CHARACTERIZATION OF HSP47 EXPRESSION IN *XENOPUS LAEVIS* CELL CULTURE AND EMBRYOS

By
Amanda M. Hamilton

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

Abstract

The heat shock or stress response is a transient response to stressful stimuli that protects vital cellular proteins from damage and irreversible aggregation. Heat shock proteins (Hsps) are molecular chaperones that bind to unfolded protein and inhibit their aggregation, thereby maintaining their solubility until they can be refolded to their native conformation. Hsp47 is an endoplasmic reticulum (ER)-resident protein that serves as a molecular chaperone during collagen production. Collagen is the major class of insoluble fibrous protein found in the extracellular matrix and in connective tissues. It is the single most abundant protein of the animal kingdom; at least 14 different forms exist, each with distinct structures and binding properties. The various types of collagen all possess protein regions with the distinct triple helical conformation. This complex physical structure requires very organized assembly and HSP47 has been established as an integral component of this process for collagen types I-V. Most of the previous studies examining the expression and function of hsp47 have been conducted with mammalian cultured cells. The present study represented the first investigation of the expression of hsp47 in the poikilothermic vertebrate, *Xenopus laevis*.

Full-length *Xenopus* hsp47 nucleotide and amino acid sequences were obtained from Genbank and compared with hsp47 from chicken, mouse, rat, human and zebrafish. *Xenopus* HSP47 protein had an identity of approximately 77% with chicken, 73% with mouse, 72% with rat and human, and 70% with zebrafish. Most of the sequence identity between HSP47 from all investigated organisms occurred centrally in the amino acid sequence and in several carboxyl terminal regions. Three key features were conserved between HSP47 proteins from most species investigated: a hydrophobic leader sequence,

two potential glycosylation sites and the ER-retention signal, RDEL.

A partial cDNA clone encoding *Xenopus* hsp47 was obtained from the American Type Culture Collection (ATCC) and used to generate hsp47 antisense riboprobe for the purpose of investigating hsp47 mRNA accumulation in *Xenopus* A6 kidney epithelial cells and embryos. Northern blot analysis detected hsp47 mRNA constitutively in A6 cells. The expression pattern for hsp47 mRNA was compared with two other *Xenopus* heat shock proteins that have been previously characterized in our laboratory: hsp70, a cystolic/nuclear hsp and BiP, an ER-resident hsp. The results of hsp47 mRNA accumulation in A6 cells suggested that the expression pattern for *Xenopus* hsp47 was unique but, with respect to some stressors, resembled that of a cytosolic hsp rather than an ER-resident hsp.

HSP47 protein levels were also examined in A6 cells. Heat shock, sodium arsenite and β-aminopropionitrile fumerate treatments enhanced hsp47 accumulation. In some experiments, western blot analysis revealed the presence of two closely sized protein bands. It is possible that minor differences in HSP47 protein size may be due to post-translational modification, namely phosphorylation or glycosylation.

The present study also examined the accumulation and spatial pattern of hsp47 mRNA accumulation during *Xenopus laevis* early development. Hsp47 was constitutively expressed throughout *Xenopus* early development. Constitutive levels of hsp47 mRNA in unfertilized eggs, fertilized eggs and cleavage stage embryos indicated that these transcripts were maternally inherited. Constitutive hsp47 mRNA accumulation was enhanced in neurula and tailbud embryos compared to earlier stages. This finding may be explained by the shift towards organogenesis during these stages. Whole mount *in situ* hybridization revealed hsp47 message along the dorsal region of the embryo, in the notochord and

somites, as well as in the head region including the eye vesicle. Hsp47 mRNA induction in *Xenopus* embryos was also examined in response to heat shock. Hsp47 mRNA accumulated in response to heat shock immediately following the midblastula transition (MBT). In tailbud stages, hsp47 mRNA accumulated in the notochord, somites and head region. Northern blot analysis and whole mount *in situ* hybridization results revealed an expression pattern that coincided well with the development of collagen-rich tissues thereby substantiating the proposed role of HSP47 as a procollagen molecular chaperone.

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1. Introduction

1.1 Heat Shock Response

The heat shock or stress response is a transient response to stressful stimuli that serves to protect vital cellular proteins from damage and irreversible aggregation (Katschinski, 2004). Once the cell returns to normal conditions, denatured proteins are refolded into their native, functional state. This protective change in cellular activity was first observed in response to heat shock in the fruit fly *Drosophila hydei* (Ritossa, 1962). Elevated temperatures caused chromosomal puffs in the larval salivary gland, which was later found to represent an increase in the transcription and production of heat shock proteins (HSPs; Nover and Scharf, 1991).

Since the discovery of the heat shock response, almost every organism studied to date, ranging from bacteria to humans, has been found to possess this crucial response and the proteins responsible for it. Further investigation has also revealed that, in addition to heat shock, other stressors can induce the heat shock response. Environmental stresses such as UV light, anoxia, heavy metals and a variety of chemicals; pathophysiological and disease states such as inflammation and viral or bacterial agents; and non-stress conditions such as growth factors, cellular differentiation, development or oncogene activation can all induce heat shock protein production (Katschinski, 2004; Morimoto *et al*, 1994; Parsell and Lindquist, 1993).

1.1.1 Heat Shock Proteins

Heat shock proteins are divided into several families based on sequence similarity and size. To date six HSP families have been characterized, these include the small heat shock proteins (sHSPs), HSP40, HSP60, HSP70, HSP90 and HSP100 families (Burdon, 1986; Schlesinger, 1994; Katschinski, 2004). The majority of heat shock protein families

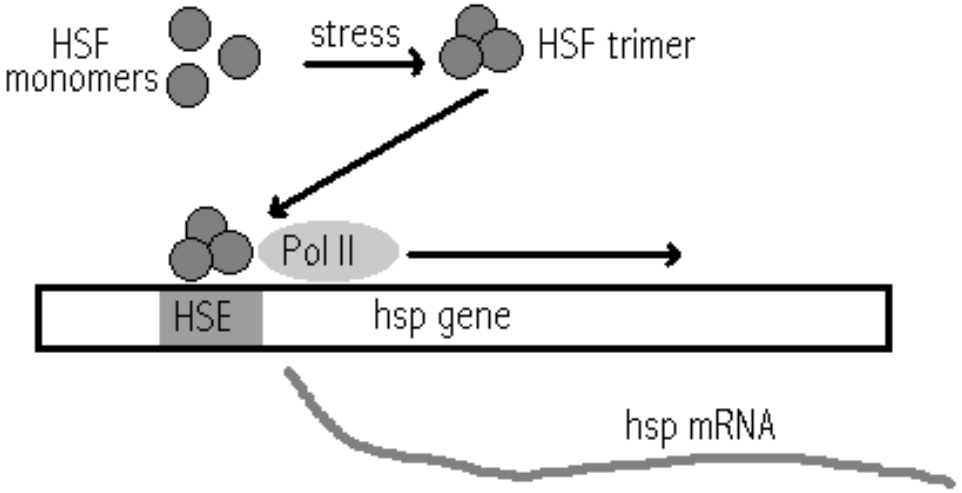
are strictly stress-inducible, however, constitutively expressed members do exist. Constitutive heat shock proteins function as molecular chaperones by assisting in the folding, assembly and intracellular transport of proteins (Parsell and Lindquist, 1993).

Elevated expression of heat shock protein genes is mediated at multiple levels: transcription, mRNA stability and translation efficiency (Katschinski, 2004). In the case of most HSPs induction is regulated by transcription factors known as heat shock factors (HSFs) (Wu *et al*, 1994). HSFs are constitutively expressed and maintained in the cytoplasm as latent, inactive monomers with low DNA-binding affinity. In response to stress, HSF monomers trimerize into a DNA-binding active complex (Figure 1). Once activated, the homotrimers will bind heat shock elements (HSE) consisting of inverted repeats of [nGAAn] that are highly conserved in the 5' upstream regions of stress-inducible genes (Fernandes *et al*, 1994). The net result of this interaction is increased transcription of hsp genes.

1.1.2 The hsp70 Family

The hsp70 family is the most heavily studied heat shock protein family and consists of several functional homologs found in different compartments of the cell. Family members include cytosolic stress-inducible hsp70, cytosolic constitutively expressed hsc70, mitochondrial p75 and ER-resident protein, immunoglobin binding protein (BiP, Grp78) (Morimoto, 1998). All of the hsc/hsp70 isoforms act as molecular chaperones that protect nascent or denatured proteins from aggregation and assist in the proper folding or refolding of cellular proteins into their correct conformations. Extensive sequence comparison

Figure 1: Transcriptional induction of heat shock proteins (HSPs). Heat shock factor (HSF) monomers present in the cytoplasm homotrimerize in response to stress. The HSF trimer migrates to the nucleus were it binds heat shock elements (HSE) found in the cis-regulatory regions of HSPs. Pol II, RNA polymerase II.


HSF
monomers
stress
HSF trimer
Pol II
HSE
hsp gene
hsp mRNA

between hsp70 family members has identified two functional domains: the amino terminal domain possessing a high affinity ATP-binding site and the carboxyl terminal domain responsible for intracellular localization and substrate interactions (Morimoto and Milarski, 1990). Studies of cytosolic hsp70 have identified the synthesis of this protein during various stress conditions including elevated temperature, chemical and heavy metal exposure. HSP70 acts to protect cells from these stress conditions that would otherwise lead to an accumulation of unfolded protein. Constitutive ER-resident BiP functions as an endoplasmic reticulum chaperone that assists in translation, folding and assembly of nascent oligomeric proteins (Lee, 1987).

1.2 Discovery of hsp47

In 1984 Kurkinen and colleagues discovered a glycoprotein from mouse embryo parietal endoderm cells that bound both native collagen and gelatin (denatured aggregates of type I and III collagens). This 47 kDa protein was termed 'colligin' and was postulated as a cell surface-associated protein due to its ability to be labelled by lactoperoxidase-iodination (Kurkinen *et al*, 1984). The exact location and function of the protein was not known at this time, although it was found to possess N-linked oligosaccharide side chains which bound native type IV collagen. Kurkinen and coworkers (1984) also showed that undifferentiated F9 embryonal carcinomal cells synthesized low levels of the collagen-binding protein and that its synthesis was significantly increased with retinoic acid and dibutyryl cAMP.

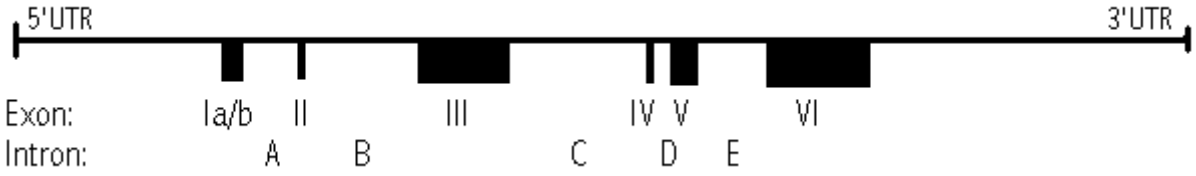
'Colligin' was discovered to be a heat shock protein (HSP) two years later when Nagata *et al* (1986) analyzed the heat shock inducibility of chick embryo fibroblast proteins using SDS polyacrylamide gradient gel electrophoresis (PAGE) (Nagata *et al*,

1986). Several well-characterized proteins were found, but also a 47 kDa protein that corresponded to the recently-discovered collagen-binding protein. This protein has been known by several names including colligin, J6, gp46 and CB48 (Hirayoshi *et al*, 1991), but since its discovery as a heat shock protein, hsp47 is now considered the uniform name. Two-dimensional (2D) PAGE revealed that HSP47 was unusually basic, with a pI of 9.0 (Nagata *et al*, 1986). The basic nature of HSP47 is one of several reasons sited for the delayed discovery of this protein as an HSP. Other HSPs are acidic with typical pIs between 5 and 6 (Schelsinger, 1985) and up until the discovery of HSP47, investigation of HSPs had been performed using 2D gels examining a pH range between 4 and 7 (Nagata *et al*, 1986). Another speculated reason for the previous absence of characterization of HSP47 was that early studies failed to use gradient gels that are necessary to obtain sufficient resolution for separation of the HSP47 band from the large actin band.

Hsp47 is related to a family of proteins called serine protease inhibitors (serpins). The genomic organization of the *hsp47* gene is similar to the α_1AT group of serpins but is sufficiently distinct to be classified into its own group within the serpin superfamily (Hosokawa *et al*, 1993).

The discovery of 'colligin' as a heat shock protein marked the first discovery of an HSP with a defined protein binding activity (Nagata *et al*, 1986). The existence of collagen-binding activity in HSP47 suggested that the well-characterized and widely studied heat shock proteins might possess a diverse range of specific biochemical activities in addition to rendering stress tolerance.

Figure 2: The gene structure of *hsp47* in mice. *hsp47* consists of 6 exons separated by 5 introns. Figure adapted from Hosokawa *et al*, 1993.


5'UTR
3'UTR
Exon:
Ia/b
II
III
IV
V
VI
Intron:
A
B
C
D
E

1.2.1 *Hsp47* Gene and Promoter Structure

The *hsp47* gene has been found in a range of organisms since its discovery: mouse, rat, chicken, zebrafish and humans (Pearson *et al*, 1996; Ikegawa *et al*, 1995; Nagata *et al*, 1986; Nagata and Yamada, 1986). In most investigated organisms, *hsp47* exists as a single copy within the genome, with the exception of human in which two *hsp47* genes have been cloned (Ikegawa and Nakamura, 1997; Ikegawa *et al*, 1995). The gene and promoter structure of *hsp47* has been investigated using the murine gene. This gene spans approximately 7.8 kb and consists of 6 exons (I-VI) separated by 5 introns (A-E) (Figure 2; Hosokawa *et al*, 1993). The mouse *hsp47* gene promoter has also been examined and several key features have been found. The TATA box was positioned at –31, however no CAAT box was found (Hosokawa *et al*, 1993). A complete heat shock element (HSE) consensus sequence was found between nt –61 and –79. The HSE consists of three repeats of NGAAN and allows increased expression of hsp47 following the binding of a homotrimer of heat shock factor (HSF) to the element. Between –156 and –215 three Sp-1 binding sites exist. Promoter mutagensis revealed that one of these Sp-1 binding sites was at least partially responsible for the tissue-specific expression of hsp47 (Hosokawa *et al*, 1993). One AP-1 binding site is located at –514 and a retinoic acid response element is also present within the regulatory region of murine *hsp47*, however its precise location has not been indicated in current literature. Computer analysis of the promoter region did not show any significant similarity to other vertebrate promoters.

1.2.2 Hsp47 Regulation: Transcription Factors and Alternative Splicing

The expression of heat shock proteins has been studied extensively and most regulation has been attributed to transcriptional control mediated by *trans*-acting heat

shock factors binding to heat shock elements. The *hsp47* promoter possesses a heat shock element and hsp47 has been clearly shown to accumulate in response to stressors whose effects are mediated by HSF activation (Hosokawa *et al*, 1993).

Post-transcriptional control is also thought to play an important role during the heat shock response, since, with the exception of heat shock proteins, protein synthesis decreases in heat-shocked cells of many organisms (Sorger, 1991). Hsp47 provides evidence that some hsps are at least partially controlled at the post-transcriptional level. In mice, three alternatively spliced mRNAs have been detected, differing only in their 5' non-coding region (Takechi *et al*, 1992; Wang and Gudas, 1990). One of the alternatively spliced mRNAs has only been detected after heat shock and is 169 nucleotides longer in the 5' UTR than hsp47 mRNA expressed under control conditions (Takechi *et al*, 1994). The altered splicing pattern caused the 'skipping' of exon II and splice donor site alteration of exon I. Several other stressors including sodium arsenite were investigated for the induction of altered splicing patterns, but so far, heat shock has been the only stressor to induce this splicing phenomenon.

The alternatively spliced 5' region of heat induced hsp47 mRNA has been suggested to be biologically significant since this transcript is more efficiently translated at elevated temperatures (Takechi *et al*, 1994). In *Drosophila* hsp70, the 5' non-coding region is necessary for efficient translatability of the gene during heat shock (Klemenz *et al*, 1985). Interestingly, the splice donor site of exon I found in the heat induced hsp47 5' UTR shares 50.8% sequence identity with the human hsp70 5' non-coding region (Takechi *et al*, 1994).

Heat shock impairs the cap-binding protein complex necessary for normal translation and the possibility that hsps may possess cap-independent internal ribosome binding sites has been suggested (Joshi-Barve *et al*, 1992). Takechi and colleagues (1994) proposed that the alternatively added region of exon I may contain an alternative ribosome binding site, but this theory has yet to be proven.

HSP47 is expressed in specific tissues and this specificity has been studied in transgenic mice (Hirata *et al*, 1999). Yasuda and colleagues (2002) used luciferase reporter and gel mobility shift analysis to identify three specific regulatory regions necessary for the *hsp47* expression pattern. The Sp-1 binding site (-210 bp) in the *hsp47* promoter region, the BS5-B element in the first intron and the EP7-D element in the second intron are all necessary for basal expression (Yasuda *et al*, 2002). Yeast one-hybrid analysis was used to identify the key transcription factors that bound to these elements. Sp1, Sp2, Sp3 and several Kruppel-like factors (KLF) proteins were found to be involved in the regulation of *hsp47*. Zf9 (KLF-6), a reported positive regulator of collagen α1, was demonstrated to *trans*-activate *hsp47* gene expression through interactions with the intron region (Yasuda *et al*, 2002). Sp1 family members have also been implicated in the transcriptional control of collagen. As well as the Sp1 binding site in the promoter, Sp2 and Sp3 were found to bind to the BS5-B and EP7-D elements. These findings suggest that the coexpression of collagen and hsp47 is due to shared *cis*-elements and the actions of specific *trans*-factors in the regulatory regions of each respective gene. KLF-3, which has been reported to have repressor activity, was suggested to be involved in the repression of hsp47 expression in hsp47-nonproducing cells (Yasuda *et al*, 2002).

1.2.3 Expression of HSP47 Correlates with Collagen

Many heat shock proteins are conserved across evolution and are expressed in nearly all cell types (Bukau *et al*, 1998). These HSPs perform essential housekeeping activities, such as protein folding, protein complex assembly and intracellular sorting, to maintain homeostasis within each cell (Katschinski, 2004). HSP47 is an example of a heat shock protein with a specialized function and, as such, it is expressed selectively with the ER of cells that synthesize and secrete collagen (Nagata, 1998).

Steady-state levels of HSP47 and type I collagen are increased in tissues undergoing pathological fibrosis (Nagata, 1998), indicating that increased collagen synthesis induces increased HSP47 synthesis by some unknown mechanism. Similarly, the production of type I collagen is decreased if HSP47 expression is inhibited (Sauk *et al*, 1998). The use of antisense hsp47 to decrease constitutive levels of HSP47 also results in a net decrease of collagen secretion from the affected cells. The only exception to the correlation of HSP47 and collagen expression is the case of heat shock. Collagen synthesis decreases under heat-shock conditions (5-8^{o}C above the organism's natural temperature), similar to many other proteins, whereas HSP47 synthesis is increased even after short periods of increased temperature treatment (Hirayoshi *et al*, 1991).

1.2.4 HSP47 Protein Structure

After the cleavage of the signal peptide, mature human HSP47 is 400 amino acids long and possesses several key elements. At its carboxyl terminus, HSP47 possesses an endoplasmic reticulum (ER)- retention signal (RDEL) that maintains this molecular chaperone in the ER and the intermediate compartments between the ER and the Golgi

apparatus (Satoh *et al*, 1996). HSP47 has two glycosylation sites at its amino terminus that are fully glycosylated with high-mannose oligosaccharides in the mature form of the protein (Satoh *et al*, 1996; Natsume *et al*, 1994). This supports the finding that HSP47 is an ER-resident glycoprotein. There are also a number of potential phosphorylation sites in the HSP47 sequence and phosphorylated forms have been detected in some but not all cell types (Nakai *et al*, 1990; Nagata and Yamada, 1986). The function and importance of HSP47 phosphorylation is as yet unknown. In fact, neither HSP47 glycosylation nor phosphorylation are required for HSP47/collagen interactions, since native and recombinant HSP47 produced in *Eshcerichia coli* have similar collagen binding characteristics (Jain *et al*, 1994; Natsume *et al*, 1994).

Amino acid sequence analysis has revealed that HSP47 is not only a heat shock protein but also belongs to the serine protease inhibitor (serpin) superfamily (Hosokawa *et al*, 1993), but has no protease inhibitor activity (Hirayoshi *et al*, 1991). Serpin family members have a distinct and highly conserved secondary structure comprised of three core β sheets surrounded by nine α helices (Sharp *et al*, 1999; Elliott *et al*, 1996; Schreuder *et al*, 1994). The central β sheet, otherwise known as the A-sheet, exists in a 5- or 6- stranded form. The 5-stranded form is a result of normal folding and is metastable (Dafforn *et al*, 2001). The 6-stranded A-sheet is hyperthermostable and is formed as a result of proteolytic cleavage or partial protein denaturation of the 5-stranded form.

Well-characterized members of the serpin superfamily include α1-antitrypsin, α1-antichymotrypsin, antithrombin III and ovalbumin (Davids *et al*, 1995). HSP47 has highest homology with serpin human protein C inhibitor (31%) (Hirayoshi *et al*, 1991). Although the crystal structure of HSP47 has not been solved, a three-dimensional molecular model

based on the structure of protein C inhibitor predicted a long cleft that could accommodate collagen chains (Davids *et al*, 1995).

Serpins can self-assemble to form homopolymers via β strand/β strand interactions (Sharp *et al*, 1999). A number of serpin family members are known to undergo such oligomerizations. HSP47 is unique in that it assembles into only two forms: a monomer and a trimer. The absence of the intermediate dimer is very unusual (Dafforn *et al*, 2001).

Monomeric HSP47 possesses the metastable 5-strand A-sheet conformation. Investigation of this region in recombinant mouse HSP47 has revealed that the amino acid sequence of the 'hinge region' of monomeric HSP47 contains a large number of large side chained residues that prevent the formation of the hyperstable 6-stranded A-sheet (Dafforn *et al*, 2001). Although monomeric HSP47 is incapable of reaching this state, trimeric HSP47 is hyperstable and possesses the same binding affinities and biological activity as the monomeric form.

Monomeric and trimeric recombinant mouse HSP47 were both able to bind monomeric and partially folded conformations of collagen mimic peptides (Dafforn *et al*, 2001). Upon binding, HSP47 has the ability to induce the peptide backbone of these monomeric prolyl peptides to fold into a prolyproline type II conformation. Induction of this conformation results in the association of mimic peptides into higher order assemblies with increased stability.

1.2.5 Substrate Binding Specificity

HSP47 binds to various types of procollagen and collagen (types I – VI) therefore it was expected to function through interactions with sequences common to the various types of collagen (Koide *et al*, 2002). The triple helical region of procollagen is comprised of

characteristic repeats of Xaa-Yaa-Gly where Xaa and Yaa are any residue with the exception of glycine (Gly), cysteine or aromatic amino acid residues (which are unfavourable in the native collagen sequence) (Koide *et al*, 1999). Proline (Pro) and prolyl 4-hydroxyproline (Hyp) are most frequently found at the Xaa and Yaa positions, respectively. The binding specificity of HSP47 has been investigated for several years and two components of procollagen were originally implicated in the binding of HSP47. $(Pro\text{-}Pro\text{-}Gly)_n$ repeats and the triple helical conformation were both considered important to HSP47 recognition (Tasab *et al*, 2000; Koide *et al*, 1999).

HSP47 binds individual procollagen α chains almost immediately after translocation into the ER (Sauk *et al*, 1994). Hence, the binding of HSP47 to procollagen is favoured before the establishment of the triple helical conformation and so this physical formation cannot be pivotal to HSP47 binding. HSP47 has been found to have increased binding affinity for highly stable triple helical procollagen and so the formation of the triple helical conformation is still considered to be a contributing factor in the HSP47 interaction (Tasab *et al*, 2002).

Solid-phase pull down assays using commercially available peptides mimicking the collagen model sequence indicated that a minimum of seven Pro-Pro-Gly triplet repeats were sufficient for HSP47 interaction and binding increased with longer chain length (Koide *et al*, 1999). HSP47 did not require amino acid residues with charged side-chains for binding and hydrophobic interactions were suggested as the main contributor to HSP47 substrate binding. Results also demonstrated that the glycine residue present every third residue was important for substrate recognition. The glycine residues in the procollagen sequence are integral to the construction of the proper triple helical conformation through

the formation of interchain hydrogen bridges (Bella *et al*, 1994). In the biosynthetic pathway of procollagen Pro-Pro-Gly repeats are quickly converted to Pro-Hyp-Gly repeats before triple helical formation (Tasab *et al*, 2002). This repeat has reduced binding affinity to HSP47 and so further research was conducted to find a more likely binding site for HSP47. Using a range of synthetic collagen model peptides, a common sequence of Xaa-Arg-Gly triplets was discovered and the affinity of HSP47 to this sequence was found to be much stronger than other previously identified sequences (Koide *et al*, 2002). An arginine residue at the Yaa position in Xaa-Yaa-Gly repeats has been shown to enhance the thermal stability of the procollagen triple helix to a similar degree as prolyl 4-hydroxyproline (Hyp). The conclusion that Xaa-Arg-Gly triplets are the prominent binding site for HSP47 also coincides with the previous suggestion that hydrophobic interactions are important to procollagen-HSP47 binding. Koide *et al* (2002) suggest that the hydrophobic ring formed by the arginine side chain would directly interact with the hydrophobic cleft in the HSP47 molecule. Tasab *et al* (2002) showed that one Xaa-Arg-Gly repeat was required for HSP47 binding. The Xaa-Arg-Gly triplet occurs in high frequency in the procollagen sequence (13%; Thomson *et al*, 2003) and is found approximately every 30 residues. The importance of arginine residues to HSP47 binding also points to HSP47 as an inhibitor of lateral aggregation in the ER. The charged side-chain of arginine is presumed to be involved in the formation of higher order collagen structures (Knupp and Squire, 2001), hence HSP47 may act to mask the arginine residues until the procollagen triple helices are releasing into the Golgi apparatus (Koide *et al*, 2002).

Thomson and colleagues (2003) were the first to investigate HSP47 binding using natural fragments of type I and II collagen. Strongest binding sites were found towards the

amino terminus of the mature collagens (Thomson *et al*, 2003). HSP47 was capable of binding collagen at several sites with different binding affinities but in general, HSP47 was found to bind mature collagen at binding sites that matched those in procollagen.

1.2.6 Localization of HSP47

Membrane, secretory and lysosomal proteins enter the endoplasmic reticulum (ER) as they are translated and are subsequently targeted for the secretory pathway. The ER membrane and lumen contains many resident proteins that are actively involved in the processing of secretory proteins (Nakai *et al*, 1992). Several of these ER-resident proteins are heat shock proteins that serve as molecular chaperones and associate with folding intermediates or misfolded proteins to prevent irreversible aggregations and promote the proper folding process (Hartl, 1996; Morimoto *et al*, 1994).

Within the ER, general molecular chaperones such as Grp78 (BiP) and Grp94 are believed to facilitate translocation, folding, oligomeric assembly and sorting of proteins (Bergeron *et al*, 1994). Unlike these chaperones that do not possess specific substrates, HSP47 is a molecular chaperone that specifically binds collagen and its precursor procollagen.

HSP47 has only been detected in collagen-producing cells (Saga *et al*, 1987). Immunoprecipitation and immunostaining of HSP47 and procollagen have both showed that these proteins are colocalized in the ER as well as the intermediate compartments between the ER and the Golgi apparatus (Nakai *et al*, 1992; Nagata and Hosokawa, 1996). These results suggest that HSP47 dissociates from procollagen as it enters the *cis*-Golgi network and this finding has been confirmed by several other factors. The dissociation of HSP47 from procollagen is concentration dependent, however, HSP47 undergoes pH-

induced conformational changes that inhibit substrate binding (El-Thaher *et al*, 1996). In the acidic environment of the Golgi compartment, HSP47 would be incapable of binding procollagen and hence, must dissociate before this point (Thomson *et al*, 2003). HSP47 also possesses a RDEL (Arg-Asp-Glu-Leu) sequence at its carboxyl terminus that functions as an ER-retention signal much like the well-characterized KDEL (Lys-Asp-Glu-Leu) sequence. Deletion of this sequence results in the secretion of mutant HSP47 out of the cell (Satoh *et al*, 1996).

1.2.7 Possible Functions of HSP47 in the ER

The ER membrane and lumen contain many resident proteins involved in the processing of membrane, lysosomal and secretory proteins. HSP47 is a rough ER protein that was found to bind collagen *in vitro* (Nagata, 1996). The location and collagen binding activity of HSP47 led to speculation that HSP47 may participate in collagen processing (Nakai *et al*, 1992). HSP47 acts as a primary stimulator of type I collagen production; selectively increasing HSP47 expression increased the steady-state levels of both intracellular and extracellular type I procollagen (Rocnik *et al*, 2002). However, the precise function of HSP47 during procollagen biosynthesis has yet to be established. In the ER, HSP47 has been found to bind nascent type I procollagen chains (Ferreira *et al*, 1994), fully translated procollagen α chains (Satoh *et al*, 1996), non helical or poorly hydroxylated procollagen trimers (Nakai *et al*, 1992) as well as well-hydroxylated triple helical procollagen (Tasab *et al*, 2000). The wide range of binding abilities of HSP47 has lead to the suggestion of several roles for this molecular chaperone.

The production and assembly of procollagen is a complex multi-step process. With the example of type I collagen: once translated and translocated into the ER, two

proα1(I)collagen chains and one proα2(I)collagen chain first associate at their globular carboxyl termini (Engel and Prockop, 1991). This process is facilitated by protein-disulfide isomerase and the association is stabilized by the formation of interchain disulfide bonds (Wilson *et al*, 1998). The triple helical domain then winds in the carboxyl to amino direction. The helical folding reaction is dependant on the hydroxylation of proline residues by prolyl-4-hydroxylase (Kivirillo *et al*, 1989). Once the complete triple helical heterotrimer is formed, procollagen is transported to the Golgi apparatus where procollagen triple helices mature into collagen fibrils by forming large-scale aggregates. These aggregates are then brought to the cell surface and are secreted as mature collagen.

Trimerization of procollagen is a very organized and complex process. For it to precede correctly nascent procollagen α chains must be maintained in an unfolded state until trimerization (Nagata and Hosokawa, 1996). Once trimerized, procollagen triple helices must not aggregate prior to entering the Golgi network. Several roles have been suggested for HSP47 including aiding the translocation of nascent procollagen α chains into the ER, prevention of premature folding of procollagen α chains, stabilization of the procollagen triple helix, prevention of the lateral aggregation of mature procollagen and quality control during stress.

1.2.7.1 Translocation of Nascent Procollagen α Chains into the ER

HSP47 association with procollagen is initiated during the co-translational import of procollagen into the ER as demonstrated by the co-immunoprecipitation of HSP47 with nascent, polysome-associated proα1(I)collagen chains (Sauk *et al*, 1994). The association of HSP47 with actively-translating membrane-bound polysomes has lead to the suggestion that HSP47 may aid the translocation of procollagen chains into the rough ER.

1.2.7.2 Prevention of Premature Folding

During the maturation of procollagen into helical heterotrimers, the triple helix is carefully formed in the direction of the carboxyl to amino terminus (Nagata and Hosokawa, 1996). Nascent polypeptide α chains should be maintained unfolded until the completion of translation; otherwise the triple helical formation would be prevented. HSP47 binds procollagen α chains as they enter the ER, thus it has been suggested that this association serves to prevent premature folding or random aggregation of the procollagen chains before the formation of the triple helix (Thomson *et al*, 2003).

1.2.7.3 Stabilization of the Procollagen Triple Helix

Unlike most molecular chaperones, HSP47 remains bound to the mature form of its substrate. In addition to binding nascent procollagen α chains, HSP47 binds mature procollagen triple helices (Tasab *et al*, 2000). This binding pattern led researchers to suggest that HSP47 may bind and stabilize thermally unstable regions of the procollagen triple helix.

1.2.7.4 Prevention of Lateral Aggregation

The formation of procollagen aggregates in the Golgi network coincides with its release from HSP47. This suggests that the association between HSP47 and mature procollagen may serve to control and limit lateral aggregation in the early secretory pathway (Tasab *et al*, 2002). HSP47 was in fact shown to prevent collagen fibril formation *in vitro* (Thomson and Ananthanarayanan, 2000). As pH levels decrease, the conformation of HSP47 changes and inhibition of fibril formation by HSP47 ceases by pH6.4. This is biologically significant since the pH of the ER (where HSP47 is active) is near neutral and

the average pH of the Golgi apparatus (where HSP47 releases procollagen) is 6.4 (Kim *et al*, 1998). Since this discovery, several studies observed trimeric forms of HSP47 that formed 'rings' around small bundles of procollagen triple helices (Dafforn *et al*, 2001; Thomson and Ananthanarayanan, 2001). These ring formations appeared sufficient to limit large-scale lateral aggregation.

The prevention of procollagen lateral aggregation is a very important potential function of HSP47. The formation of aggregates within the ER would prevent the transport of procollagen through the remainder of the secretory pathway and hence the presence of HSP47 would be required for proper protein trafficking (Tasab *et al*, 2002). Also, if uncontrolled aggregation were to occur in the ER, it could potentially interfere with the expression of other proteins.

1.2.7.5 Quality Control During Stress

HSP47 is a stress-inducible heat shock protein and is believed to participate in quality control mechanisms during stressful conditions, including the prevention of the secretion of procollagen with abnormal conformation (Nagata, 1998). Procollagen within the cell is more thermostable than the isolated protein, suggesting that the intracellular environment could protect the procollagen triple helix from heat denaturation.

Collagen is a very abundant and essential structural protein in the extracellular matrix of numerous cell types. Hence, the importance of its correct production is undoubtedly necessary to the survival and structural integrity of many cells and tissues. Although the exact function of HSP47 has yet to be clearly seen, it has been shown to be key to the proper assembly of collagen.

1.2.8 The Role of HSP47 in Various Fibrotic Diseases

Altered expression of various collagen genes has been revealed to cause or aggravate many genetic and developed diseases (Nagai *et al*, 2000). Since HSP47 expression has been shown to be so closely related to that of collagen, the expression of HSP47 has also been examined in many fibrotic diseases.

1.2.8.1 HSP47 in Developed Fibrotic Diseases

Fibrosis characteristically occurs in the advanced stages of chronic inflammatory diseases and is frequently the determinant factor in disease prognosis (Hagiwara *et al*, 2003). Fibrotic lesions originate by the immigration of fibroblasts into a wounded site and develop through the deposition of excess extracellular matrix proteins by these immigrated fibroblasts (Cotran *et al*, 1999). Fibrotic lesions therefore consist mainly of collagen and hence the expression of HSP47 in diseases involving fibrosis has been largely studied.

Since 1994, investigation of HSP47 has turned to examine its involvement in a wide range of fibrotic diseases, using both clinical samples and experimental animal models. In fact, comprehension of the *in vivo* relevance of HSP47 has largely come from its identification in organs undergoing fibrosis. Examined diseases have included vascular fibrosis (Rocnik *et al*, 2001), pulmonary fibrosis (Ishii *et al*, 2003; Iwashita *et al*, 2000; Razzaque *et al*, 1998), liver cirrhosis (Kawada *et al*, 1996; Masuda *et al*, 1994), glomerulosclerosis (Sunamoto *et al*, 1998; Moriyama *et al*, 1998), rheumatic autoimmune diseases (Yakota *et al*, 2003; Hattori *et al*, 1998, 2000, 2003), systemic sclerosis (Kuroda *et al*, 1998), Keloid (Naitoh *et al*, 2001), Cicatricial Pemphigoid (Razzaque and Ahmed, 2002) and fibrosis associated with diabetes (Liu *et al*, 2001).

All investigated fibrotic diseases have set characteristics in common: the accumulation of excessive amounts of extracellular matrix components, the upregulation of at least one type of collagen, both on the mRNA and protein level and a correlated upregulation of HSP47 in collagen-producing cells. Immunohistological staining has been used to demonstrate the correlated expression of collagen and HSP47, suggesting that HSP47 may be key in the excessive assembly and synthesis of collagens during the progression of fibrosis (Iwashita *et al*, 2000; Razzaque *et al*, 1998). The harmful role of HSP47 in rheumatic autoimmune diseases has further been exemplified by the discovery of both increased HSP47 protein and autoantibodies to HSP47 in patients' sera (Yokota *et al*, 2003).

The evident role of HSP47 in fibrotic diseases has lead to the search for specific therapies against this collagen molecular chaperone. Current investigation has primarily used antisense oligonucleotides or ribozymes against HSP47 to downregulate collagen in fibrotic diseases such as rheumatoid arthritis and glomerulonephritis (Hattori *et al*, 2003; Sunamoto et al, 1998). Research continues to try to limit HSP47 expression as a means to control the progression of fibrosis.

1.2.8.2 HSP47 in Tissue transplantation

In current medicine, extreme damage or failure of specific organs or tissues is rectified through tissue transplantation. The transplantation of organs or grafting of tissues is not flawless; rejection is still prevalent and associated with it are pathological features such as inflammation and fibrosis (Abe *et al*, 2000). Abe and colleagues (2000) examined the interstitial expression of HSP47 in relation to renal allograft failure. Results indicated that HSP47 expression significantly correlates with the degree of interstitial fibrosis and the

distribution pattern of HSP47 was similar to that of type I collagen. These findings support the idea the HSP47 has a role in the pathogenesis of fibrosis that results in chronic graft rejection.

1.2.8.3 HSP47 in Wound Healing

After tissue damage, macrophages co-ordinate repair by secreting growth factors that stimulate fibroblast recruitment, proliferation and collagen deposition (Bleacher *et al*, 1993). Scar tissue is characterized by abundant, disorganized collagen bundles and can cause other detrimental effects such as defective growth and functional impairment (Diegelmann *et al*, 1981). Masuda and colleagues (1994) were the first to observe that hsp47 is co-expressed with collagen in scarred regions of tissue.

Wound healing in fetuses and in adults differs in that fetal wound healing lacks an inflammatory response and occurs in the absence of neovascularization (Lovvorn *et al*, 1998; Longaker *et al*, 1994). These differences have been linked to the regulation of collagen production and deposition (Soo *et al*, 2000). Wang and coworkers (2002) examined the differences in HSP47 and type I collagen expression between fetal and neonatal rats. They concluded that the scarless healing of fetal skin was related to the lack of change in HSP47 expression and suggested that if collagen synthesis was strictly controlled during healing then scar formation could be prevented. Researchers suggest that suppression of HSP47 expression following surgery may enable scarless wound healing in adult tissues.

1.2.9 HSP47 During Development

Hsp47 and collagen genes are co-ordinately expressed in several different organisms including chicken, mouse, rat and zebrafish (Miyaishi *et al*, 1992; Shroff *et al*,

1993; Pak *et al*, 1996; Lele *et al*, 1997). The role of HSP47 during embryonic development has mostly been examined in the murine system. In the mouse embryo, HSP47 is expressed mainly in mesoderm and mesoderm derived tissues: cartilage, bone, notochord and somites (Masuda *et al*, 1998). Its expression correlates both temporally and spatially with that of type I and II collagen. HSP47 is strongly expressed in cartilaginous regions, particularly those producing prominent levels of type II collagen, namely chondrocytes in the proliferation and maturation zones of epiphyseal growth plates (Satoh *et al*, 1996). During ossification, type I collagen and HSP47 are colocalized to the extracellular matrix and osteoblasts in the vascularization zone.

Kambe *et al* (1994) reported HSP47 expression in the epiphyseal cartilage and in cultured chondrocytes of chick embryos. Lele and colleagues (1997) used whole mount *in situ* hybridization analysis to establish that following stress induction hsp47 mRNA is expressed predominantly in precartilagenous cells and several other connective tissue cell populations. HSP47 has also been detected during embryological development of the heart, lung and kidneys (Pak *et al*, 1996a, 1996b; Masuda *et al*, 1998).

1.2.9.1 Hsp47 Knockout Mice

The *hsp47* gene exists as a single copy in most vertebrates including mouse, rat, chicken and zebrafish (Nagai *et al*, 2000). In 2000, Nagai and coworkers established *hsp47* -/- knockout mice by disrupting the *hsp47* alleles by homologous recombination. The knockout mice mesenchymal tissues were severely deficient in fibril structures and mature, propeptide-processed type I collagen. The molecular form of type IV collagen was also affected and as a result, the formation of basement membranes, whose major constituent is

type IV collagen, was disrupted in homozygous *hsp47* -/- mice. The net result of these abnormalities was abnormally oriented epithelial tissues and disrupted blood vessels.

Mov13 mice are defective in type I collagen production and die before 13.5 days postcoitus (dpc) with the mesenchymal necrotic cell death phenotype (Schnieke *et al*, 1983; Lohler *et al*, 1984). The phenotype of *hsp47* -/- mice resembled that of Mov13 mice but was more severe. By 9.5 dpc, deficient fibril formation and growth retardation was apparent and the disruption of *hsp47* was lethal before 11.5 dpc (Nagai *et al*, 2000). Nagai and colleagues suggested the more severe phenotype of *hsp47* -/- mice might reflect pleiotropic roles for HSP47 during the maturation of not only type I collagen but also types II to V collagen based on the binding properties of HSP47 *in vitro*.

Nagai *et al* (2000) also established *hsp47* -/- fibroblastic cell lines to examine the importance of HSP47 in the formation of triple helical type I procollagen. The integrity of triple helical formation was monitored by examining the protease sensitivity of secreted type I collagen. Type I collagen from *hsp47* +/+ or +/- cells were resistant to protease digestion, whereas those secreted from *hsp47* -/- cells were sensitive to the treatment (Nagai et al, 2000). The *hsp47* -/- procollagen did not form correctly aligned triple helices. Transfection of the *hsp47* gene into *hsp47* -/- cells restored proper procollagen triple helix formation and provided strong evidence for the importance of HSP47 in the correct processing of procollagen in the ER.

1.3 *Xenopus laevis* as a Model Organism

The South African clawed frog, *Xenopus laevis*, is a widely studied amphibian model organism. As an experimental animal, *Xenopus laevis* is easy to obtain and maintain in a laboratory setting and has been extensively used to examine various molecular

mechanisms, particularly those involved in early vertebrate development. A single *Xenopus* female can be induced to produce hundreds of eggs that are easy to fertilize and maintain externally. *Xenopus* eggs are relatively large (1-1.2 mm in diameter) making them particularly amenable to manipulation and microinjection studies. The stages of *Xenopus* development have been well characterized at both the cellular and molecular level and serve as a valuable resource for developmental studies (Nieuwkoop and Faber, 1967). Another tool for molecular examination of *Xenopus laevis* is the *Xenopus* A6 kidney epithelial cell line. This somatic cell line is simple to propagate and has a quick growth rate, thereby allowing easy investigation of gene expression.

1.3.1 *Xenopus laevis* Early Development

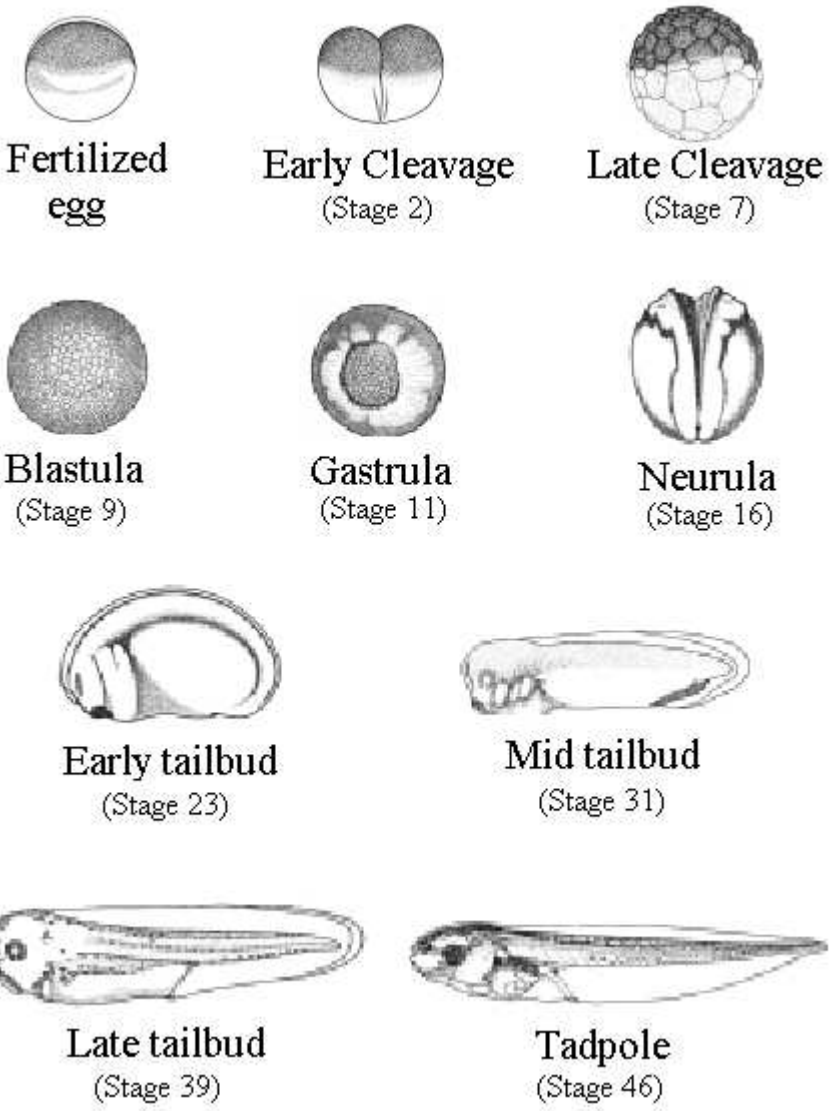
In *Xenopus laevis*, the egg is responsible for initiating and directing early development. The animal-vegetal polarity of the unfertilized egg is visually recognizable by the pigmented animal half and the unpigmented vegetal half (Gilbert, 1994). The sperm entry point at fertilization determines the dorso-ventral polarity of the embryo; the dorsal region forming opposite the sperm entry point. The initial stages of *Xenopus* embryo development consist of rapid and synchronous cleavages. During this period of development the embryonic genome is quiescent and any protein produced is translated from maternally inherited mRNA. At stage 8.5, the midblastula transition (MBT), the embryonic genome is activated and transcription is thereon possible.

At blastula (stage 9) the inner cell mass is segregated into presumptive ectoderm, mesoderm and endoderm (Nieuwkoop and Faber, 1967). Embryo development progresses into the gastrula stage, characterized by extensive cell and tissue movement. Gastrulation is initiated in the dorsal part of the marginal zone between the animal and vegetal

hemisphere. Local endoderm cells invaginate to form the blastopore lip and become the presumptive pharyngeal cells of the foregut. Ectoderm cells remain on the surface of the embryo with mesoderm in between the two layers. The interaction between the dorsal mesoderm and overlaying ectoderm commences organogenesis: the formation of specific tissues and organs. Ectoderm will give rise to the skin, brain, spinal cord and represents the origin of neural crest cells. Endoderm will form the linings of the digestive and respiratory tubes. Mesoderm will differentiate into muscle, cartilage, bone, heart, the urogenital system, blood cells and most of the internal organs.

The chordamesoderm initiates neurulation by inducing ectoderm to form the neural tube. During the early neurula fold stages; neural crest cells originating at the dorsal region of the neural tube begin to segregate from the neural plate. These cells migrate extensively to generate various different cell types including the neurons and glial cells of the sensory, sympathetic and parasympathetic nervous system, medullar cells of the adrenal gland, melanocytes, skeletal and connective tissue constituents of the head and the musculo-connective tissue wall of the arteries (Nieuwkoop and Faber, 1967). At stage 15, two presumptive heart-forming regions start to develop. The digestive tube begins to form from the primitive gut archenterons. Buds from this tube will develop into the liver, gall bladder and pancreas. The respiratory tube and pharynx will also form from outgrowths of the digestive tube. The first organ to differentiate is the cement gland, which is begins to develop during neurula (stage 15) and is fully formed by late neurula stages (stage 19). The cement gland is a temporary mucus-secreting ectodermal organ that allows developing embryos to attach to solid structures. At stage 17, mesodermal cells that are not involved in notochord formation migrate laterally along each side of the notochord and neural tube to

Figure 3. An overview 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 39)
Tadpole
(Stage 46)

form somites (Gilbert, 1994). These structures will give rise to cells that form the vertebrae, ribs, dermis of the dorsal skin and skeletal muscles of the back and limbs. At stage 20, the brain regionally segregates into the archencephalon and deuterencephalon (Nieuwkoop and Faber, 1967). In the following stage, the primary eye vesicles and olfactory placodes develop from the forebrain and hindbrain, respectively. At this time (stage 21), the intermediate mesodermal region develops into the pronephric tubule, the precursor of both the kidneys and genital ducts. Organogenesis proceeds throughout tailbud and early tadpole stages and a heartbeat is evident by late tailbud (stage 33/34).

Early tadpole stages (starting at stage 43) marks the beginning of limb development from mesenchymal cells released from the lateral plate mesoderm and somites. Rear limb buds and fore limb buds become visible at stage 45 and stage 48, respectively. Metamorphosis begins at stage 50 following the initial development of the fore limbs. Select *Xenopus laevis* developmental stages are depicted in Figure 3.

1.3.1.1 Gene Expression During *Xenopus laevis* Development

As earlier discussed, the genome of *Xenopus laevis* embryos is quiescent until MBT. Previous to this stage of development, the embryo survives on cellular factors present in the oocyte before fertilization. MBT is characterized by an increased duration of the cell cycle, loss of synchronous cell divisions and decreased DNA synthesis (Newport and Kirschner, 1982). It is only after this stage that select hsps become heat-inducible.

Changes in transcriptional regulation during development are most likely due to several transitions occurring before or at MBT. A number of studies discovered the presence of RNA polymerase II and also a number of relevant transcription factors in pre-MBT embryos (Newport and Kirschner, 1982). These findings suggested that it was not

the absence of transcriptional machinery but possibly the presence of rapid cell divisions prior to MBT that prevented RNA transcription. However, experimental investigation into chemically-induced premature transcription in *Drosophila* and *Xenopus* have suggested that a high rate of synthesis was not sufficient to explain transcriptional repression during early development (Edgar and Schlubiger, 1986; Kimelmen *et al*, 1987). Another theory proposed that zygotic transcription could only be initiated in the embryo when a critical nucleus to cytoplasm ratio was met (Newport and Kirschner, 1982). An examination of the *Xenopus* unfertilized egg revealed that it has an unusually high ratio of histones to DNA. For example, a single egg possesses enough histones to assemble up to 20 000 nuclei (Woodland and Adamson, 1977). This large pool of histones is thought to compete with basal transcriptional machinery during early development (Hair *et al*, 1998). Recent, experimental findings have suggested that the assembly of nucleosomes is largely favoured over the formation of transcriptional complexes in pre-MBT embryos (Hair *et al*, 1998). Interestingly, the concentration of maternal factors including histones does not significantly change during early embryogenesis, however, the ratio of DNA to nuclear protein changes from 1:30 000 from egg to gastrula. Thus providing strong evidence for a role of histone concentration and chromatin organization in the activation of the zygotic genome.

1.3.1.2 Hsp70 in *Xenopus* Development

In 1984, the first set of *hsp* genes isolated from *Xenopus laevis* was four members of the *hsp70* family (hsp70A, B, C and D; Bienz, 1984). DNA sequence analysis of the *hsp70A* gene revealed that it had high identity with *Drosophila hsp70* and also that it contained a HSE in the 5' regulatory region necessary for stress induction (Bienz, 1986). HSP70 has a nuclear localization signal that has been found in several organisms and has

specifically been established as functional with immunolocalization studies in *Xenopus* oocytes and early development (Herberts *et al*, 1993). In mammalian species, HSP70 possesses a carboxyl terminal sequence EEVD, which is necessary for several chaperone activities such as substrate binding and intramolecular coupling of ATP. *Xenopus* HSP70 has also been found to possess this sequence, thereby suggesting it to have similar chaperone activities to its mammalian counterparts.

Several studies have found both hsp70 mRNA and protein to be present constitutively during *Xenopus* oogenesis (Bienz, 1984; Bienz and Gurdon, 1982; Browder *et al*, 1987; Davis and King, 1989; Horrell *et al*, 1987). Hsp70A and hsp70B transcripts were maintained through oocyte maturation, fertilization and early cleavage stages of development. However, in subsequent studies, heat shock-induced HSP70 was not detectable in oocytes, suggesting that earlier findings may have been due to follicular cell contamination (Horrell *et al*, 1987; King and Davis, 1987). During *Xenopus* embryogenesis, hsp70 transcript was found to be heat inducible at all stages following MBT, with particularly elevated levels during the midtailbud stage (Lang *et al*, 2000). Whole mount *in situ* hybridization results established that heat shock treatment at 33^{o}C induced hsp70 mRNA accumulation throughout the whole embryo during gastrulation. Also, immunocytochemical analysis of gastrula embryos has detected a differential pattern of HSP70 synthesis (Herberts *et al*, 1993). HSP70 was concentrated in the nucleus and perinuclear region of involuted cells of the marginal zone. At neurula stage, heat induced transcript accumulation was distributed over the surface of the embryo (Lang *et al*, 2000). In heat shocked midtailbud embryos, hsp70 mRNA was found across the embryo surface

and enriched in the anterior region of the embryo including the heart, cement gland, olfactory pit, lens placode, pronephros, somites, spinal cord and proctodeum.

In order to determine the mechanism by which *hsp* genes were induced by stress after MBT Ovsenek and Heikkila (1990) examined the presence of HSF during *Xenopus* development by performing DNA mobility shift experiments with an oligonucleotide corresponding to the proximal HSE of the *hsp70B* gene. HSF binding activity was found in all investigated stages of development including cleavage stage embryos that are transcriptionally inactive and unable in elicit a heat shock response. The finding of HSF binding activity in unfertilized eggs and cleavage stage embryos established this transcription factor as maternally inherited. In 1995, Stump *et al* isolated the cDNA encoding *Xenopus* HSF1 (XHSF1). Analysis of the protein-coding region revealed XHSF1 to be comparable in size and possess similar putative DNA-binding and trimerization domains to other vertebrate HSF proteins. More recently, a HSF2 homologue was found in *Xenopus* that had previously only been identified in humans, mouse and chicken (Hilgarth *et al*, 2004). This HSF was found to undergo the same modifications as its human counterpart. Also within this study, analysis of *Xenopus* expressed sequence tag clones identified at least two other HSF family members distinct from XHSF1 and XHSF2 that have yet to be characterized.

Bienz *et al* (1984) found that all examined adult tissues of *Xenopus laevis* displayed heat-inducible expression of hsp70 mRNA at approximately the same level. Hsp70 mRNA is not constitutively expressed in the A6 kidney epithelial cell line but is inducible by various stressors including heat, sodium arsenite and heavy metals (Briant *et al*, 1997).

1.3.1.3 BiP in *Xenopus* Development

The full-length cDNA for *Xenopus* immunoglobulin binding protein, BiP, was found to possess greater identity with BiP from other vertebrates than with other members of the *Xenopus* hsp70 family (Miskovic *et al*, 1997). High sequence identity between amino-terminal ATP-binding domains of mammalian BiP and the N-terminal portion of *Xenopus* BiP suggests that *Xenopus* BiP has ATP-binding activity. *Xenopus* BiP also possessed a carboxyl terminal sequence, KDEL, which has been reported in BiP proteins from other organisms as well as other ER-resident proteins such as glucose-regulated protein 94. This sequence has been established as an ER-retention signal that serves to retrieve ER proteins from post-ER compartments.

During *Xenopus* development, BiP mRNA is present at constitutive levels from the unfertilized egg through to 4-day-old tadpoles, with elevated constitutive levels during tailbud stages (Miskovic and Heikkila, 1999). Whole mount *in situ* hybridization established that BiP mRNA is enriched along the surface of the embryo during gastrulation. At neurula, accumulation was localized to the neural plate, neural folds and blastopore. As embryos develop into tailbud stages, BiP mRNA was found primarily along the dorsal plane of the embryo, specifically in the somatic region, spinal cord, cranial nerves, optic vesicle and forebrain. BiP mRNA was also found in the heart, liver, diverticulum, pronephros, pronephric duct and around the anus. Elevated levels of BiP mRNA were first heat inducible at gastrula stage. At early tailbud stage, heat shock elevated levels in the somites, forebrain, tail, anus and along the spinal cord.

In *Xenopus* tissue culture cells, BiP is expressed constitutively and is induced by inhibitors of glycosylation, sulfhydryl-reducing agents and glucose starvation conditions (Winning *et al*, 1989). The relative levels of BiP transcript has also been examined in select *Xenopus* adult tissues (Miskovic *et al*, 1997). Constitutive levels were found in all examined tissues; however, the relative transcript level differed dramatically between tissue types. Results suggested that the expression of *BiP* genes varied according to the cellular requirements of each particular tissue. It has also been suggested that *BiP* genes may be differentially expressed during *Xenopus* development (Heikkila *et al*, 1997). Three putative isoforms of BiP have been detected using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) studies (Winning *et al*, 1991). One of these isoforms was observed solely in embryos, another was adult-specific and the third was found in both embryo and adult cells.

1.4 Objectives

Hsp47 is a heat shock protein that had not yet been examined in *Xenopus laevis* and the majority of hsp47 research had been performed in mammalian organisms. Through the course of this study, various cellular, ER-specific or procollagen-specific stressors were employed to determine their effects on the induction of hsp47 mRNA and protein in *Xenopus laevis* somatic cells and embryos.

Objectives included:

1) Sequence analysis of the hsp47 cDNA and comparison with hsp47 from chicken, mouse, rat, human and zebrafish
2) Northern blot analysis of hsp47 mRNA levels in response to cellular, ER or procollagen specific stressors in A6 cells and embryos
3) Western blot analysis of HSP47 protein levels in response to cellular, ER or procollagen specific stressors in A6 cells
4) Whole mount *in situ* hybridization analysis to localize hsp47 mRNA levels in *Xenopus* embryos in response to heat shock

Experimental Procedures

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

2.1.1 Culturing of *Xenopus laevis* A6 kidney epithelial cells

The *Xenopus laevis* A6 kidney epithelial cell line [American Type Culture Collection (ATCC), Rockville, MD] was cultured in 55% (w/v) Liebovitz's L-15 medium (Sigma, Oakville, Ontario) supplemented with 10% (v/v) fetal bovine serum (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) at 22°C. When cells reached confluence, old media was aspirated off, 2 ml Versene [0.02%(w/v) KCl, (0.8%(w/v) NaCl, 0.02%(w/v) KH_2PO_4, 0.115%(w/v) Na_2HPO_4, 0.02%(w/v) Na_2EDTA] was added to the flasks for 2 to 5 min and then aspirated off. Cells were lifted off the surface of the flask using 1 ml of 1X trypsin (Sigma) in 100% Hank's balanced salt solution (HBSS; Sigma) for approximately 30 sec and then aspirated off. Free cells were resuspended in fresh media and then distributed evenly into additional tissue culture flasks.

2.1.2 Treatment of *Xenopus* A6 kidney epithelial cells

Cell treatments on the A6 cell line were performed with cells that reached 90-100% confluence. The cells were treated with various potential hsp47 inducers for periods up to 48 h. The final concentrations used to examine transcript levels were as follows: sodium arsenite (NaAs; 50 μM for 1, 3, 6, 12 and 24 h); tunicamycin (1 μg/ml for 4, 6 and 24 h); calcium ionophore A23187 (0.7 μM for 4, 6 and 24 h); β-aminopropionitrile fumerate (βAPN; 1 mM for 1, 2, 4, 6 and 24 h). To examine the effect of heat shock on hsp47 mRNA induction, A6 cells were incubated for 1 h at 27, 30, 33, 35 and 37°C. Time courses were also performed at 33 and 35°C using 30 min, 1, 2, 4 and 8 h time points. Some stressors were also examined in combination. A6 cells were exposed to sodium

arsenite (10 μM) and temperature (30°C) for 2 h to observe any synergistic effects. Hsp47 mRNA accumulation in response to long term exposures to A23187 (24 and 36 h) coupled with a 1 h heat shock at 35°C was also examined.

The final concentrations of stressors used to examine HSP47 protein levels were the same as that of RNA induction, however, timing of treatment varied as follows: sodium arsenite (3, 6, 9, 12 and 24 h), tunicamycin (4, 6, 8 and 24 h), A23187 (4, 6 and 24 h) and APN (2, 4, 6, 8 and 24 h). To examine the effect of heat shock on HSP47 protein induction, A6 cells were incubated for 2 h at 27, 30, 33, 35 and 37°C and then allowed to recover at 22°C for an additional 2 h. Time courses were also performed at 33 and 35°C using 1, 2, 4 and 8 h time points followed by 2 h recovery time at 22°C. HSP47 protein accumulation in response to long term exposures to A23187 (24 and 48 h) coupled with a 2 h heat shock at 35°C (with 2 h recovery at 22°C) was also examined.

2.1.3 Harvesting of A6 epithelial cells

Following all heat shock and/or chemical treatments, the media was aspirated off and 2 to 5 ml of 65% HBSS was added to rinse the cells. 1.5 ml of 100% HBSS was then added, the cells were scraped from the bottom of the flask and transferred to a 1.5 ml microcentrifuge tube. After the cells were pelleted by centrifugation in an Eppendorf Centrifuge 5415D microcentrifuge (Brinkmann Instruments Ltd, Mississauga, ON) for 1 min at 13 200 rpm the supernatant was removed and the cells were stored at –80°C.

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

Xenopus eggs were obtained and fertilized according to the method outlined in Heikkila *et al* (1985). Pigmented or albino female *Xenopus laevis* frogs (Boreal, St. Catherine's, ON and Ward's Biology, St. Catherine's, ON; respectively) were primed with

50 units (IU) of human chorionic gonadotropin (hCG; Sigma) resuspended in sterile 0.65% (w/v) NaCl by injection into the dorsal lymph sac 5 days prior to induction. Approximately 10 h prior to egg collection, 1000 IU of hCG was injected to induce superovulation. By applying gentle pressure to the abdomen of the frogs, eggs were collected 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]. A male was sacrificed, the testes were excised and placed in a petri dish with 1X MBS. All MBS was removed from the eggs, the testes were divided into segments and fertilization was achieved by physically touching a segment of testis to each individual egg. The eggs were then covered with 0.1X MBS [8.8 mM NaCl, 0.1 mM KCl, 0.07 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM HEPES-KOH, 0.25 mM $NaHCO_3$, pH 7.8] and left to shake at room temperature on an orbital shaker for 20 to 30 min to allow for sperm penetration. Fertilized eggs became evident by cortical rotation where the animal pole rotated to face upward. After fertilization, the embryos were dejellied with 2% (w/v) L-cysteine (Sigma) in 0.1X MBS pH 8.0. The embryos were subsequently washed 7 to 8 times with fresh 0.1 X MBS to remove any residual L-cysteine. In some cases, the embryos were maintained at 22^{o}C during the entire course of development. On a few occasions, to promote survival through early stages, the embryos were maintained at 16^{o}C until gastrulation after which they were kept at 22^{o}C. Any dead or deformed embryos were promptly removed and 0.1 X MBS was periodically changed during development. Developmental stages were determined by morphological changes as outlined in tables established by Nieuwkoop and Faber (1967).

2.1.5 Treatment and storage of *Xenopus laevis* embryos

Embryos to be heat shocked were placed in large beakers containing 0.1X MBS. The beaker was covered with parafilm and placed in a heated water bath at 33°C. Control embryos were maintained in 35 X 10 mm petri dishes in a 22°C incubator. Pigmented embryos were used for RNA isolations and subsequent Northern analysis and were heat shocked for 1 h. After heat shocking, heat shocked and control embryos were staged, transferred to 1.5 ml microcentrifuge tubes and immediately frozen in liquid nitrogen. Embryos were then stored at –80°C until further processing could be completed. Pigmented late tailbud (ltb) or early tadpole (TP) embryos were also used for treatment with 0.7 μM A23187 for 3 or 6 h. A23187-treated embryos were then either collected or heat shocked for 1 h at 33°C. After treatment, all embryos were collected and stored as described above.

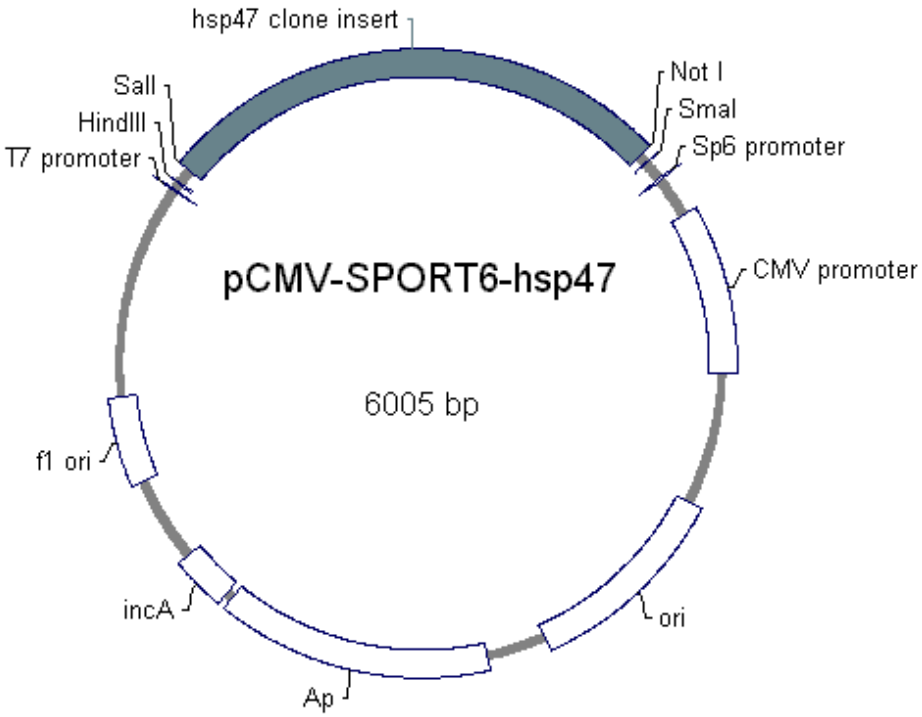
Albino embryos were used for whole mount *in situ* hybridization. Control and heat shocked (1 h at 33°C) embryos were transferred into 20 ml plastic specimen vials and fixed for 2 h in MEMFA [0.1 M MOPS pH 7.4, 2 mM EDTA, 1 mM $MgSO_4$, 4% (w/v) Paraformaldehyde] at room temperature. After fixation, the MEMFA was removed, the embryos were rinsed twice with cold 100% MetOH and stored at –20°C.

2.2 Characterization of hsp47 cDNA

2.2.1 hsp47 cDNA

The *Xenopus laevis* hsp47 cDNA clone was obtained from the American Type Culture Collection (ATCC). The gene was isolated from a cDNA library prepared from whole embryos and was cloned into a pCMV-SPORT6 phagemid vector at the *Not*I and

Figure 4. The hsp47 cDNA. The *Xenopus laevis* hsp47 cDNA clone was obtained from the American Type Culture Collection (ATCC). The gene was isolated from a cDNA library prepared from whole embryos and was cloned into a pCMV-SPORT6 phagemid vector at the *Not*I and *Sal*I restriction endonuclease sites. To synthesize anti-hsp47 riboprobes, the pCMV-SPORT6 plasmid vector containing the hsp47 insert was isolated and then linearized as described below. The antisense probe was generated by linearization with *Sma*I and transcribed *in vitro* with T7 polymerase. The sense probe was generated by linearization with *Hind*III and transcribed *in vitro* with SP6 polymerase.


hsp47 clone insert
Not I
SmaI
Sp6 promoter
CMV promoter
ori
Ap
incA
f1 ori
SalI
HindIII
T7 promoter
pCMV-SPORT6-hsp47
6005 bp

*Sal*I restriction endonuclease sites (Klein *et al*, 2002). Plasmid vectors containing the hsp47 cDNA insert were then transformed into *Escherichia coli* DH10 cells.

2.2.2 Isolation of plasmid DNA

E. coli DH5αcells containing the appropriate plasmid were used to asceptically streak LB agar plates [1% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bacto-agar, pH7.5] supplemented with 100 µg/ml ampicillin (BioShop, Burlington, ON). Plates were incubated inverted overnight at 37°C. The following morning plates were incubated at 4°C until later that evening. Single colonies were asceptically transferred to 5 ml of LB broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] supplemented with 100 µg/ml ampicillin. Broth cultures were incubated with shaking overnight at 37°C. Cells were pelleted by centrifugation at 5 000 rpm for 10 min at 4°C in an Eppendorf Centrifuge 5810R (Brinkmann Instruments Ltd) using a swinging bucket rotor. The supernatant was removed and plasmids were isolated using one of the following methods.

2.2.2.1 Plasmid Isolation by phenol-chloroform

Pelleted cells were resuspended in 200 µl of ice-cold solution I [50 mM glucose, 25 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)] and transferred to a microcentrifuge tube. The cells were lysed by the addition of 200 µl of freshly prepared solution II [0.2 N NaOH, 1% (w/v) SDS], mixed by gentle inversion and incubated at room temperature for 5 min. The sample was precipitated by the addition of 200 µl of ice-cold solution III [3 M potassium acetate, 5 M glacial acetic acid], mixed by inversion and incubated on ice for 5 min. The tubes were centrifuged at 14 000 rpm for 15 min at 4°C. Supernatants were transferred to new tubes and any RNA present within the each sample was eliminated by the addition of

10 µl of RNase A (10 mg/ml; BioShop) and incubated at 37°C for 2 h. After RNase A digestion, 600 µl of phenol:chloroform (1:1) was added to each tube, vortexed for 30 sec and centrifuged at 4°C for 2 min at 14 000 rpm. The top aqueous phase was transferred to a fresh tube and the phenol:chloroform step was repeated. After the second centrifugation, the top aqueous phase was transferred to another fresh tube and 600 µl of chloroform as added. As before, the solution was vortexed, centrifuged and the top phase was transferred to a fresh tube to which 60 µl of 3 M sodium acetate (pH 5.2) and 1 ml of 100% ice-cold ethanol was added per tube, mixed by vortexing and precipitated at –80°C for 30 min to overnight. The precipitated plasmid DNA was pelleted by centrifugation at 14 000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 120 µl of 70% (v/v) ethanol. The pellet was centrifuged at 14 000 rpm for 3 min at 4°C, the ethanol was removed and pellet was allowed to air-dry. Each pellet was resuspended in 30-50 µl DEPC-treated water and stored at –20°C.

2.2.2.2 Plasmid Isolation Using the QIAgen QIAprep Miniprep Kit

Plasmid DNA was also isolated from pelleted cells using a QIAprep Miniprep Kit (QIAgen, Mississauga, ON) as detailed in the QIAprep Miniprep Handbook QIAprep Spin Miniprep Kit Protocol using a microcentrifuge (2002) and as described below. Pelleted bacterial cells were resuspended in 250 µl Buffer P1 containing RNase A and the suspension was transferred to a microcentrifuge tube. An equal volume of Buffer P2 was added to each tube and mixed by gently inverting each tube several times. To each lysis reaction 350 µl of Buffer N3 was added and again gently mixed by inversion. The tubes were centrifuged at for 10 min. All centrifugations were performed at 13 200 rpm in a Eppendorf Centrifuge 5415 D microcentrifuge (Brinkmann Instruments Ltd). After

centrifugation, supernatants were applied to the QIAprep column and pellets were discarded. Columns were spun for 1 min and the flow-through was discarded. The QIAprep spin column was washed by addition of 500 µl Buffer PB and centrifugation for 1 min. Flow-through was discarded and 750 µl of Buffer PE was added to each column. Columns were centrifuged for 1 min at 13 200 rpm and flow-through was discarded. To remove any residual wash buffer, columns were centrifuged for an additional minute. To elute plasmid DNA, columns were transferred to 1.5 ml microcentrifuge tubes and 50 µl of Buffer EB [10 mM Tris-Cl (pH 8.5)] was added. The columns were allowed to sit for 1 min and then centrifuged for 1 min. Eluted plasmids were stored at –20^{o}C.

2.2.3 DNA Sequencing

The QIAprep Miniprep Kit was used, as described above, to isolate hsp47 phagemid. This phagemid was first sequenced by automated sequencing at MOBIX (McMaster University, Hamilton, ON) using standard T7 and SP6 primers (taatacgactcactataggg and gatttaggtgacactatag, respectively). After the initial sequencing, custom internal primers were designed using Gene Tools Lite 1.0 (BioTools Incorporated, 2000) to continue sequencing (primers: hsp47A forward primer: attcagcagctctccagtttctaa; hsp47B reverse primer: ccaagctcttctacgtcgacca). Computer analysis of DNA sequences was performed with Gene Tools Lite 1.0. The DNA sequence obtained was used to search the Genbank DNA sequence database. The corresponding translated protein sequences for *Xenopus* hsp47 were compared with those of chicken, mouse, rat, human and zebrafish hsp47. Analysis of *Xenopus* hsp47 nucleotide and amino acid sequences was performed using DNassist, ClustalW, NetNGlyc 1.0 (Technical University of Denmark, 2004) and Gene Tools Lite 1.0.

2.3 Preparation of Digoxygenin (DIG)-labelled riboprobes

To synthesize hsp47 riboprobes, the pCMV-SPORT6 plasmid vector containing the hsp47 insert was isolated as described above. The antisense probe was generated by linearization with *Sma*I and transcription with T7 polymerase. The sense probe was generated by linearization of the plasmid with *Hind*III and transcription with SP6 polymerase (as described below).

The coding region of the hsp70 gene was cloned into the *Sma*I and *Pst*I sites of pSP72 (Promega, Napean, ON), creating pSP72-hsp70 (present from Dr. Tim Mohun). Hsp70 antisense probe was generated by linearization of the plasmid with *Mlu*NI and transcription with SP6 polymerase.

The BiP cDNA was cloned into the *Xho*I and *Eco*RI sites of pBluescript KS+ (Gibco/BRL Laboratories, Burlington, ON) (Miskovic *et al*, 1997). BiP antisense riboprobe was generated by linearization of the plasmid with *Bam*HI and transcription with T7 polymerase.

The L8 coding region was cloned into pBluescript (present from Dr. Yun-Bo Shi) to generate pBlue-L8. L8 antisense riboprobe was generated by linearization of the plasmid with *XhoI* and transcription with T3 polymerase.

2.3.5 *In vitro* transcription

Digoxygenin (DIG)-labelled riboprobes were generated using *in vitro* transcription reactions with the linearized vectors previously described. With the exception of RNA polymerase and RNase inhibitors, all components of the *in vitro* transcription reaction

Figure 5. Hsp70 template for *in vitro* transcription. To generate hsp70 riboprobes, the coding region of the hsp70 gene was cloned into the *Sma*I and *Pst*I sites of pSP72 creating pSP72-hsp70. To synthesize antisense riboprobe, the vector was linearized with *Mlu*NI and transcribed *in vitro* with SP6 polymerase.

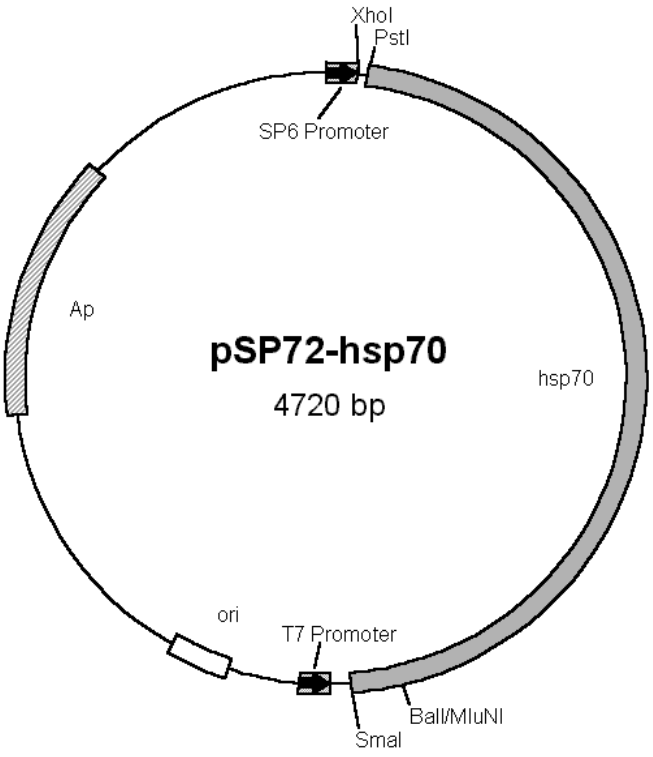

XhoI
PstI
SP6 Promoter
Ap
pSP72-hsp70
4720 bp
hsp70
ori
T7 Promoter
BalI/MluNI
SmaI

Figure 6. BiP template for *in vitro* transcription. To synthesize BiP riboprobes, the BiP cDNA was cloned into the *Xho*I and *Eco*RI sites of pBluescript KS+. To generate antisense riboprobe, the vector was linearized with *Bam*HI and transcribed *in vitro* with T7 polymerase.

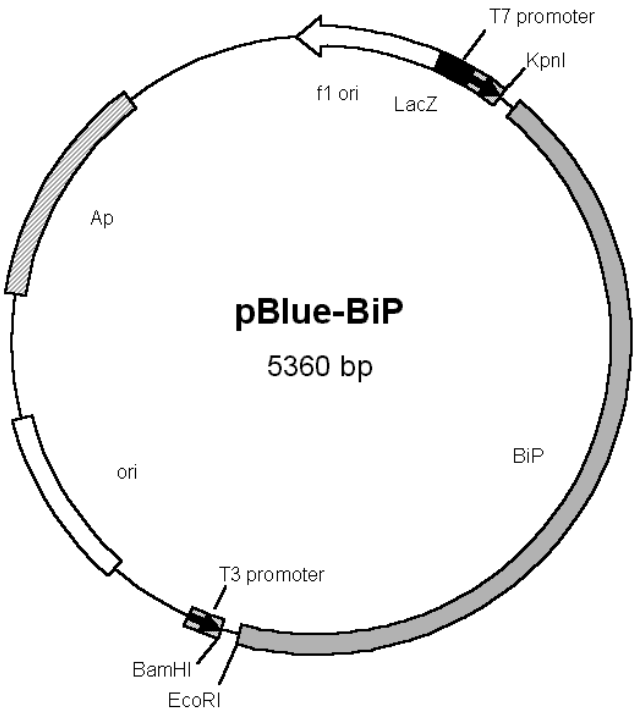
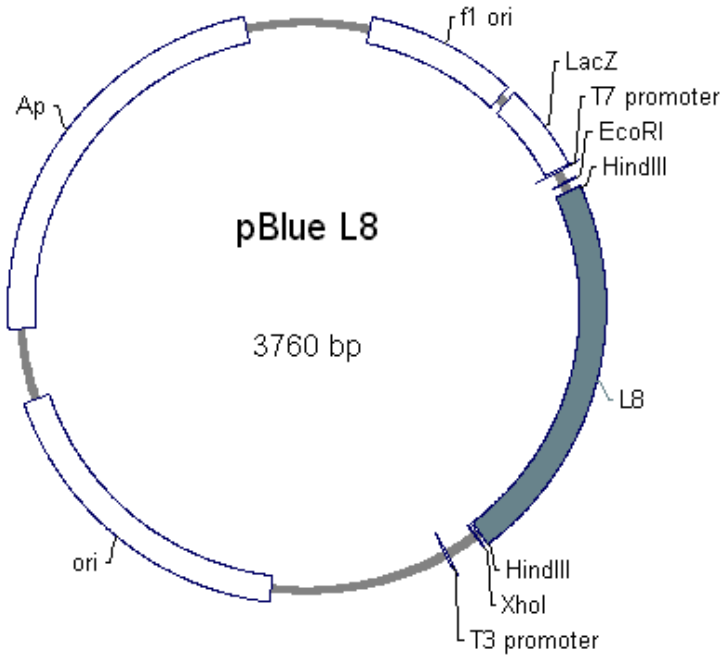

T7 promoter
KpnI
f1 ori
LacZ
Ap
pBlue-BiP
5360 bp
BiP
ori
T3 promoter
BamHI
EcoRI

Figure 7. L8 template for *in vitro* transcription. The L8 coding region was cloned into pBluescript to generate pBlue-L8. Linearizing pBlue-L8 using *Xho*I and transcription with T3 polymerase generated antisense probe.


f1 ori
LacZ
T7 promoter
EcoRI
HindIII
pBlue L8
3760 bp
L8
HindIII
XhoI
T3 promoter
ori
Ap

were allowed to warm to room temperature. For each reaction, 4 µl of linearized DNA template, 4 µl of rNTP mix [2.5 mM rGTP, 2.5 mM rATP, 2.5 mM rCTP, 1.625 mM rUTP (Promega), 0.875 mM DIG-11-UTP (Roche Molecular Biochemicals, Laval, QE)], 1.5 µl DEPC-treated water, 4 µl of 100 mM DTT (final 20 mM; Promega), 4 µl 5 X transcription buffer (final 1 X; MBI Fermentas, Burlington, ON), 0.5 µl RNase inhibitor (MBI Fermentas) and 40 IU of the appropriate RNA polymerase [SP6 RNA polymerase (Roche Molecular Biochemicals), T7 RNA polymerase, T3 RNA polymerase (MBI Fermentas)] into a microcentrifuge tube. The reaction was incubated at 37^{o}C for 1 h and then an additional 40 IU of the appropriate polymerase was added and incubated for 1 h. The DNA template was then removed from the reaction by the addition of 2 µl of RNase-free DNase I (Roche Molecular Biochemicals) for 10 min at 37^{o}C. After the digestion, the *in vitro* transcription product was ethanol precipitated out of solution as described below. 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 combined and incubated at –80^{o}C for 30 min to overnight. The precipitate was pelleted by centrifugation for 15 min at 14 000 rpm at 4^{o}C in an Eppendorf Centrifuge 5810R (Brinkmann Instruments Ltd). The supernatant was removed by pipetting and the pellet was air dried. The pellet was resuspended in 21 µl of DEPC-treated water of which 1 µl was removed for electrophoretic analysis. The remainder was kept at –80^{o}C until needed for Northern blot analysis or *in situ* hybridization.

2.4 RNA Isolation and Northern Blot Analysis

2.4.1 RNA Isolation from A6 Cells

RNA from A6 cell treatments was isolated using the QIAgen RNeasy Mini Kit (QIAgen) as detailed in the RNeasy Mini Handbook Animal Cell Protocol (2001) and also as described below. Stored frozen cells were first combined with 600 µl Buffer RLT containing β-mercaptoethanol, vortexed and homogenized by passing the lysate ten times through a 20 ½ -gauge needle fitted to a sterile syringe. An equal volume of sterile 70% EtOH was added to the lysate and mixed by pipetting. 700 µl of each sample was then applied to an RNeasy mini column placed in a 2 ml collection tube. The columns were spun at 13 200 rpm for 30 s in an Eppendorf Centrifuge 5415 D microcentrifuge (Brinkmann Instruments Ltd) and the flow-through was discarded. This was repeated for the remainder of the sample. 700 µl of Buffer RW1 was added to each column and centrifuged at 13 200 rpm for 30 s. The collection tube and flow-through were both discarded. The columns were transferred to new collection tubes, 500 µl of Buffer RPE was added and the columns were spun at 13 200 rpm for 30 s. The flow-through was discarded and an additional 500 ml of Buffer RPE was added and the columns were centrifuged at 13 200 rpm for 2 min. To elute the RNA, the columns were transferred to 1.5 ml Eppendorf tubes, 50 µl of DEPC-treated water was pipetted directly onto the membrane of each column and then spun at 12 000 rpm for 1 min.

2.4.2 RNA Isolation from *Xenopus* embryos

Total RNA was isolated from embryos using the GIT/CsCl centrifugation method as described by Chirgwin *et al* (1979). Embryos were homogenized in 9 ml of GIT buffer [4 M guanidine isothiocyanate (BioShop), 0.025 M sodium acetate (pH 6.0), 0.835% (v/v)

β-mercaptoethanol] using a PowerGen 125 homogenizer (Fisher Scientific, Napean, ON). The homogenate was layered on top of 3.3 ml cesium chloride solution [5.7 M cesium chloride (BioShop), 0.025 M sodium acetate (pH 6.0)] in 14 X 89 mm ultra-clear ultracentrifuge tubes (Beckman). Samples were centrifuged for 20-23 h at 30 000 rpm at 21^{o}C in a Optima L-90L Ultracentrifuge (Beckmann Instruments Ltd) using a SW41 Ti rotor. The RNA pellets were then washed with 70% EtOH and resuspended in 360 □l TES buffer [10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 1% (w/v) SDS]. The RNA was ethanol precipitated by the addition of 40 µl 3 M sodium acetate (pH 5.2) and 1 ml 100% EtOH and incubated for at least 30 min (maximum overnight) at –80^{o}C. Tubes were centrifuged at 14 000 rpm for 10 min at 4^{o}C in a Eppendorf Centrifuge 5810 R (Brinkmann Instruments Ltd). The pellet was dissolved in 360 µl of DEPC-treated water, followed by an addition ethanol precipitation and centrifugation. The resulting RNA pellet was dissolved in 80 µl of DEPC-treated water and stored at –80^{o}C.

2.4.3 Quantification of RNA

Samples of isolated RNA were diluted 1:200 in water and quantified by ultraviolet-spectrophotometry at 260 nm in a Cary 50 Bio UV-visible spectrophotometer (Varian, Mississauga, ON). RNA integrity was checked and concentration reconfirmed by electrophoretic analysis using a 1 µg aliquot of each sample on a 1.2% (w/v) formaldehyde agarose gel [1.2 % (w/v) agarose, 10% (v/v) 10 X MOPS (pH 7.0; 0.2 M 3-(morpholino) propane sulfonic acid, 50 mM anhydrous sodium acetate, 10 mM EDTA, pH 8.0) and 16% (v/v) formaldehyde]. Each RNA sample was supplemented with 1 µl 10 X MOPS, 1.6 µl formaldehyde, 2 µl loading dye [0.2% (w/v) bromophenol blue, 1 mM EDTA (pH 8.0) and 50 % (v/v) glycerol], 5 µl formamide and 0.5 µg/ml ethidium bromide. Samples were heat

denatured for 10 min at 68°C, cooled on ice for 5 min, loaded into the gel and run for 1-2 h at 90 V. Distinct 28S and 18S rRNA staining with ethidium bromide indicated intact RNA.

2.4.4 Northern blot analysis

Due to varying levels of specific mRNAs present within A6 cells, 15 µg of RNA was used to investigate hsp47 mRNA and BiP mRNA expression patterns and 10 µg of RNA was used to investigate hsp70 mRNA and L8 rRNA expression patterns. Each sample was dried in a Speedvac for 15 min and then separated by formaldehyde agarose gel electrophoresis as previously described but with the following exceptions. Ethidium bromide was omitted from the loading buffer and the gel was electrophoresed for 3-4 h at 65 V. After electrophoresis, the gel was soaked in 0.05 N NaOH for 20 min at room temperature to denature the RNA. The gel was rinsed with DEPC-treated water and then soaked for 20 min in 20 X SSC buffer [3 M sodium chloride, 300 mM sodium citrate (pH 7.0)]. The SSC wash was repeated a second time.

The RNA as transferred to a positively charged nylon membrane (Roche Molecular Biochemicals) by capillary elution as described below. The gel was inverted onto a 20 X SSC-pre-soaked blotting paper (VWR, West Chester, PA) wick positioned on a Plexiglas support over a Pyrex® dish containing approximately 500 ml 20 X SSC. An appropriately sized piece of nylon membrane was placed on top of the gel and two equally sized pieces of 10 X SSC-pre-soaked blotting paper were placed on top of the membrane. A 10 cm stack of paper towels was placed on top, followed by a glass support and approximately 500 g of weight to aid transfer. The base of the apparatus was wrapped in plastic wrap to avoid evaporation. Transfer was allowed to proceed overnight. Following transfer, paper towel and blotting paper layers were discarded and the correct side of the nylon membrane

was marked with pencil. RNA was fixed to the membrane by UV cross-linking twice with ither a GS-Gene linker (program 'C3' 150mJ; BioRad, Mississauga, ON) or an UVC-515 Ultraviolet Multilinker (120 000 $\mu J/cm^2$; UltraLum Inc).

To check the efficiency of transfer and to verify equal loading, the membrane was stained with 1 X Reversible Blot Stain (Sigma). Before adding the stain, the membrane was soaked in 10% (v/v) glacial acetic acid for 5 min with shaking. The acetic acid was removed and 1 X Blot Stain added for 5 min with shaking. The membrane was then destained by washing several times with DEPC-treated water. The blot stain was photographed and then the membrane was transferred to a hybridization bag (SealPAK pouches, VWR).

Approximately 50 ml pre-heated prehybridization buffer [50% (v/v) formamide, 5 X SSC, 0.02 % SDS, 0.01% N-lauryl sarcosine, 2% Blocking Reagent] was added to the hybridization bag. Prehybridization proceeded in a Shake'N'Bake Hybridization Oven (Boekel Scientific) for at least 4 h at 65^{o}C for hsp47, hsp70 and BiP and at 60^{o}C for L8. After prehybridization, the buffer was replaced with hybridization buffer (as described above) containing the appropriate DIG-labelled antisense RNA probe. Hybridization was allowed to proceed overnight at the same temperature as prehybridization.

Following hybridization, the membrane was washed to remove unbound probe. The blot was first washed twice for 5 min each in 2 X SSC and 0.1% (w/v) SDS at room temperature. This was followed by a 15 min wash in 0.5 X SSC and 0.1% (w/v) SDS at hybridization temperature (60 or 65^{o}C). The membrane was then washed in 0.1 X SSC and 0.1% SDS, also at hybridization temperature. The blot was equilibrated for 1 min at room temperature in washing buffer [100 mM maleic acid, 0.3% (v/v) Tween 20] and incubated

in blocking solution [2% (w/v) blocking reagent, 10% (v/v) maleic acid buffer (pH 7.5)] for 30 to 60 min at room temperature. Blocking solution was replaced with fresh blocking solution containing 1:8000 dilution of Anti-DIG-alkaline phosphatase-conjugated Fab fragments antibody (Roche Molecular Biochemicals) for 30 min at room temperature. To remove any unbound antibody, the membrane was washed twice for 20 min each in washing buffer at room temperature. It was then equilibrated in detection buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl] for 2 min at room temperature. CDP-Star (Roche Molecular Biochemicals) was applied evenly over the membrane and placed in a hybridization bag. Bubbles were removed and the chemiluminescent reaction was allowed to develop for 10 min at room temperature. Chemiluminescence was detected using a Fluorchem 8000 Chemiluminescence and Visible Imaging System (filter position 1; Alpha Innotech Corp, San Leandro, CA) for up to 30 min depending on the strength of signal. Densitometric analysis was performed on sodium arsenite and heat shock, tunicamycin, and A23187 and heat shock Northern blots using NIH Image 1.62 software. Statistical analysis of the densitometry results was performed in triplicate using GraphPad InStat 3.

2.5 Whole Mount *in situ* Hybridization

Whole mount *in situ* hybridization was performed using *Xenopus laevis* albino embryos (the product of both an albino male and female) using a protocol developed by Harland (1991), including modifications of Dr. Tom Drysdale (Lawson Institute, London, ON). As previously described, treated embryos were fixed for 2 h in MEMFA [0.1 M MOPS pH 7.4, 2mM EDTA, 1mM $MgSO_4$, 4% Paraformaldehyde] at room temperature. After fixation, the MEMFA was removed; the embryos were rinsed twice with cold 100% MetOH and stored at -20^{o}C. Embryos were allowed to warm to room temperature and

were then transferred to Sigmacoated (Sigma) 6 ml glass vials for the remainder of the procedure. Unless otherwise stated, the entire in situ procedure was performed at room temperature using a nutator (VWR).

The embryos were first rehydrated with decreasing concentrations of methanol [75% (v/v) and 50% (v/v) methanol in water and 25% (v/v) in TTw (200mM NaCl, 50 mM Tris pH 7.4 and 0.1% (v/v) Tween 20)] for 5 min each. This was followed by three 5 min washes of 100% TTw. Embryos were then treated with 5 µg/ml Proteinase K for 20 min to remove mucous membranes from the surface of the embryos. The treatment was followed by a 10 min TTw wash and two 5 min washes with 0.1 M Triethanolamine (Sigma) pH 7-8. The Triethanolamine was then replaced with 2 ml of fresh Triethanolamine and 5 µl of acetic anhydride (Sigma) for 5 min. This serves to block positively charged groups on the embryos from non-specifically binding probe. An additional 5 µl of acetic anhydride was added for 5 more minutes. Following two 5 min TTw washes, the embryos were re-fixed in MEMFA for 20 min. After fixation, embryos were washed in TTw five times for 5 min each.

Prehybridization was carried out in hybridization buffer [50% (v/v) Formamide, 5 X SSC, 1 mg/ml Torula RNA (Boehringer Mannheim, Laval, QE), 1 X Denhart's solution, 0.1% (v/v) Tween 20, 5 mM EDTA, 100 µg/ml heparin] for 2 h at 65^{o}C in a Shake'N'Bake Hybridization Oven (Boekel Scientific). Prehybridization buffer was replaced with hybridization buffer containing the appropriate DIG-labelled antisense probe and incubated overnight at 60^{o}C.

The next day, hybridization solution was removed and excess probe was rinsed out with 10 min washes of decreasing concentrations of hybridization buffer in 2 X SSC (100%

hybridization buffer, 50% (v/v) hybridization buffer 50% (v/v) 2 X SSC, 25% (v/v) hybridization buffer 75% (v/v) 2 X SSC) at 60°C. Following two 20 min washes in 2 X SSC at 37°C, embryos were treated with 1 µl/ml RNase A in 2 X SSC for 30 min also at 37°C. Embryos were then rinsed in 2 X SSC for 10 min at room temperature before two 1 h washes of 0.2 X SSC at 60°C.

In preparation for antibody detection, TTw was added for 10 min, followed by another 10 min wash in TBT [200 mM NaCl, 50 mM Tris pH 7.4, 2 mg/ml BSA (ICN), 0.1% (v/v) Triton X-100 (ICN)] and 1 h incubation in TBT containing 20% heat-treated lamb serum (Gibco/BRL Laboratories). A 1:3000 dilution of anti-DIG-alkaline phosphatase-conjugated Fab fragments antibody (Roche Molecular Biochemicals) in TBT containing 20% (v/v) heat-treated lamb serum was prepared and embryos were incubated in this overnight at 4°C. Excess antibody was removed with twelve 30 min washes of TBT at room temperature and then embryos were treated with alkaline phosphatase buffer [100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% (v/v) Tween 20, 2 mM Levamisol] twice for 15 min. The embryos were incubated overnight with nitro blue tetrazolium (NBT; 1.375 µl/ml of buffer) and 5-bromo-4-chloro-3-iodoyl-phosphate (BCIP; 0.875 µl/ml of buffer). The colourmetric reaction was stopped with washes of increasing concentrations of methanol (25 %, 50% and 75% in water) each for 5 min. The embryos were left in –20°C 100% methanol without shaking until background staining was sufficiently reduced (usually 2 h). The embryos were then rehydrated with decreasing concentrations of methanol (50% and 25%, each for 5 min) and counterstained overnight with Bouin's Fixative (VWR). The following day, the embryos were dehydrated with increasing concentrations of methanol (25%, 50% and 100% twice) each for 5 min and

then cleared for viewing in benzyl alcohol/benzyl benzoate (BABB; one part benzyl alcohol two parts benzyl benzoate). Representational photographs were taken of the embryos using a Nikon COOLPIX995 digital camera (Nikon Inc, Melville, NJ) attached to a Nikon Eclipse stereoscopic microscope. For long term storage embryos were washed several times in 100% methanol and stored at –20^{o}C.

2.6 Protein Isolation and Western Blot Analysis

2.6.1 Protein Isolation from A6 Cells

A6 cell pellets were lysed by the addition of 500 µl of lysis buffer [160 mM sucrose, 1.6 mM EGTA, 0.8 mM EDTA, 32 mM NaCl, 24 mM HEPES, 1% (w/v) SDS, 100µg /ml PMSF, 1 µg /ml aprotnin, 0.5 µg /ml leupeptin, pH 7.4], vortexed and then homogenized using a Teflon pestle. Each sample was then sonicated using a Branson Sonifer 250 (15 pulses, duty cycle 65%, output control 4.5; Branson Sonic Power Co, Danbury, CT). All samples were centrifuged at 14 000 rpm for 30 min at 4^{o}C in an Eppendorf Centrifuge 5810R. After centrifugation, supernatants were removed by pipetting, transferred to new Eppendorf tubes and kept at –20^{o}C.

2.6.2 Protein Quantification

The concentrations of all isolated proteins were calculated using a bicinchoninic acid (BCA) protein assay according to the manufacture's instructions (Pierce, Rockford, IL) and as described below. Dilutions of bovine serum albumin (BSA) ranging from 0 to 2 mg/ml were prepared in sterile water from a 2 mg/ml stock. Aliquots of isolated protein samples were diluted 1:10 and 1:20 in sterile water. 10µl of each BSA standard was loaded onto a polystyrene 96 well assay plate in triplicate. An equal volume of each sample protein dilution was also loaded onto the plate in duplicate. A 50:1 mixture of BCA

reagent A to reagent B (Pierce) was prepared and 80 µl of the mixture was added to each well. The plate was incubated at 37°C for 30 min and then allowed to cool at room temperature for 10 min. The plate was read using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, CA) at 562 nm and using a soft max pro program. The BSA standards were used to construct a standard curve that was in turn used to determine the concentration of each protein sample.

2.6.3 Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were cast in a Mini Protean II gel apparatus (BioRad, Mississauga, ON). Separating gels [10% (v/v) acrylamide, 0.27% (v/v) n'n'-bis methylene acrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS), 0.15% (v/v) n,n,n'n'-Tetramethylethylenediamine (TEMED)] were prepared, poured and overlayed with 100% ethanol for 30 min. Once polymerized, the ethanol layer was removed and stacking gels [4% (v/v) acrylamide, 0.11% (v/v) n'n'-bis methylene acrylamide, 0.125 M Tris pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.2% (v/v) TEMED] were layered on top. Combs were inserted to create wells and gels were allowed to polymerize for another 30 min. During gel polymerization, protein samples were prepared by combining the volume for 40 µg of each protein sample with water (to equilibrate volumes) and loading buffer [0.0625 M Tris pH6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue] to a final concentration of 1 X. All protein samples and protein markers were boiled for 10 min, allowed to cool and pulse-spun in a microcentrifuge before loading into the gel. After gel polymerization, combs were removed and the gels were assembled in the gel apparatus. The tank was filled with 1 X electrophoresis buffer

[25mM Tris, 0.2 M glycine, 1 mM SDS], samples were loaded and electrophoresed at 90 V until samples reached the separating gel. Electrophoresis was then continued at 120-170 V until the dye front reached the bottom of the gel. While the gels were running, one Polyvinylidene fluoride (PVDF) membrane (Millipore corp., Napean, ON) and 6 blotting papers (VWR, West Chester, PA) per gel were cut to 5.5 cm X 8.5 cm. Membranes were activated in 100% methanol for 10 sec and then equilibrated in cold transfer buffer [25mM Tris, 192 mM glycine, 20% (v/v) methanol] for 30 min. Following electrophoresis, gels were also soaked in transfer buffer for 10-15 min. Proteins were transferred to the PVDF membrane using a Trans-Blot Semi-dry Transfer Cell (BioRad) at 25 V for 40 min. After transfer, blots were stained with Ponceau-S stain [0.19% (w/v) Ponceau-S, 5% (v/v) acetic acid] for 10 min, destained in water for 5 min and then scanned with a Hewlett Packard ScanJet 3300C. Blots were then incubated in 5% blocking solution [2 mM Tris (pH 7.5), 30 mM NaCl, 5% (w/v) Nestle® Carnation skim milk powder] for 1 h at room temperature. The blocking solution was then replaced with monoclonal mouse anti-rat-HSP47 primary antibody (Cat# SPA-470; Stressgen, Victoria, BC) at a concentration of 1 μg /ml in 5% blocking solution for 1 h at room temperature. Blots were washed with TBS-T [2 mM Tris (pH 7.5), 30 mM NaCl, 0.1% (v/v) Tween-20] once for 15 min and then twice for 10 min. After the third wash, blots were incubated with a goat anti-mouse IgG (H+L)-horse radish peroxidase conjugated secondary antibody (BioRad) at a 1:3000 dilution in 5% blocking solution for 1 h. Blots were washed with TBS-T once for 15 min and then twice for 5 min. Detection of HSP47 was accomplished using an ECL plus Western Blotting Detection System (RPN2132; Amersham Biosciences, Piscataway, NJ) as per manufacture's instructions. A 40:1 solution A to solution B reagent mixture was prepared, placed on

membranes and allowed to develop for 5 min. Chemiluminescence was detected using a Fluorchem 8000 Chemiluminescence and Visible Imaging System (filter position 1; Alpha Innotech) for up to 15 min depending on the strength of signal. Densitometric analysis was performed in triplicate on sodium arsenite and heat shock, tunicamycin, and A23187 and heat shock western blots using NIH Image 1.62 software. Statistical analysis of the densitometry results was performed using GraphPad InStat 3.

3. Results

3.1 Sequence analysis of hsp47 nucleotide and amino acid sequence.

Full length published nucleotide and amino acid sequences for *Xenopus* hsp47 mRNA and protein were obtained from Genbank (Klein *et al*, 2002; Figure 8). The protein coding region of *Xenopus* hsp47 is 1257 bp long and the HSP47 protein consists of 419 amino acids with a predicted molecular weight of 47 326 Da. Examination of the 3' untranslated region (3' UTR) revealed the presence of three potential instability consensus elements, TATTTA. This sequence is believed to confer instability to a number of mRNAs (Brawerman, 1987). The *Xenopus* HSP47 protein sequence had a predicted basic pI of 8.36, which is consistent with HSP47 from other organisms (Nagata, 1996). Examination of the predicted amino acid sequence revealed the presence of a hydrophobic N-terminal leader sequence (MWMIKLLALSILLVV; Figure 8). An initial hydrophobic sequence on the amino terminal is necessary for the translocation of newly synthesized protein into the endoplasmic reticulum (ER; Alberts *et al*, 1994). The amino terminal of the *Xenopus* HSP47 protein also possessed two possible asparagine-associated glycosylation sites. It was previously been established that human HSP47 (colligin-2) possessed these two glycosylation sites that were fully glycosylated with high-mannose oligosaccharides in the mature form of the protein (Satoh *et al*, 1996). The carboxyl terminal of the *Xenopus* HSP47 protein possessed an ER-retention sequence consisting of 4 amino acids, arg-asp-glu-leu (RDEL). This sequence is important for the sequestering of proteins in the ER and has been found on HSP47 from all investigated organisms.

Figure 8. Nucleotide and amino acid sequence of *Xenopus* hsp47. Full length *Xenopus laevis* hsp47 nucleotide and amino acid sequences were obtained from Genbank nucleotide and protein databases, respectively. The encoded amino acids are listed above the nucleotide sequence. The stop codon is indicated with an asterisk. The C-terminal RDEL sequence is shown in bold.

ggcagagcgcaagaggacatttgggagagaccattcactcagccttcccagctctttaggtgttccagtt 70

M W M I K L L A L S I L L V V D
tgctccctattactgaagccaccatgtggatgatcaagcttctagccctcagtatcctcctggttgtgga 140

A A V N K K P I A E K K V E P P L E Q K M S Q
tgccgccgtaaacaagaagccaatagcagagaaaaaagtggagcctccactagagcagaagatgagccaa 210

H A N V L A D K S A G L A F N L Y Q I M A K D K
catgcaaatgtactagcggacaagagtgcaggcctggctttcaacctctaccaaatcatggccaaggaca 280

K V E N I L L S P V V V A S S L G L V S M G G
agaaagtggagaatatacttctctctccagtagtggttgcctcttctcttggcctggtgagtatgggtgg 350

Q A S T A A Q A K T V L N A E K L S D E H I H
acaagccagcactgcagcccaagctaaaaccgtccttaatgcggagaaactcagcgatgaacacatccac 420

S G L A E L L N E V S N S T A R N V T W K M G N
tccggccttgctgagctactcaatgaagttagcaactccactgcccgcaacgtcacctggaagatgggga 490

R L Y G P S S I S F S D N F V K D S K K H Y N
accgcctgtatggccccagctccatcagcttctccgacaactttgtgaaggacagtaagaagcactacaa 560

Y E H S K I N F R D K R S T L R S I N E W A A
ctatgaacactccaagataaactttagagacaagagaagtaccttgagatcaatcaatgaatgggctgcc 630

Q T T D G K L P E V T S D V E K T D G A L I V N
cagaccactgatggcaagctgcctgaggtaaccagtgatgtggagaagactgatggagctctcattgtca 700

A M F F K P H W D E R F H H Q M V D N R G F M
acgctatgtttttcaagcctcactgggatgaacgattccaccatcagatggttgacaaccgtggcttcat 770

V T R S F T V S V P M M H R T G L Y K Y T D D
ggtgacccgttctttcaccgtgtctgtccccatgatgcaccgcacagggctgtacaaatacactgatgat 840

E T N N L Q I L E M P L A H K L S S M I I I M P
gagacaaataacctccagatcctggagatgccactagcccacaagctctccagcatgatcatcatcatgc 910

Y H V E P L E R L E K L L T R E Q V N A W D G
cctaccacgtggagcccttggagaggttggagaagctcctgacaagagaacaagtcaatgcttgggatgg 980

K M K K R A V A V S L P K V S L E V S H D L Q
aaagatgaagaagagagcagtggctgtgtctctgcctaaagtcagtttggaagtcagccatgatctccag 1050

K H L G D L G L T E A I D K S K A D L S K I S G
aaacatctaggggaccttggtctgactgaggccattgacaaatctaaagctgatctctccaaaatctctg 1120

K K D L Y L A S M F H A A A L E W D T E G N P
gcaagaaggacctctacctggccagcatgttccacgccgctgccctggagtgggacacagagggaaatcc 1190

F D S D L Y S R E E L R S P K L F Y A D H P F
atttgattctgatctctacagccgagaggagctcaggtcacccaagctcttctatgccgaccatcccttc 1260

V F L I K D N K T D S I L F I G R L V R P K G D
gtcttcctcatcaaggacaacaagactgattctatcctctttatcggcagactcgtgaggccgaagggag 1330

K M **R D E L** *
acaaaatgcgagatgaattatagtagattaaggagttctagggttggggaggagggctgggtagtcttta 1400

aaaataatttctgtttgtgaacaagagaatggtttatggatcacgttaggatttacttcttcagagcatt 1470

ccaccaaagcaggaggttgtaatgcctttggtcttacctaaaataggtggtagctggtgctaaaggggat 1540

catttaatagatgttcttatctcaggagatttcactgggctggggatatttattatatctggttaattca 1610

aaaatgtggatattatcagtgttcttattacatgtatgggatctaacaactgggaaccataaagctcgga 1680

gcataagtgcatggccagaaaagcccaggtatcatgagtttctatacctgtaatttaatctttaaaagtg 1750

cacttcagagtttaactaaggtcagttacatctatctatcaagccaatcaggctccatttactactggcc 1820

gacagctctgattgatgccaaacaagtcataaccgttcaactcctctgtccatgacctgagattccggcc 1890

aagaatgtgaccaatgaatgatctcccctgaccactggtcactttgttcaattcatgatatttagagttg 1960

tttctaagtgaatttgctactggaaattcatggtgatgacatggcggaggcaggccttggatatgatgtt 2030

atttaaagcatttttaaagtatagactacacgtggccctttaggtgttgttcaactaaaactctcagatt 2100

tttgctatcaggggcctgaatcccagagctggggtgagatatgagtgagaatgaatgggtgcatgttagt 2170

gtatgggggggggctgtatggattgaagtattaaataaacaaaaatacatttaaaaaaaatgattttgc 2240

tttactttcaccatgtggattcacaaaatctttattttgtaaaagttacaatgatgtccgagagaaactg 2310

acctgtgtgtgttttatttgcaataaaactttaaaaaaaaaaaaaaaaaaaaaa 2365

3.1.1 Amino acid sequence comparison of *Xenopus* HSP47 with HSP47 from other species.

A search of the Genbank protein database revealed that the *Xenopus* HSP47 amino acid sequence (Klein *et al*, 2002) has the highest identity with chicken (*Gallus gallus*; Hirayoshi *et al*, 1991) HSP47 protein. A comparison of the entire predicted amino acid sequence of *Xenopus* HSP47 with chicken (*Gallus gallus*), mouse (*Mus musculus*; Wang and Gudas, 1990), rat (*Rattus norvegicus*; Clarke *et al*, 1991), human (*Homo sapiens*; Clarke and Sanwal, 1992) and zebrafish (*Danio rerio*; Pearson *et al*, 1996) is shown in Figure 9 and summarized in Table 1. The *Xenopus* HSP47 protein sequence exhibited 77% identity with chicken, 73% with mouse, 72% with rat and human, and 70% with zebrafish. Most of the sequence identity of HSP47 from all investigated organisms occurred centrally in the amino acid sequence and in several carboxyl terminal regions. Compared to chicken, *Xenopus* HSP47 has 89 amino acid substitutions and 14 additions. All 6 HSP47 proteins have a hydrophobic N-terminal leader sequence and share the carboxyl terminal ER-retention sequence, RDEL.

3.1.2 Characterization of the hsp47 cDNA clone.

The *Xenopus laevis* hsp47 cDNA clone was obtained from the American Type Culture Collection (ATCC) in DH10 *Escherichia coli* cells. The phagemid was isolated from broth cultures using a QIAgen QIAprep Miniprep Kit as described in Experimental Procedures and sequenced by automated sequencing at MOBIX (McMaster University, Hamilton, ON). Sequence analysis revealed that the hsp47 cDNA clone was missing approximately the first 900 nucleotides of the protein coding sequence (Figure 10). The 1.6 kb insert contained the 3' UTR of *Xenopus* hsp47 as well as 461 bp of the protein

Figure 9. A comparison of the amino acid sequence of HSP47 proteins. The *Xenopus* HSP47 amino acid sequence published on Genbank was aligned with HSP47 protein sequences from chicken (*Gallus gallus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), human (*Homo sapiens*) and zebrafish (*Danio rerio*) using ClustalW version 1.82. Asterisks (*) denote complete amino acid identity between all sequences, colons (:) denote conserved strong amino acid groups and periods (.) denote conserved weak groups.

```
Xenopus         MWMIKLL-ALSILLVVDAAVNKKPIAEKKVEPPLEQKMSQHANVLADKSAGLAFNLYQIM 59
Chicken         MQIFLVL-ALCGLAAAVPSEDRK--------------LSDKATTLADRSTTLAFNLYHAM 45
Mouse           MRSLLLG-TLCLLAVALAAEVKKPLEA--AAPGTAEKLSSKATTLAERSTGLAFSLYQAM 57
Rat             MRSLLLG-TLCLLAVALAAEVKKPVEA--AAPGTAEKLSSKATTLAERSTGLAFSLYQAM 57
Human           MRSLLLLSAFCLLEAALAAEVKKPAAA--AAPGTAEKLSPKAATLAERSAGLAFSLYQAM 58
Zebrafish       MWVSSLI-ALCLLAVAVSGEDKK--------------LSTHATSMADTSANLAFNLYHNV 45
                *    :  ::. * .. ..  :*              :* :*  :*: *: ***.**: :

Xenopus         AKDKKVENILLSPVVVASSLGLVSMGGQASTAAQAKTVLNAEKLSDEHIHSGLAELLNEV 119
Chicken         AKDKNMENILLSPVVVASSLGLVSLGGKATTASQAKAVLSADKLNDDYVHSGLSELLNEV 105
Mouse           AKDQAVENILLSPLVVASSLGLVSLGGKATTASQAKAVLSAEKLRDEEVHTGLGELLRSL 117
Rat             AKDQAVENILLSPLVVASSLGLVSLGGKATTASQAKAVLSAEKLRDEEVHTGLGELVRSL 117
Human           AKDQAVENILVSPVVVASSLGLVSLGGKATTASQAKAVLSAEQLRDEEVHAGLGELLRSL 118
Zebrafish       LK-KGLENIFISPVVVASSLGMVAMGSKSSTASQVKSILKADALKDEHLHTGLSELLTEV 104
                 * : :***::**:*******:*::*.:::***:*.*::*.*: * *: :*:**.**: .:

Xenopus         SNSTARNVTWKMGNRLYGPSSISFSDNFVKDSKKHYNYEHSKINFRDKRSTLRSINEWAA 179
Chicken         SNSTARNVTWKIGNRLYGPASINFADDFVKNSKKHYNYEHSKINFRDKRSALKSINEWAA 165
Mouse           SNSTARNVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAS 177
Rat             SNSTARNVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAS 177
Human           SNSTARNVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAA 178
Zebrafish       SDPQTRNVTWKISNRLYGPSSVSFAEDFVKNSKKHYNYEHSKINFRDKRSAINSINEWAA 164
                *:. :*******:..*****:*:.*:::**:.**:*** ************::.******:

Xenopus         QTTDGKLPEVTSDVEKTDGALIVNAMFFKPHWDERFHHQMVDNRGFMVTRSFTVSVPMMH 239
Chicken         QTTDGKLPEVTKDVEKTDGALIVNAMFFKPHWDEKFHHKMVDNRGFMVTRSYTVGVPMMH 225
Mouse           QTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDERFHHRMVDNRGFMVTRSYTVGVTMMH 237
Rat             QTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKFHHKMVDNRGFMVTRSYTVGVTMMH 237
Human           QTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKFHHKMVDNRGFMVTRSYTVGVMMMH 238
Zebrafish       KTTDGKLPEITKDVKNTDGAMIVNAMFFKPHWDEKFHHKMVDNRGFLVTRSHTVSVPMMH 224
                :*********:*.**:.*****:*************:***:*******:****.**.* ***

Xenopus         RTGLYKYTDDETNNLQILEMPLAHKLSSMIIIMPYHVEPLERLEKLLTREQVNAWDGKMK 299
Chicken         RTGLYNYYDDEAEKLQVVEMPLAHKLSSMIFIMPNHVEPLERVEKLLNREQLKTWASKMK 285
Mouse           RTGLYNYYDDEKEKLQMVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKAWMGKMQ 297
Rat             RTGLYNYYDDEKEKLQLVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKTWMGKMQ 297
Human           RTGLYNYYDDEKEKLQIVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKIWMGKMQ 298
Zebrafish       RTGIYGFYEDTENRFLIVSIALAHKKSSMIFIMPYHVEPLDRLENLLTRQQLDTWISKLE 284
                ***:* : :*  :.: ::.:.**** **:*::** *****:*:*:**.:::*:. * .*::

Xenopus         KRAVAVSLPKVSLEVSHDLQKHLGDLGLTEAIDKSKADLSKISGKKDLYLASMFHAAALE 359
Chicken         KRSVAISLPKVVLEVSHDLQKHLADLGLTEAIDKTKADLSKISGKKDLYLSNVFHAAALE 345
Mouse           KKAVAISLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFE 357
Rat             KKAVAISLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFE 357
Human           KKAVAISLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFE 358
Zebrafish       ERAVAISLPKVSMEASHDLQKHLGELGLTEAVDKSKADLSNISGKKDLYLSNVFHASSLE 344
                :::**:****  :*.:*******. ******:**.*****.:*********.:***:::*

Xenopus         WDTEGNPFDSDLYSREELRSPKLFYADHPFVFLIKDNKTDSILFIGRLVRPKGDKMRDEL 419
Chicken         WDTDGNPYDADIYGREEMRNPKLFYADHPFIFMIKDSKTNSILFIGRLVRPKGDKMRDEL 405
Mouse           WDTEGNPFDQDIYGREELRSPKLFYADHPFIFLVRDNQSGSLLFIGRLVRPKGDKMRDEL 417
Rat             WDTEGNPFDQDIYGREELRSPKLFYADHPFIFLVRDNQSGSLLFIGRLVRPKGDKMRDEL 417
Human           LDTDGNPFDQDIYGREELRSPKLFYADHPFIFLVRDTQSGSLLFIGRLVRPKGDKMRDEL 418
Zebrafish       WDTEGNPFDPSIFGSEKMRNPKLFYADHPFIFLVKDNKTNSILFIGRLVRPKGDKMRDEL 404
                 **:***:* .::. *:::*.**********:*:::*.::.*:******************
```

Table 1. A comparison of HSP47 amino acid sequences*

	Percent identity with *Xenopus* HSP47
Chicken HSP47	77%
Mouse HSP47	73%
Rat HSP47	72%
Human HSP47	72%
Zebrafish HSP47	70%

*An amino acid sequence comparison of the Genbank *Xenopus laevis* HSP47 with HSP47 from chicken (*Gallus gallus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), human (*Homo sapiens*) and zebrafish (*Danio rerio*).

Figure 10. A diagrammatical representation of the full length *Xenopus* hsp47 nucleotide sequence and the ATCC *Xenopus* hsp47 cDNA clone insert. The full length *Xenopus* hsp47 nucleotide sequence was obtained from the Genbank nucleotide database. The protein coding region is 1257 bp long and encodes 419 amino acids. The *Xenopus* hsp47 cDNA clone insert is approximately 1.6 kb in length and contains the 3'UTR of *Xenopus* hsp47 as well as 461 bp of the protein coding sequence including the highly conserved carboxyl terminus.

Full length Xenopus hsp47

ATCC Xenopus hsp47 cDNA clone

1.6 kb insert contained the 3' UTR of *Xenopus* hsp47 as well as 461 bp of the protein coding sequence including the highly conserved carboxyl terminus. This cDNA clone was used to generate DIG-labeled antisense hsp47 riboprobes for the purpose of investigating hsp47 mRNA accumulation by Northern blot analysis and whole mount *in situ* hybridization. Although the ATCC clone was truncated, the partial sequence was more than enough to serve as a template for the production of hsp47 riboprobes.

3.2 Characterization of hsp47 mRNA and protein accumulation in A6 cells.

The availability of a *Xenopus laevis* A6 kidney epithelial cell line (A6 cells) permitted the examination of hsp47 expression in somatic cells. Northern blot and Western blot analysis were used to examine the relative levels of hsp47 mRNA and protein, respectively. HSP47 is an ER-resident heat shock protein that was found to have induction patterns more similar to general cytosolic/nuclear HSPs than HSPs localized in the ER (Lele *et al*, 1997). It is for this reason that relative levels of *Xenopus* hsp47 mRNA were compared with hsp70 (a cytosolic/nuclear hsp) and BiP (an ER-resident hsp) mRNA. The mRNA encoding *Xenopus* large ribosomal subunit protein 8 (L8), a constitutively expressed protein, was used as a control for all Northern blot analysis. For Western blot analysis, a *Xenopus* anti-HSP47 antibody was unavailable. Therefore, relative levels of HSP47 protein were detected using a mouse anti-rat HSP47 monoclonal antibody (Stressgen). Rabbit anti-actin polyclonal antibody (Sigma) was used to examine the relative levels of actin protein.

3.2.1 Characterization of hsp47 mRNA and protein accumulation in response to elevated temperature in A6 cells.

A6 cells were heat shocked at 27, 30, 33, 35 or 37^{o}C for 1 h. Northern blot analysis of total RNA revealed that hsp47 mRNA was present constitutively in A6 cells (Figure 11). Enhanced accumulation of this transcript was found at 30^{o}C, although optimal induction was seen at 35^{o}C with lower levels at 37^{o}C. Hsp70 mRNA was maximally induced at 33^{o}C but enhanced accumulation was also observed at 35 and 37^{o}C. Figure 11 demonstrates that BiP mRNA was also expressed constitutively. This transcript increased in response to heat shock at all investigated temperatures ranging from 27 to 37^{o}C. L8 mRNA was expressed at approximately the same level at all temperatures examined.

To examine the effect of elevated temperature on HSP47 protein levels, A6 cells were heat shocked at 27, 30, 33, 35 or 37^{o}C for 2 h and then allowed to recover at 22^{o}C for 2 h. Figure 12, shows that HSP47 protein was expressed constitutively in A6 cells. The anti-HSP47 antibody detected two distinct bands closely related in size, approximately 51 and 54 kDa. The upper HSP47 band accumulated slightly in response to temperatures of 30, 33 and 35^{o}C. The lower protein band accumulated optimally after heat shock at 30^{o}C, however, elevated protein levels were also evident at 33 and 35^{o}C. Two HSP47 protein bands may be due to post-translational modifications and will be discussed later.

To further characterize hsp47 expression in response to heat shock, the pattern of hsp47 mRNA and protein accumulation was examined in A6 cells exposed to 33^{o}C over time. A6 cells were heat shocked at 33^{o}C for 0.5, 1, 2, 4, or 8 h. Northern blot analysis revealed that hsp47 transcripts were heat inducible at 33^{o}C after 30 min (Figure 13).

Figure 11. The effect of elevated temperature on hsp47, hsp70 and BiP mRNA accumulation. A6 cells were heat shocked for 1 h at 27, 30, 33, 35 or 37^{o}C. Control cells were maintained at 22^{o}C. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labelled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain. Transcript sizes: hsp47, 2.7 kb; hsp70, 2.4 kb; BiP, 2.7 kb and L8, 1.0 kb.

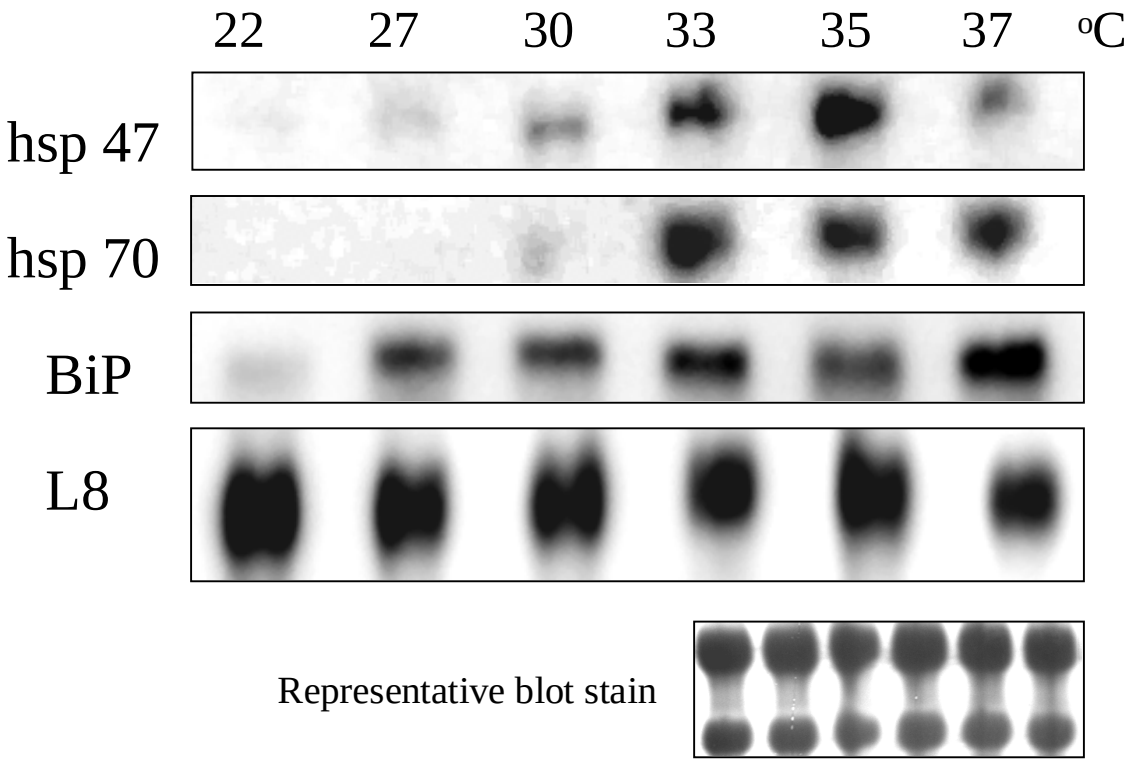

22
27
30
33
35
37
°C
hsp 47
hsp 70
BiP
L8
Representative blot stain

Figure 12. HSP47 protein accumulation in response to elevated temperature in A6 cells. A6 cells were heat shocked for 2 h at 27, 30, 33, 35 or 37^{o}C and then allowed to recover for 2 h at 22^{o}C. Control cells were maintained at 22^{o}C. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein). Size of protein bands: 51 and 54 kDa.

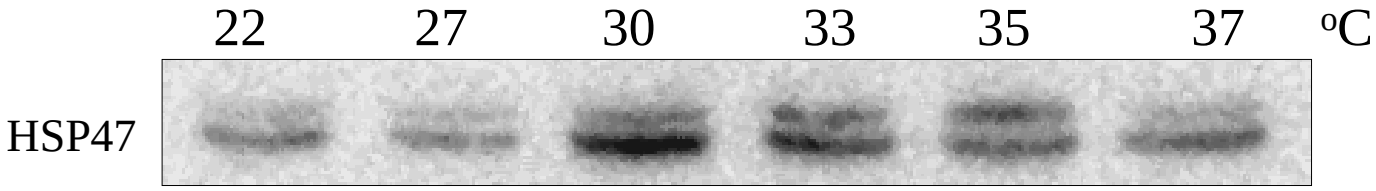
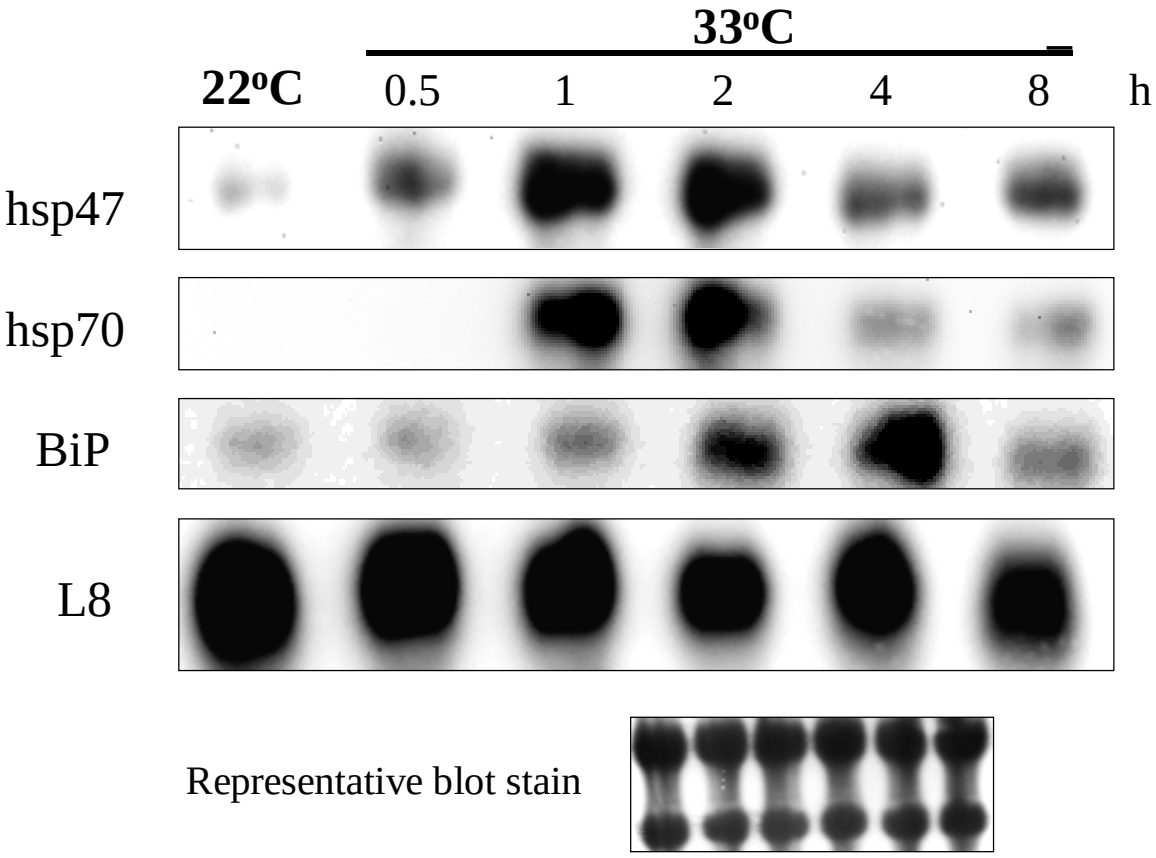

22
27
30
33
35
37
°C
HSP47

Figure 13. Hsp47, hsp70 and BiP mRNA accumulation at 33°C over time in A6 cells. A6 cells were heat shocked at 33°C for 0.5, 1, 2, 4 or 8 h. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labelled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.


33°C
22°C
0.5
1
2
4
8
h
hsp47
hsp70
BiP
L8
Representative blot stain

Optimal induction was observed at 1 h but hsp47 accumulated relative to control at all investigated time points ranging from 0.5 to 8 h. Hsp70 mRNA accumulated in response to 33^{o}C at 1 and 2 h. BiP transcripts accumulated slightly after a 1 h exposure at 33^{o}C with maximal induction at 4 h. L8 transcript levels were relatively unaffected by incubation at 33^{o}C over time. To examine HSP47 protein levels A6 cells were heat shocked at 33^{o}C for 1, 2, 4, or 8 h and then allowed to recover at 22^{o}C for 2 h. Western blot analysis showed that the upper HSP47 protein band accumulated after exposure to 33^{o}C for 2 and 4 h (Figure 14). The lower protein band levels decreased slightly after 2, 4, and 8 h of heat shock at 33^{o}C.

The temporal pattern of hsp47 mRNA and protein accumulation was also examined at 35^{o}C. Northern blot analysis revealed that heat shock-induced hsp47 mRNA accumulation occurred from 30 min to 4 h, with optimal induction at 1 h. Hsp70 exhibited the same mRNA accumulation pattern as hsp47 at 35^{o}C. BiP mRNA showed optimal induction at 2 h and elevated accumulation at 4 and 8 h. In contrast, L8 mRNA levels were unaffected by 35^{o}C exposure. Western blot analysis revealed that the upper HSP47 band accumulated within 2 h exposure to 35^{o}C (Figure 16). The upper protein band was optimally expressed after a 4 h exposure to 35^{o}C and remained relatively constant up to 8 h of heat shock. The level of the lower HSP47 protein band decreased after 4 and 8 h of heat shock at 35^{o}C. In comparison, actin protein production was unaffected by this temperature over time.

Figure 14. Transient accumulation of HSP47 protein in A6 cells during prolonged incubation at 33°C. A6 cells were heat shocked at 33°C for 1, 2, 4 or 8 h and then allowed to recover for 2 h at 22°C. Control cells were maintained at 22°C. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

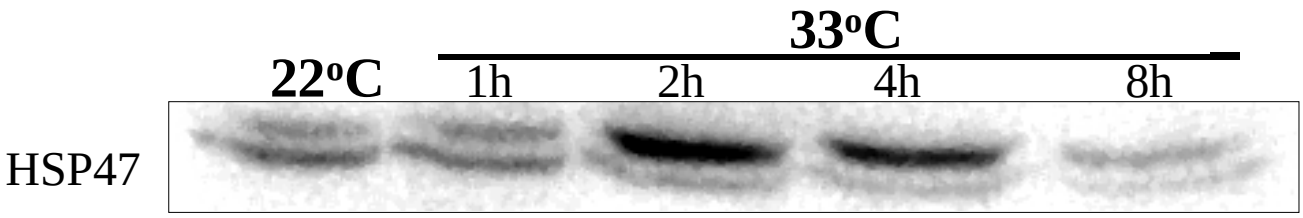

33°C
22°C
1h
2h
4h
8h
HSP47

Figure 15. Hsp47, hsp70 and BiP mRNA accumulation at 35°C over time in A6 cells. A6 cells were heat shocked at 35°C for 0.5, 1, 2, 4 or 8 h. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labelled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

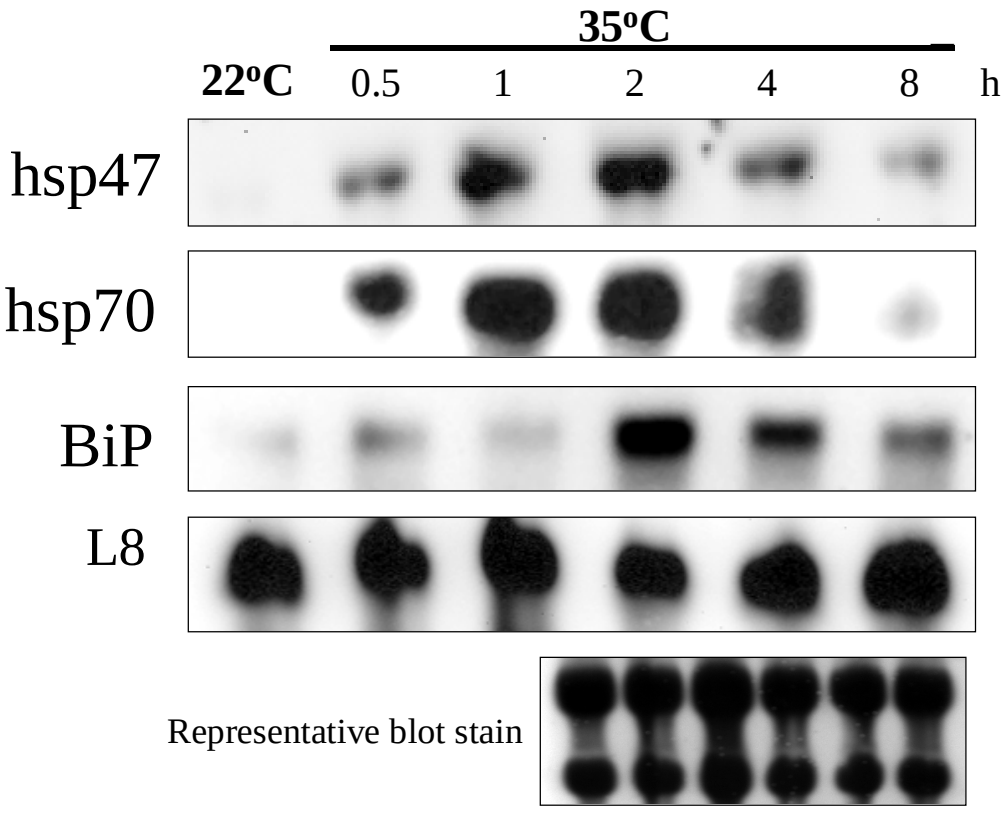
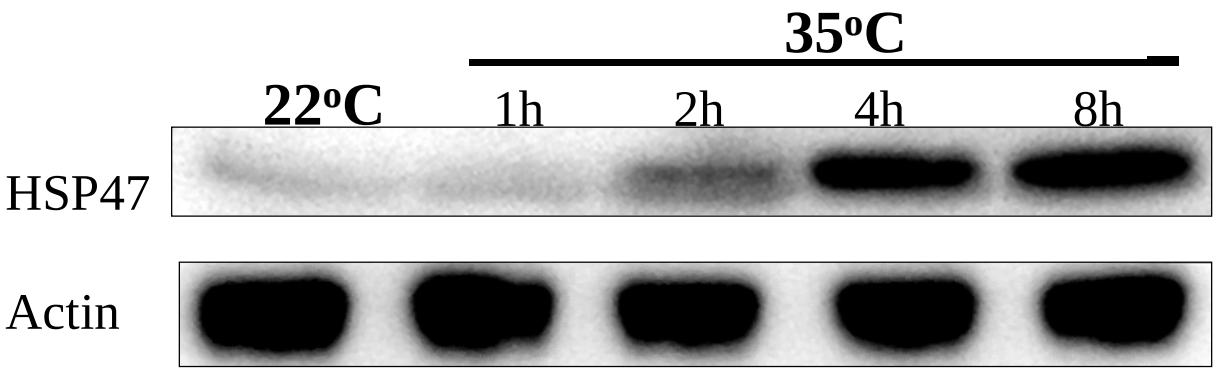

35ºC
22ºC
0.5
1
2
4
8
h
hsp47
hsp70
BiP
L8
Representative blot stain

Figure 16. HSP47 protein accumulates over time upon exposure to 35^{o}C in A6 cells. A6 cells were heat shocked at 35^{o}C for 1, 2, 4 or 8 h and then allowed to recover for 2 h at 22^{o}C. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein). A polyclonal rabbit anti-actin antibody was used to examine the relative levels of actin protein by western blot analysis (using 20 μg total protein).


35°C
22°C
1h
2h
4h
8h
HSP47
Actin

3.2.2 Hsp47 mRNA and protein accumulation in response to 50 μM sodium arsenite in A6 cells.

Sodium arsenite (NaAs) is an alternative cellular stressor that has been used to examine heat shock protein gene expression. NaAs is suspected to act on nascent polypeptides and inhibit proper protein folding, although its specific mode of action is still unknown (Bernstam and Nriagu, 2000). In the present study, A6 cells were treated with 50 μM NaAs for 1, 3, 6, 12 or 24 h at 22°C. Hsp47 mRNA accumulated within the first 3 h of exposure, with optimal induction at 6 h (Figure 17). Hsp70 and BiP transcripts were both optimally expressed with 24 h of 50 μM NaAs exposure. L8 mRNA levels were unaffected by NaAs exposure. To examine HSP47 protein levels in A6 cells, cells were treated with 50 μM NaAs for 3, 6, 9, 12 or 24 h at 22°C. The lower HSP47 band accumulated slightly after a 6 h exposure to NaAs and optimal induction was evident by 12 h as seen in Figure 18. The upper protein band remained at relatively the same level during the course of treatment.

3.2.3 The combined effect of temperature and sodium arsenite on hsp47 mRNA and protein in A6 cells.

A6 cells were exposed to heat shock and sodium arsenite treatments individually and in combination. Cells were heat shocked for 2 h at 30 or 33°C or treated with 10 μM NaAs for 2 h at 22°C. Additionally, cells were concurrently exposed to 30°C and 10 μM NaAs for 2 h. As seen in Figures 19 and 20, hsp47 mRNA was induced 1.4 fold in response to a 2 h 30°C heat shock and 2.5 fold to a 2 h 33°C heat shock compared to control. Exposure to 10 μM NaAs for 2 h appeared to have no detectable effect on hsp47 mRNA accumulation. When A6 cells were exposed to 30°C and 10 μM NaAs in concert, hsp47

Figure 17. Hsp47, hsp70 and BiP mRNA accumulation in response to 50 μM NaAs exposure in A6 cells. A6 cells were exposed to 50 μM NaAs for 1, 3, 6, 12 or 24 h at 22^{o}C. Control cells were maintained at 22^{o}C. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labeled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

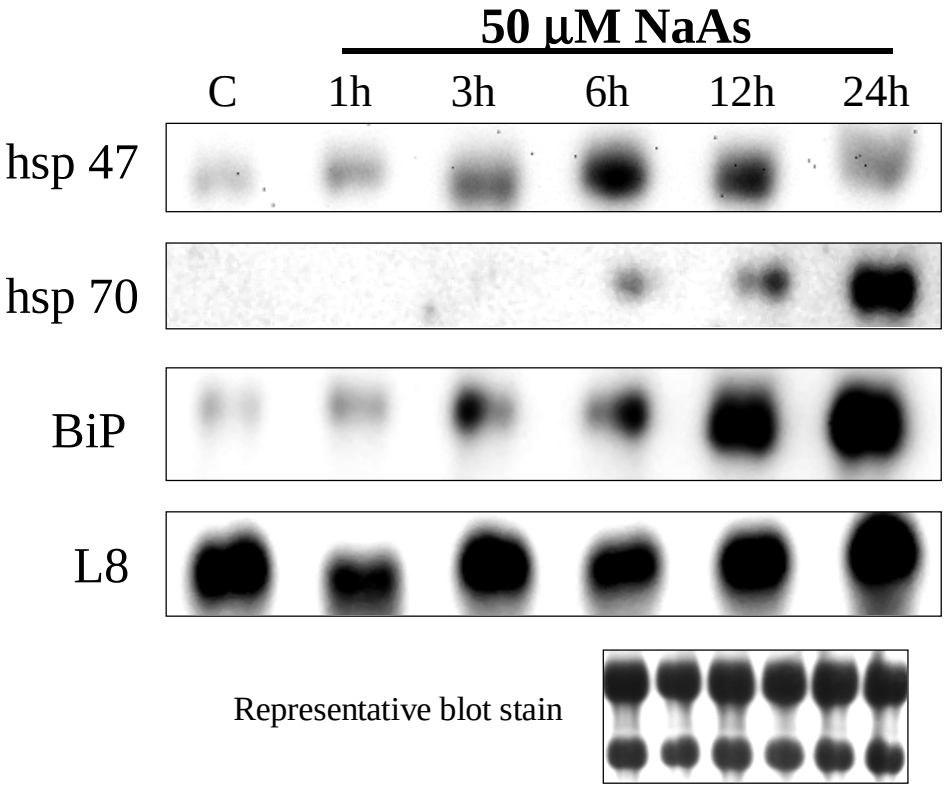

50 μM NaAs
C
1h
3h
6h
12h
24h
hsp 47
hsp 70
BiP
L8
Representative blot stain

Figure 18. HSP47 protein accumulation in response to 50 μM NaAs exposure in A6 cells. A6 cells were exposed to 50 μM NaAs for 3, 6, 9, 12 or 24 h at 22°C. Control cells were maintained at 22°C. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

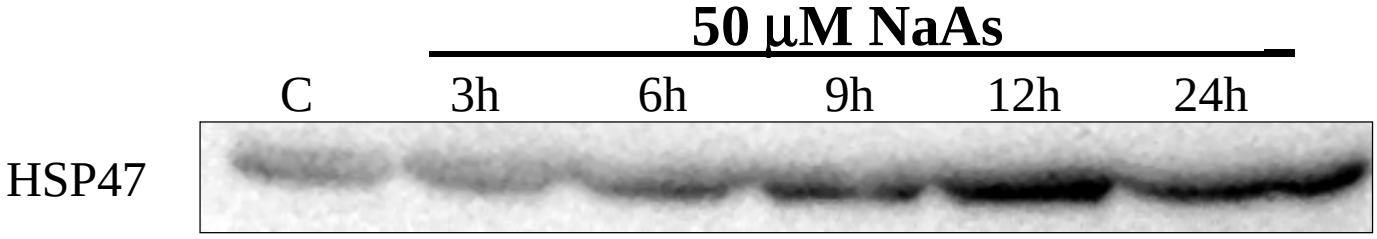

50 μM NaAs
C
3h
6h
9h
12h
24h
HSP47

Figure 19. The effect of combined heat shock and sodium arsenite treatment on hsp47, hsp70 and BiP mRNA accumulation in A6 cells. Cells were exposed to 30^{o}C or 33^{o}C for 2 h or treated with 10 μM NaAs for 2 h at 22^{o}C. Other cells were treated with 10 μM NaAs at 30^{o}C for 2 h. Following various treatments, cells were harvested and total RNA was isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labelled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

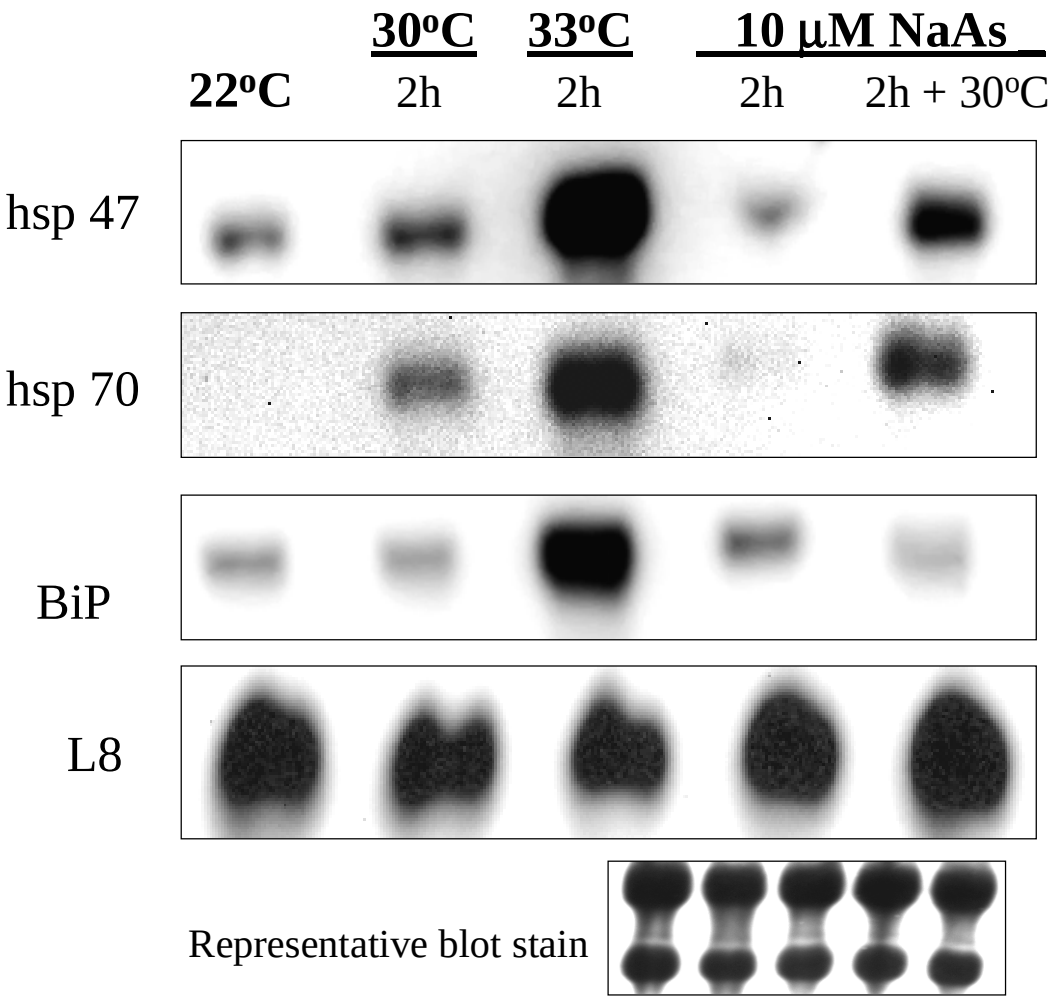
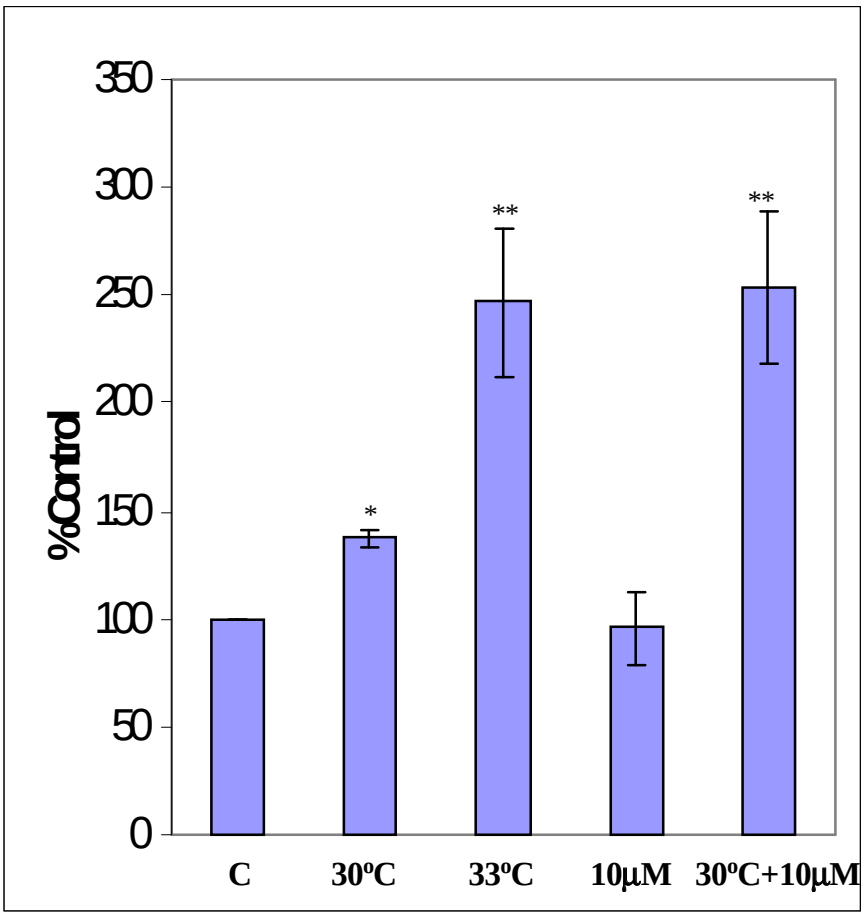

30ºC
33ºC
10 µM NaAs
22ºC
2h
2h
2h
2h + 30ºC
hsp 47
hsp 70
BiP
L8
Representative blot stain

Figure 20. Densitometric analysis of the combined effect of heat shock and sodium arsenite treatment on hsp47 mRNA accumulation in A6 cells. The relative densities of hsp47 mRNA bands on Northern blot images were measured using NIH Image 1.62 software. The data was expressed as a percentage of the control hybridization obtained with each blot/probe combination. Graphed data were expressed as mean +/- standard error. *, $P < 0.05$; **, $P < 0.01$, significantly different from corresponding untreated cells.


350
300
250
200
150
100
50
0
%Control
*
**
**
C
30ºC
33ºC
10µM
30ºC+10µM

mRNA expression was 2.5 fold higher than the expression level seen with each independent stressor (Figures 19 and 20). This pattern of expression was also found for hsp70 mRNA, the only difference being that hsp70 was not constitutively expressed. Elevated BiP mRNA levels were not observed in response to heat shock and NaAs. Increased BiP transcript was only evident in 33^{o}C heat shocked cells. L8 mRNA levels were unaffected by any of the treatments.

To observe the effect of heat shock and/or sodium arsenite treatment on HSP47 protein accumuation, A6 cells were exposed to the following treatments: 2 h at 30^{o}C with 2 h recovery at 22^{o}C, 2 h at 33^{o}C with 2 h recovery at 22^{o}C, 10 μM NaAs for 6 h and 10 μM NaAs for 6 h with 2 h at 30^{o}C and 2 h recovery at 22^{o}C. Western blot analysis revealed that under the conditions investigated heat shock and/or sodium arsenite treatment had no detectable combined effect on HSP47 protein accumulation in A6 cells (Figure 21). This finding was substantiated with densitometric analysis (Figure 22).

3.2.4 The effect of the ER-specific stressors tunicamycin and A231871 on hsp47 mRNA and protein accumulation in A6 cells.

Tunicamycin is an ER-specific stressor that inhibits the amino-glycosylation of newly synthesized proteins (Lee, 1987). Tunicamycin (dissolved in methanol) was added to flasks of A6 cells at a final concentration of 1 μg/ml for 4, 6 or 24 h at 22^{o}C. Other cells were treated with 0.1% methanol for 6 h to serve as a solvent control. Figure 23 shows that hsp47 and hsp70 mRNA expression was not affected by exposure to 1 μg/ml tunicamycin. Densitmetric analysis of hsp47 Northern results substantiated that no significant change in hsp47 mRNA was detected (Figure 24). However, BiP mRNA accumulated in response to a 24 h exposure of 1 μg/ml tunicamycin (Figure 23). L8 mRNA decreased slightly over the

Figure 21. The effect of combined heat shock and sodium arsenite treatment on HSP47 protein accumulation in A6 cells. Cells were exposed to 30°C or 33°C for 2 h and allowed to recover at 22°C for 2 h or exposed to 10 μM NaAs for 6 h at 22°C. Additionally, cells were treated with 10 μM NaAs for 6 h and concurrently heat shocked at 30°C for 2 h and allowed to recover at 22°C for 2 h. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

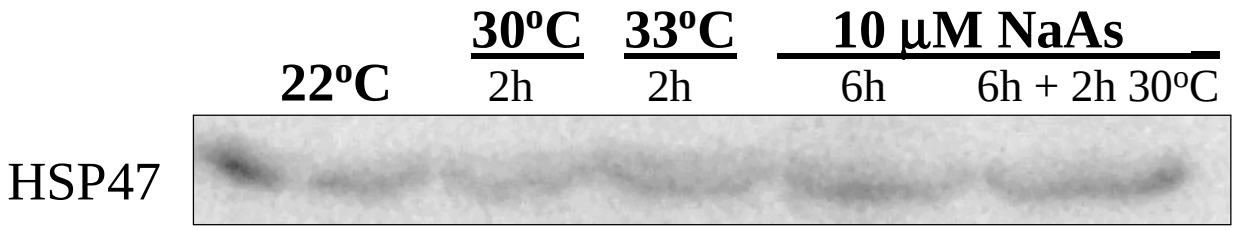

30ºC
33ºC
10 μM NaAs
22ºC
2h
2h
6h
6h + 2h 30ºC
HSP47

Figure 22. Densitometric analysis of the combined effect of heat shock and sodium arsenite treatment on HSP47 protein accumulation in A6 cells. The relative densities of hsp47 protein bands on Western blot images were measured using NIH Image 1.62 software. The data was expressed as a percentage of the control hybridization obtained with each blot/antibody combination. Graphed data were expressed as mean +/- standard error. Experimental data was not significantly different form corresponding untreated cells.

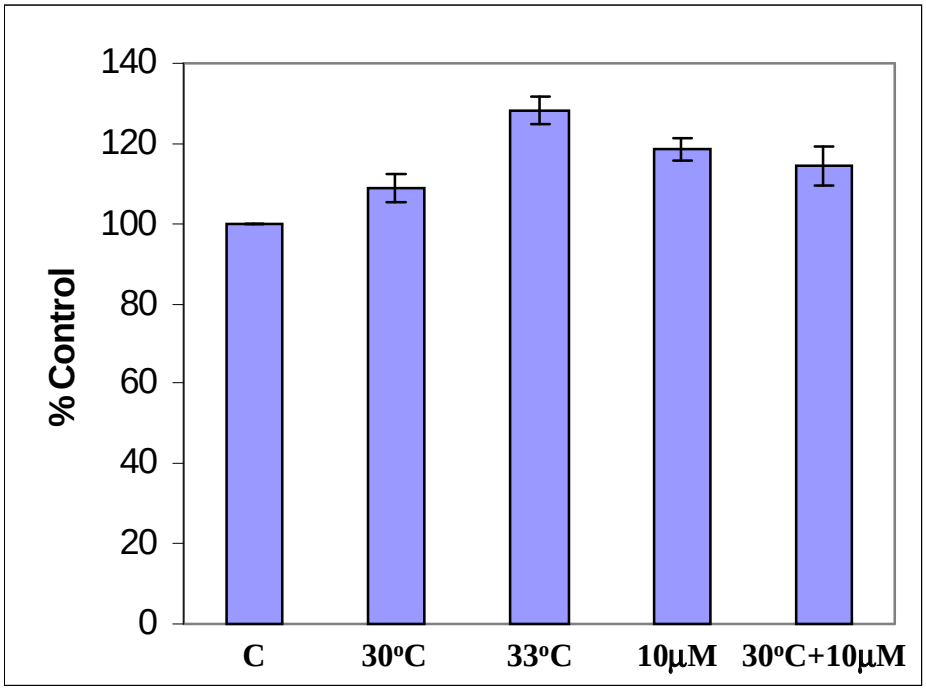

140
120
100
80
60
40
20
0
% Control
C
30°C
33°C
10µM
30°C+10µM

Figure 23. The effect of 1 μg/ml tunicamycin on hsp47, hsp70 and BiP mRNA accumulation. A6 cells were treated with 1 μg/ml tunicamycin for 4, 6 or 24 h at 22^{o}C. A 6 h cell treatment of 0.1% methanol served as a solvent control. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labelled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

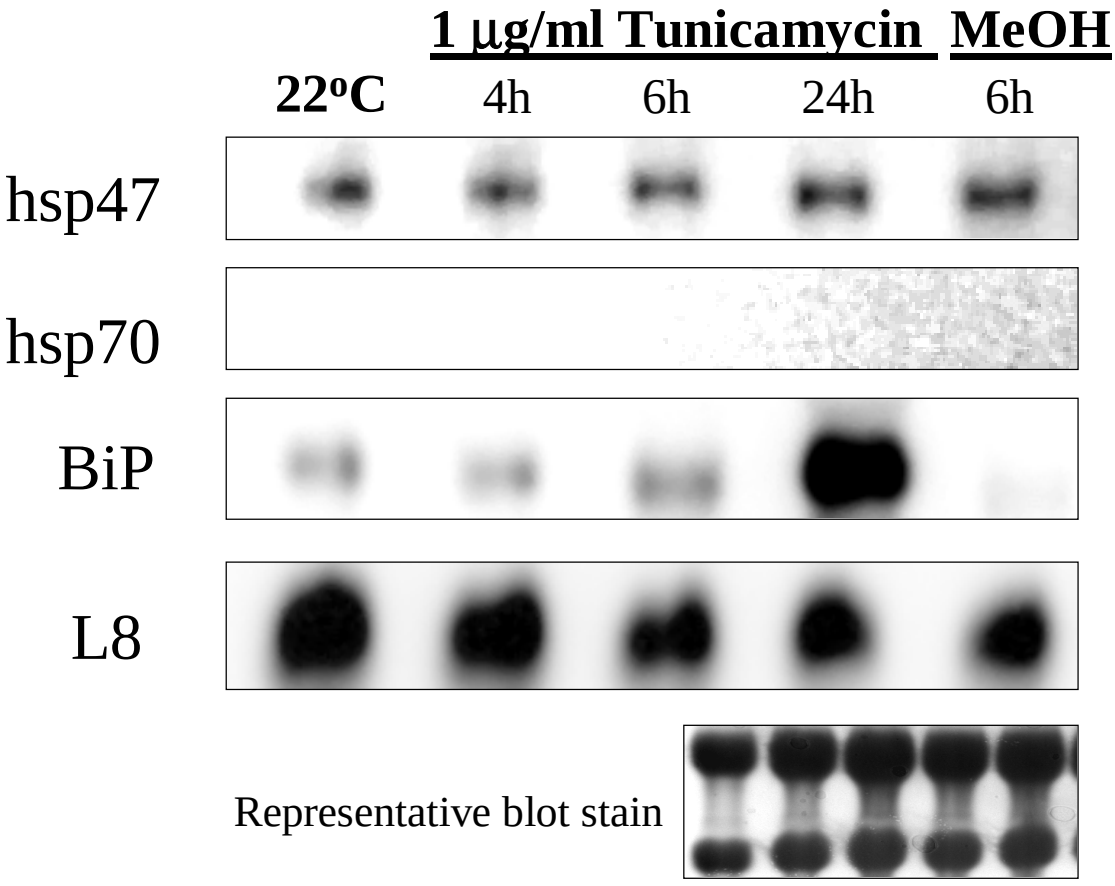

1 μg/ml Tunicamycin
MeOH
22ºC
4h
6h
24h
6h
hsp47
hsp70
BiP
L8
Representative blot stain

Figure 24. Densitometic analysis of 1 μg/ml tunicamycin on hsp47 mRNA accumulation in A6 cells. The relative densities of hsp47 mRNA bands on Northern blot images were measured using NIH Image 1.62 software. The data were expressed as a percentage of the control hybridization obtained with each blot/probe combination. Graphed data were expressed as mean +/- standard error. Experimental data was not significantly different form corresponding untreated cells.

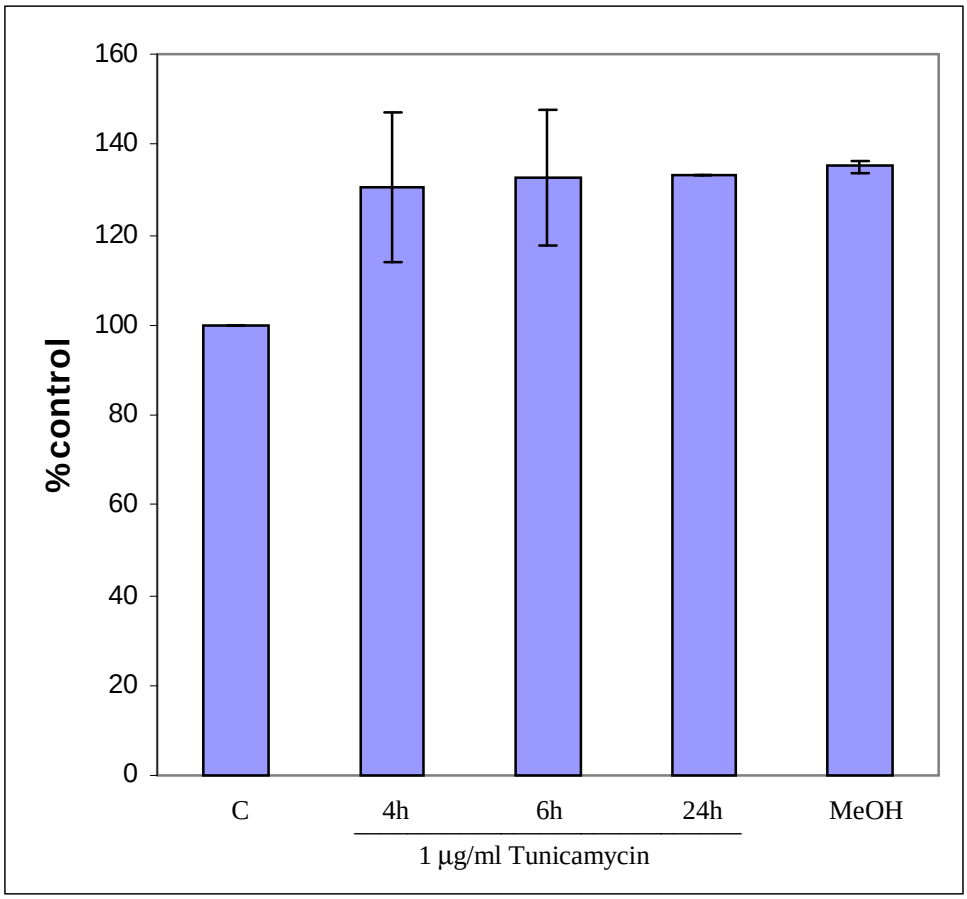

160
140
120
100
80
60
40
20
0
%control
C
4h
6h
24h
MeOH
1 µg/ml Tunicamycin

Figure 25. The effect of 1 μg/ml tunicamycin on HSP47protein accumulation. A6 cells were treated with 1 μg/ml tunicamycin for 4, 6, 8 or 24 h at 22^{o}C. A 6 h cell treatment of 0.1% methanol served as a solvent control. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

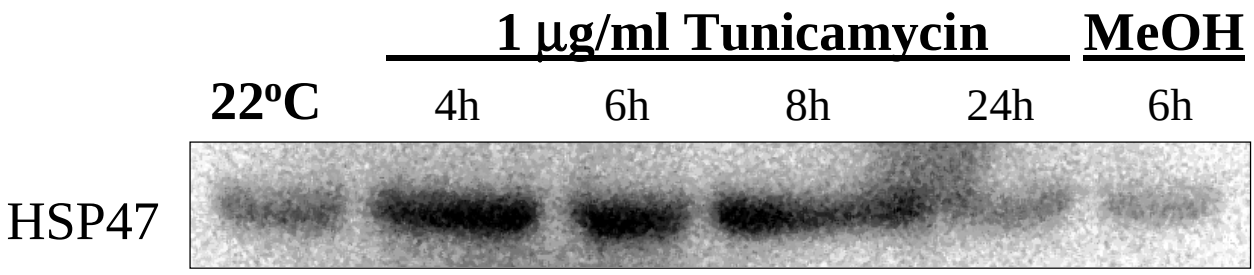
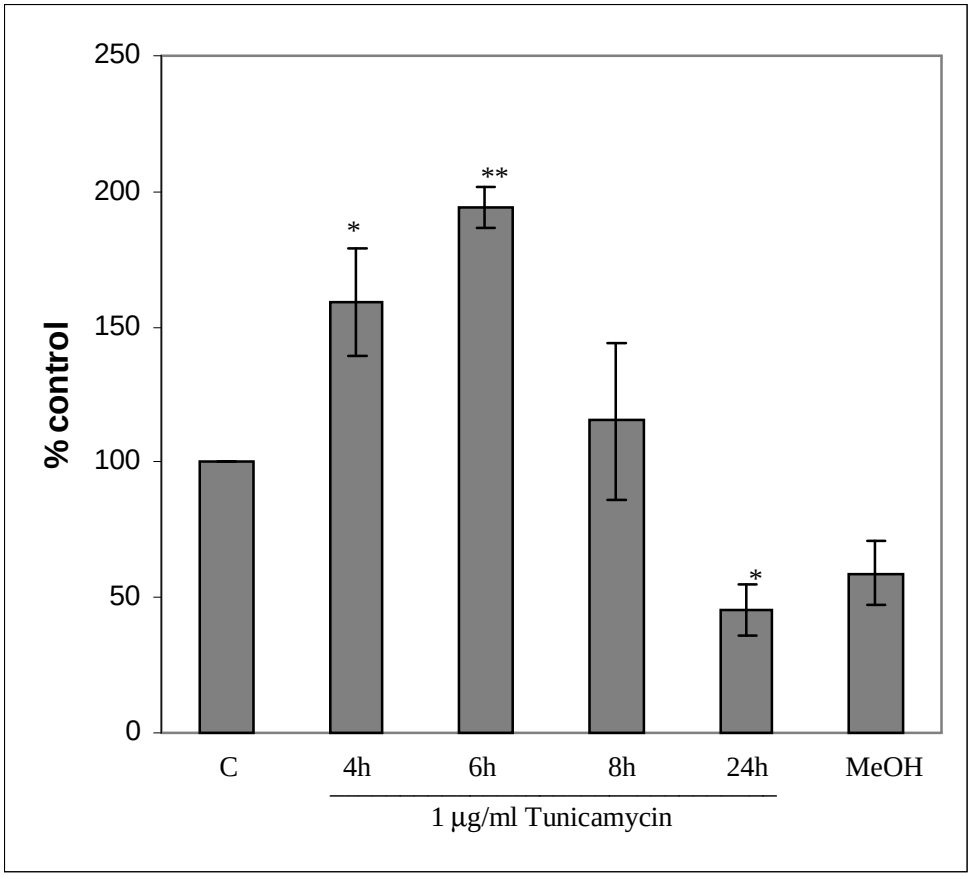

1 μg/ml Tunicamycin
MeOH
22ºC
4h
6h
8h
24h
6h
HSP47

Figure 26. Densitometric analysis of the effect of 1 μg/ml tunicamycin on HSP47 protein accumulation in A6 cells. The relative densities of HSP47 protein on Western blot images were measured using NIH Image 1.62 software. The data was expressed as a percentage of the control hybridization obtained with each blot/antibody combination. Graphed data were expressed as mean +/- standard error. *, $P < 0.05$; ** $P < 0.01$, significantly different from corresponding untreated cells.


250
200
150
100
50
0
% control
*
**
*
C
4h
6h
8h
24h
MeOH
1 µg/ml Tunicamycin

course of treatment. As shown in Figure 25, enhanced lower HSP47 protein band accumulation was observed within 4 h of exposure to tunicamycin and remained elevated up to 8 h of treatment. The upper HSP47 band observed with other treatments was not evident with exposure to tunicamycin. As seen in Figure 26, densitometric analysis revealed that HSP47 protein levels elevated 1.6 and 1.9 fold at 4 and 6 h, respectively. A significant decrease in HSP47 protein was noted with 24 h of tunicamycin exposure.

A23187 is a calcium ionophore and known ER stressor (Elia *et al*, 1996). A6 cells were treated with 0.7 □M A23187 for 4, 6 or 24 h or exposed to 0.07% ethanol for 6 h (solvent control). For a positive control, A6 cells were heat shocked for 1 h at 33^{o}C. As shown in figure 27, enhanced hsp47 mRNA accumulation was observed only in cells heat shocked at 33^{o}C. In fact, a decrease in the relative level of hsp47 mRNA occurred in response to a 24 h exposure to 0.7 μM A23187. Hsp70 mRNA accumulation was not induced in response to 0.7 μM A23187. However, BiP mRNA accumulated at 6 h treatment with 0.7 μM A23187 to similar levels observed with exposure to heat shock at 33^{o}C for 1 h. L8 mRNA levels were unaltered by A23187 treatment. In contrast to hsp47 mRNA expression patterns, HSP47 protein appeared to accumulate slightly in response to long-term exposure to 0.7 μM A23187 (Figure 28).

The finding that hsp47 mRNA appeared to decrease in response to long-term exposure to 0.7 μM A23187 (Figure 27) and published results showing that hsp70 mRNA expression was inhibited by exposure to A2387 in human cells (Ella *et al*, 1996) led to an experiment designed to observe whether hsp47 induction was affected by long-term exposure to 0.7 μM A23187. To examine relative mRNA levels, A6 cells were heat shocked at 35^{o}C following long-term exposure (24 or 36 h) to 0.7 μM A23187 in ethanol.

Figure 27. The effect of 0.7 μM A23187 on hsp47, hsp70 and BiP mRNA accumulation in A6 cells. Cells were treated with 0.7 μM A23187 for 4, 6 or 24 h at 22°C. Solvent control cells were exposed to 0.07% ethanol for 6 h. For a positive control, A6 cells were heat shocked for 1 h at 33°C. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labeled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

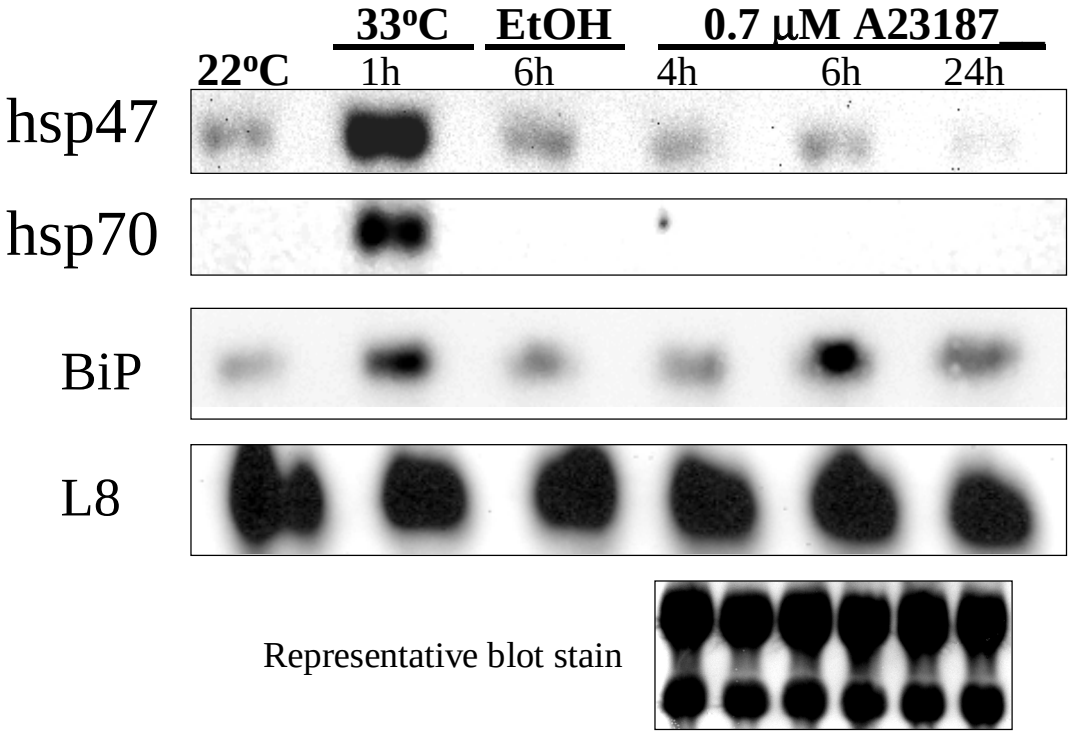

33ºC
EtOH
0.7 µM A23187
22ºC
1h
6h
4h
6h
24h
hsp47
hsp70
BiP
L8
Representative blot stain

Figure 28. The effect of 0.7 μM A23187 on HSP47 protein accumulation in A6 cells. Cells were treated with 0.7 μM A23187 for 4, 6 or 24 h at 22°C. Solvent control cells were exposed to 0.07% ethanol for 6 h. For a positive control, A6 cells were heat shocked for 2 h at 33°C and allowed to recover at 22°C for 2 h. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

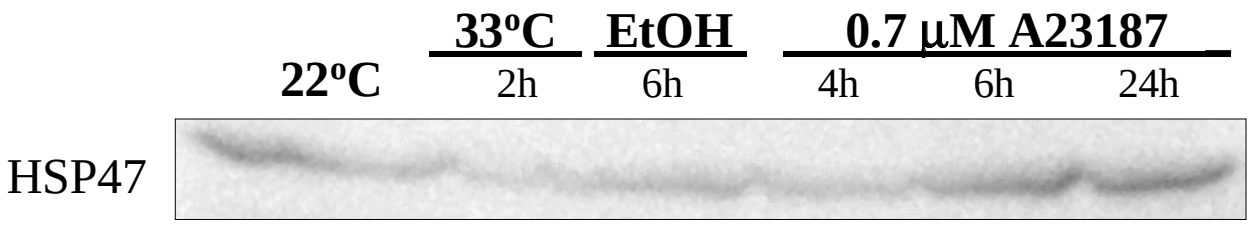

33ºC
EtOH
0.7 µM A23187
22ºC
2h
6h
4h
6h
24h
HSP47

Figure 29. The effect of long term 0.7 μM A23187 exposure and heat shock on hsp47, hsp70 and BiP mRNA accumulation in A6 cells. Cells were treated with 0.7 μM A23187 in ethanol for 24 or 36 h and then heat shocked or collected. Heat shocked cells (control and treated) were exposed to 35^{o}C for 1 h (Control A6 cells were maintained at 22^{o}C). Solvent control cells were exposed to 0.07% ethanol for 36 h and then heat shocked or collected. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labeled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

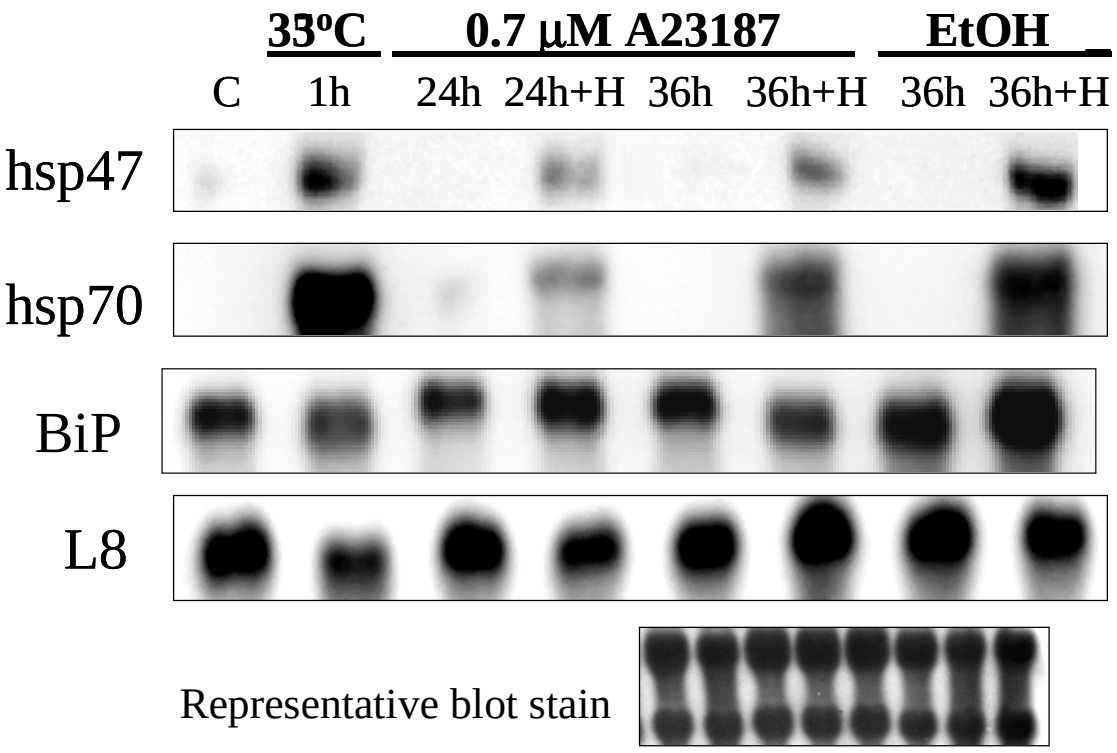
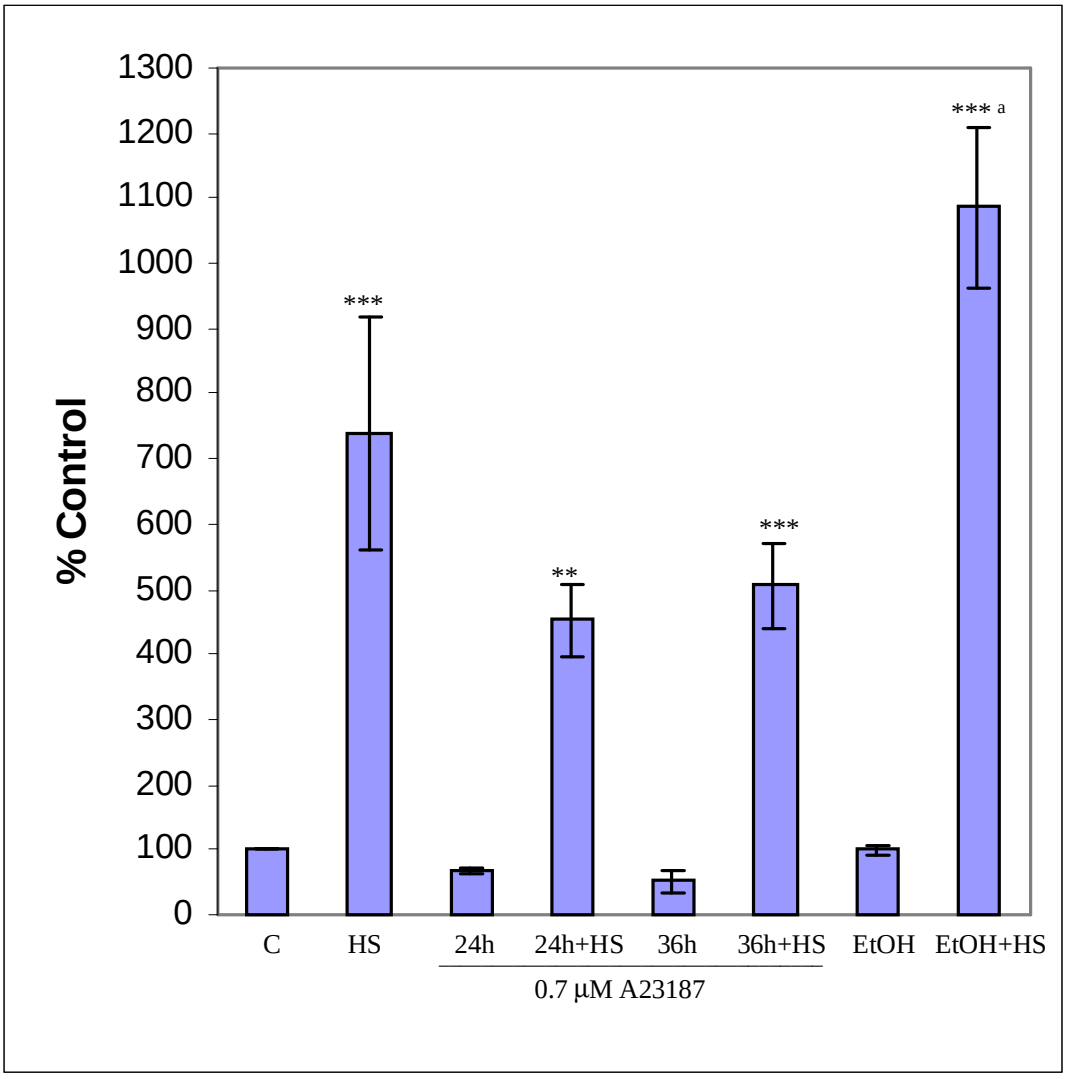

33ºC
0.7 μM A23187
EtOH
C
1h
24h
24h+H
36h
36h+H
36h
36h+H
hsp47
hsp70
BiP
L8
Representative blot stain

Figure 30. Densitometric analysis of the long-term effect of 0.7 μM A23187 exposure and heat shock on hsp47 mRNA accumulation in A6 cells. The relative densities of hsp47 mRNA bands on Northern blot images were measured using NIH Image 1.62 software. The data was expressed as a percentage of the control hybridization obtained with each blot/probe combination. Graphed data were expressed as mean +/- standard error. **, $P < 0.01$; ***, $P < 0.001$, significantly different from corresponding untreated cells. a, $P < 0.001$, significantly different from corresponding heat shocked cells.


1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
% Control
***
**
***
*** a
C
HS
24h
24h+HS
36h
36h+HS
EtOH
EtOH+HS
0.7 µM A23187

Solvent control cells were exposed to 0.07% ethanol for 36 h. Figures 29 and 30 show that 24 and 36 h exposures to 0.7 μM A23187 reduced the enhancement of hsp47 mRNA accumulation in response to a 1 h heat shock at 35°C by 2.9 and 2.3 fold, respectively compared to heat shocked cells. The ethanol control samples showed the same induction pattern as control samples. The accumulation pattern observed for hsp70 mRNA was very similar to that observed with hsp47; hsp70 transcript induction was reduced in 0.7 μM A23187 exposed samples and ethanol controls mimicked the results observed for control samples. Both BiP and L8 transcript expression appeared unaltered by treatment with 0.7 μM A23187. Western blot analysis showed that the upper HSP47 protein band accumulated in response to a 35°C heat shock with and without long-term exposure to 0.7 μM A23187 (Figures 31 and 32). Optimal HSP47 protein accumulation was observed in response to a 48 h 0.7 μM A23187 treatment followed by 2 h heat shock at 35°C. Desitometric analysis revealed a 2.8 fold increase in both the upper and lower protein bands (in comparison to the control lower protein band) with this treatment (Figure 32). Elevated accumulation of the lower HSP47 protein band was observed in response to 24 h exposure to 0.7 μM A23187 (Figure 31).

3.2.5 The effect of 1 mM βAPN on hsp47 mRNA and protein accumulation in A6 cells.

β-amionpropionitrile (βAPN) is a procollagen-specific stressor that inhibits lysyl oxidase (Rocha *et al*, 1986). By hindering the function of this enzyme, the lysyl hydroxylation of procollagen α-chains is blocked, resulting in the inhibition of mature collagen triple helix formation. Figure 33 shows that treatment of A6 cells with βAPN resulted in hsp47 mRNA accumulation with short-term exposure to 1 mM βAPN. Hsp70, BiP and L8 transcript expression was unaltered by 1 mM βAPN. Western blot analysis

Figure 31. The effect of long term 0.7 μM A23187 exposure and heat shock on HSP47 protein accumulation in A6 cells. Control A6 cells were maintained at 22°C. Cells were treated with 0.7 μM A23187 in ethanol for 24 or 48 h and then heat shocked or collected. Heat shocked cells (control and treated) were exposed to 35°C for 2 h and then allowed to recover at 22°C for 2 h (Control A6 cells were maintained at 22°C). Solvent control cells were exposed to 0.07% ethanol for 48 h and then heat shocked or collected. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).

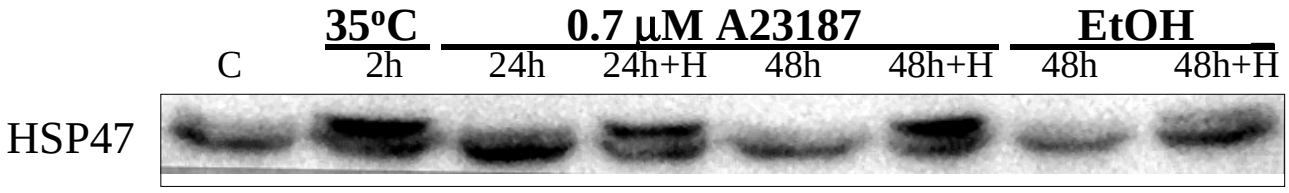
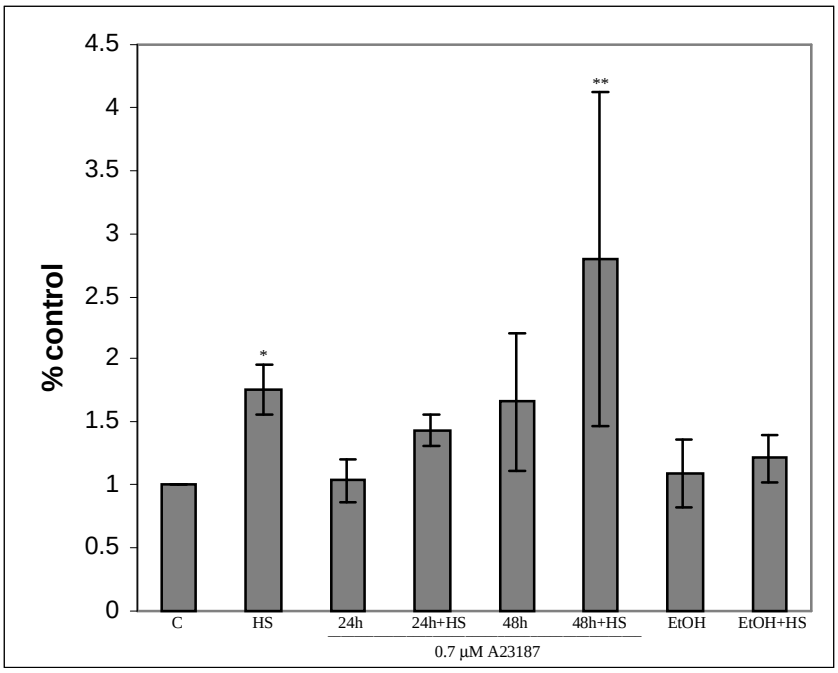

35ºC
0.7 μM A23187
EtOH
C
2h
24h
24h+H
48h
48h+H
48h
48h+H
HSP47

Figure 32. Densitometric analysis of the long-term effect of 0.7 μM A23187 and heat shock on HSP47 protein accumulation in A6 cells. The relative densities of HSP47 protein bands on Western blot images were measured using NIH Image 1.62 software. A) The lower protein band data was expressed as a percentage of the control lower band hybridization obtained with each blot/antibody combination. B) The upper protein band data was expressed as a percentage of the control upper band. Graphed data were expressed as mean +/- standard error. *, $P < 0.05$; **, $P < 0.01$, significantly different from corresponding untreated cells.

A


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% control
*
**
C
HS
24h
24h+HS
48h
48h+HS
EtOH
EtOH+HS
0.7 µM A23187

B

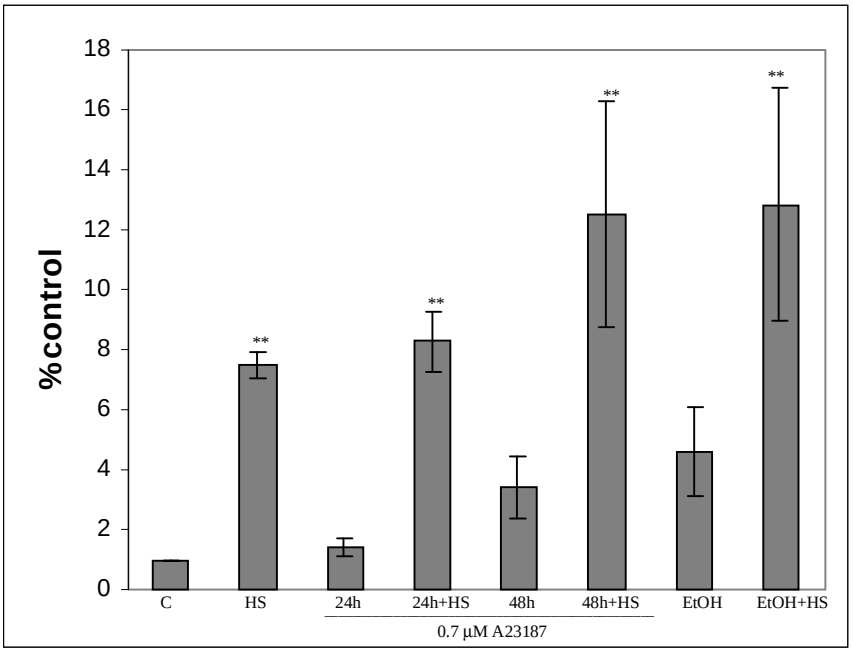

18
16
14
12
10
8
6
4
2
0
% control
**
**
**
**
C
HS
24h
24h+HS
48h
48h+HS
EtOH
EtOH+HS
0.7 µM A23187

Figure 33. The effect of 1mM βAPN on hsp47, hsp70 and BiP mRNA accumulation. A6 cells were treated with 1 mM βAPN for 1, 2, 4, 6 or 24 h. Experimental and control cells were maintained at 22°C. Cells were harvested and total RNA isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labeled antisense riboprobes to examine relative levels of hsp47 (using 15 μg total RNA), hsp70 (10 μg total RNA), BiP (15 μg total RNA) and L8 (10 μg total RNA) mRNA. The bottom panel is a representative blot stain.

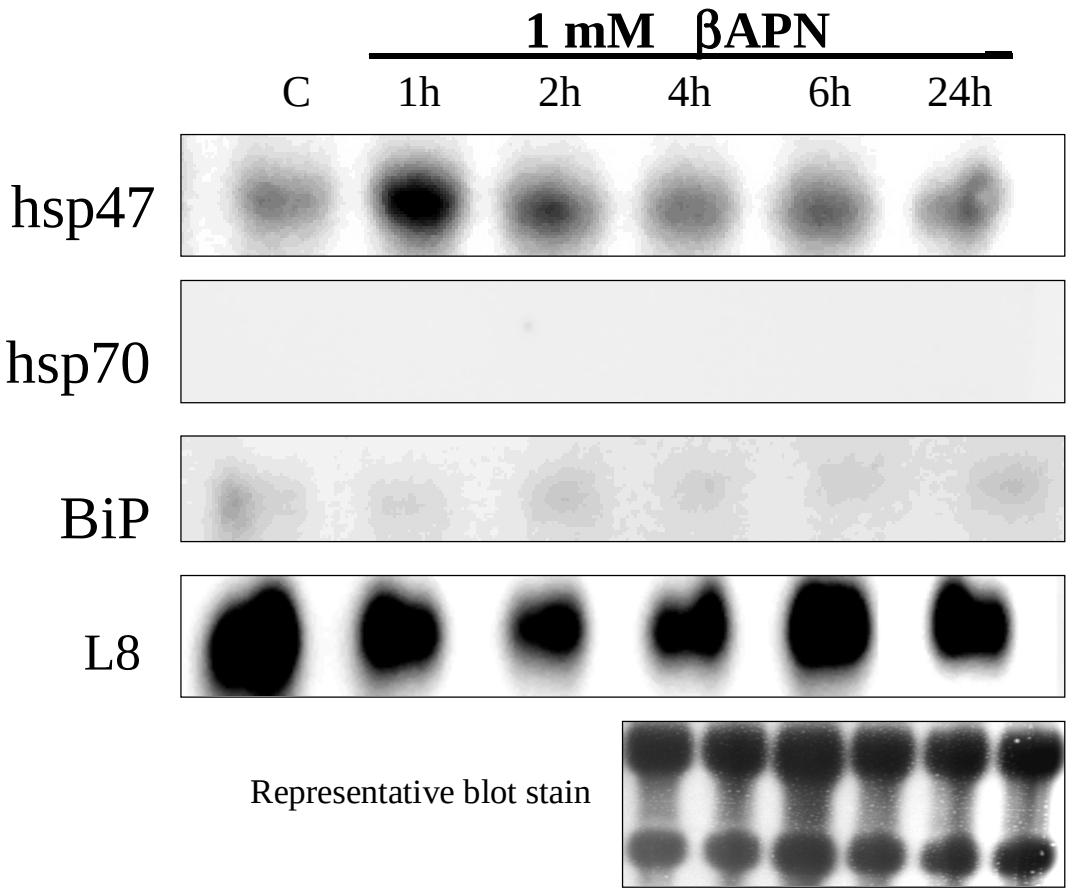
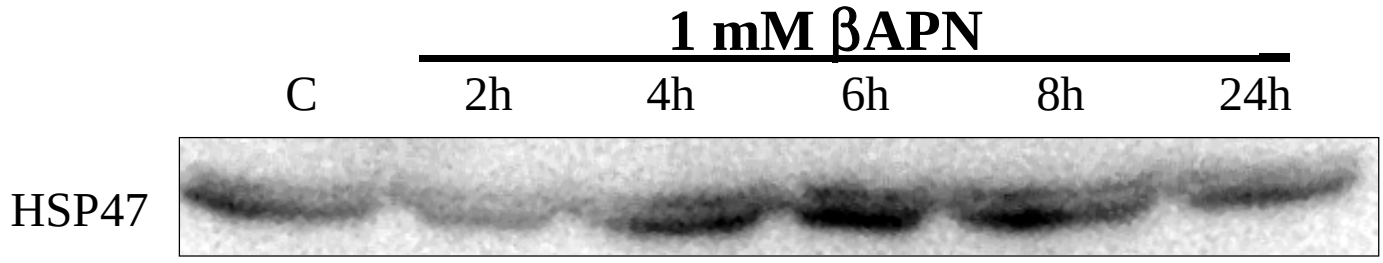

1 mM βAPN
C
1h
2h
4h
6h
24h
hsp47
hsp70
BiP
L8
Representative blot stain

Figure 34. HSP47 protein accumulation in response to 1 mM βAPN in A6 cells. A6 cells were exposed to 1 mM βAPN for 2, 4, 6, 8 or 24 h at 22^{o}C. Control cells were maintained at 22^{o}C. Cells were harvested and total protein isolated as detailed in Experimental Procedures. Western blot analysis was performed with monoclonal mouse anti-rat HSP47 antibody to examine the relative levels of HSP47 (using 40 μg total protein).


1 mM βAPN
C
2h
4h
6h
8h
24h
HSP47

revealed HSP47 protein lower band accumulated slightly at times ranging from 4 to 8 h, with optimal induction after a 6 h exposure (Figure 34). The levels of the HSP47 upper protein band remained relatively constant with exposure to βAPN.

3.3 Characterization of hsp47 mRNA accumulation during *Xenopus laevis* embryo development

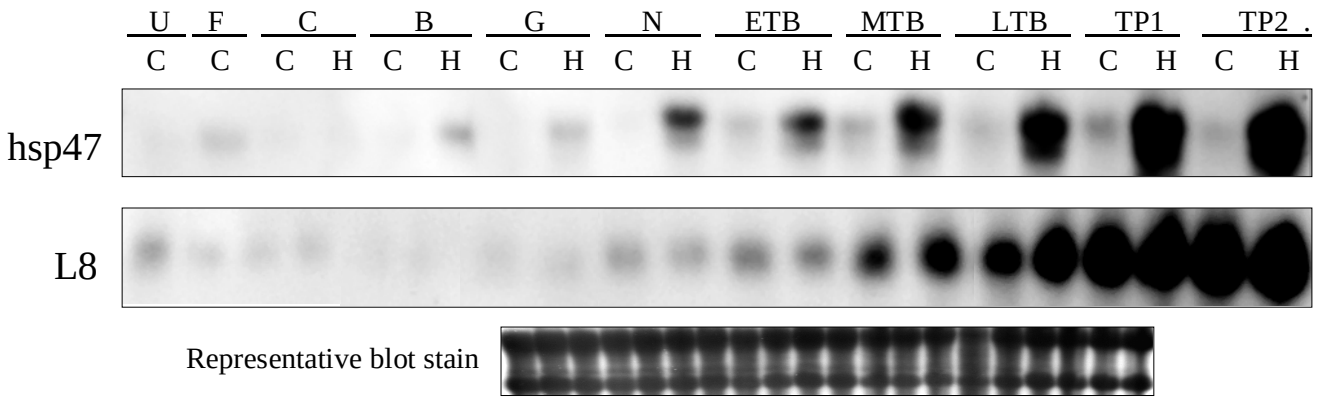
3.3.1 Constitutive expression of hsp47 mRNA during *Xenopus* development

Northern blot analysis was utilized to examine the relative levels of hsp47 mRNA during *Xenopus* development. Constitutively expressed hsp47 mRNA was detectable at all investigated stages of early development from unfertilized egg to 5-day-old tadpole (Figure 35). Relative hsp47 mRNA levels rose slightly during tailbud stages and peaked at early tadpole. Interestingly, 5-day-old tadpoles (stage 46) were found to have lower constitutive levels of hsp47 mRNA than 4-day-old tadpoles (stage 43). The *Xenopus* large ribosomal subunit protein 8 (L8) was used as a control in this experiment. L8 mRNA was found present at all investigated stages and its relative mRNA level increased throughout the course of development.

3.3.2 Effect of heat shock on hsp47 mRNA accumulation in *Xenopus* embryos

To examine the heat inducibility of hsp47 mRNA through early *Xenopus* development, embryos were heat shocked for 1 h at 33^{o}C at selected stages. Northern blot analysis revealed that hsp47 mRNA was heat inducible immediately following MBT and at every investigated stage thereafter (Figure 35). Accumulation of hsp47 transcript in response to heat rose steadily during the course of development. No heat inducible accumulation was observed for control L8 mRNA.

Figure 35. Heat shock-induced hsp47 mRNA accumulation during early *Xenopus laevis* development. *X. laevis* embryos were maintained in 0.1 XMBS at 22^{o}C. At select stages, control (C) and heat shocked (H; 1 h at 33^{o}C) embryos were collected and total RNA was isolated as outlined in Experimental Procedures. Northern blot analysis was performed with DIG-labeled antisense riboprobes to examine relative levels of hsp47 in *Xenopus* unfertilized eggs (U), fertilized eggs (F) and embryos at cleavage (C; stage 7), blastula (B; stage 9), gastrula (G; stage 12), neurula (N; stage 17), early tailbud (ETB; stage 24), mid-tailbud (MTB; stage 32), late tailbud (LTB; stage 39) and two early tadpole (TP1 and TP2; stages 43 and 46, respectively) stages. As a control the relative levels of L8 mRNA was also examined by Northern analysis at the same stages.


U F C B G N ETB MTB LTB TP1 TP2 .
C C C H C H C H C H C H C H C H C H C H
hsp47
L8
Representative blot stain

3.3.3 Spatial pattern of hsp47 accumulation during *Xenopus* development

The spatial distribution of hsp47 gene expression during *Xenopus* early development was investigated using whole mount *in situ* hybridization with DIG-labelled antisense hsp47 probe (Figure 36, 37 and 38). Blue staining indicated the presence of hsp47 message. As shown in Figure 36, hsp47 mRNA was detectable in embryos prior to MBT. After the midblastula transition, hsp47 mRNA levels were enhanced in response to heat shock (Figure 36, 37 and 38; 1 h at 33^{o}C). At blastula (Figure 36; stage 9) and gastrula (Figure 37; stage 11), hsp47 mRNA was found throughout control and heat shocked embryos. The spatial distribution of hsp47 mRNA was more specifically localized at neurula and all investigated stages thereafter. At neurula (Figure 37; stage 16), hsp47 mRNA was found along the anterior of the embryo, specifically along the neural folds. In control early and mid tailbud stage embryos (Figure 38; stages 23 and 31, respectively), hsp47 transcript was found along the dorsal region of the embryo, in the notochord and somites, as well as in the head region, specifically in the eye vesicle. In heat shocked embryos at the same stages (Figure 38; stages 23 and 31, respectively), the expression pattern of hsp47 was also along the dorsal plane of the embryo but at a much greater abundance particularly in the somites, notochord and in the head region excluding the cement gland. Hsp47 expression in late tailbud control embryos (Figure 38; stage 39) was reduced in the dorsal region of the embryo but remained prominent in the head region. Hsp47 mRNA continued to be heat inducible along the dorsal plane and head region of the embryo at late tailbud (Figure 38; stage 39).

Figure 36. The spatial pattern of hsp47 mRNA accumulation in cleavage and blastula stages of early *Xenopus laevis* development. *X. laevis* embryos were maintained in 0.1 X MBS at 22^{o}C. At select stages, control and heat shocked (1 h at 33^{o}C) embryos were collected and fixed as described in Experimental Procedures. Whole mount *in situ* hybridization with DIG-labeled hsp47 antisense riboprobes was performed with *Xenopus* embryos at cleavage (stage 7) and blastula (stage 9) stages.

Control **Heat Shock**

Cleavage

Blastula

Figure 37. The spatial pattern of hsp47 mRNA accumulation in gastrula and neurula stages of early *Xenopus laevis* development. *X. laevis* embryos were maintained in 0.1 X MBS at 22^{o}C. At select stages, control and heat shocked (1 h at 33^{o}C) embryos were collected and fixed as described in Experimental Procedures. Whole mount *in situ* hybridization with DIG-labeled hsp47 antisense riboprobes was performed with *Xenopus* embryos at gastrula (stage 11) and neurula (stage 16) stages.

Control **Heat Shock**

Gastrula

Neurula

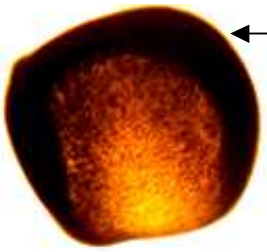
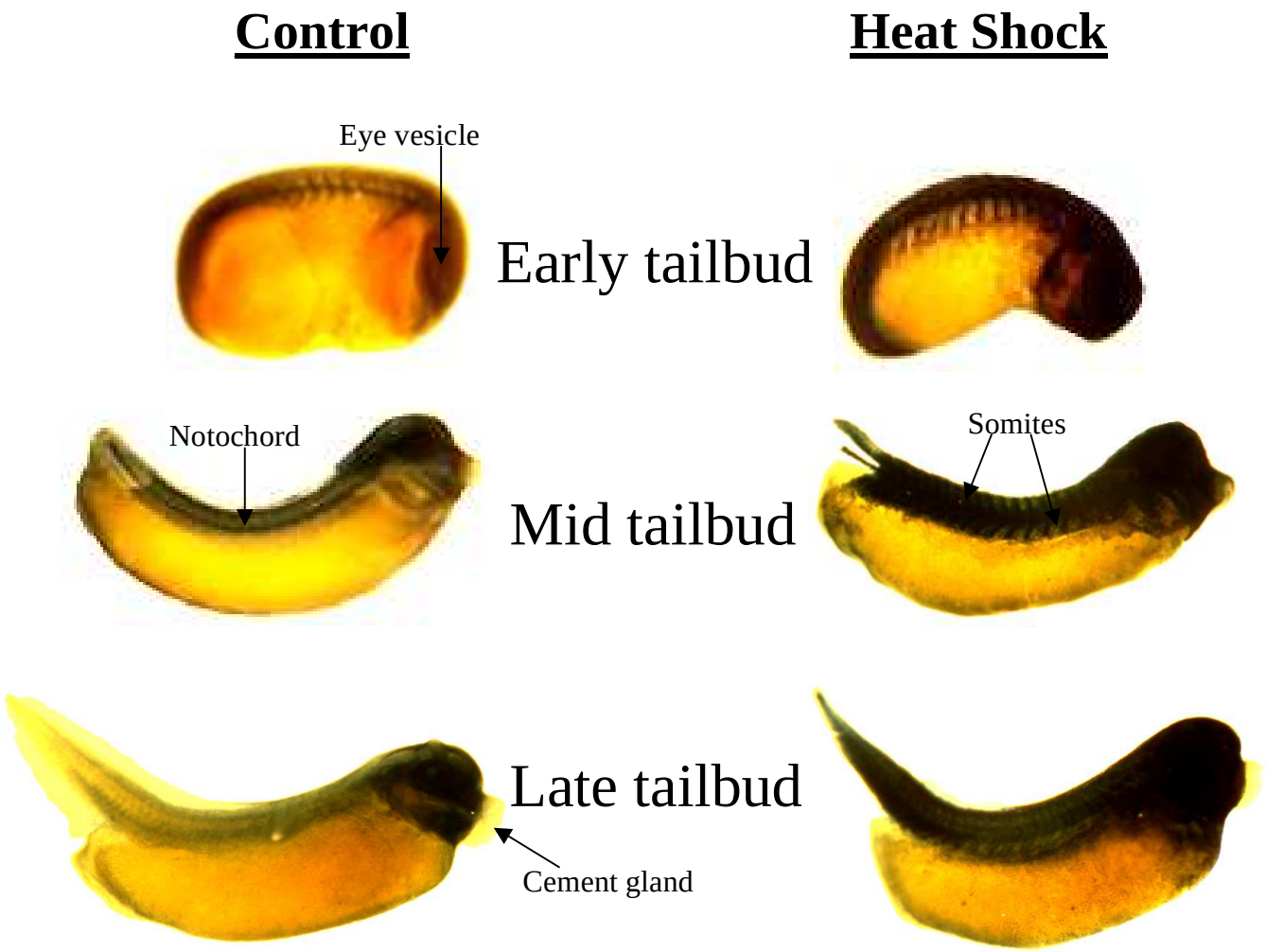


Figure 38. The spatial pattern of hsp47 mRNA accumulation in tailbud stages of early *Xenopus laevis* development. *X. laevis* embryos were maintained in 0.1 X MBS at 22^{o}C. At select stages, control and heat shocked (1 h at 33^{o}C) embryos were collected and fixed as described in Experimental Procedures. Whole mount *in situ* hybridization with DIG-labeled hsp47 antisense riboprobes was performed with *Xenopus* embryos at early (stage 23), mid (stage 31) and late (stage 39) tailbud stages.


Control
Heat Shock
Eye vesicle
Early tailbud
Notochord
Somites
Mid tailbud
Late tailbud
Cement gland

4. Discussion

This study represents the first examination of hsp47 mRNA and protein accumulation in an amphibian species, namely, *Xenopus laevis*. Previous studies investigated the expression of the hsp47 gene in chicken, mouse, rat, human and zebrafish (Pearson *et al*, 1996; Clarke and Sanwal, 1992; Clarke *et al*, 1991; Hirayoshi *et al*, 1991; Wang and Gudas, 1990). Sequence comparison of the hsp47 cDNA clone with the full-length hsp47 cDNA published in Genbank (Klein *et al*, 2002) revealed that the clone obtained from ATCC was not complete. The hsp47 cDNA clone consisted of the 3'UTR of *Xenopus* hsp47 as well as 461 bp of the protein coding sequence and was sufficient for the generation of hsp47 antisense riboprobes for northern blot and whole mount *in situ* hybridization analysis. The Genbank hsp47 nucleotide sequence consists of a small portion of the 5'UTR, the entire protein coding sequence and a large segment of 3'UTR. The protein coding sequence of the *Xenopus* cDNA encodes a 47,326 Da protein that has a predicted isoelectric point of 8.4. HSP47 proteins investigated in other organisms also possess a basic pI of approximately 9 (Nagata, 1996).

The coding region of *Xenopus* HSP47 shared an identity of approximately 77% with chicken, 73% with mouse, 72% with rat and human, and 70% with zebrafish. Most of the sequence identity between HSP47 from all investigated organisms occurs centrally in the amino acid sequence and in several carboxyl terminal regions. Despite great deviations in amino acid sequence at the amino terminus, two key components are conserved. The N-terminal amino acid sequence of each HSP47 protein is rich in hydrophobic amino acids, which is typical for leader sequences in ER-resident proteins (Alberts *et al*, 1994). In comparison with human HSP47, the signal peptide of *Xenopus* HSP47 may be cleaved after

the valine in position 19 (Satoh *et al*, 1996). Human HSP47 has two asparagine-associated glycosylation sites at its amino terminus that are fully glycosylated with high-mannose oligosaccharides in the mature form of the protein (Satoh *et al*, 1996). Investigation of potential glycosylation sites in *Xenopus* HSP47 revealed that it also possesses two possible asparagine residues in the same location as human HSP47. In fact all investigated HSP47 amino acid sequences possess these asparagine residues with the exception of zebrafish HSP47 that only possesses one of the residues (Pearson *et al*, 1996).

As mentioned earlier, the carboxyl portion of the HSP47 proteins is highly conserved. All investigated HSP47 proteins share the carboxyl terminal ER-retention sequence, RDEL. This signal is related to the well-established KDEL ER-retention signal and has been found to maintain HSP47 in the ER and the intermediate compartment between the ER and the Golgi apparatus (Satoh *et al*, 1996). In mammals, KDEL-tagged proteins bind the membrane receptor Erd2p found at a steady state in the Golgi apparatus and ER (Griffiths *et al*, 1994). Upon ligand binding, the receptor-ligand complex undergoes a conformational change that triggers the transport of the complex from the Golgi apparatus to the ER (Lewis and Pelham, 1992). The exact mechanism of ER retention by the RDEL signal has not been examined, but this variant of the KDEL signal was found to direct intracellular retention (Andreas *et al*, 1990).

Investigation of the 3'UTR of the hsp47 cDNA revealed the presence of three potential mRNA instability consensus elements (TATTTA). The mRNA instability element, UAUUUA, was shown to be involved in mRNA instability in interferon, c-fos and c-myc mRNAs (Vriz and Mechali, 1989; Brawerman, 1987; Shaw and Kamen, 1986).

This sequence was also found in *Xenopus* hsp30 and BiP mRNAs (Miskovic *et al*, 1997; Krone and Heikkila, 1988).

An examination of hsp47 mRNA levels in *Xenopus* A6 kidney epithelial cells revealed that this message was present constitutively and that its levels were altered by a variety of cellular, ER or procollagen specific treatments. The results of hsp47 mRNA accumulation studies in A6 cells strongly suggest that the expression pattern for *Xenopus* hsp47 resembles that of a cytosolic heat shock protein rather than an ER-resident hsp. With respect to heat shock, both hsp47 and hsp70 (a cytosolic/nuclear hsp) mRNAs exhibited enhanced accumulation at 33 and 35^{o}C whereas BiP mRNA (an ER-resident hsp) accumulated to elevated levels at a broader range of temperatures, 27 to 37^{o}C. The gene promoters of hsp47 and hsp70 have been studied in other organisms and each contained functional HSE in the upstream promoter region (Fiszer-Kierzkowska *et al*, 2003; Hosokawa *et al*, 1993). It is not known if a HSE is present in the promoter region of the *Xenopus* BiP gene since only a BiP cDNA is available (Miskovic *et al*, 1997). Thus, in *Xenopus* A6 cells differences in mRNA accumulation in response to temperature between each of the investigated transcripts may be due to the induction of hsp47 and hsp70 by the heat shock response as opposed to BiP that may be induced by the unfolded protein response (Haas, 1994). During time courses at 33 and 35^{o}C hsp47 and hsp70 mRNA accumulation displayed a very similar temporal pattern with optimal accumulation at 1 and 2 h, respectively. BiP expression was enhanced at 2 and 4 h. In experiments examining the combined effect of heat shock and sodium arsenite treatments, both hsp47 and hsp70 exhibited increased mRNA accumulation when exposed to elevated temperature and sodium arsenite treatments in concert than seen with each stressor independently. BiP

expression was unaffected by each stressor (30^{o}C and 10 μM NaAs), both independently and in combination.

Similar expression patterns between *Xenopus* hsp47 and hsp70 were also noted with ER-specific treatments. Tunicamycin acts to inhibit the N-glycosylation of newly synthesized proteins in the ER (Lee, 1987). *Xenopus* hsp47 expression was unaltered at the mRNA level after exposure to tunicamycin. Basal levels of hsp47 mRNA were expressed irrespective of the treatment. Tunicamycin did not induce hsp70 mRNA. BiP transcript levels were enhanced after 24 h exposure to tunicamycin compared to control. It has been suggested that hsp47 mRNA accumulates at times when there is a greater need for HSP47 protein (Nagata, 1998). The specific substrate for HSP47, namely procollagen, is not a glycoprotein and hence its assembly would be unaffected by the stress-inducing action of tunicamycin. Without a stress on procollagen triple helix formation, there was no increased need for HSP47 protein and therefore no hsp47 mRNA accumulation. BiP protein, however, does not have a specific substrate in the ER and has a role in the folding and assembly of a variety of newly synthesized proteins (Miskovic *et al*, 1997). Thus, BiP mRNA most likely accumulated due to an increased need for BiP protein in order to avoid aggregation of proteins affected by the tunicamycin treatment in the ER.

Hsp47 message levels were also not enhanced by A23187; in fact, long-term exposure to A23187 resulted in decreased hsp47 mRNA levels. Hsp70 transcripts were not constitutively expressed and were not induced by A23187 treatment. Elia and colleagues (1996) previously examined the effect of A23187 on hsp70 mRNA and protein synthesis in K562 human erythroleukemia cells. A23187 inhibited sodium arsenite-induced hsp70 mRNA accumulation. This inhibition was proposed to be partially due to the inhibitory

effects of A23187 on the phosphorylation and trimerization of HSF1 (Elia *et al*, 1996). It was postulated that inhibition of HSF1 trimerization hindered the transcriptional activation of hsp genes that possess HSEs including hsp70. Although the *hsp47* gene promoter was not examined in *Xenopus*, the mouse *hsp47* gene promoter has a complete HSE consensus sequence upstream of the protein-coding region (Hosokawa *et al*, 1993). To examine the effect of long-term exposure of A23187 on *Xenopus* hsp47 mRNA induction, A6 cells were treated with A23187 and then challenged with heat shock at 35^{o}C. Both hsp47 and hsp70 exhibited reduced mRNA accumulation in response to heat shock following 24 and 36 h exposures to A23187. Therefore, heat shock induced *Xenopus* hsp47 and hsp70 gene expression was inhibited by A23187 as found in human erythroleukemia cells. It is possible that A23187 might have had an effect on *Xenopus* HSF phosphorylation and trimerization. BiP mRNA accumulation was unaltered by long-term A23187 exposure under the experimental conditions used in the present study.

In some investigated treatments hsp47 mRNA exhibited a unique expression pattern compared to both of the other hsps examined. Exposure of A6 cells to sodium arsenite led to optimal accumulation of hsp47 message at 6 h which was much sooner than accumulation patterns observed with hsp70 and BiP transcripts that were both optimally expressed after 24 h. Short-term treatments with βAPN (a procollagen-specific stressor) resulted in slight accumulation of hsp47 mRNA. Neither hsp70 nor BiP message levels were affected by this treatment. This was expected since βAPN specifically blocks the proper assembly of procollagen α-chains by inhibiting the function of lysyl oxidase (Rocha *et al*, 1986). Lysyl oxidase is an amine oxidase that initiates the biosynthesis of lysine-derived cross-links in collagen and elastin (Tang *et al*, 1983). βAPN is an active site-

directed inhibitor that irreversibly inactivates the lysyl oxidase by competing with the enzyme substrate and covalently binding the enzyme. HSP47 has been established as having a role in the formation of procollagen triple helices whereas neither HSP70 nor BiP has been implicated in the assembly of most types of collagen. The induction of hsp47 mRNA by βAPN is likely to occur independent of the heat shock response since hsp70 mRNA accumulation was unaffected. Investigation of murine *hsp47* revealed *cis*-elements for several transcription factors including Sp-1, Sp-2, Sp-3 and KLF-6 in the promoter region and first two introns of the gene (Yasuda *et al*, 2002). The Sp-1 family of transcription factors were necessary for the basal expression of hsp47 in BALB/c 3T3 cells. KLF-6 was found to transactivate *hsp47* gene expression by interacting with *cis*-elements in the intron region. It is likely that the induction of hsp47 mRNA by βAPN is though the action of a transcription factor such as KLF-6 rather than the heat shock response.

In the present study, HSP47 protein levels in A6 cells were examined in response to cellular, ER and procollagen-specific stressors as previously used to examine hsp47 transcript levels. Elevated temperature, sodium arsenite (NaAs) and β-aminopropionitrile fumerate (βAPN) treatments induced the accumulation of HSP47 protein. Treatments with these stressors resulted in HSP47 protein accumulation at later time points than observed with mRNA accumulation. For example hsp47 mRNA optimally accumulated after a 6 h exposure to sodium arsenite whereas HSP47 protein optimally accumulated after a 12 h treatment. Previous studies have also noted delays in HSP protein production in comparison to hsp mRNA accumulation. Manzerra and colleagues (1993) noted a delay in HSP70 protein accumulation in hyperthermia-treated rabbit cerebral tissues. They suggested that constitutively expressed HSC70 functions to prevent protein aggregation at

basal levels and that HSP70 is only induced in response to prolonged stress. Thus, the delayed accumulation of HSP47 protein in comparison to hsp47 mRNA accumulation was likely due to the ability of constitutive levels of HSP47 to prevent the improper aggregation of procollagen for limited periods of stress. HSP47 protein accumulation likely occurred in response to stress beyond the capability of constitutive HSP47 levels.

In A6 cell treatments with ER stressors different results were observed with respect to hsp47 mRNA and protein accumulation. Hsp47 mRNA accumulation was unaltered by exposure to tunicamycin, however HSP47 protein accumulated after 4 to 8 h of treatment. Tunicamycin acts to inhibit the N-glycosylation of newly synthesized proteins in the ER (Lee, 1987). As an ER-resident glycoprotein, HSP47 is likely affected by the inhibitory effects of tunicamycin (Kurkinen *et al*, 1984). Hughes and colleagues (1987) examined the patterns of murine HSP47 glycosylation in mouse parietal endoderm cells. Murine HSP47 was shown to possess two asparagine-linked oligosaccharides that were sensitive to tunicamycin. As discussed earlier, amino acid sequence comparison performed in this study, indicated that *Xenopus* HSP47 possessed two asparagine residues in the same location as those previously found in murine and human HSP47. Thus, *Xenopus* HSP47 may also be glycosylated at these sites and glycosylation of the protein may be inhibited by tunicamycin. Changes in glycosylation do not directly explain the accumulation of HSP47 protein that was observed with western blot analysis. Since no hsp47 mRNA accumulation was detected with tunicamycin treatment, HSP47 protein accumulation could be due to increased translation or decreased protein degradation.

Different trends were also observed in the hsp47 mRNA and protein accumulation results observed with A23187 treatments in A6 cells. Hsp47 mRNA levels decreased in

response to a 24 h exposure to A23187. However, HSP47 protein accumulated slightly with the same treatment. A similar trend was observed in treatments of long-term A23187 exposure coupled with heat shock. Exposures with A23187 reduced the enhancement of hsp47 mRNA accumulation in response to heat shock, whereas HSP47 protein levels elevated in response to a 24 h exposure to A23187 and also accumulated in response to heat shock. As previously stated, hsp47 mRNA may decrease with long-term A23187 exposure due to inhibition of HSF1 phosphorylation and trimerization. The accumulation of HSP47 protein may be explained by another effect of the A23187 treatment. A23187 is a calcium ionophore that is known to permeabilize the plasma membrane and induce the leakage of Ca^{2+}, but has also been shown to cause the discharge of intracellular ER Ca^{2+} stores (Li *et al*, 1993). This ER Ca^{2+} discharge was found to cause the induction of GRP genes including BiP. The discharge of Ca^{2+} from the ER may cause sufficient metabolic stress to warrant the accumulation of HSP47 protein. Since a reduction in hsp47 mRNA levels were found in response to A23187 treatments, HSP47 accumulation may be due to enhanced translation or reduced degradation of the protein.

Another key feature observed during western blot analysis was the occurrence of more than one HSP47 protein band. As previously stated, the amino terminal of the *Xenopus* HSP47 protein possessed two asparagines that may be subject to glycosylation as found previously in human and murine HSP47 (Satoh *et al*, 1996; Hughes *et al*, 1987). Studies investigating the glycosylation of human HSP47 revealed that the asparagines were fully glycosylated with high-mannose oligosaccharides in the mature form of the protein (Satoh *et al*, 1996). The inhibition of mannose-labelling of murine HSP47 resulted in alterations in the size of the protein and reduced its M_r by approximately 4000 (Kurkinen *et*

al, 1984). Unglycosylated murine HSP47 was still able to bind native type IV collagen as well as gelatin, thereby suggesting that the high-mannose oligosaccharides were not required for proper HSP47 function (Hughes *et al*, 1987). Therefore, the two HSP47 protein bands observed in this study may reflect HSP47 with different levels of glycosylation. This possibility could be examined by incubating HSP47 with peptide-N-Glycosidase. This enzyme is an asparagine amidase that releases intact N-linked oligosaccharides from glycoproteins or glycopeptides (Twining, 1984). It should be noted that only one protein band was observed with tunicamycin treatments and that tunicamycin has already been found to inhibit the glycosylation of murine HSP47 (Hughes *et al*, 1987). Another potential source of multiple protein bands is changes in protein size due to post-translational phosphorylation. This possibility is unlikely since significant protein phosphorylation would be necessary to account for the difference in size between protein bands. A number of potential phosphorylation sites have also been identified in murine HSP47 and phosphorylated forms of the protein have been identified in some cell types (Nakai *et al*, 1990; Nagata and Yamada, 1986). The phosphorylation of HSP47 has not been specifically studied in any organism to date but does not appear to be necessary for HSP47 protein function.

Hsp47 gene expression has been examined in mouse, chicken and zebrafish embryos, but until this study *hsp47* expression during development had not been investigated in an amphibian organism. This study examined hsp47 mRNA accumulation and its spatial pattern of distribution in developing *Xenopus laevis* embryos. Northern blot analysis revealed that hsp47 mRNA was present from unfertilized egg to tadpole. Whole mount *in situ* hybridization studies determined that hsp47 mRNA was present throughout

the embryo in cleavage, blastula and gastrula stage embryos. The finding of constitutive levels of hsp47 mRNA in unfertilized eggs, fertilized eggs and cleavage stage embryos indicated that these transcripts were maternally inherited since the zygotic genome is not transcriptionally active until after the midblastula stage of development (Gilbert, 1994). This finding was expected given the establishment of a role for HSP47 protein in collagen production in other organisms and the fact that collagens are key structural elements in most tissues and extracellular matrices (Nagata, 1998; Lodish *et al*, 1995). The presence of hsp47 mRNA in pre-MBT embryos probably reflects the need of early embryos to produce HSP47 protein to interact with newly synthesized procollagen α chains in the ER.

Northern blot analysis results revealed that constitutive hsp47 mRNA expression was enhanced further in neurula and tailbud embryos compared to earlier stages. At the neurula stage, hsp47 mRNA accumulation was preferentially enriched along the neural folds. This pattern was maintained in early and mid tailbud stage embryos where hsp47 mRNA was distributed primarily along the dorsal part of the embryo in the somites, spinal cord and head region including the optic vesicle. The increase in hsp47 mRNA in neurula and tailbud stage embryos and the preferential localization of hsp47 message may be explained by the shift towards organogenesis during these stages. The formation of the notocord and somites begins during neurula stages (Nieuwkoop and Faber, 1967). These preliminary structures will give rise to cells that form the spinal cord, vertebrae, ribs, dermis of the dorsal skin and skeletal muscles of the back and limbs. Collagen is a primary constituent of bone, cartilage and epidermal tissue. Nascent collagen is formed from procollagen triple helices that must first be assembled in the ER and transiently interact with HSP47 for proper helix trimerization (Nagata and Hosokawa, 1996). Thus, the

increased synthesis of hsp47 mRNA during neurula and tailbud stages most likely reflects increased production of HSP47 protein needed to mediate the formation of mature collagen in several tissue types during organogenesis. This probability is supported by investigations of hsp47 expression using knockout mice. The *hsp47 -/-* knockout mice mesenchymal tissues were severely deficient in fibril structures and mature type I collagen (Nagai *et al*, 2000). The molecular form of type IV collagen was also affected. The net result of these abnormalities was incorrectly oriented epithelial tissues and disrupted blood vessels and basement membranes. By 9.5 days-post-coitus (dpc) deficient fibril formation and growth retardation was apparent and the disruption of *hsp47* gene expression was lethal by 11.5 dpc. It has therefore been established in mice that hsp47 is necessary for the proper development of collagen-containing tissues and structures. Hence, the expression of hsp47 during similar stages of development in *Xenopus* likely reflects a need for this molecular chaperone in the proper formation of several tissues during *Xenopus* early development. The tissue specific expression of HSP47 was studied in transgenic mice. An Sp-1 binding site in the *hsp47* promoter region, a BS5-B element in the first intron and an EP7-D element in the second intron were all necessary for basal expression (Yasuda *et al*, 2002). Kruppel-like factor (KLF) proteins KLF-3 and KLF-6, as well as Sp-1, Sp-2 and Sp-3 were all transcription factors implicated in the regulation of *hsp47*. KLF-6 and Sp-1 are also positive regulators of collagen transcription. Hence, researchers suggested that the coexpression of collagen and hsp47 was due to shared *cis*-elements and the actions of specific *trans*-factors in the regulatory regions of each respective gene. KLF-3 has been reported to have repressor activity and was suggested as a repressor of *hsp47* expression in hsp47-nonproducing cells (Yasuda *et al*, 2002). The tissue-specific expression of *Xenopus*

HSP47 is likely due to gene regulation by the aforementioned transcription factors although this probability cannot be examined until the upstream region of the *Xenopus hsp47* gene has been isolated and analyzed.

In the present study, hsp47 mRNA induction in *Xenopus* embryos was also examined in response to heat shock. Northern blot analysis determined that heat shock-induced hsp47 transcript accumulation began at late blastula stage, immediately following MBT. As stated earlier, the activation of the zygotic genome at this point in development likely occurred upon the establishment of a critical DNA to nuclear protein ratio (Newport and Kirschner, 1982). Initial high concentrations of histones present in maternal eggs are decreased during rapid cell division in the initial stages of development, resulting in changes in chromatin organization and subsequent activation of the zygotic genome. *In situ* hybridization analysis revealed that heat shock enhanced hsp47 mRNA levels were preferentially located along the neural folds of neurula stage heat shocked embryos. This pattern was preserved in early, mid and late tailbud heat shocked embryos with particularly enhanced accumulation in the head region, spinal cord and somites. Given these results it is possible that heat shock-induced *hsp47* gene expression serves to protect collagen synthesis from thermal stress in collagen-rich tissues of the embryo. This possibility is supported by investigation of thermal stability of procollagen and collagen in mammalian organisms. In mammals, the melting temperature of native collagen helices is close to body temperature and even slight increases in temperature can affect the integrity of collagen triple helices (Bruckner and Eikenberry, 1984). Thus, the ability of HSP47 to thermally stabilize collagen has been considered a very important function of this molecular chaperone in mammalian species. *Xenopus laevis* does not possess the ability to

control its body temperature and relies on its environment to determine its resting temperature. Hence, *Xenopus* must possess mechanisms to protect temperature sensitive proteins from thermally induced aggregation. Although the melting temperature of *Xenopus* collagen was not investigated in this study, it can be presumed that a protective function of HSP47 during thermal stress would be important to the integrity of collagen in this poikilothermic organism.

This study served as an initial characterization of *Xenopus* hsp47. There are many avenues of research that could now be taken to further our understanding of the expression pattern and function of HSP47 in *Xenopus laevis*. The gene promoter of *Xenopus hsp47* has not been sequenced or analyzed. RACE PCR could be used to isolate the entire *hsp47* protein coding sequence as well as the 5'upstream region. The 5' region could then be examined for the possible presence of a HSE and other transcription factor binding sites. Also once the entire *hsp47* protein coding sequence is isolated it could be cloned into a pRSET vector and recombinant *Xenopus* HSP47 protein could then be generated. It has been established in other organisms that post-translational modifications were not necessary for the proper functioning of HSP47 protein (Jain *et al*, 1994; Natsume *et al*, 1994). Thus, recombinant *Xenopus* HSP47 could be used in *in vitro* binding studies with synthetic procollagen α chains and other potential substrates to determine whether it has the same binding specificity as HSP47 investigated in other organisms. The anti-HSP47 antibody used in this study was weak and had to be used at a very high concentration. Recombinant *Xenopus* HSP47 protein could also be used to induce the production of anti-*Xenopus* HSP47 antibodies. These specific antibodies could be used for further western blot analysis, immunoprecipitation or immunocytochemical studies in A6 cells or *Xenopus*

embryos. The anti-*Xenopus* HSP47 antibodies could be used in immunoprecipitation studies to investigate the proteins that associate with HSP47 *in vivo*. Immunocytochemical investigation in A6 cells could verify the localization of *Xenopus* HSP47 in the ER. Anti-*Xenopus* HSP47 antibodies could be used in *Xenopus* embryos to identify the localization of this protein throughout early development. *Hsp47* knock-out studies in mice established that the disruption of the *hsp47* gene was lethal before 11.5 dpc (Nagai *et al*, 2000). Morpholino oligonucleotides against hsp47 could be used in *Xenopus* embryos to block the translation of hsp47, thereby decreasing the concentration of functional HSP47 protein. The effects of disrupted HSP47 production could then be observed and help establish the importance of this protein in proper *Xenopus* development.

In conclusion, the findings of this study have suggested that the pattern of stress-induced hsp47 gene expression is more similar to that of hsp70 than BiP. The gene promoters of hsp47 and hsp70 have been examined in other organisms and a functional HSE was identified in the upstream promoter regions of each of these heat shock proteins (Fiszer-Kierzkowska *et al*, 2003; Hosokawa *et al*, 1993). Therefore, similarities in mRNA and protein accumulation between *Xenopus* hsp47 and hsp70 are likely due to the possession of HSEs in their promoter regions and the ability of these proteins to be induced by the heat shock response. Conversely, a HSE has not been identified in the promoter region of *Xenopus* BiP to date (Miskovic *et al*, 1997). It has been suggested that BiP gene expression is activated by the unfolded protein response in which increases in unfolded or denatured protein in the ER enhances the relative levels of BiP mRNA and protein (Haas, 1994). Thus the expression patterns of hsp47 and BiP likely differ because each of these hsps is induced by a different stress response pathway. Although the patterns of hsp47

mRNA and protein accumulation were similar to hsp70 in some cases, the expression pattern of hsp47 is clearly unique in comparison to other hsps characterized in our laboratory. HSP47 was the first heat shock protein identified with a defined protein binding activity (Nagata *et al*, 1986). Unlike other investigated HSPs, HSP47 is the only identified HSP that possesses a specific substrate. HSP47 is also one of a small subset of HSPs found to localize in the ER (Morimoto *et al*, 1994). The unique expression pattern of hsp47 found in this study was likely due to the binding specificity and ER localization of HSP47.

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