnow HSP27 (AAB46593)	53%
Xenopus HSP30C (P30218)	19%
Xenopus HSP30D (P30219)	19%
Topminnow HSP30B (AAB46594)	17%
Bullfrog HSP30 (AAB04143)	17%
Chicken HSP25 (BAD34492)	16%

*An amino acid sequence comparison of *Xenopus laevis* HSP27 with HSP30, HSP27, and/or HSP25 from chicken, human, rat, zebrafish, mouse, topminnow, bullfrog and *Xenopus*. Genbank accession numbers are indicated within the brackets.

Figure 34: Phylogenetic analysis of the nucleotide sequences encoding regions of selected HSP27 and HSP30s.

Sequences were aligned using the Clustal W program and a phylogenetic tree was generated with the computer program MEGA2 using the neighbour-joining method with pairwise deletion and the Jukes and Cantor (1969) correction.

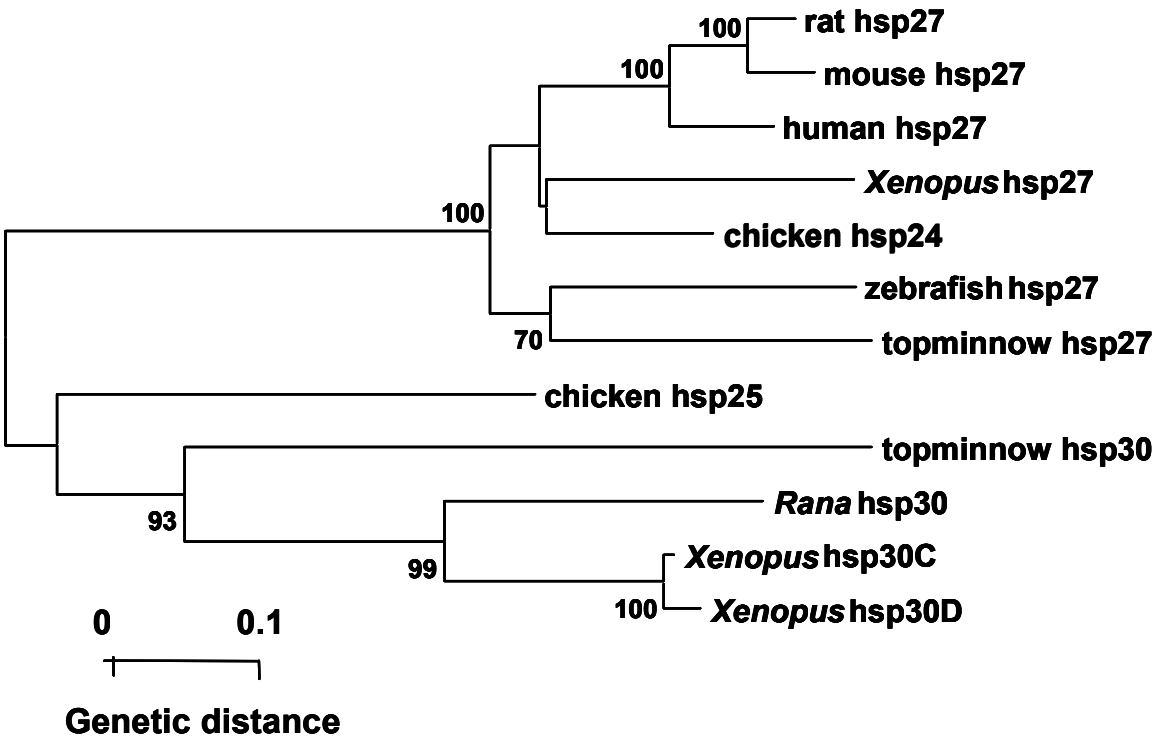

100
rat hsp27
100
mouse hsp27
human hsp27
Xenopus hsp27
100
chicken hsp24
zebrafish hsp27
70
topminnow hsp27
chicken hsp25
topminnow hsp30
Rana hsp30
93
99
Xenopus hsp30C
100
Xenopus hsp30D
0
0.1
Genetic distance

Figure 35: Purification of the *Xenopus* HSP27 recombinant protein.

Xenopus HSP27 was overexpressed in *E. coli* by induction with IPTG. The lysates from these cells were then passed over a nickel affinity column. The recombinant protein, which contains a six histidine tag, was bound to the column and then purified as outlined in the Experimental Procedures. Purified protein was then analysed by SDS-PAGE and visualised by Coomassie Brilliant Blue R-250 staining. Lane 1 contains molecular weight markers, masses shown in kDa. The bacterial lysates, not passed through a nickel affinity column are in lanes 2 and 3: uninduced (lane 2) and induced (6 h, lane 3) bacterial lysates, both containing the recombinant pRSETB-*Xenopus hsp27* construct. Lane 4 shows the purified recombinant *Xenopus* HSP27 protein. The asterisk indicates the position of the HSP27 protein.

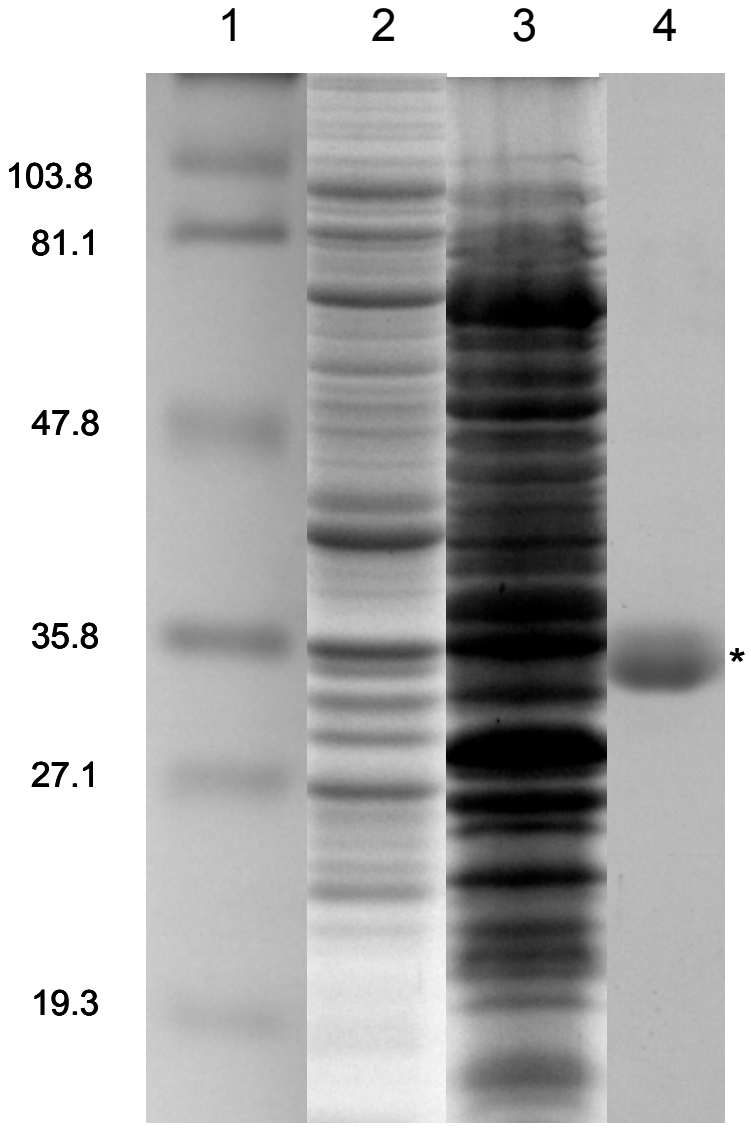
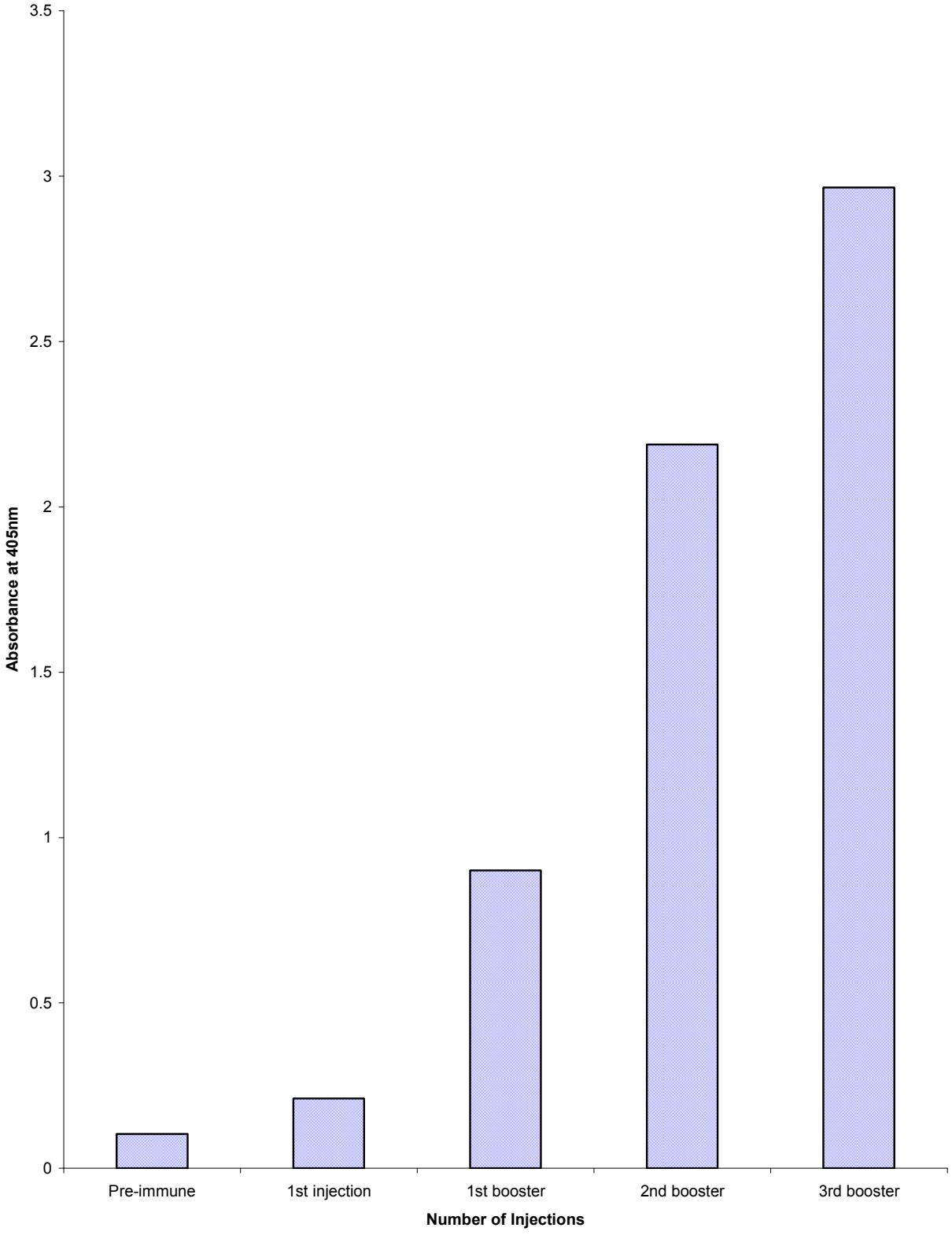

1
2
3
4
103.8
81.1
47.8
35.8
27.1
19.3
*

Figure 36: Antibody titre using an enzyme-linked immunosorbent assay.

Serum from the rabbit used to generate an anti-*Xenopus* HSP27 antibody was tested for reactivity to *Xenopus* HSP27 before (Pre-immune) and after a number of re-immunization injections. The relative antibody titre was gauged by the absorbance at 405 nm in an ELISA. The levels of antibody present in the serum continued to rise, even after the second booster. The serum did not cross-react with BSA when used as the antigen in the ELISA (data not shown).


3.5
3
2.5
2
1.5
1
0.5
0
Absorbance at 405nm
Pre-immune
1st injection
1st booster
2nd booster
3rd booster
Number of Injections

was shown to contain the polyclonal antibody against *Xenopus* HSP27 in an ELISA with an increasing titre after three subsequent injections (Figure 36). BSA did not cross-react with the serum, nor did the pre-immune serum react with recombinant *Xenopus* HSP27 (data not shown). The antibody was purified on a nickel affinity column bound with the recombinant *Xenopus* HSP27 antigen and tested with protein extracts from A6 cells as well as with *Xenopus* HSP27 recombinant protein as a positive control.

3.3.3 *Xenopus* HSP27 was detected constitutively and heat inducibly in tadpole stage embryos

Studies of *Xenopus* HSP27 accumulation were conducted in both *Xenopus laevis* A6 kidney epithelial cells and developing embryos. Western blot analysis of A6 cells exposed to heat shock or control conditions failed to show any accumulation of HSP27 protein (data not shown). However, analysis of tadpole-stage embryos (stage 44) showed a constitutive and heat-inducible accumulation of HSP27 (Figure 37). No accumulation of HSP27 was detected prior to this stage, contrary to results obtained with *Xenopus* HSP30, which was heat-inducible at midtailbud stage (stage 26).

3.4 Characterization of *Xenopus* HSP27 protein function

3.4.1 *Xenopus* HSP27 forms large multimeric aggregates

Recombinant *Xenopus* HSP27 protein was produced using the pRSETB vector system and induced using IPTG as outlined in Section 3.1. Recombinant *Xenopus* HSP27 protein was then used to determine the possibility of multimeric complex formation by means of native pore exclusion limit electrophoresis (Figure 38). Recombinant HSP27 was

Figure 37: *Xenopus laevis* HSP27 protein accumulates and is heat-inducible in embryos at the tadpole stage.

Xenopus laevis embryos were heat shocked for 2 h at either 33^{o}C, followed by a 2 h recovery period at 22^{o}C. Control embryos were maintained at 22^{o}C until being collected. Embryos were immediately frozen in liquid nitrogen following recovery and total protein was isolated and quantified as outlined in Experimental Procedures. Western blot analysis was performed using rabbit anti-*Xenopus* HSP27 or HSP30 antibodies to observe the relative levels of HSP27 and HSP30C (using 40 μg of total protein). HSP30C is not expressed constitutively in *Xenopus*, unlike HSP27, and is heat-inducible at mid-tailbud stage (MTB; stage 26 shown here) and at late tailbud stage (LTB; stage 34 shown here), two and a half days earlier than tadpole stage embryos (TP; stage 44 shown here) when HSP27 is seen both constitutively and the signal increases when heat-induced. The arrows indicate the position of the two proteins. The bottom panel is a representative Ponceau S stain of a portion of a stained blot.

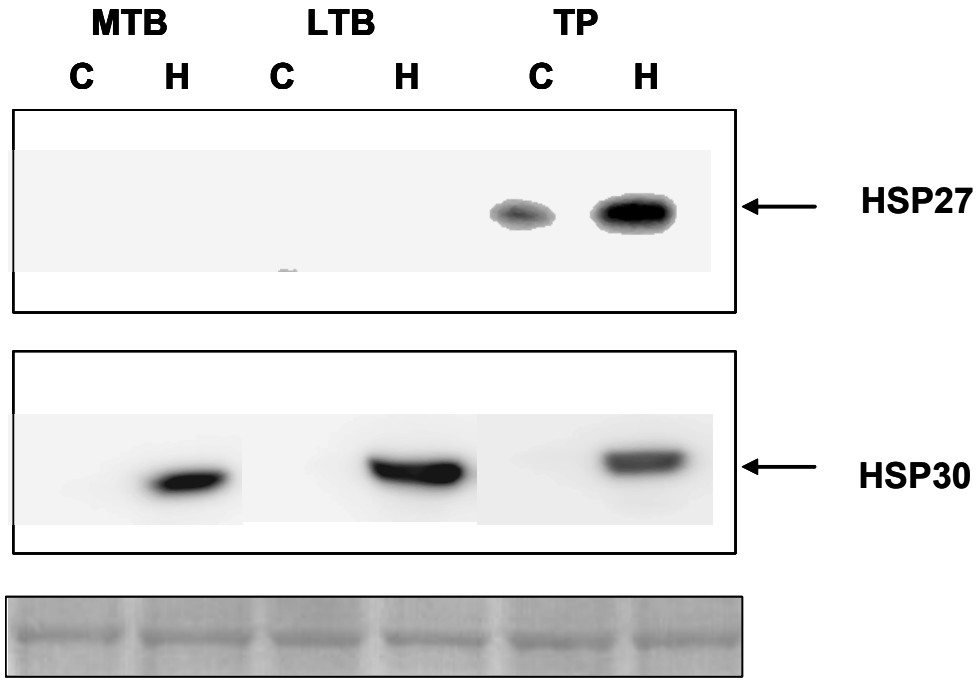
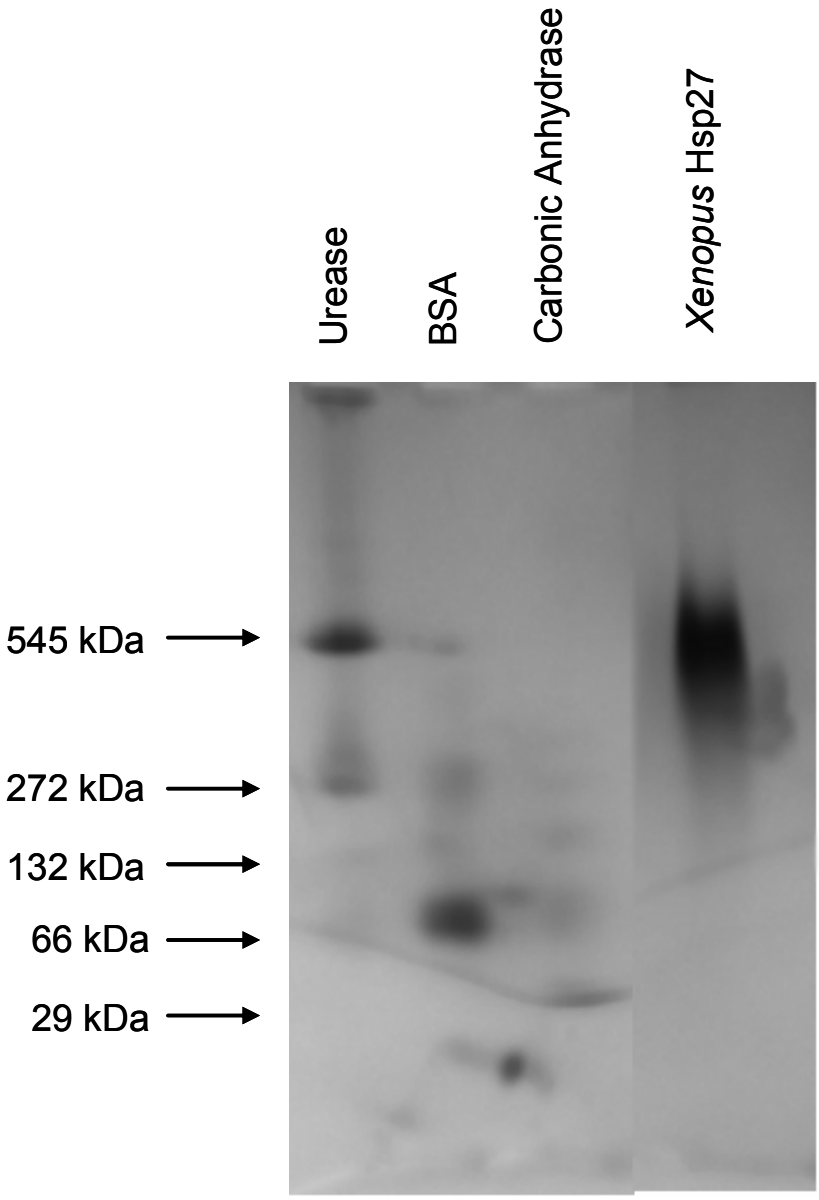

MTB
LTB
TP
C
H
C
H
C
H
HSP27
HSP30

Figure 38: *Xenopus laevis* HSP27 forms multimeric complexes.

Three proteins of known molecular weights (lane 1: urease, lane 2: bovine serum albumin, lane 3: carbonic anhydrase) were separated on a 4-20% non-denaturing gradient acrylamide gel in addition to 15µg of purified *Xenopus* HSP27 recombinant protein (lane 4). Proteins were visualized by Coomassie Brilliant Blue R-250 staining. Arrows indicate molecular masses, in kDa, of some of the bands in the protein standards.


Urease
BSA
Carbonic Anhydrase
Xenopus Hsp27
545 kDa
272 kDa
132 kDa
66 kDa
29 kDa

present as an aggregate with an approximate size of 545 kDa, similar to the size of the molecular weight standard, urease (Figure 38, lane 1).

3.4.2 Recombinant *Xenopus* HSP27 inhibits the thermal aggregation of target proteins

Analysis of the function of *Xenopus* HSP27 involved the determination of whether it was capable of inhibiting the heat-induced thermal aggregation of citrate synthase, luciferase or malate dehydrogenase *in vitro*. The amount of heat-induced aggregation was determined by measuring the increase in the amount of light scattering at 320 nm at 42^{o}C (CS) or 45^{o}C (LUC and MD) (Figures 39-41). Incubation of CS with varying molar ratios of *Xenopus* HSP27 showed its ability to prevent aggregation of CS. A 0.1:1 (HSP:CS) ratio resulted in a 30% reduction in aggregation. An additional 32% reduction was observed when a 1:1 ratio was used. Increasing the molar ratio to 3:1 showed a 90% reduction in aggregation. Incubation of *Xenopus* HSP27 with LUC confirmed its ability to prevent aggregation. A molar ratio of 0.5:1 (HSP:LUC) resulted in a 20% reduction in aggregation. A molar ratio of 3:1 resulted in a 60% reduction in aggregation over the course of the experiment. Finally, incubation of *Xenopus* HSP27 in varying molar ratios with MD indicated a role as molecular chaperone for *Xenopus* HSP27, as it prevented aggregation of MD. A ratio of 0.1:1 (HSP:MD) resulted in a 40% reduction in aggregation, while a 3:1 ratio resulted in 90% reduction of aggregation when compared to MD alone.

Figure 39: Inhibition of heat-induced aggregation of citrate synthase by *Xenopus* HSP27.

The effect of various molar quantities of recombinant *Xenopus* HSP27 on the heat-induced aggregation of citrate synthase (CS) at 42^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Citrate synthase was heated alone (♦) or combined in different molar ratios with *Xenopus* HSP27 (HSP:CS) at 3:1 (▲), 2:1 (X), 1:1 (*), 0.5:1 (|) and 0.1:1 (■). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of CS after 60 minutes and were expressed as the mean ± S.E.

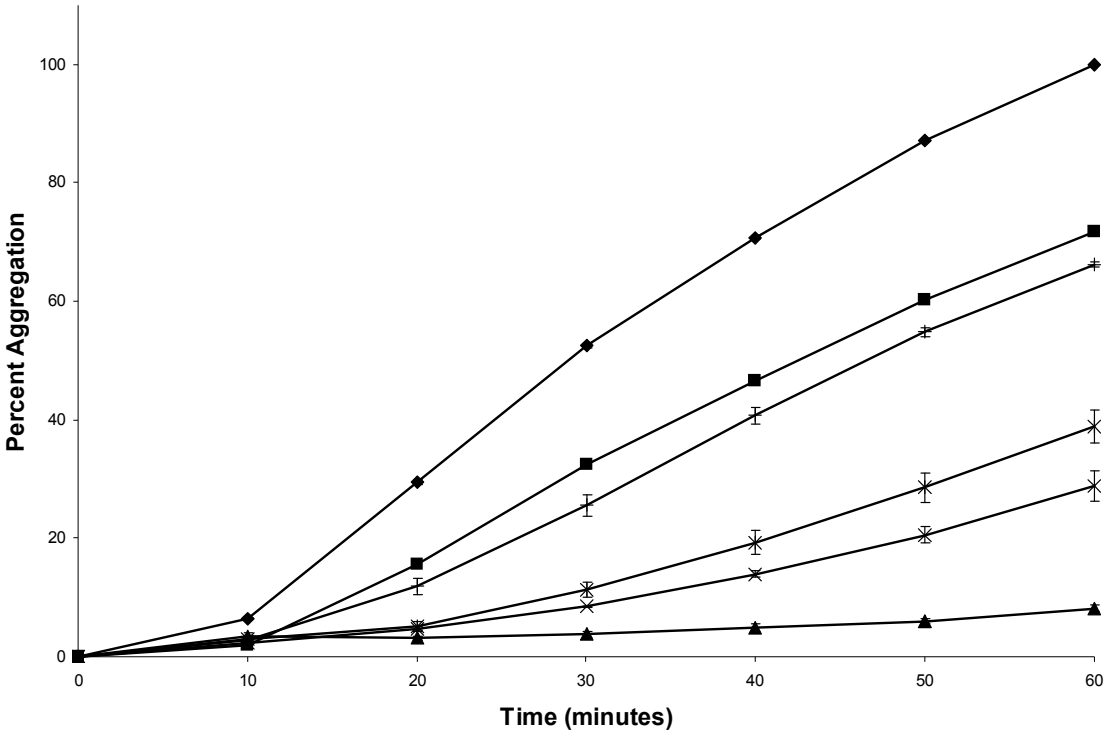

Percent Aggregation
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Figure 40: Inhibition of heat-induced aggregation of luciferase by *Xenopus* HSP27.

The effect of various molar quantities of recombinant *Xenopus* HSP27 on the heat-induced aggregation of luciferase (LUC) at 45^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Luciferase was heated alone (♦) or combined in different molar ratios with *Xenopus* HSP27 (HSP:LUC) at 3:1 (▲), 2:1 (*), 1:1 (|) and 0.5:1 (–). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of LUC after 60 minutes and were expressed as the mean ± S.E.

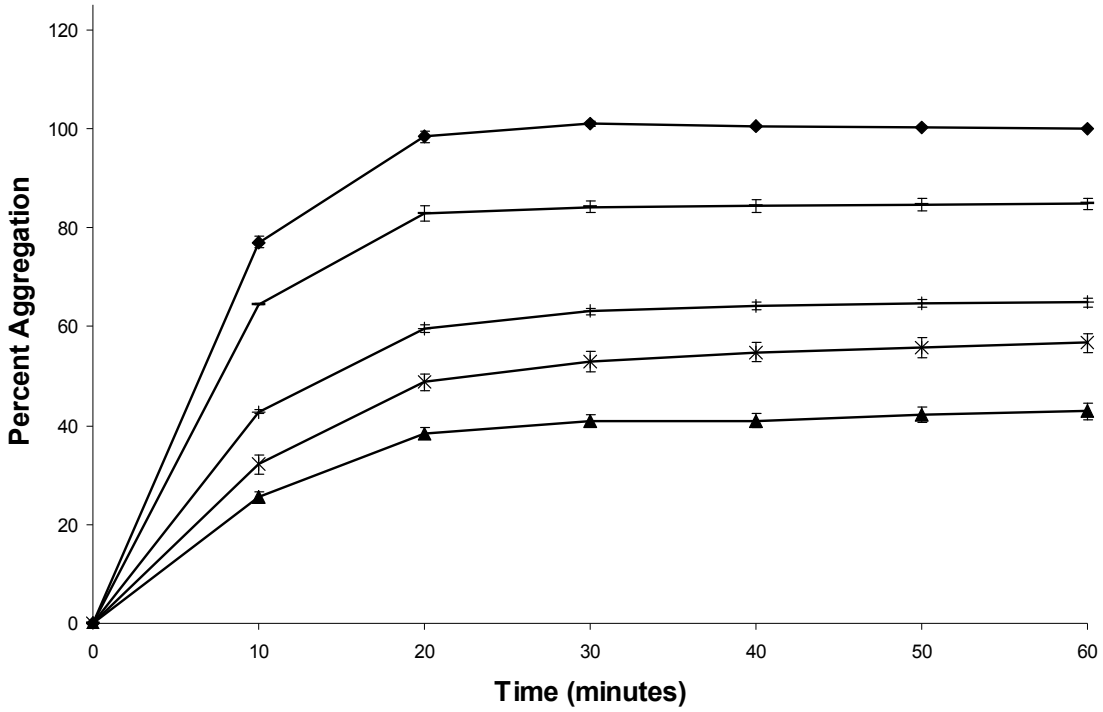
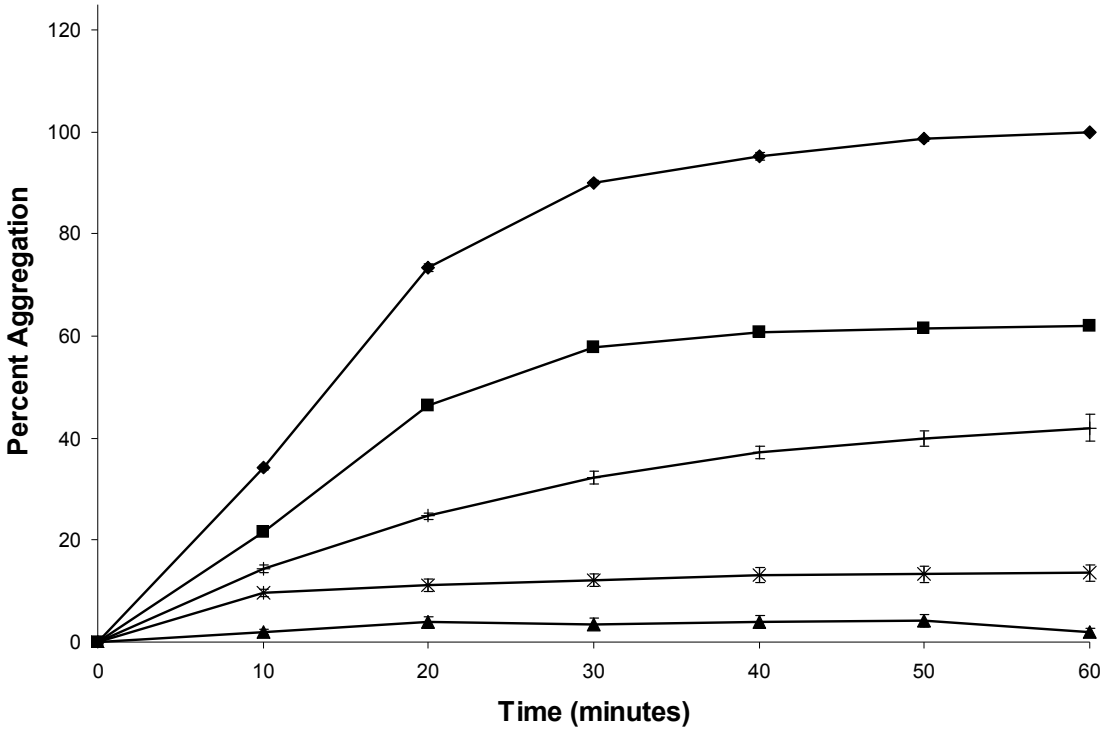

Percent Aggregation
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Figure 41: Inhibition of heat-induced aggregation of malate dehydrogenase by *Xenopus* HSP27.

The effect of various molar quantities of recombinant *Xenopus* HSP27 on the heat-induced aggregation of malate dehydrogenase (MD) at 45^{o}C was determined by measuring the amount of light scattered at 320 nm, as outlined in Experimental Procedures. Malate dehydrogenase was heated alone (♦) or combined in different molar ratios with *Xenopus* HSP27 (HSP:MD) at 3:1 (▲), 2:1 (*), 1:1 (ı) and 0.1:1 (■). Data are representative of 3 trials and were calculated as a percentage of the maximum aggregation of MD after 60 minutes and were expressed as the mean ± S.E.


120
100
80
60
40
20
0
Percent Aggregation
0
10
20
30
40
50
60
Time (minutes)

4 Discussion

The present study investigated the expression and functional properties of the *Rana catesbeiana* sHsp, HSP30, as well as the *Xenopus laevis* sHsp, HSP27. In my examination of *Rana hsp30* mRNA accumulation, an antisense riboprobe against *Rana hsp30* mRNA was produced. Determination of *hsp30* mRNA levels in *Rana* FT cells by Northern blot analysis revealed that this message was not detectable in cells maintained at 22^{o}C but that *hsp30* message accumulated in response to heat shock. Heat shock treatment of FT cells resulted in a transient accumulation of *hsp30* mRNA after 2 h of treatment at either 33^{o}C or 35^{o}C. Heat shock induced accumulation of *hsp30* mRNA in *Rana catesbeiana* was also reported by Helbing *et al*. (1996) who found that a heat shock temperature of 36^{o}C was sufficient to induce the accumulation of *Rana hsp30* mRNA in heat shocked isolated red blood cells and liver tissue. The accumulation of *Rana hsp30* mRNA was most likely the result of HSE-HSF interaction. This was suggested by the work of Helbing *et al*. (1996) who screened a *Rana* genomic DNA library for members of the *hsp30* gene family and found that the *hsp30* gene contained a highly conserved TATA box that was preceded by one complete HSE. As with other Hsps, it was likely that HSF interacted with the HSE that has been found in the 5' upstream promoter region of most heat shock protein genes studied to date, which mediates transcription (Heikkila, 2003), initiating the RNA polymerase II transcription of the hsp gene.

Rana hsp30 mRNA was detected in heat-treated FT cells but not in cells maintained at 22^{o}C. This finding was similar to results found with *Xenopus laevis* A6 cells in which *hsp30* mRNA was not present constitutively, but was stress-inducible (Lang *et al*., 1999; Fernando and Heikkila, 2000). The temporal pattern of *Rana hsp30* mRNA

accumulation at 33°C and 35°C displayed optimal accumulation at 2 h, but with a greater extent of accumulation at 35°C. In Northern blot analysis, *hsp30* message occurred as a doublet. This may have been due to the presence of different, but closely related genes, as previously described by Helbing *et al*. (1996). Southern blot analysis revealed two or more gene sequences that recognized the ORF of the *hsp30* gene isolated from *Rana catesbeiana.* Also in the latter study, *in vitro* translation of hybrid-selected *hsp30* mRNAs encoded at least ten different proteins that ranged in size from 25 to 30 kDa, indicating that *Rana hsp30* may be a member of a multigene family (Helbing *et al*., 1996).

Analysis of *Rana* HSP30 protein accumulation by Western blot analysis and immunocytochemistry required an antibody. To this end, the coding region of the *Rana hsp30* gene was cloned into a plasmid vector and over-expressed in bacteria. The purified recombinant protein was employed to generate an anti-*Rana* HSP30 antibody in rabbits which cross-reacted with HSP30 in cultured heat-treated *Rana* FT cells. The anti-*Rana* HSP30 antibody also cross-reacted weakly with heat-inducible HSP30 protein in *Xenopus* A6 kidney epithelial cells. This result was consistent with the degree of identity (52%) between the *Xenopus* and *Rana* HSP30 amino acid sequences.

A temperature profile, conducted to determine optimal experimental temperatures for HSP30 protein accumulation, revealed that HSP30 was undetectable in FT cells at heat shock temperatures below 35°C. This was a higher temperature than found for *Xenopus laevis*, which accumulated HSP30 at heat shock temperatures of 33°C (Lang *et al*., 1999). As found with *Rana hsp30* mRNA, *Rana* HSP30 protein was undetectable in control samples but was heat shock-inducible after as little as 2 h and optimal at 4 h. However, treatment with sodium arsenite (10 – 100 μM), a known stressor and inducer of heat shock

proteins in *Xenopus laevis* (Gellalchew and Heikkila, 2005; Hamilton and Heikkila, 2006), failed to elicit detectable accumulation of either *hsp30* mRNA or protein. This finding was consistent with similar findings in salmon (*Salmo salar*), in which branchial lamellae and hepatic tissues did not mount a heat shock or stress response after exposure to concentrations of sodium arsenite ranging from 25 to 200 μM (Smith *et al*., 1999).

Heat shock treatments resulted in HSP30 accumulation at time points later than observed with mRNA accumulation. This was most likely due to the time required to process the mRNA for translation into protein. Previous studies have also indicated delays in heat shock protein production in comparison to heat shock protein mRNA accumulation. For example, in heat-treated rabbit cerebral tissues, a delay was observed between accumulation of *hsp70* mRNA and its respective protein (Manzerra *et al*., 1993). Interestingly, *Rana* HSP30 protein also occurred as a double band in Western blot analysis. There are a number of possibilities to account for this finding including multiple *hsp30* gene products. *Xenopus laevis* was reported to have multiple HSP30 isoforms (Tam and Heikkila, 1995). For example, Western blot analysis using an anti-*Xenopus* HSP30 antibody revealed a total of 13 sHsps at the early tadpole stage of development and a total of eight sHsps in cultured A6 cells, all of which were only heat-inducible (Tam and Heikkila, 1995). Of these sHsps, there were some that were common to both embryos and cultured cells while others were unique to one or the other. It is also possible that post-translational modification could account for two *Rana* HSP30 protein bands. Further studies will have to be done to determine possible phosphorylation sites or other post-translational modifications which would result in the appearance of more than one band in Western blot analysis.

Confocal laser scanning microscopy (CLSM) analysis of immunostained cells examined the intracellular localization of HSP30 protein after heat shock in *Rana catesbeiana* FT cells. Immunocytochemical analysis of heat shocked cells revealed that HSP30 was enriched in the nucleus with diffuse localization in the cytoplasm. While heat shock treatment below 35^{o}C did not produce detectable HSP30 in *Rana* FT cells, incubation at 35^{o}C resulted in accumulation in the nucleus and cytoplasm after as little as 2 h. Increasing incubation time resulted in a greater concentration of HSP30 in the nucleus compared to the cytoplasm. An 8 h incubation at 35^{o}C resulted in almost no cytoplasmic localization of HSP30, but a high relative amount within the nucleus. Upon closer examination of HSP30 accumulation in *Rana* FT cells, a punctate pattern similar to that found previously in *Xenopus* A6 cells was observed (Gellalchew and Heikkila, 2005). *Rana* HSP30 was previously found to form high molecular weight aggregates in our laboratory (Kaldis *et al*., 2004). It is very likely, then, that this punctate pattern is a reflection of the formation of these sHsp multimeric aggregates *in vivo*. This phenomenon has been previously observed in other eukaryotic systems following heat shock treatment (Arrigo and Landry, 1994; Gellalchew and Heikkila, 2005). Heat shock treatment at 35^{o}C did not induce any detectable alteration in FT cell shape or F-actin structure. There was also no obvious co-localization of HSP30 and actin under heat shock conditions. Studies conducted in *Xenopus laevis* A6 kidney epithelial cells revealed that 35^{o}C heat treatment caused localized areas of F-actin disorganization in the cytoplasm and that these regions contained HSP30 (Gellalchew and Heikkila, 2005). Similarly, rat αB-crystallin and HSP20 have both been found to associate with actin in cardiac myocytes (Pipkin *et al*., 2003). Contrary to previously observed patterns of small heat shock protein accumulation

(Vincent and Tanguay, 1979; Collier and Schlesinger, 1986; Arrigo and Landry, 1994; Pipkin *et al*., 2003), *Rana* HSP30 appears to preferentially accumulate in the nucleus and not the cytoplasm or perinuclear regions. Additional recovery time following a 4 h heat shock treatment at 35^{o}C resulted in an increase in nuclear localization of HSP30. The nuclear localization of HSP30 following extended heat shock and recovery suggest that its primary protective function may involve the protection of nuclear proteins. In the future, it would be interesting to study whether *Rana* HSP30 co-immunoprecipitates with known nuclear proteins.

In an effort to elucidate the molecular chaperone properties of *Rana catesbeiana* HSP30, a recombinant *Rana* HSP30 protein was produced. One characteristic of a molecular chaperone is the ability to prevent the heat-induced aggregation of target proteins. Thus, *Rana* HSP30 was tested with a variety of target proteins. While heat treatments of citrate synthase (CS), luciferase (LUC) and malate dehydrogenase (MD) resulted in extensive aggregation, incubation of these target proteins with *Rana* HSP30 resulted in the successful inhibition of heat-induced aggregation in the presence of molar ratios of 1:1 or more of *Rana* HSP30. Previous studies conducted in our laboratory examined the chaperone function of *Xenopus* HSP30C and HSP30D. These studies found that a 5:1 molar ratio of either HSP30C or HSP30D were required to reduce aggregation by 90% (Fernando and Heikkila, 2000; Abdulle *et al*., 2002). Studies with sHsps from other organisms have yielded similar results. For example, Lee *et al*. (1995, 1997) determined that a 2:1 molar ratio of plant sHsp was sufficient to achieve inhibition of aggregation of CS, MD or glyceraldehyde-3-phosphate dehydrogenase. Comparison of *Rana* HSP30 chaperone function with that of *Xenopus* HSP30C revealed that there was no major

difference between the two proteins in the inhibition of heat-induced aggregation of target proteins.

In conclusion, this study has shown that *Rana hsp30* mRNA was expressed under heat shock conditions in FT cells. In addition, *Rana* HSP30 was localized primarily in the nucleus with additional diffuse cytoplasmic localization. *Rana* FT cells were unresponsive to sodium arsenite as a stressor. Future studies with other known chemical stressors are required to determine whether this apparent insensitivity to chemical stress is unique to sodium arsenite. It was also found that *Rana* HSP30 functioned as a molecular chaperone by inhibiting the thermal aggregation of a variety of target proteins. Previous studies showed that *Rana* HSP30 was capable of maintaining luciferase in a folding competent state, another indicator of molecular chaperone activity (Kaldis *et al*., 2004). Based on the present and previous studies, it is most likely that HSP30 functions to inhibit stress-induced protein aggregation (Helbing *et al*., 1996; Arrigo, 1998; Kaldis *et al*., 2004).

The present study has also analysed the cDNA sequence and expression of the small heat shock protein gene, *hsp27*, in *Xenopus laevis* embryos. Amino acid sequence comparison of the coding region of *Xenopus* HSP27 with HSP27 from avian, fish and mammalian species revealed an identity of 53-71%, *Xenopus* HSP27 having the highest identity with chicken HSP24. Most of the identity was found in the α-crystallin domain. However, *Xenopus* HSP27 only shared a 19% identity with either *Xenopus* HSP30C or HSP30D, a finding that is in keeping with the severe lack of conservation of small heat shock protein sequences among and between species (Arrigo and Landry, 1994; Lindner *et al.*, 1998; van den Ijssel *et al*., 1999; Stromer *et al*., 2003). As with *Xenopus*, chicken sHsps are not well-conserved within the species. Chicken HSP25 has a high identity with

Xenopus HSP30C and HSP30D (Krone *et al*., 1992), topminnow HSP30B (Norris *et al*., 1997), and salmon HSP30 (unpublished, SP accession No. P49231), but is less related to chicken HSP24, a member of the HSP27 family (Katoh *et al*., 2004).

Purified recombinant HSP27 protein was employed to generate an anti-*Xenopus* HSP27 antibody in rabbits. Western blot analysis results using this antibody revealed that HSP27 protein was not detectable in *Xenopus* A6 kidney epithelial cells under control or heat shock conditions. This result was in agreement with preliminary findings in our laboratory that *hsp27* mRNA was neither constitutive nor heat-inducible in A6 cells (Julie Gauley, unpublished results). In contrast, Western blot analysis of embryos revealed a constitutive protein band that cross-reacted with the anti-*Xenopus* HSP27 antibody at tadpole (stage 44). HSP27 was also heat-inducible at this stage. No accumulation was detected prior to this stage of development. The accumulation pattern of HSP27 protein was distinct from *Xenopus* HSP30, which was heat-inducible at midtailbud stage 26, approximately two and a half days earlier in development. Northern blot studies using mRNA isolated from embryos during early development indicated that *hsp27* mRNA was present both constitutively and was heat-inducible as early as midtailbud (stage 26) (Julie Gauley, unpublished results). The pattern of HSP27 accumulation in *Xenopus laevis* embryos may indicate that its role was not protection during the formation of essential structures within the early embryo, but one of maintenance once these structures were formed. Further analysis of HSP27 function during embryogenesis and cellular localization will have to be conducted to elucidate its role during development. A differential pattern of heat-inducible accumulation of HSP30 family members was also observed in *Xenopus laevis* embryos, suggesting that they may have distinct functions at specific developmental

stages (Tam and Heikkila, 1995). In zebrafish, constitutive levels of HSP27 were detected in embryos as early as 5 – 8 h post fertilization, a time which corresponds to mid-gastrula stage in *Xenopus laevis* development (Mao *et al*., 2005). The accumulation of this sHsp was also heat-inducible. Due to the early detection time of constitutive and heat-inducible HSP27, it is highly probable that HSP27 may play a role in early embryogenesis in zebrafish.

To examine the molecular chaperone properties of *Xenopus laevis* HSP27, a recombinant *Xenopus* HSP27 protein was produced. Previous studies have shown that most molecular chaperones require the formation of multimeric complexes for functionality (Freeman and Yamamoto, 2002; Azzoni *et al*., 2004). In the cyanobacterium *Synechocystis*, HSP16.6 was shown to require changes in oligomerization to function as a molecular chaperone both *in vivo* and *in vitro* (Giese and Vierling, 2002). Consequently, native pore exclusion limit electrophoresis was conducted on *Xenopus* HSP27 to determine its ability to form these complexes. Native pore exclusion limit electrophoresis of the recombinant *Xenopus laevis* HSP27 revealed the formation of a large molecular weight complex of approximately 545 kDa in size. Native pore exclusion limit electrophoresis studies conducted with *Rana* HSP30 and size exclusion chromatography with *Xenopus* HSP30C, revealed comparable results indicating that both Hsps formed large multimeric aggregates ranging in size from 130 kDa to 900 kDa (Kaldis *et al*., 2004; Fernando and Heikkila, 2000).

Finally, studies were conducted with recombinant *Xenopus* HSP27 protein to determine its ability to prevent the heat-induced aggregation of target proteins. As with *Rana* HSP30, *Xenopus* HSP27 successfully inhibited the heat-induced aggregation of

citrate synthase, luciferase and malate dehydrogenase. However, higher molar ratios of 3:1 or more were required to achieve inhibition of aggregation by 90% or more. Similar findings have been obtained with HSP27 from other organisms. For example, chicken HSP24 and human HSP27 inhibited thermal aggregation of CS in a dose-dependent manner (Chabaud *et al*., 2003; Chávez Zobel *et al*., 2005). Furthermore, mouse HSP27 bound and protected denatured CS from thermal aggregation (Thériault *et al*., 2004). The finding that HSP27 was detected constitutively in tadpole stage embryos suggests a role as a molecular chaperone in normal cellular maintenance. Also, heat-induction of HSP27 indicates a possible molecular chaperone function in the protection of cells from external stressors. Other research revealed that HSP27 protected cells against oxidative stress, which can occur naturally under normal physiological conditions (Arrigo *et al*., 2005). It is not known whether a similar function can be ascribed to *Xenopus* HSP27 during early development.

In conclusion, this study has shown that *Xenopus* is among several organisms that possess more than one sHsp subfamily of the α-crystallin/sHsp superfamily. Researchers studying the desert topminnow, *P. lucida*, discovered that, in addition to HSP30, *P. lucida* also had a heat-inducible HSP27 (Norris *et al*., 1997). Characterization of both cDNAs revealed that both were members of the α-crystallin/sHsp superfamily but were members of separate lineages; HSP30 being more closely-related to *Xenopus* and salmon HSP30s and *P. lucida* HSP27 was more closely related to mammalian and avian HSP27 sHsps. Chicken was also determined to possess members from both the HSP30 and HSP27 lineages. Chicken HSP24 was a member of the HSP27 family, while HSP25 was more closely related to the HSP30 family, as determined by amino acid sequence and

phylogenetic analyses (Katoh *et al*., 2004). There are a number of possible explanations for the presence of more than one sHsp family. Multiple sHsps with overlapping functions could protect the organism from genetic mutation. Also, the presence of more than one sHsp family ensures that the organism is adequately protected from environmental and chemical stressors. Gene knock-out experiments in which entire families of sHsps are rendered ineffective may shed some light on their function. Currently, we know that *Rana catesbeiana* possesses a member of the HSP30 subfamily as well as a member of the α-crystallin subfamily. In future studies, it may be of interest to determine whether it also contains a member of the HSP27 family as found with *Xenopus*, chicken and topminnow.

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