AN EXAMINATION OF SMALL HEAT SHOCK PROTEIN GENE EXPRESSION IN *XENOPUS LAEVIS* EMBRYOS AND A6 KIDNEY EPITHELIAL CELLS

By

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A thesis

presented to the University of Waterloo

in fulfilment of the thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 1997

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ABSTRACT

In this study, using Northern blot analysis, the presence of very low levels of hsp30 messenger RNA was detected in late blastula stage *Xenopus laevis* embryos exposed to heat shock. The relative levels of these pre-tailbud (PTB) hsp30 mRNAs increased at the gastrula and neurula stage followed by a dramatic enhancement in heat-shocked tailbud and tadpole stage embryos (20- to 40-fold relative to late blastula). Interestingly, treatment of blastula or gastrula embryos at a temperature of 37°C for 1 h, or with cycloheximide, a protein synthesis inhibitor, followed by heat shock led to enhanced accumulation of the pre-tailbud (PTB) hsp30 mRNAs. Hsp70, hsp87, and actin messages were not stabilized at high temperatures or by cycloheximide treatment. Finally, hsp30D mRNA, which has been previously shown to be first heat-inducible at the mid-tailbud stage using RT-PCR analysis, was not detected by RT-PCR analysis of cycloheximide-treated, heat-shocked blastula stage embryos confirming that it is not a member of the PTB hsp30 mRNAs. This indicates that differential gene expression and mRNA stability are involved in the regulation of hsp30 gene expression during early *Xenopus laevis* development.

In addition, the use of non-equilibrium pH gradient gel electrophoresis (NEPHGE) SDS-PAGE in this study has confirmed the preliminary discovery of seven basic small heat shock proteins in *Xenopus* A6 kidney epithelial cells. These small HSPs are distinct from the previously described acidic small HSPs based on the inability of the HSP30C antibody, which reacts with eight acidic small HSPs, to cross-react with the basic small HSPs. Also, the constitutive synthesis of six of the seven basic small HSPs is another distinctive feature of

these proteins, as acidic small HSP synthesis has only been detected under stress conditions. A *P. lucida* hsp27 cDNA, which encodes a basic small HSP, was used as a heterologous probe in an attempt to examine small hsp mRNA accumulation by Northern blot analysis. The hsp27 cDNA was not observed to cross-react with *Xenopus* small hsp mRNA.

Two-dimensional analysis revealed that, similar to the patterns of acidic small HSP synthesis, the basic small HSPs are induced by heat shock, sodium arsenite, and herbimycin A, and are not significantly induced by hydrogen peroxide, cadmium chloride, or zinc chloride. The effect of two inducers, including mild heat in conjunction with either mild sodium arsenite or herbimycin A concentrations, revealed a synergistic effect on protein synthesis for both the acidic and basic small HSPs. The synergism resulted from an increase in the individual levels of small HSP synthesis, as well as from the induction of the full sets of small HSPs.

The presence of other basic stress-inducible proteins was also examined using NEPHGE analysis. The effect of tunicamycin and dithiothreitol, agents which induce acidic BIP protein synthesis in A6 cells, revealed a tunicamycin-induced 45 kDa basic protein. As well, analysis of basic HSP synthesis in *Xenopus* gastrula embryos revealed the induction of two 50 kDa heat-inducible proteins.

Also in this study, an examination of heat-induced protein aggregation using pore exclusion limit electrophoresis identified the presence of four heat-induced aggregates. Immunoblot analysis of the aggregates using an HSP30C antibody revealed that two of the aggregates (510 and 350 kDa) contained

small HSPs. One-dimensional analysis of the 510 kDa aggregate proteins under denaturing conditions confirmed the presence of a 30 kDa heat-induced protein. Two-dimensional analysis of the 510 kDa aggregate revealed that all eight acidic small HSPs were present.

ACKNOWLEDGEMENTS

First, I have to thank my supervisor, Dr. John Heikkila. I have been very fortunate to have had the opportunity to work in your laboratory and your patience and support was much appreciated. I also have to thank my committee members Dr. Barb Moffatt (for both my M.Sc. and Ph.D.!) and Dr. Bernie Glick, as well as Dr. Morton Globus (for stepping in for Bernie at the last moment for my defense) and Drs. John Honek and Tim Westwood for acting as internal/external and external committee members.

It doesn't feel like it is possible for words to convey how I feel about the lab mates that I have been lucky enough to work with during the past three-and-a-half years: Adnan Ali, Ying Tam, Doug Briant, Dragana Miskovic, Donna Phang, Pasan Fernando, Lisa Lang, and Liz Joyce - thank you for your friendship - without it I could never have made it (and I hope that it will not end when my Ph.D. does!).

Thanks also goes to everyone in Biology 1 and 2, especially the people in Bernie's and Barb's lab - Yuwen, Charles, Li, Sansun, Hao, Cheryl, Yvonne, Henry, Mike, to name but a few. I also have to thank Sue Moore, Henry, Heather, and Jennifer (coolest 499 students). I also have to thank Karen Trevors, Karen Pike, Gracia, Janet Waite, Elizabeth Harnum, and Fran and Chris.

I also cannot forget to mention my family - Thanks Mom, Thanks Dad, and Thanks Ian (again and again and again) as well as my 'new' family - Maria,

Elias, Nick, Sue, Irene, Nicky, and Cath - you have all so freely given me so much love and support, without which I could never have made it.

And last but most definitely not least, I have to thank 'My Reason For Living' (as she is more commonly known around the lab) - my wife Dorothy. You have suffered alongside me each day and night (not always just suffering I hope!) as I struggled to get everything done, and I am aware of the toll that it took on you - Thank you Dorothy for your unfailing love and support. I love you and I cannot wait to get on with our lives! (I can't forget to mention here - a thank you extending to Jie and Liming for first introducing Dorothy and I to each other).

I have obviously had the good fortune of working with a lot of great people during the course of my Ph.D. - and, I suppose like many things in life, it is the friendships that were formed, and the memories that were made during the course of this work that are ultimately more meaningful than anything written in these pages and which will last long after this thesis is sitting in the library collecting dust.

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LIST OF ABBREVIATIONS

2-D SDS-PAGE	two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
A6 cells	*Xenopus* kidney epithelial cell line
ARE	AU-rich element
BiP	immunoglobulin binding protein (see GRP)
CHX	cycloheximide
CPE	cytoplasmic polyadenylation element
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ER	endoplasmic reticulum
GRP	glucose regulated protein (see BiP)
HSC	heat shock cognate
HSE	heat shock element
HSF	heat shock transcription factor
HSG	heat shock granule
hsp	heat shock protein gene/ mRNA
HSP	heat shock protein
IEF	isoelectric focusing
IRE	iron-responsive element
IRE-BP	iron-responsive element-binding protein
MBT	mid-blastula transition
mRNA	messenger RNA

NEPHGE	non-equilibrium pH gradient electrophoresis
PCR	polymerase chain reaction
PTB	pre-tailbud
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
UTR	untranslated region

1. Introduction

The heat shock response, whereby an organism responds with the enhanced synthesis of various families of heat shock proteins in the presence of stresses such as heat shock or exposure to heavy metals, is a highly conserved, virtually universal response. Since Ritossa (1962) first noted the appearance of puffs on the salivary gland chromosomes of *Drosophila* exposed to heat (which were not present in untreated flies), much research has led to the identification and characterization of numerous stress-inducible genes (reviewed in: Waters *et al.*, 1996; Casper *et al.*, 1995; Sax and Piatigorsky, 1994), many of the regulatory factors involved in their expression (reviewed in: Mager and de Kruijff, 1995; Wu, 1995; Voellmy, 1994), and clues as to their functions (reviewed in: Hartl, 1996; Boelens and de Jong, 1995; Jacob and Buchner, 1994; Schlesinger, 1994; Parsell and Lindquist, 1993; Welch, 1993; Walsh *et al.*, 1991) in cells. This information has given insights into different mechanisms of gene regulation, as well as the evolution of different organisms. Finally, studies of the functions of the heat shock proteins has provided a focus for research on cellular mechanisms such as protein folding and protein translocation. Many potential clinical applications of the heat shock proteins are currently being pursued.

1.1 *Xenopus laevis*: A Model Embryonic System

In our laboratory we use the African clawed frog, *Xenopus laevis*, as a system in which to study the vertebrate heat shock response. *Xenopus* has

been used extensively as a system with which to study various aspects of vertebrate embryogenesis. There are several advantages to working with *Xenopus*. They are commercially available and easy to maintain. The females are capable of producing hundreds-to-thousands of eggs throughout the year, and the eggs are relatively large (1-1.2 mm in diameter) which makes them easy to manipulate. The eggs are fertilized externally and the development of the embryos can be observed with low power microscopes. Development is reasonably rapid, with the embryos reaching the tadpole stage within 3 to 4 days. As well, a detailed Normal Table describing and defining the stages of development is available to provide a standard for researchers (Nieuwkoop and Faber, 1967).

1.2 *Xenopus laevis*: Embryonic development

A large store of maternal ribosomal and messenger RNA, as well as proteins are produced early during *Xenopus* oogenesis (Davidson and Hough, 1971). Near the end of oogenesis, the egg is arrested at the prophase of meiosis I. Pituitary gonadotropins cause the oocyte to proceed into metaphase II of meiosis, and the germinal vesicle ruptures, releasing the egg into the oviduct. As the egg passes through the oviduct it is coated with jelly (Brun, 1975).

Fertilization of the egg occurs when a sperm enters the animal hemisphere. The entry-point of the sperm determines the dorso-ventral polarity of the embryo. The animal hemisphere will give rise to the ectoderm, the vegetal hemisphere will give rise to the endoderm, and a marginal zone positioned between the animal and vegetal hemispheres will give rise to the mesoderm.

The first cleavage begins approximately 90 minutes following fertilization. Subsequent cell divisions are rapid and synchronous involving approximately 30 minute cycles, comprising alternating phases of DNA synthesis and mitosis (Newport and Kirschner, 1982a, b). By the thirteenth cycle (approximately 4000 cell stage), cell divisions become asynchronous and slower. This stage is referred to as the mid-blastula transition (MBT), and is the point at which the zygotic genome first becomes transcriptionally activated (Newport and Kirschner, 1982). It is thought that the rapid cycling between synthesis (S) and mitosis (M) phases in the cleaving embryo may prevent RNA transcription, until the MBT at which point the cell divisions become slower (Kimelman *et al.*, 1987). Alternatively, it has also been postulated that the ratio of nucleus to cytoplasm may be involved. In this case, as the cells divide, the ratio of nucleus to cytoplasm increases, and this may cause the titration of a finite maternal factor which must now interact with a greater quantity of DNA. Following the MBT, genes such as the actin gene (Mohun *et al.*, 1984) and GS 17 gene (Krieg and Melton, 1985) are activated. As well, inducible genes such as hsp70, hsp90, BiP78, and ubiquitin can now be expressed, and/or stress-induced (Miskovic *et al.*, in press; Ali *et al.*, 1996; Krone and Heikkila, 1989; Ovsenek and Heikkila, 1988; Bienz, 1984a).

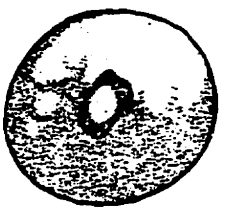
The mid-blastula transition is followed by gastrulation. Gastrulation involves the formation of the endoderm, mesoderm, and ectoderm. The interactions of these germ layers results in differentiation and organogenesis which will ultimately give rise to the tissues and organs in the adult frog. Gastrulation leads into neurulation, in which neural tube formation occurs and eventually gives rise to the spinal cord and brain. During the subsequent tailbud

stage, the embryo hatches and organs such as the developing eye and mouth appear. By the fourth day, the embryo is a free-swimming tadpole, and must now rely on external sources of food, as its yolk sac has been depleted. The aforementioned stages are depicted in Figure 1. The tadpoles eventually metamorphose (including limb formation and the loss of their tail) into young adults.

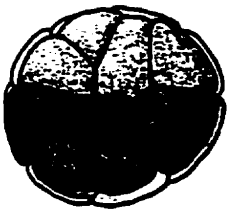
During *Xenopus* embryogenesis, a number of gene families are developmentally regulated, including the actin genes (Mohun *et al.*, 1984). For example cytoskeletal and cardiac actin genes are first transcribed at the end of the gastrula stage, and only in mesodermal cells that will ultimately form muscle (Mohun *et al.*, 1984). Subsequent analysis revealed different isoforms of the cytoskeletal actins, which were denoted as type-5 and type-8. Type-5 actin was found to be present in all regions of the embryo, while type-8 was equated with the aforementioned muscle-specific actin (Mohun and Garrett, 1987). A third muscle-specific actin gene was identified, and its mRNA accumulation was not detected until after neurulation (Mohun and Garrett, 1988). Thus the actin genes are differentially regulated during *Xenopus* development both spatially and temporally. Other examples of developmentally regulated genes in *Xenopus* include activin (Thomsen *et al.*, 1990), insulin (Shuldiner *et al.*, 1991) and integrin (Whittaker and DeSimone, 1993).

In addition to *Xenopus* embryos, a *Xenopus* kidney epithelial cell line, A6, has also been used by our laboratory and others to facilitate the study of the vertebrate heat shock response. Previous studies of the heat shock response in other *Xenopus* cell lines, such as primary cultures of liver, lung, and testis cells

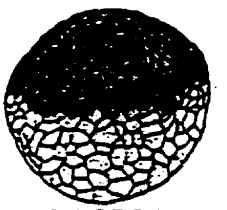
FIGURE 1. An overview of the developmental stages of *Xenopus laevis*, from fertilized egg to tadpole (adapted from Nieuwkoop and Faber, 1967).



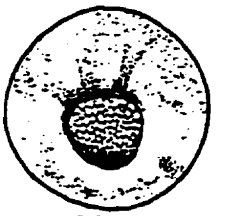
FERTILIZED EGG (Stage 1)



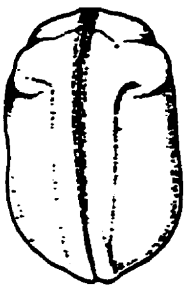
CLEAVAGE (Stage 5)



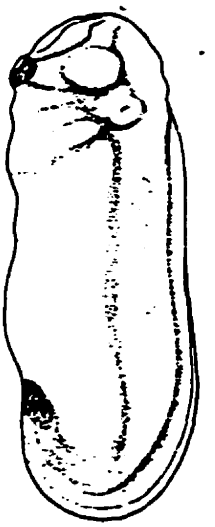
BLASTULA (Stage 8)



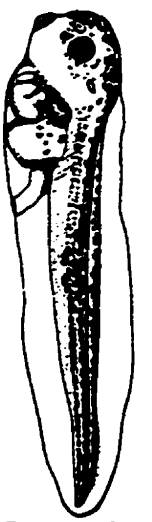
GASTRULA (Stage 11)



NEURULA (Stage 21)



TAILBUD (Stage 27)



TADPOLE (Stage 42)

have proven to be hampered by the inability of these cells to express small HSPs (Wolffe *et al.*, 1984). A6 cells have been demonstrated to produce virtually all of the major HSP family members in *Xenopus,* in response to heat, sodium arsenite, and herbimycin A among others, as well as inducers of the immunoglobulin binding stress proteins (or glucose regulated proteins) such as 2-deoxyglucose and tunicamycin (Tam and Heikkila, 1996; Briant, 1995; Winning *et al.*, 1989; Darasch *et al.*, 1987, 1988; Ketola-Pirie and Atkinson, 1983; Voellmy and Rungger, 1982). The ability to employ A6 cells in the study of heat shock proteins has provided an efficient means with which to study the effects of different inducers in an adult tissue, and has acted as a basis for comparison with the hsp response in embryos.

1.3 Regulation of Eukaryotic Gene Expression: mRNA stability

DNA is transcribed into RNA, which is in turn translated into protein, the main functional unit in the cell. Control of protein production can be achieved by trans-acting factors which interact with cis-acting elements at several points along this pathway. The most fundamental level of regulation is the activation or repression of transcription, and as such, it has been the major focus of research on gene expression. Many trans-acting transcription factors which bind to cis-acting DNA elements, such as the TATA box and CCAAT box (bound by TATA binding protein and CCAAT binding protein transcription factors, respectively) have been identified.

More recently, regulation by post-transcriptional/translational events has come to the forefront, due in part to the inability of transcriptional regulation

alone to account for all occurrences of protein production. As well, the wide variations in messenger RNA half-lives, ranging from minutes to days, suggests the possible role of mRNA stability as a means of regulating gene expression (reviewed in Ross, 1995; Sachs, 1993; Brawerman, 1993). While it may seem inefficient to continually produce a message that is rapidly degraded, such a system would allow a very rapid response by the cell. The use of transcriptional, translational, as well as post-translational levels of regulation would permit very complex modulation of protein production.

To illustrate how mRNA stability is used to regulate gene expression, three well-documented examples will be described. The first example involves the regulation of iron uptake by the transferrin receptor and ferritin. This will be followed by the regulation of gene expression of two proto-oncogenes, c-*fos* and c-*myc*, and thirdly, β-tubulin regulation will be described. This will be followed by a discussion of mRNA stability in *Xenopus* oocytes and mRNA stability and heat shock.

All organisms require iron for growth and cellular metabolism. Thus, cells employ a membrane-bound transferrin receptor to transport iron into the cell as required. This uptake cannot be indiscriminant, as excess iron can react with oxygen to form hydroxy radicals which are toxic to the cell. In order to overcome this hazard, the cell utilizes an additional protein, ferritin, to sequester unused iron (Theil, 1987). A balance must be maintained between the production of transferrin receptor (to increase the intake of iron when required) and the production of ferritin (to sequester iron molecules when they are present in

excess). Recent research has revealed that mRNA stability is integral to maintaining this balance (reviewed in Klausner *et al.*, 1993; Harford, 1993).

The first evidence that post-transcriptional regulation is involved came from studies in which it was found that cells grown in excess iron in the presence of actinomycin D, a transcriptional inhibitor, were still able to produce elevated levels of ferritin protein (Zahringer *et al.*, 1976). This indicated that ferritin protein expression could occur in the absence of transcription. The subsequent cloning of the human ferritin gene permitted deletion studies which identified a 30 nucleotide region in the 5' UTR necessary for translational regulation by iron levels (Hentze *et al.*, 1987b). Further studies involving the insertion of the 5' UTR of the ferritin genes in heterologous mRNA confirmed the necessity of this region for correct translational control, as did mutational studies (Hentze *et al.*, 1987a). Examination of the potential secondary structure of the 30 nucleotide region suggested the formation of a stem-loop in the 5' UTR. This sequence in the ferritin mRNA is referred to as the iron responsive element (IRE).

Analysis of the transferrin receptor indicated that the major means of iron regulation involved a 678 nucleotide region in the 3' UTR of the message (Casey *et al.*, 1988a, b). This was determined by employing constructs generated from the human growth hormone gene and various regions of the transferrin receptor gene. Most interestingly, examination of the potential secondary structure in this region suggested the presence of 5 stem-loop structures very similar (although not perfectly identical) to the iron responsive element found in ferritin. This region appears to be involved in the stabilization of the message. Furthermore, more detailed analyses utilizing more refined

deletion mutants identified the presence of instability elements (within the same stabilizing 678 nucleotide sequence) which are involved in the rapid degradation of the transferrin receptor mRNA (Casey *et al.*, 1989; Harford and Klausner, 1990). It is thought that this instability region could be susceptible to endonuclease attack.

The presence of the IREs in the 5' UTR of ferritin and 3' UTR of the transferrin receptor suggested the distinct possibility of a common regulatory factor. By employing RNA mobility shift assays in human cell lysates, a so-called iron responsive element-binding protein (IRE-BP) was identified (Rouault *et al.*, 1988). Furthermore, in vitro studies of the interaction of the IRE-BP with ferritin indicated its ability to prevent ferritin translation (Walden *et al.*, 1988; 1989) and the ability to stabilize the transferrin receptor mRNA.

A model based on the aforementioned data has been suggested. The absence of iron triggers the IRE-BP to bind to the IRE in the 5' UTR of the ferritin mRNA thereby preventing further translation of its protein. This will prevent the sequestering of more iron under iron-deficient conditions. At the same time, the IRE-BP can bind to the 3' UTR of the transferrin receptor mRNA, which will block the instability elements present within the same region, and stabilize the mRNA facilitating its translation. The increased transferrin recepter protein synthesis will ultimately allow the entry of more iron into the cell. In both cases the IRE-BP is acting as a repressor (Harford, 1993).

c-*fos* and c-*myc* are examples of proto-oncogenes which are very short-lived and which are expressed rapidly in response to extracellular stimuli. They are thought to produce proteins important in the regulation of gene expression.

The requirement for the ability to rapidly activate and cease protein production could be met by regulation via mRNA stability (reviewed in Greenberg and Belasco, 1993; Schiavi *et al.*, 1992). Stability studies began with the observation that there are AU-rich regions found in the 3' UTR of many early response genes, including c-*fos*, c-*myc* and the granulocyte monocyte-colony stimulating factor (GM-CSF; Shaw and Kamen, 1986). Other studies have implicated AU-rich regions as instability elements. When the expression of constructs consisting of the rabbit β-globin gene with a substituted GM-CSF 3' UTR was analyzed in mouse fibroblast cells, the half-life of the message was reduced to 30 minutes, as compared to its normal half-life of more than 2 hours (half-life was measured by first inhibiting transcription with actinomycin D and monitoring the presence of the mRNA over time utilizing RNase protection assays). To make sure that the construct did not somehow affect its rate of transcription, nuclear run-on assays were employed. Thus, an AU-rich element (ARE) was identified as an instability element. Similarly, a neomycin gene with a substituted c-*myc* 3' UTR produced an unstable message. If the AU-rich region was removed, the message was stable (Jones and Cole, 1987). Comparison of AU rich regions in various 3' UTR from different genes suggested that an AUUUA pentanucleotide was a recurring motif. For example, it was noted that c-*fos* has 3 of the pentanucleotides, c-myc has 2, and GM-CSF has 7.

It is interesting to note that while the addition of an ARE to a normally stable mRNA can destabilize the message, the removal of an ARE from c-*fos*, for example, does not stabilize its normally unstable message (Shyu *et al.*, 1991). This suggested the presence of additional instability elements in the c-*fos*

message. Shyu *et al.* generated various ß-globin/c-*fos* constructs to identify the additional element(s). They found that the construct which included the β-globin 5' and 3' UTRs surrounding the c-*fos* coding region was destabilized. Thus, c-*fos* mRNA instability is regulated by AREs in its 3' UTR as well as by sequences within the carboxy-terminal coding region. A similar finding was made with c-*myc* mRNA (Wisdom and Lee, 1991).

c-*fos* mRNA has also been found to be stabilized in the presence of cycloheximide, a translation inhibitor which prevents elongation and leaves the ribosomes associated with the mRNA (Wilson and Treisman, 1988). This suggests the association of the degradation of the message with the process of translation. The two most obvious explanations for this correlation are either i) the inhibition of translation prevents the association of a degradative enzyme with the mRNA (an enzyme which could be associated with the ribosomes), or ii) the global prevention of translation prevents the production of a degradative protein, which would itself have to be very unstable, as the observed effects of cycloheximide seem to be almost immediate. While there is evidence for both possibilities (and it is likely that both pathways occur in different systems), a rather interesting experiment to test which one affects c-*fos* expression will be described here. This experiment utilized knowledge of the aforementioned iron response system. Koeller *et al.* (1991) constructed a chimeric gene which involved the addition of an iron responsive element (IRE) to the 5' UTR of a gene that possessed the 3' c-*fos* ARE present in its 3' UTR (thereby causing the message to be unstable). The presence of the IRE allowed the very specific control of the translation of the chimeric gene by iron levels. This bypasses the

global inhibition of translation conferred by cycloheximide and thus allows the continued synthesis of any putative degradative factors. Under these conditions, the resultant mRNA was still rapidly degraded even though translation of the message was blocked, providing evidence for the second hypothesis; namely, that the degradation of the message is due to a trans-acting factor and does not depend on translation of the unstable message per se. This does not eliminate the first possibility as an alternative pathway, and evidence for the association of translation with mRNA instability will be discussed in the following section about tubulin mRNA.

α- and β-tubulins are the subunits which form microtubule structures in the cell, playing important roles as cytoskeletal elements involved in cell motility, shape and division. There is a dynamic balance between the amount of unassembled tubulin subunits and the amount of assembled tubulin polymers. Furthermore, this balance is autoregulated by the tubulin subunits (Cleveland *et al.*, 1983). Evidence that this autoregulation occurs at the post-transcriptional level was provided by the use of enucleated cells (Caron *et al.*, 1985; Pittenger and Cleveland, 1985). When enucleated cells were treated with colchicine, which binds to the tubulin subunits thereby preventing polymer assembly, the cytoplasts produced less β-tubulin (in order to compensate for the increased quantity of unassembled tubulin). Thus the cells could still regulate tubulin levels even in the absence of any nuclear material, consistent with post-transcriptional regulation (reviewed in Theodorakis and Cleveland, 1993).

In order to localize the region of the β-tubulin message involved in the autoregulation, constructs of various regions of the β-tubulin gene and the

herpes simplex virus thymidine kinase revealed that the first 13 nucleotides encoding the first 4 codons of the β-tubulin protein were necessary (Yen *et al.*, 1988a). Conversely, deletion of any of these amino acids resulted in the loss of the β-tubulin autoregulatory capacity.

Interestingly, treatment with different translation inhibitors produced different effects on autoregulation. Specifically, cycloheximide treatment enhanced β-tubulin mRNA degradation whereas pactamycin stabilized the message. Cycloheximide prevents translation initiation, but leaves polysomes on the mRNA, whereas pactamycin acts by disrupting polysomes. Thus, the presence of the translational machinery is required for the mRNA degradation to occur (Pachter *et al.*, 1987).

This suggested two possible modes of post-transcriptional regulation: i) tubulin (or another cellular factor) binds directly to the 13 nucleotide region of the β-tubulin message, or ii) it binds to the nascent polypeptide (the first 4 amino acids) as they are being translated. In order to resolve this, Yen *et al.* (1988a and b) utilized the following reasoning: if mutations are made in the 13 nucleotide region which still maintain codon fidelity, if model (i) is true, the message should remain stable since the proposed protein factor could not bind to the mutated RNA. If model (ii) is true, mRNA instability should be maintained. A preliminary examination of yeast, chicken and human β-tubulin sequences revealed conservation of the 4 N-terminal amino acids, but divergent mRNA sequences, supporting model (ii) (i.e. binding to the nascent polypeptide). Experimentally, point mutational analysis confirmed the binding of the protein factor to the nascent polypeptide. Thus, β-tubulin mRNA instability requires translation of at least the first 4 codons.

The next step involved determining if it is the tubulin subunit which interacts with the polypeptide, or if it acts indirectly through an additional cellular factor. An attempt to identify an affinity betwen tubulin and the amino terminal of another tubulin molecule has not been fruitful (Theodorakis and Cleveland, 1992). It is interesting that they postulate that a molecular chaperone such as HSP60 or HSP70 may be this mystery factor. The molecular chaperones play a role in protein folding and thus could be present as the protein is being produced. However, it is difficult to draw any conclusions because the chaperones can bind to many different proteins. Either way, it is still not clear how the binding of a protein to the polypeptide facilitates the degradation of the message. Possibly a ribosome-associated nuclease is involved, but no conclusive evidence has been presented.

The aforementioned examples of messenger RNA stability reveal some of the diverse mechanisms that are employed by the cell to regulate gene expression post-transcriptionally. The iron response system involves mRNA secondary structure (stem-loops), 3' UTR stabilizing and destabilizing elements, and the interaction of a specific trans-acting factor (IRE-BP). c-*fos* and c-*myc* provide examples of another 3' UTR destabilizing element (ARE), as well as a coding region determinant. They also provide evidence of a destabilizing factor involved in the subsequent mRNA degradation and suggest that it may be activated by the ARE in the 3' UTR to promote rapid deadenylation, a step thought to be necessary for final mRNA degradation. Finally, β-tubulin is an example of another type of coding region instability determinant, however

unlike that found in the proto-oncogenes, it is directly coupled to the translation of at least 4 codons. An autoregulated feedback system is also suggested.

1.3.1 Messenger RNA Stability in *Xenopus laevis*

Xenopus oocytes contain maternal mRNAs which carry them through oocyte development and following fertilization, through cleavage stages to the mid-blastula transition. Some of the maternal messages are translationally activated, while others remain dormant. There are two major switch-over points at which the expression of different mRNAs become activated and deactivated; namely, during the progesterone induced meiotic maturation period when the oocyte becomes an unfertilized egg, and following fertilization. Since there is very little transcription that occurs during these periods, post-transcriptional and translational control must be the main mechanisms involved (reviewed in Wormington, 1994; Richter, 1993; Richter *et al.*, 1990). There appears to be a strong correlation between polyadenylation and translational activation of the maternal messsages. The loss of polyadenylation probably plays an important role in the subsequent degradation of many of the maternal messages following the mid-blastula transition.

In *Xenopus* there are two 3' UTR polyadenylation signals: i) the AAUAAA hexanucleotide, which seems to be necessary to determine the site of nuclear pre-mRNA cleavage and polyadenylation, and ii) U-rich cytoplasmic polyadenylation elements (CPE) typically located 4 to 13 nucleotides upstream of (i). It is thought that differences in these sequences in conjunction with spatial differences between the elements within the 3' UTR could account for variations

in the timing of polyadenylation and in the length of the poly (A) tail (Baker, 1993). Variations in the two cis-elements would affect regulation by modulating the interactions of CPE-binding proteins. The regulation of maternal mRNA polyadenylation seems to be under translational control, since the presence of cycloheximide inhibits its expression (Simon and Richter, 1994).

As an example of a gene regulated by mRNA stability in *Xenopus* embryos, Eg2 mRNA stability will be examined. Bouvet *et al.* (1991) have observed that in *Xenopus*, some messenger RNAs in the oocyte remain adenylated following fertilization, while others become deadenylated. Those messages which become deadenylated have been termed Eg mRNAs. It is interesting to note that while the deadenylation event occurs upon fertilization, the messages are not actually degraded until the mid-blastula transition. This temporal distinction can facilitate studies of the association of the two events. Bouvet *et al.* (1991) began by examining Eg2 mRNA stability. They found that chimeric messages containing the proximal 497 nucleotide region of the Eg2 3' UTR were unstable in embryos. Furthermore, the presence of cycloheximide stabilized the messages. The stabilization effect could be due to the prevention of translation and thus any translationally associated mRNA degradation factors, or it could be due to the inhibition of the expression of an mRNA-degrading protein. When they added a 5' hairpin loop to specifically inhibit translation of the construct, they found that the message was not stabilized. Thus, they concluded that the degradation of the message requires the translation of another message encoding a trans-acting mRNA degrading factor. Similarly, Duval *et al.* (1990) found that post-MBT treatment with cycloheximide

stabilized the Eg messages, however in addition, they noted that treatment with the RNA synthesis inhibitor actinomycin D did not stabilize the messages. Thus the destabilization event is not due to the transcription of a new gene at the mid-blastula transition.

1.3.2 Heat Shock and mRNA Stability

The heat shock response involves a general reduction in mRNA and protein synthesis in conjunction with the specific enhanced induction of heat shock protein synthesis. Thus there are two potential avenues to explore in terms of heat shock and mRNA stability; namely, is the reduction of general protein synthesis controlled by some general heat-induced instability of mRNA? As well, is the induction of heat shock protein synthesis correlated with a temperature-mediated enhanced stability of heat shock mRNA?

The reduction of general protein synthesis upon heat shock does not appear to be due to the degradation of the messages, as treatment of heat-shocked cells with actinomycin D did not prevent the rapid resumption of translation of general mRNAs following the stress (Lindquist, 1981). This suggests that the messages are somehow protected during the stress and reactivated following it, allowing minimal disruption of cellular activities. Many experiments indicate that messenger RNA instability does not appear to play a major role in the repression of general translation, instead this response has been attributed to various aspects of translation, including modifications of the ribosome, inactivation of cap-binding factors (which may not be necessary for heat shock translation but are for regular translation), post-translational

modifications of initiation factors, and possible competition by heat shock mRNA for ribosomes (reviewed by Sierra and Zapata, 1994; Lindquist and Petersen, 1990).

Of more immediate interest is the apparent role of mRNA instability in the regulation of heat shock protein expression. The expression of HSPs is reduced or non-existent in unstressed cells as compared to stressed cells (as a general note: 'hsp' will be used to refer to genes or mRNA, whereas 'HSP' will be used to refer to proteins). It seems plausible that the heat shock messages are actually unstable in the absence of stress, and somehow are stabilized during the stress allowing their enhanced synthesis. Evidence for this hypothesis has been demonstrated with hsp70 mRNA in *Drosophila* (Petersen and Lindquist, 1988, 1989). The expression of a chimeric hsp70 gene under the control of the copper inducible metallothionein promoter in *Drosophila* cells was examined under control and heat-shock conditions. At normal temperatures, the half-life of the message was 15 to 30 minutes. When heat-shocked, the half-life was longer than 4 hours. In order to determine which component of the message confers this instability, constructs comprised of the alcohol dehydrogenase (ADH) gene with various segments replaced with related hsp70 gene fragments were utilized. Substitution of the 3' UTR of ADH with the 3'UTR of the hsp70 message resulted in the destabilization of the ADH message under control conditions. Heat shock resulted in enhanced ADH production. Thus the *Drosophila* hsp70 message seems to be naturally unstable and this instability is conferred by its 3' UTR under stress conditions.

Following stress, the synthesis of heat shock proteins returns to their former lower levels. The role of mRNA stability in this regulated repression is

logical. Petersen and Lindquist (1989) fused the 5' UTR of the hsp70 gene to the coding and 3' UTR of the ADH gene. Recovery from a mild heat shock did not diminish the production of ADH message or protein synthesis, whereas the endogenous hsp70 message was rapidly degraded. An ADH/3' UTR hsp70 construct resulted in the destabilization and degradation of the chimeric mRNA during recovery from heat shock. Thus it appears that mRNA instability plays a role in the repression of HSP70 protein synthesis during recovery from heat shock in *Drosophila*, and that this instability element is in the 3' UTR of the message.

It is very interesting to note that the 3' UTR of the *Drosophila* hsp70 message is very AU-rich and possesses 2 close matches to the AUUUA pentanucleotide thought to stabilize other mRNAs such as c-*fos*, c-*myc* and GM-CSF (Petersen and Lindquist, 1989). Furthermore, c-*myc* and c-*fos* have been reported to be stabilized by heat shock (Sadis *et al.*, 1988) suggesting that a common mRNA instability regulatory system is involved. Studies of human hsp70 half-life and stability in the presence of translation inhibitors such as cycloheximide and pactamycin also support a role for mRNA stability in human hsp70 regulation (Theodorakis and Morimoto, 1987). The presence of a single AUUUA pentanucleotide and several similar sequences in the human hsp70 3' UTR were also noted, although the authors did not test their possible roles.

In an attempt to bring together the information known about *Drosophila* hsp70 expression, Lindquist (1993) postulated both transcriptional and post-transcriptional mechanisms are required in order to regulate the rapid induction upon heat shock of the HSP70 protein and its subsequent degradation following the stress. It has been shown that at the transcriptional level, the

chromatin at the 5' ends of the hsp70 genes are accessible at normal temperatures, which permits the binding of RNA polymerase II and allows it to remain transcriptionally engaged, but paused, until the heat shock factor is activated by heat to bind to the heat shock elements, permitting the completion of transcription of the hsp70 genes. The hsp70 gene contains no introns, and thus is exempt from heat-induced inhibition of mRNA splicing. As well, as was previously mentioned, the transcript is stabilized in the presence of heat due to sequences in its 3' UTR, allowing the translation of the protein to high levels. According to Lindquist, following heat shock, transcription of the hsp70 gene is repressed, and the once heat-stabilized messages are now subject to more rapid degradation. Lindquist further suggested that there is degrading machinery which acts on hsp70 transcripts (probably on others as well) during control conditions which is lost during heat shock. Following heat shock the HSP70 proteins may help repair this machinery and in so doing, facilitate their own autoregulated degradation (Lindquist, 1993).

Finally, Dellavalle *et al.* (1994) have found that deadenylation plays a role in the post-transcriptional regulation of the expression of the *Drosophila* hsp70 gene. Their experiments indicated that approximately 40% of the hsp70 transcripts produced during a standard heat shock lacked poly (A) tails. Furthermore, it appears that this is not due to failure in adenylating the message, but actually to an active deadenylation of previously adenylated hsp70 transcripts. The deadenylated transcripts are translated inefficiently. Following heat shock, the deadenylated hsp70 messages are rapidly degraded. Thus, there appears to be an important role for deadenylation in the post-transcriptional regulation of hsp70 mRNA expression.

This is probably only one of several possible regulation mechanisms. Studies of hsp70 mRNA in chicken reticulocyes indicate a very slight increase in mRNA abundance in heat-shocked versus control cells, and that the quantity of protein produced is regulated at the level of translation; specifically in the rate of elongation of the HSP70 peptide (Theodorakis *et al.*, 1988). Edington and Hightower (1990) found both post-transcriptional and translational levels of regulation were utilized by the chicken hsp23 gene. While transcription remained fairly constant between control and heat shock conditions, the levels of mRNA were found to increase 4-fold upon heat shock, suggesting some sort of post-transcriptional stabilizing event. Examination of the protein under control conditions revealed a half-life of 2 hours, whereas during heat shock it was 13 hours, suggesting a post-translational event also stabilizes the protein product.

1.4 Heat Shock Proteins

The study of the regulation of gene expression is complicated by the presence of many rapid cascades of events occurring simultaneously in a cell at any given time, making it difficult to examine a particular aspect. The heat shock response provides an opportunity to study the regulation of gene expression in a more controlled manner, in which an external stimulus (heat), the application of which is determined by the experimenter, can be used to activate the expression of specific genes. The finding that the heat shock response occurs in virtually every organism studied, from bacteria, to plants, to human beings, has provided the opportunity to compare the responses in different organisms.

Indeed, the heat shock protein genes found in these organisms exhibit a high degree of sequence similarity, suggesting the conservation of the response throughout evolution.

During heat shock, cells undergo several changes (reviewed in Schlesinger, 1994). These changes include the halting of DNA replication and the condensing of chromatin, the slowing of ribosomal RNA processing, incomplete splicing of mRNA, and the collapse of the intermediate filament assembly. Amongst this general shutting down of the cell, there is the enhanced activation of the heat shock proteins whose primary role is to protect the cellular components during the stress.

1.5 HSP Inducers

Interestingly, the study of heat shock proteins has revealed that the activation of their synthesis is induced by stresses other than heat, such as exposure to sodium arsenite (targets protein sulfhydryl groups; Kato *et al.*, 1993; Lee *et al.*, 1991; Darasch *et al.*, 1987, 1988), heavy metals (such as cadmium and zinc; Ovelgonne *et al.*, 1995; Heikkila *et al.*, 1982), herbimycin A (a tyrosine kinase inhibitor; Hedge *et al.*, 1995; Murakami *et al.*, 1991), and hydrogen peroxide (Compton and McCarthy, 1978). Due to the response of heat shock proteins to many different inducers, the hsps are also commonly referred to as stress proteins (Gething and Sambrook, 1992). Typically, these inducers all negatively affect the folding of proteins (Nover, 1991). It appears that the subsequent heat-shock response relates to the ability of the HSPs to act as molecular chaperones and aid in the correct folding of proteins.

Interestingly, herbimycin A, a tyrosine kinase inhibitor, has been shown to induce the enhanced synthesis of constitutively expressed stress proteins in animal cells even though herbimycin A does not appear to affect cellular activities normally interrupted by stresses like heat shock (Hedge *et al.*, 1995). For example, herbimycin A was not observed to affect the maturation of proteins, solubility of proteins, integrity of the intermediate filament cytoskeleton, or cell viability. Thus, the authors suggest that herbimycin A may short-circuit the pathways involved in the regulation of HSP expression, specifically regarding transcription factors normally involved in the constitutive expression of HSPs. In terms of the heat shock factor (HSF) which regulates the stress-induced expression of HSPs, the authors noted that in the presence of herbimycin A, HSF1 activation was not observed in rodent cells, but was observed in primate cells (Hedge *et al.*, 1995). Thus the effect of herbimycin A may differ in different cell types.

Immunoglobulin binding proteins (BiP, also referred to as glucose regulated proteins, GRP) are also categorized as stress-inducible proteins, responding mainly to glucose starvation. BiP inducers include 2-deoxyglucose, 2-deoxygalactose, tunicamycin (all of which interfere with the ability of the cell to use glucose), the calcium ionophore A23187 (which deprives cells of calcium), and dithiothreitol (interferes with disulfide bond formation) among others. These agents are thought to result in the production of abnormal proteins, primarily within the endoplasmic reticulum, where BIP proteins reside (Nover, 1991).

An interesting observation noted with different inducers of heat shock proteins is that the inducers do not necessarily induce the same subsets of HSPs. For example, Darasch *et al.* (1987) found that sodium arsenite and heat

shock induce a number of common HSPs in *Xenopus* A6 cells, including HSP87, HSP70, and HSP30, as well as inducer-specific HSPs; for example, heat shock specifically induced HSP51, and HSP54, whereas sodium arsenite specifically induced HSP37, HSP57, HSP68 and HSP100. Similar findings have been reported for Chinese hamster ovary cells treated with heat shock and sodium arsenite. Heat shock induced the synthesis of five major HSPs, whereas sodium arsenite induced four major HSPs plus an additional stress protein (Lee *et al.*, 1991). In the case of human skin fibroblasts, it has been shown that sodium arsenite induces the synthesis of the major HSPs, as well as a 32 kDa protein, whereas hydrogen peroxide induces the small HSPs and a 32 kDa protein (Keyse and Tyrrell, 1987). This differential pattern of HSP synthesis from different inducers may relate to different regulatory mechanisms involved in the response (Nover, 1991).

1.6 Heat Shock Protein Families

There are three major families of heat shock proteins which have been categorized according to their molecular weights. Thus there is the high molecular weight heat shock protein family (80-110 kDa, predominantly hsp90), the hsp70 family (68-73 kDa), and the low molecular weight hsp family (16-36 kDa; small hsps). It seems that virtually all organisms possess at least one representative from each of the three families; however there is much variety in the particular hsp family members produced in any one kind of organism (reviewed by Lindquist, 1986). Thus in yeast cells there is one small hsp, whereas nematodes produce three, and *Drosophila* produce four. In *Xenopus*,

there are as many as 16 small HSPs produced from at least seven small hsp genes (Darasch *et al.*, 1988; Krone *et al.*, 1992) and more than 20 small HSPs are detected in soybeans (Schoffl and Key, 1982).

1.6.1 The High Molecular Weight Family (HSP90)

The high molecular weight family includes stress-induced proteins ranging between 80 and 110 kDa (Nover and Scharf, 1991). In *Drosophila*, a single hsp90 gene (hsp82) is present. In higher eukaryotes such as humans, two hsp90 genes have been isolated, and are referred to as hsp90α and hsp90β. The immunoglobulin binding protein BiP94 is also included in the hsp90 family. The sequences of these genes and proteins are highly conserved between organisms (reviewed in Parsell and Lindquist, 1993). For example, an antibody produced against the chicken HSP90 protein is capable of cross-reacting with HSP90 proteins in human cells, *Drosophila*, and frogs among others (Kelley and Schlesinger, 1982). Unlike most other heat shock genes, the hsp90 family members possess introns. A PCR amplified genomic DNA fragment has been recently isolated in *Xenopus* using degenerate primers whose sequences were based on comparisons of common regions in hsp90 genes isolated from other organisms (Ali *et al.*, 1996). Amino acid sequence comparison has revealed a stronger identity between HSP90βs of humans and zebrafish, as compared to their HSP90α proteins.

The hsp90 genes are expressed constitutively as well as being heat-inducible, as has been demonstrated, for example, with HSP90α and β in mammals (Welch, 1990). In *Xenopus*, hsp90β mRNA has been detected

constitutively in oocytes (present as maternal mRNA) as well as throughout early embryogenesis. The hsp90β mRNA first exhibits heat-inducible accumulation following the mid-blastula transition (Ali *et al.*, 1996). In general, the HSP90 proteins are present at relatively high levels in the cytoplasm of cells.

In terms of function, HSP90 has been found to interact with a number of proteins, including calmodulin, actin, tubulin, kinases, and steroid receptors (reviewed in Jakob and Buchner, 1994). Unlike HSP70, the variety of proteins HSP90 interacts with appears to be more limited, and it is generally considered a more discriminating heat shock protein than HSP70. Functions ascribed to the HSP90 family members include regulation of steroid hormone receptors and oncogenic kinases (such as v-Src), and more recently they have been shown to exhibit general chaperone activity. For example, it was recently shown that HSP90 in conjunction with HSP70 and ATP was capable of reactivating a previously heat-inactivated luciferase enzyme (Schumacher *et al.*, 1994).

1.6.2 The HSP70 Family

The hsp70 family members are probably the most studied of the heat shock proteins. It is a large family encompassing many different hsp subfamilies, including the hsp70s, the heat shock cognate (hsc) 70, and the BiP78 genes (Nover and Scharf, 1991; Lindquist and Craig, 1988). The genes are highly conserved, exhibiting 45-50% amino acid sequence identity between the prokaryotic and eukaryotic hsp70, hsc70 and BiP, for example. In eukaryotes, BiP78 proteins exhibit 60-70% sequence identity with HSP70 and

HSC70. At least eight hsp70 family members have been identified in mammals, and 4 in *C. elegans*. In *Xenopus*, four hsp70 genes, two hsc70 cDNAs, and a BiP78 cDNA have been isolated (Miskovic *et al.*, in press; Ali *et al.*, 1996; Bienz, 1984). It was found that BiP78 from *Xenopus* has a higher identity with BiP proteins from other organisms than with other members of the hsp70 family in *Xenopus*, as has been found in other organisms (Haas, 1994). In general, the hsp70 proteins have two domains: a highly conserved N-terminal domain which has a high affinity ATP-binding site, and a less conserved C-terminal domain which is thought to interact with substrate proteins (reviewed in Nover, 1991).

Specific HSP70 proteins are localized to various compartments such as the endoplasmic reticulum (BiP78), mitochondria, chloroplasts, and more generally in the nucleus and cytoplasm (Hendrick and Hartl, 1993). Mitochondrial and chloroplast HSP70 proteins are more similar to bacterial DnaK (HSP70 homolog) than eukaryotic HSP70, at the amino acid level. The hsc70 genes in human and mouse possess introns whereas their hsp70 counterparts do not. A tissue specific hsc70 has also been identified in the testis (hst70). The BiP78 protein possesses a KDEL sequence in its carboxy-terminus which is responsible for the maintenance of its presence in the endoplasmic reticulum (Munro and Pelham, 1986).

The hsc70 genes are expressed constitutively, whereas the hsp70 genes are predominantly only stress-induced, although in some cases, low basal levels of synthesis are detected. The BiP78 genes are expressed constitutively, and exhibit enhanced protein synthesis upon exposure to glucose starvation or agents which interfere with their ability to use glucose, such as tunicamycin, as

well as agents which reduce the availability of calcium ions, such as calcium ionophore A23187 (Lee, 1992).

The hsp70 proteins have the ability to bind and release hydrophobic segments of an unfolded polypeptide chain in an ATP-hydrolytic reaction cycle (reviewed by Hartl, 1996; Welch, 1993). They function as molecular chaperones, whereby they stabilize translating and newly synthesized proteins until the required regions are available for correct folding. They are also involved in the degradation of misfolded proteins. As well they play a role in the translocation of proteins across membranes, in which compartmentalized hsp70 family members (in the mitochondria or endoplasmic reticulum, for example) aid in the translocation of proteins through the compartment membranes (Hartl, 1996).

1.6.3 The Small HSP Family

The family of small hsps is made up of 16-30 kDa proteins. All organisms studied to date possess at least one small hsp, and the number can vary dramatically between species. For example, in *C. elegans*, there are 6 small hsp genes (Candido *et al.*, 1989). Four small hsp genes have been isolated in *Drosophila* (hsp22, 23, 26, 27), one in mammals (hsp27), at least seven in *Xenopus*, and more than six subfamilies made up of at least 20 small hsps have been discovered in plants (reviewed by Waters *et al.*, 1996). Many of the small hsp genes are present in clusters, including *C. elegans* small hsp genes which are present in two clusters, and *Drosophila* and *Xenopus* small hsp genes, which are present at a single chromosomal locus, and in at least 1 to 2 clusters,

respectively. The greatest sequence diversity among heat shock proteins is encountered in the small hsp genes. The amino-terminus of small HSPs contain a highly conserved region of approximately 80-100 amino acid residues, which account for most of the similarity between species' small hsps. For example, this region exhibits 70% identity between the human HSP27 and *Drosophila* HSP27 proteins.

This conserved region is sometimes referred to as the α-crystallin domain, relating to the presence of a similarly conserved region present in α-crystallins, an observation first noted by Ingolia and Craig (1982; reviewed by Caspers *et al.*, 1995). Alpha-crystallin is one of the most abundant proteins in vertebrate lenses, comprising up to 50% of the total water soluble fraction (de Jong *et al.*, 1993). The recruitment of housekeeping genes to serve as structural proteins in the lens has been observed for other crystallin genes. For example, S-crystallin was derived from the glutathione S-transferase gene, and delta-crystallin was derived from the argininosuccinate lyase gene (for review, see Caspers *et al.*, 1995; de Jong *et al.*, 1994; Wistow, 1993a, b). Alpha-crystallin is made up of αA- and αB-crystallin polypeptides, each having a molecular weight of around 20 kDa. Unlike α-crystallin, many small hsps lack introns. However, some small hsp genes do have introns. For example, the small hsp genes in *Caenorhabditis elegans* have a single intron. Interestingly, the position of the intron matches the position of the first intron in the α-crystallins (Piatigorsky and Zelenka, 1992). As will be shown, the α-crystallins share many properties with small hsps, such that they are considered to be members of the small hsp family.

The small hsps seem to be localized to different regions of the cell in different species. For example *Drosophila* HSP27 has been found to be localized in the nucleus, chicken HSP25 in the cytoplasm, and *Neurospora crassa* HSP30 is present in mitochondria. In human cells, HSP27 has been localized to the perinuclear region and exists as a 200-800 kDa aggregate, where, upon heat shock, the small HSPs move to the nucleus and form a 2 MDa aggregate (Arrigo *et al.*, 1988). As mentioned previously, in higher plants, six families of small HSPs have been categorized. Interestingly, these families are localized to different regions; for example, different small HSP families are found in the cytoplasm, chloroplasts, endoplasmic reticulum, and mitochondria (Waters *et al.*, 1996; Helm *et al.*, 1993).

The small HSPs are, of course, stress inducible, however they also exhibit varying degrees of constitutive expression. Constitutive expression of small HSPs has been observed in *Drosophila* (Sirotkin and Davidson, 1982; Cheney and Shearn, 1983; Arrigo and Pauli, 1988), mouse (Walsh *et al.*, 1991; Gaestel *et al.*, 1993), and yeast (Kurtz *et al.*, 1986), as well as in cultures of human HeLa cells and monkey COS cells (Arrigo and Landry, 1994). Often, constitutive small HSP expression is regulated by transcription factors other than the heat shock factor (see below). In *Xenopus* embryos and A6 cells, no constitutive small hsp mRNA accumulation or protein synthesis has been observed (Krone *et al.*, 1992; Ali *et al.*, 1993).

As has been found for other hsps, the heat-induced regulation of small HSP expression is under the control of the heat shock factor (HSF) which interacts with the heat shock elements present in small hsp gene promoters

(see Section 1.7). Interestingly, other promoter elements have been identified, which are mainly involved in small hsp gene expression under unstressed conditions. This has been studied in detail in *Drosophila*, in which it has been shown that the regulation of levels of small HSP expression are under the control of β-ecdysterone, a molting hormone (reviewed in Arrigo and Landry, 1994). Thus, the *Drosophila* small hsp genes also possess a *cis*-acting binding site for the hormone. Similarly, estrogen-responsive elements have also been found in human tissues sensitive to estrogen (for example, the uterus, vagina, and cervix), including breast tumors (Oesterreich *et al.*, 1996). Estrogen responsive elements have also been found in the promoter of the mouse hsp25 gene (Gaestel *et al.*, 1993).

The αA-crystallin gene lacks a heat shock element (HSE) and has not been found to be stress-inducible. αB-crystallin possesses a HSE in its 5' flanking region and has been found to be inducible by a variety of stresses which also induce small HSPs, including heat, sodium arsenite, and cadmium, in a variety of cell types. For example, Northern and Western blot analysis of human glioblastoma cells exposed to heat shock or sodium arsenite revealed that the expression of both αB-crystallin and hsp28 were analogously enhanced (Kato *et al.*, 1993). Some stresses have been found to induce both small hsp and αB-crystallin expression, while others will only induce one. For example, rat astrocytes exposed to heat-shock induced both αB-crystallin and hsp27 expression, whereas hypertonic stress only enhanced αB-crystallin mRNA synthesis (Head *et al.*, 1994).

1.6.3.1 Developmental regulation

Developmental regulation is observed for both small hsp and αB-crystallin expression. In *Drosophila*, small hsp expression is developmentally regulated by the steroid hormone ecdysterone, resulting in a complex pattern of expression of various small hsps at different times and concentrations in different tissues (Arrigo and Pauli, 1988; Cheney and Shearn, 1983). In plants, developmental regulation of small hsps are observed during pollen development and seed maturation (Waters *et al.*, 1996). Expression of the small hsps in *Xenopus laevis* also exhibit heat-inducible developmental regulation. The heat-induced accumulation of hsp30 mRNA first appears at the early tailbud stage of development at which point the hsp30C mRNA is detectable, followed by hsp30D mRNA at the mid-late tailbud stage of development, as determined by Northern blotting and reverse-transcription polymerase chain reaction (RT-PCR; Ohan and Heikkila, 1995; Ali *et al.*, 1993 ; Krone and Heikkila, 1989). Analysis of the levels of αB-crystallins using immunoassays have revealed that their levels increase during development in rat muscle and kidney tissues (Kato *et al.*, 1991). αB-crystallin levels in the brain remained low, not increasing until later in development, as compared to the muscle and kidney.

1.6.3.2 Phosphorylation

Small hsps can undergo post-translational modifications; for example, when exposed to various stimuli, including growth factors, arsenite, or calcium

ionophores, mammalian hsps become phosphorylated at serine residues located within the recognition sequence Arg-X-X-Ser, which is recognized by the calmodulin-dependent protein-kinase II. More recently, MAP kinase activated protein kinase-2 (MAPKAP kinase-2) was identified as an important enzyme in small hsp phosphorylation (Stokoe *et al.*, 1992). Phosphorylation of hsp28 in HeLa cells has been correlated with hsp oligomerization (Mehlen and Arrigo, 1994). When hsp28 was dephosphorylated in serum-starved cells, only small aggregates of hsp28 were observed. Replenishing the cells with serum resulted in the phosphorylation of the proteins, and the subsequent formation of larger aggregates. It is thought that phosphorylation may also enhance chaperone activity.

Similarly, the α-crystallins can undergo several post-translational modifications, including phosphorylation. Up to 30% of bovine lens αA- and αB-crystallins are phosphorylated. The proteins are phosphorylated by cAMP-dependent protein kinases on one to three serine residues, as was observed with the small HSPs. In mature lens fiber cells, the phosphorylation of the α-crystallins appears to be irreversible whereas this is not the case in the lens fiber epithelial cells. In other tissues phosphorylation of α-crystallin is reversible, implying a possible regulatory mechanism (de Jong *et al.*, 1993). The phosphorylation state does not appear to affect the association of the αA- and αB-crystallins with one another (Augusteyn *et al.*, 1989). The apparent lack of phosphorylation in chicken α-crystallin and in other organisms' crystallins (Groenen *et al.*, 1994) suggests that the role of phosphorylation is not a general one.

1.6.3.4 Aggregation

A characteristic property of small heat shock proteins is their ability to form aggregates which, during stress gather in and around the nucleus (reviewed in Arrigo and Landry, 1994). These aggregates have a native molecular mass of around 400-800 kDa and appear as 10-15 nm granules when viewed under an electron microscope. In sucrose gradients, the aggregates have sedimentation coefficients ranging from 15-20S. Similarly, α-crystallins exist as large homo- or heteropolymers, forming aggregates from 300 to 800 kDa (comprising about 30-40 subunits). At the extreme, complexes greater than 10000 kDa have been isolated (Clauwert *et al.*, 1989; examples of these heat shock granules HSGs, have been described for small HSPs in *Drosophila*, Arrigo *et al.*, 1988; and tomato cells, Nover *et al.*, 1989). Electron microscopy has revealed that they form 14-18 nm heterogeneous globular proteins. Aggregates made up of αB-crystallin and small hsps occur naturally in adenovirus-transformed cells (Zantema *et al.*, 1992). They have also been co-eluted from an affinity column using antibodies against αB-crystallin in human astroglioma U373 MG cells (Kato *et al.*, 1993).

The common ability to form aggregates suggests that small HSP's and α-crystallin's conserved carboxyl-terminals are likely to be involved in this activity. Comparison of hydropathy plots in the conserved carboxyl-terminal of the small hsps and α-crystallins have revealed a region (amino acids 45-55) which is highly hydrophilic which could act as a connecting peptide (Lindquist and Craig, 1988). Based on the conserved regions between α-crystallins and small hsps, and the structural information available, it seems likely that the functional

region of the proteins are found in the carboxyl-terminal and that the amino terminal serves to distinguish individual family members (de Jong *et al.*, 1995).

The function of these aggregates has not been determined. In plants, mRNA has been found in association with the aggregate, and it has been postulated that the structure may protect mRNAs from stress. As well, their localization to the nucleus has prompted a role for the aggregate as a protector of nuclear structure, perhaps through an association with actin filaments.

1.6.3.5 Function

The function of small HSPs is a rather enigmatic issue (Parsell and Lindquist, 1993). A number of studies have provided evidence for several different possible roles for the small HSPs. This probably relates to the evolution of diverse functions for the small HSPs in different species (and even within species) which is probably reflected in the relatively low levels of sequence similarity encountered between small HSPs (de Jong *et al.*, 1993). In yeast, the small HSPs do not appear to be necessary for any particular function (Petko and Lindquist, 1993). In other systems, they have been found to act as molecular chaperones, similar to the hsp70 family, where they are capable of preventing heat-induced aggregation of proteins and can renature denatured proteins (Jakob *et al.*, 1993; reviewed in Jakob and Buchner, 1994). They also seem to be important in thermotolerance, whereby cells exposed to a low level of stress, are subsequently more resistant to normally lethal levels of stress (see below). More recently they have been postulated to play a role in the growth

and differentiation of cells, which may relate to their presence constitutively within some cells (Arrigo and Landry, 1994).

There is evidence that small HSPs may be involved in protecting the microfilament network (Lavoie *et al.*, 1993). Perhaps the greatest evidence for small HSP/microfilament interactions comes from work with crystallin proteins. αB-crystallin has been observed in association with intermediate filaments. In the lens, α-crystallins have a strong non-covalent association with the fiber cell cytoskeleton as determined by the co-precipitation of α-crystallins with vimentin from the lens (Nicholl and Quinlan, 1994). The association with intermediate filaments is not limited to lens tissue. In Alexander's disease, a human neurodegenerative disorder, αB-crystallin was found associated with intermediate filaments of astrocytes (Iwaki *et al.*, 1989). In heart and slow muscle, αB-crystallin is found with desmin filaments in the Z bands (Boelens and de Jong, 1995). During early chicken embryogenesis αB-crystallin is present and appears to play a role in cytomorphological reorganization (Scotting *et al.*, 1991). Treatment of glioma cells with αB-crystallin antisense mRNA caused a change in the morphology of the cells suggesting a correlation between the αB-crystallins and the assembly of intermediate filaments (Iwaki *et al.*, 1994). Finally, αA- and αB-crystallin were found to bind with actin on an affinity column. Thus it is possible that small HSPs, including the α-crystallins are involved in the remodelling and protection of intermediate filaments (Boelens and de Jong, 1995; Arrigo and Landry, 1994).

The role of small hsps in generating thermotolerance has been well-documented. For example, Berger and Woodward (1983) showed that in *Drosophila* tissue culture cells in which relatively high levels of small HSPs are present constitutively, are more tolerant of higher temperatures than tissue culture cells lacking small HSPs. Additional direct evidence came from a study by Landry *et al.* (1989) in which they found that the overexpression of an hsp27 gene in transfected Chinese hamster cells resulted in a thermoresistant phenotype. As well, the presence of αB-crystallin in cultured cells can make the cells more thermoresistant. For example, the transfection of αB-crystallin into mouse NIH 3T3 cells made the cells more thermoresistant, that is, their ability to survive a normally lethal heat shock increased in proportion to the amount of αB-crystallin present in the cells (Aoyama *et al.*, 1993). In the lens, the natural stress-resistance of the crystallins would be essential, because it is not possible for the terminally differentiated lens fiber cells to produce hsps. The higher levels of αB-crystallin expression in other cells aids in their thermoresistance (makes the cells more resistant to high temperatures). Interestingly, αA-crystallin also confers thermoresistance, even though it is not heat-inducible (Boelens and de Jong, 1995).

1.7 Transcriptional Regulation of Hsp Genes

Initial studies using Dnase I hypersensitivity assays and deletion mutant analysis of the promoter region of the *Drosophila* hsp70 gene revealed the presence of a TATA box which defines the initiation position of RNA polymerase II, as well as a second region which was required for heat-inducibility (Wu,

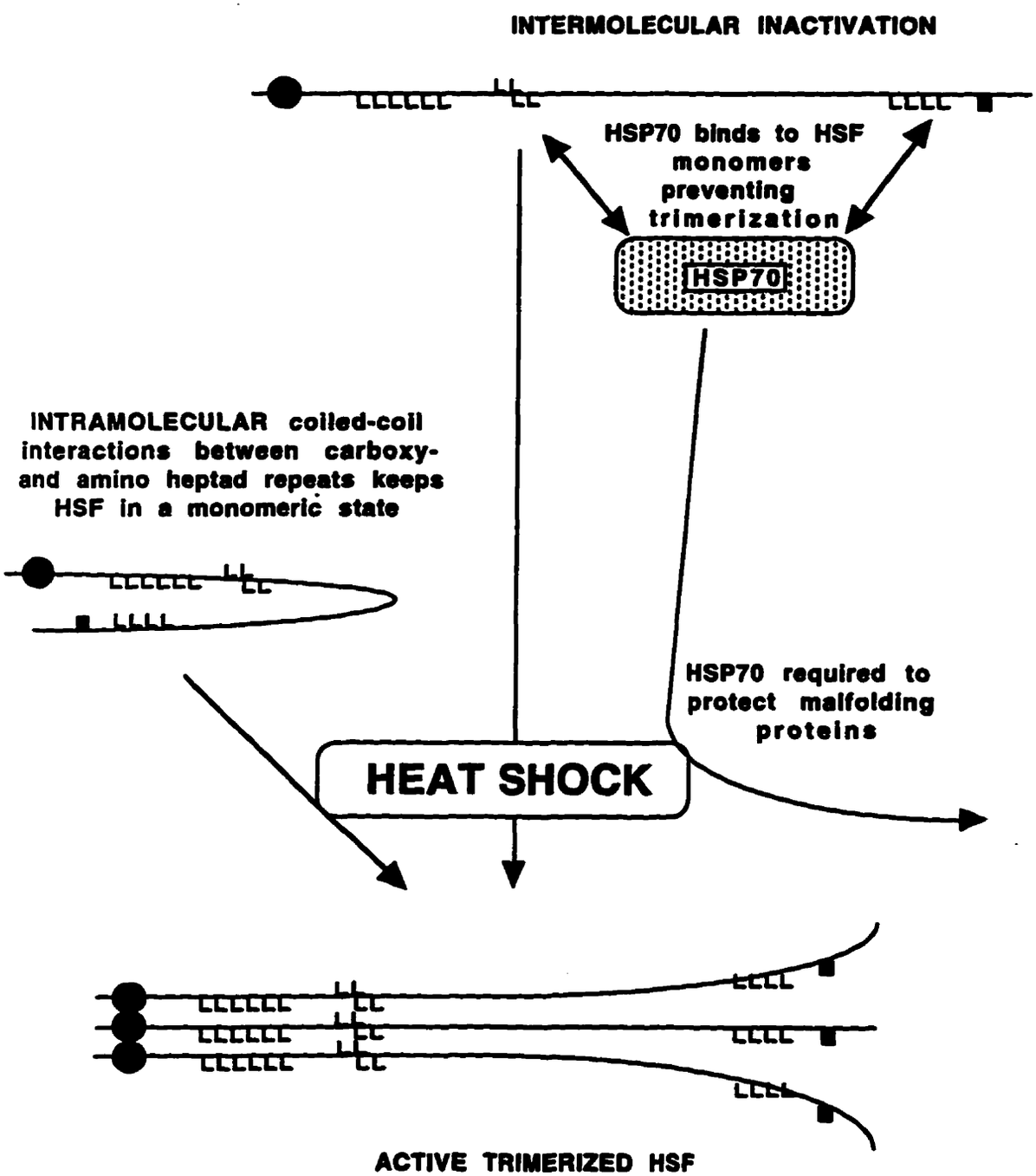
1980; Pelham, 1982). Subsequent studies of this second region in *Drosophila* and other organisms' heat shock genes more specifically defined a heat shock element (HSE) as a pentanucleotide consisting of nGAAn. A functional HSE consists of 3 of these pentanucleotides present in alternating orientations (Amin *et al.*, 1988). The HSE is bound upon heat shock, by a heat shock transcription factor (HSF) (Parker and Topol, 1984). Zimarino and Wu (1987) revealed that HSF binding occurs independently of new protein synthesis, indicating that a pre-existing stable non-HSE-binding pool of HSF is present in unshocked cells. Subsequent work led to the isolation of HSFs from *S. cerevisiae* and *Drosophila* (Wiederrecht *et al.*, 1987; Wu *et al.*, 1987). In 1990, Clos *et al.*, cloned the *Drosophila* hsf gene using degenerate oligos (whose sequences were derived from peptide analysis of the isolated *Drosophila* HSF protein to screen genomic and cDNA libraries). The isolation of additional HSFs from other organisms revealed the presence of only a few conserved domains; specifically, a helix-turn-helix DNA binding domain which recognizes the heat shock element, leucine zippers involved in the trimerization of the monomers, and in eukaryotes, a conserved C-terminal region required to maintain the inactive monomeric state (reviewed by Wu, 1995). While there is high sequence similarity between heat shock proteins in different species, this does not appear to be the case for heat shock factors.

S. cerevisiae and *Drosophila* each have a single heat shock factor (Sorger and Pelham, 1987; Clos *et al.*, 1990). Interestingly, analysis of higher eukaryotic organisms has revealed the presence of multiple heat shock factors: tomatoes, chickens, and humans have three HSFs (Scharf *et al.*, 1990, Nakai and Morimoto, 1993; Rabindran *et al.*, 1991; Schuetz *et al.*, 1991), mice have

two (Sarge *et al.*, 1991) and to date, a single HSF has been isolated in *Arabidopsis* (Hubel and Schoffl, 1994) and *Xenopus* (Stump *et al.*, 1995). Different properties have been associated with the different HSFs. For example, unshocked HSF1 is present as a monomer, whereas inactive HSF2 is present as a dimer. HSF1 appears to be the common HSF which is induced by heat, sodium arsenite and other previously identified stressers, whereas HSF2 does not respond to these inducers but has been found to be activated by hemin induced differentiation in K562 cells. The identification of more than one HSF within a species has opened up many new avenues of research in the regulation of the heat shock response.

Nevertheless, the basic heat shock response seems to be regulated by a single HSF. A model has been proposed to account for its stress-activation (Figure 2). It has been postulated that the carboxyl-terminal leucine zipper associates directly with zippers in the amino-terminal region by intramolecular coiled-coil interactions. Stress-induced activation causes a breakdown of the intramolecular interactions, and trimerization of the HSF monomers can take place unimpeded. The trimerized HSF then accumulates in the nucleus and activates the heat shock genes. It is also possible that interactions with other molecules, such as HSP70, may prevent trimerization (as oppposed to the aforementioned intramolecular coiled-coil interactions) by blocking the oligomerization domain (Morimoto, 1993). Upon heat-shock, the HSP70 molecules would now interact with misfolding proteins, releasing the HSF so that it can trimerize and activate further hsp transcription. Evidence is available for both possibilities (inter vs. intra-molecular interactions), and it has yet to be determined which model accurately reflects the *in vivo* situation.

FIGURE 2. A model of heat shock factor (HSF) regulation. The solid circle represents the amino-terminal DNA binding domain. 'L's represent hydrophobic heptad repeats (zippers). An additional regulatory region is represented by a solid square. In the unstressed cell, HSF is postulated to be maintained in a monomeric, non-DNA binding form either through intramolecular interactions between the amino and carboxy-terminal zippers (Wu, 1995), or via intermolecular interactions between HSP70 (or possibly other molecules) and the monomeric form of HSF (Morimoto, 1993). Upon heat shock or other forms of stress, HSF assembles into a trimer either by the breaking of the intramolecular interactions, or by the release of HSF by HSP70 which now responds to the increased amount of malfolded proteins. The trimerized HSF can now interact with heat shock elements in heat shock gene promoters and activate their transcription. After the heat shock, the trimer dissociates into a monomer, or HSP70-HSF interactions resume. (adapted from Wu, 1995 and Morimoto, 1993).


INTERMOLECULAR INACTIVATION
HSP70 binds to HSF monomers preventing trimerization
HSP70
INTRAMOLECULAR coiled-coil interactions between carboxy- and amino heptad repeats keeps HSF in a monomeric state
HSP70 required to protect malfolding proteins
HEAT SHOCK
ACTIVE TRIMERIZED HSF

1.8 Small HSP expression in *Xenopus laevis* embryos and A6 cells

Research in our laboratory and others has focused on the heat shock response in *Xenopus laevis* (reviewed in Heikkila *et al.*, in press) Many heat shock genes have been isolated from *Xenopus*, including a PCR-amplified genomic DNA fragment of an hsp90 gene (Ali *et al.*, 1996b), four hsp70 genes (Bienz, 1984a,b), two hsc70 cDNAs (Ali *et al.*, 1996a), a BiP78 cDNA (Miskovic *et al.*, in press), 5 hsp30 genes and 2 hsp30 cDNAs (Bienz, 1984a,b; Krone *et al.*, 1992), as well as a heat-inducible ubiquitin cDNA (Dworkin-Rastl *et al.*, 1984). A *Xenopus* heat shock transcription factor (HSF1) has also recently been isolated (Stump *et al.*, 1995).

Bienz (1984) isolated the first cluster of hsp30 genes, comprising hsp30A and hsp30B. Further analysis of these genes revealed that hsp30A produced a truncated protein due to a frame-shift insertion causing a premature stop codon. Hsp30B appears to be a pseudogene which is not expressed. The hsp30A gene was employed to screen a *Xenopus* genomic library resulting in the isolation of an additional hsp30 gene cluster containing functional hsp30C and hsp30D genes, and the amino terminal of the coding region of hsp30E (Krone *et al.*, 1992). Analysis of the expression of hsp30 gene expression during development using Northern blot analysis revealed that hsp30 mRNA is not present constitutively in oocytes or embryos (Krone and Heikkila, 1988, 1989). Hsp30 mRNA was however, first detected in heat-shocked early tailbud stage embryos. More specific analysis of individual hsp30 family members was facilitated by the use of RNase protection assays and reverse transcription-polymerase chain reaction (RT-PCR). These techniques were required due to

the relatively high sequence identity between individual family members, ranging from 75-97% identity. RNase protection and RT-PCR revealed that hsp30A and hsp30C genes are first heat inducible at the early tailbud stage (stages 23-24; Ali *et al.*, 1993). RT-PCR analysis further revealed that hsp30D is first heat-inducible approximately 24 hours later at the mid-tailbud stage (stage 34-35; Ohan and Heikkila, 1995). This differential developmental regulation differs from other heat shock proteins in *Xenopus* development, such as hsp90, hsp70, hsc70, and ubiquitin, which are more typically first expressed following the mid-blastula transition and the activation of the zygotic genome.

Analysis of small HSP protein synthesis has also been carried out in *Xenopus* embryos and A6 cells. One dimensional SDS-PAGE studies of small HSP synthesis have revealed that they are induced by heat shock and sodium arsenite in A6 cells. As many as 16 heat-inducible small HSPs have been detected in A6 cells using 2-D SDS-PAGE (Darasch *et al.*, 1988). An HSP30C antibody was recently produced by injecting a synthetic peptide corresponding to the carboxyl end of the HSP30C protein into rabbits (predicted from the DNA sequence; Tam and Heikkila, 1996). Use of this antibody has confirmed that small HSP synthesis does not occur constitutively, nor is it detectable in heat-shocked developing embryos until the tailbud stage. At the early tailbud stage a single small HSP was present. At the late tailbud stage 5 more small HSPs were recognized by the antibody, and by tadpole a total of 13 were detected. In heat-shocked A6 cells, 8 small HSPs reacted with the HSP30C antibody. The single small HSP first detected at the early tailbud stage was specifically determined to be the HSP30C protein, by microinjecting a chimeric hsp30 gene containing a constitutively active human hsp70 promoter, into cleaving *Xenopus*

embryos. Subsequent immunoblot analysis with the HSP30C antibody of 2-D SDS-PAGE-resolved proteins from microinjected embryos at the gastrula stage (in which no HSP30 proteins are detectable by the antibody), revealed a single HSP30 protein (HSP30C). Mixing experiments permitted the subsequent realization that this protein corresponded to the single small HSP first induced by heat-shock at the early tailbud stage, identifying it as the HSP30C protein. These studies corresponded with the findings at the mRNA level and confirmed the differential expression of small HSPs in *Xenopus* development at the protein level.

1.9 Objectives

As previously mentioned, in our laboratory, a number of different heat shock protein genes have been isolated [including hsp30 (C,D,E), hsp70, hsc70 (I and II), Bip78, hsp90 (PCR-amplified fragment)], and their mRNA accumulation and protein expression characterized to varying extents (reviewed in Heikkila *et al.*, 1996). In the present study, I have endeavoured to expand our knowledge of the small hsp family at the mRNA and protein levels in *Xenopus laevis* embryos and A6 kidney epithelial cells. Specifically, the main objectives of this work were:

1. to examine small hsp mRNA accumulation during *Xenopus* development.

2. to determine if mRNA stability is a potential developmental regulatory mechanism involved in *Xenopus* small hsp gene expression.

3. to characterize a newly discovered set of basic small HSPs in *Xenopus* A6 cells and compare their expression to their acidic counterparts by exposing A6 cells to a variety of known HSP inducers.

4. to detect other basic stress-inducible proteins in *Xenopus* embryos and A6 cells using NEPHGE 2-D SDS-PAGE.

5. to examine heat-induced aggregation in *Xenopus* A6 cells and determine if small HSPs are present in these aggregates.

2. Materials and Methods

2.1 Egg Production, Fertilization, and Embryo Dejelling

Adult *Xenopus laevis* females (Xenopus I, Ann Arbor, Michigan) were induced to ovulate approximately 10-12 h following the injection of 900 I.U. of human chorionic gonadotropin (hCG; resuspended in 0.65% NaCl; Sigma, Mississauga, Ont.) into the dorsal lymph sac. The male's testes were dissected from its abdomen and crushed using forceps in 1 ml of 100% DeBoer's solution [0.11 M NaCl, 1.3 mM $CaCl_2$, 0.44 mM KCl, (pH 7.2); Grey *et al.*, 1982]. The application of gentle pressure to the abdomen of the injected females released hundreds of eggs which were collected in 55x15 mm petri dishes containing 27% DeBoer's solution. The sperm suspension was then mixed with the eggs (approximately 1 testis/1,000 eggs) for 20 min on a rotating shaker (120 rpm; American Rotator V, Canlab, Toronto, Ont.) at room temperature. Following fertilization, the embryos were transferred to a petri dish containing 2% (w/v) cysteine (pH 7.8) dissolved in Steinberg's solution [60 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1.4 mM Tris base pH 7.4] for approximately three min to remove their jelly coating (Dawid, 1965). The cysteine solution was washed away with several rinses of fresh Steinberg's solution in new petri dishes. The embryos were maintained in a 22^{o}C incubator, with periodic changes of fresh Steinberg's solution (75% or 100%). Dead and deformed embryos were removed as they appeared. Developmental stages were determined according to the external criteria delineated by Nieuwkoop and Faber (1967).

2.2 Embryo Treatments: Heat Shock, Cycloheximide, Dissociation

Embryos to be heat shocked (40 embryos for RNA, 20 for protein) were placed into a 35 x 10 mm petri dish containing Steinberg's solution which was then sealed with parafilm. The petri dish was immersed in a heated water bath (30 to 39^{o}C) for various times (1-6 h). Control embryos were maintained in 35 x 10 mm petri dishes in a 22^{o}C incubator. Following heat shock, embryos were collected in a 1.5 ml Eppendorf tube and residual Steinberg's solution was removed. The samples were then stored at -80^{o}C until required.

To inhibit protein synthesis, embryos were bathed in Steinberg's solution containing cycloheximide (Sigma, Mississauga, Ont.) at concentrations ranging from 10 to 200 µg/ml for 1 h. These cycloheximide conditions inhibit [^{35}S]-methionine incorporation into protein by 80-95% (Duval *et al.*, 1990; Kam *et al.*, 1992). The embryos were then rinsed in fresh Steinberg's solution, and heat-shocked as described above, or incubated at 22^{o}C for 1 h after which the samples were frozen at -80^{o}C.

In order to facilitate the entry of cycloheximide, some embryos were first dissociated (Gurdon *et al.*, 1984). Dissociation involved placing the embryos in Steinberg's solution lacking divalent cations [$MgSO_4 \cdot 7H_2O$ and $Ca(NO_3)_2 \cdot 4H_20$], followed by gentle shaking (40 rpm) on a shaker for 3 h. Successful dissociation was confirmed by removal of the vitelline membrane with forceps and by direct observation of the dissociated cells. Cycloheximide and heat shock treatments were then carried out as described above.

2.3 Culturing and Treatment of *Xenopus* A6 Kidney Epithelial Cells

Xenopus A6 kidney epithelial cells (American Type Culture Collection, Rockville, Maryland) were grown at 22°C in 55% Leibovitz L-15 media (Life Technologies, Burlington, Ont.) containing 10% fetal bovine serum (FBS; Life Technologies, Burlington, Ont.), 100 I.U./ml penicillin, and 100 mg/ml streptomycin (Flow Laboratories, McLean, Va.).

Heat shock treatment of A6 cells involved immersing the flask of cells (typically, T75 flasks for RNA, T25 for protein; Fisher, Ottawa, Ont.) sealed with parafilm in a heated waterbath (27 to 39°C) for various times (2 to 12 h). For sodium arsenite treatments, sodium arsenite was dissolved in water at a 100 mM working stock concentration, and added to the A6 cell media at a concentration of 50 µM for various times (2-24 hours) (Darasch *et al.*, 1988). Herbimycin A (Sigma, Mississauga, Ont.) was resuspended in dimethylsulfoxide (DMSO) at a working stock of 1 µg/µl and added to the cell media to final concentrations ranging from 0.5 to 2 µg/ml, for periods of 2 to 6 h (the treatments were carried out in the dark).

Following the treatments, cells were then rinsed with 65% Hank's balanced salt solution (HBSS; Flow Laboratories, Mississauga, Ont.). One and a half milliliters of 100% HBSS was added to the flask and the cells were scraped off of the bottom of the flask using a plastic spatula. The cells were collected in a 1.5 ml Eppendorf tube and pelleted by centrifugation for 5 min at 12,000 rpm (Eppendorf Microcentrifuge, Model 5414). The supernatant was drained and the pellets were stored at -80°C until required.

2.4 RNA Isolation

Total RNA was isolated using a modified form of the guanidine isothiocyanate/cesium chloride method of Chirgwin *et al.* (1979). In order to prevent the degradation of RNA by contaminating RNAses, all glassware was baked at 180°C overnight. All solutions (except those containing Tris or SDS) were made in unautoclaved 0.1% (v/v) diethylpyrocarbonate-treated water [0.1% (v/v) DEPC; ICN, Montreal, Que.]. After a 15 min incubation period, the solutions were autoclaved to neutralize the DEPC. Tris and SDS solutions were prepared in autoclaved 0.1 % (v/v) DEPC-treated water. Plasticware was soaked for 10 min in 3% hydrogen peroxide followed by a final rinse with autoclaved 0.1% DEPC-treated water.

Forty frozen embryos were homogenized in 7 ml of guanidine isothiocyanate buffer [GIT; 4 M guanidine isothiocyanate (ICN, Montreal, Que.), 25 mM sodium acetate pH 6.0, 120 mM ß-mercaptoethanol, in autoclaved 0.1% (v/v) DEPC-treated water] and layered onto a 3.3 ml cushion of filtered cesium chloride buffer [5.7 M cesium chloride (optical grade, ICN, Montreal, Que.), 25 mM sodium acetate pH 6.0, in autoclaved 0.1% (v/v) DEPC-treated water] in Ultra-clear ultracentrifuge tubes (14x89 mm, Beckman, Mississauga, Ont.). To pellet the RNA, the samples were centrifuged at 30,000 rpm for 23 h at 20°C using an SW41 rotor in a Beckman L8-70 ultracentrifuge. Most of the GIT and cesium chloride buffers were then gently removed using a micropipette and the tubes were inverted to drain the last ml of buffer. A hot scalpel was employed to cut the upper portion of the tubes off, approximately 1 cm from the bottom. To remove any remaining cesium chloride, the translucent RNA pellet was rinsed

with room temperature 70% (v/v) ethanol (made with 0.1% DEPC-treated water) and drained for 10 min. The pellet was resuspended in 360 µl of TES buffer [10 mM Tris pH 7.4, 5 mM EDTA, 1% (w/v) SDS] and incubated for 10 min on ice. The resuspended RNA was transferred to 1.5 ml Eppendorf tubes and precipitated with absolute ethanol in the presence of 0.3 M sodium acetate (pH 5.2) for 30 min in dry ice. The samples were then centrifuged at 13500 rpm for 10 min at 4^{o}C in a Hermle Z320K microcentrifuge. The supernatant was drained and the RNA pellet was resuspended in 360 µl of 0.1% (v/v) DEPC-treated water and subjected to a second ethanol precipitation. The final RNA pellet was left to air dry for 15 min after which it was resuspended in 84 µl of 0.1% (v/v) DEPC-treated water. The RNA was aliquoted and stored at -80^{o}C until needed.

Concentration and purity were determined by ultraviolet spectrophotometry (Beckman DU7 spectrophotometer). Concentration was calculated on the basis that 1 O.D.$_{260}$ unit = 40 µg RNA/ml. Purity was based on a 260nm / 280nm ratio greater than 1.8. To confirm that the RNA was not degraded, a volume containing 5 µg of RNA was dried under speed-vacuum for 15 min. The RNA pellet was resuspended in 2.4 µl of 0.1% (v/v) DEPC-treated water, 1.0 µl 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], 1.6 µl formaldehyde, 5.0 µl formamide, and 2 µl loading dye [0.2% (w/v) bromophenol blue, 1 mM EDTA (pH 8.0), and 50% (v/v) glycerol]. The RNA was denatured by heating at 65^{o}C for 5 min followed immediately by rapid cooling on ice for an additional 5 min. The samples were then resolved by electrophoresis in a 1.2% (w/v) formaldehyde agarose gel [1.2% (w/v) agarose, 10% (v/v) 10 X MOPS, and

16% (v/v) formaldehyde] containing 0.5 μg/ml ethidium bromide, for 3 to 4 h at 80 V. The 1 X MOPS running buffer was rotated between the electrodes every 45 min to avoid pH changes. Intact RNA was indicated by strong 28S and 18S ribosomal bands, with the 28S band appearing approximately twice as intense as the 18S band, and very little smearing. The presence of equal levels of ethidium bromide staining between samples further confirmed the prior concentration determination by ultraviolet spectrophotometry.

2.5 Northern Blotting

Fifteen micrograms of each RNA sample, and 3.5 μg of an RNA ladder (Gibco, BRL, Burlington, Ont.) were prepared and electrophoresed as described in Section 2.4; however in this case, ethidium bromide was omitted. Following electrophoresis, the RNA ladder lane was cut from the gel and stained in concentrated ethidium bromide for 5 min. The ladder was destained in water overnight, illuminated by ultraviolet light, and photographed with a Polaroid Land Camera. The remainder of the gel was rinsed twice in 0.1% (v/v) DEPC-treated water for a total of 10 min. This was followed by a 10 min wash in 10 mM sodium phosphate buffer (pH 7.0). A corner of the gel was cut for orientation. The gel was inverted onto a presoaked 3MM Whatman filter paper wick lying on a glass plate positioned over a Pyrex dish filled with 20 X SSC (pH 7.0) [3 M sodium chloride, 300 mM sodium citrate]. A piece of nylon membrane (Amersham, Oakville, Ont.) with a corner cut for orientation, was layered onto the gel. Bubbles were removed by rolling a Pasteur pipette along the membrane. Two pieces of 3MM Whatman paper were layered onto the

nitrocellulose (bubbles rolled out from each layer), followed by a 10 cm stack of paper towels. The structure was wrapped in Saran Wrap to prevent evaporation and a weight was placed on top to secure it. The transfer proceeded overnight and the wet paper towels were replaced with fresh ones in the morning. After an additional few hours, the layers of paper towels and 3MM Whatman paper up to the nylon membrane were removed, and the gel and nylon were inverted together. Lanes were marked through the wells with a pencil and the gel was discarded. The nylon membrane was placed RNA-side up in an ultraviolet cross-linker (GS Gene Linker, program 'C3' 150 mJ; BioRad, Mississauga, Ont.) and the RNA was cross-linked to the membrane. The Northern blot was stored in a plastic bag at room temperature, until ready for hybridization.

2.6 Nick Translation and Hybridization

The Northern blot was placed inside a plastic bag and 20 ml of pre-hybridization buffer {50% (v/v) formamide, 5 X SSC, 10 mM sodium phosphate buffer pH 7.0, 2.5 X Denhardt's solution [0.05% (w/v) bovine serum albumin (BSA), 0.05% (w/v) polyvinylpyrrolidone, 0.05% (w/v) ficoll], and 250 µg/ml denatured (boiled for 5 min) herring sperm DNA} were added. Air bubbles were removed and the bag was sealed using a Seal-a-Meal bag sealer (Canadian Tire, Kitchener, Ont.). The blot was incubated overnight at 42^{o}C in a water-filled container pre-equilibrated at 42^{o}C, with periodic shaking.

Nick translation was employed to label the DNA probe. One hundred to three hundred nanograms of DNA probe was added to 1 µl of 10 X nick translation buffer [500 mM Tris pH 7.5, 100 mM $MgSO_4 \cdot 7H_2O$, 1 mM

dithiothreitol, and 0.5 µg/ml BSA], 1 µl 2 mM dNTP (dATP, dGTP, dTTP), 5 µl deoxycytidine 5'-[^{32}P]-triphosphate (ICN; sp. act. 3,000 Ci/mmol), 1 µl DNA polymerase I (5 U/µl; Boehringer Mannheim, Laval, Que.) and 1 µl of DNase I (0.05 µg/ml). The reaction was incubated at 14^{o}C for 90 min after which the reaction was stopped by the addition of 2.5 µl 0.5 M EDTA (pH 8.0) and 87.5 µl sterile water. The labeled probe was then separated from unincorporated label by passing it through a 1 ml Sephadex G-50 column (ICN, Montreal; Que.) prepared in a 1 cc syringe. The reaction volume was loaded onto the top of the column and centrifuged at 3,000 rpm in an IEC clinical centrifuge. The labeled probe was collected in a 1.5 ml Eppendorf tube and was used immediately in the hybridization reaction.

The sources of DNA probe that were utilized during the course of this work are as follows: To detect hsp30 mRNA, the genomic subclone of the entire *Xenopus laevis* hsp30C or hsp30D genes (Krone *et al.*, 1992) was used. Hsp70 mRNA was detected by the entire hsp70 gene (pXL 16P; gift of Dr. M. Bienz, MRC Laboratory of Molecular Biology, Cambridge, UK; Bienz, 1984). The hsp87 probe was a genomic PCR fragment amplified from *Xenopus* genomic DNA (Ali, *et al.*,1996). A *Xenopus* cytoskeletal actin cDNA clone (pX1cA1; gift of T. Mohun, Department of Zoology, University of Cambridge, UK; Mohun *et al.*, 1983) was used to detect actin mRNA. A *P. lucida* hsp27 cDNA clone which was also utilized was a generous gift of Dr. L. Hightower (University of Connecticut).

The labeled probe DNA was added to 0.5 ml of 10 mg/ml herring sperm DNA (250 µg/ml final concentration; prepared according to Maniatis) and boiled for 5 min to denature the DNA. After boiling, the DNA was placed on ice for 5 min to cool. It was then added to 19.5 ml of hybridization buffer [50% (v/v)

formamide, 4 X SSC, 10 mM sodium phosphate buffer pH 7.0, 2.5 X Denhardt's solution, and 7.5% (w/v) dextran sulphate). The pre-hybridization buffer was drained through a cut corner of the bag and the hybridization buffer added in its place. Bubbles were removed and the bag was resealed. The reaction was incubated at 42°C for 48 h, with periodic shaking.

The hybridization buffer was drained and the blot was rinsed in increasingly stringent post-hybridization washes. Two washes over a total of 15 min were carried out at room temperature in 1 X SSC and 0.1% (w/v) SDS. This was followed by a 15 min and a 5 min wash at 42°C in 0.5 X SSC and 0.1% (w/v) SDS. Finally, two 5 min washes were carried out at 42°C in 0.1 X SSC and 0.1% (w/v) SDS. The radioactivity of the blot was monitored with a Geiger counter throughout the series of washes to prevent too stringent a washing.

The blot was placed onto a glass plate and wrapped in Saran Wrap. In the darkroom, Kodak XAR-5 film was placed on top of the blot, followed by a Cronex intensifying screen (VWR, Toronto, Ont.). The layers were sandwiched between two thick pieces of cardboard, clamped together and wrapped in black garbage bags. The film was exposed overnight at -80°C. For developing, the assembly was removed from the freezer and as quickly as possible, the film was placed into Kodak developer for 4 min, followed by a 1 min rinse in deionized water and 4 min in Kodak fixer. The autoradiogram was finally rinsed in deionized water and air dried. Re-exposure was then timed according to the observed strength of the signal in the first exposure.

In some cases, a blot was stripped in order to be re-hybridized with additional DNA probes. Stripping of the blot involved soaking it in boiling 0.1% (w/v) SDS for 5 min, the solution was drained, after which fresh boiling 0.1%

(w/v) SDS was added, and the blot soaked until the solution cooled to room temperature. The stripped blot was monitored with a Geiger counter, and/or exposed to X-ray film to check for residual radioactivity. The blot was then pre-hybridized and hybridized as described above.

Autoradiograms to be analyzed by densitometry were scanned using the Macintosh OneScanner. Densitometric analysis of the resultant computer images were carried out using NIH Image 1.55.

2.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.7.1 Reverse Transcription

Reverse transcription of total RNA was carried out according to the method of Rappolee *et al.* (1989) as modified by Shultz (1991). One microgram of lyophilized RNA was resuspended in 5 µl of RNAguard-treated (40 U/l; Pharmacia, Uppsala, Sweden) water. Five microliters of reverse transcription (Rt) master mix#1 [0.2 µl oligo dT (1 µg/µl), 1.0 µl 10 mM dNTPs, 0.5 µl 2 mg/ml BSA, 0.1 µl of 1 M DTT, 2.0 µl 5 X Rt buffer (250 mM Tris pH 8.3 at 43°C, 300 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 0.125 µl RNAguard, 24 units avian myeloblastosis virus reverse transcriptase (AMV-RT, high concentration; Promega, Ottawa, Ont.)] was added to the resuspended RNA, mixed, and briefly centrifuged. The reaction was incubated at 43°C for 1 h. The cDNA was then heat denatured for 3 min at 95°C and immediately quenched on ice. Two microliters of Rt master mix#2 (0.1 µl 10 mM dNTP, 0.05 µl 2 mg/ml BSA, 0.4 µl 5 X Rt buffer, 1 µl RNAguard water, and 15 units AMV-RT) was added and

mixed as before. Again, the reaction proceeded at 43°C for 1 h. Following this, the volume was diluted to 30 µl by the addition of 18 µl of RNAguard water and the cDNA was used immediately in the polymerase chain reaction. The remainder was stored at -20°C and could be used up to 2 to 3 days later.

2.7.2 Polymerase Chain Reaction

The polymerase chain reaction protocol was based on that of Rappolee *et al.*, (1989, 1990). Appropriate precautions were utilized to prevent carry-over contamination of PCR products (Kwok and Higuchi, 1989). A PCR master mix [4.8 µl of 10 X PCR buffer (100 mM Tris pH 8.3, 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml BSA, per sample] was prepared and aliquots for each sample made. PCR primers (1.5 µl of each primer, to a final concentration of 0.35 µg/µl) was added to each sample. The primers utilized were as follows:

5'-Hsp30D: 5' ATCTCCAGCTGTTCTCTGAT 3'

3'-Hsp30D: 5' GGGGATCTTGGACTGTTGC 3'

The expected hsp30D PCR fragment was 284 base pairs.

5'-Actin: 5' CATCCGTAAGGACCTGTATGCC 3'

3'-Actin: 5' CAACGCATCTGCTAGCAGTCCA 3'

The expected PCR fragment was 290 bp using a cDNA template and 369 bp using a DNA template (due to the presence of a 79 bp intron). Primers were designed with the aid of the Oligonucleotide Synthesizing Program (Hillier and Green, 1991) and synthesized by the Department of Genetics at the University of Guelph, Guelph, Ontario.

Following the addition of the primers, 3 µl of the freshly synthesized cDNA was added and the contents of the tube mixed. The samples were overlaid with 50 µl of mineral oil and placed in the heating block of a Perkin-Elmer Cetus DNA thermocycler. The thermal profile involved an initial denaturation period at 94°C for 4 min, during which one unit of Taq polymerase (Pharmacia, Uppsala, Sweden; diluted in PCR master mix to a final volume of 1 µl) per sample was added to create a hot start (Chou *et al.*, 1992). This was followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. A final period at 72°C for 7 min permitted the complete extension of the amplified DNA. PCR products were stored at 4°C.

PCR products were resolved on a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 100 V for 2 h in 1 X Tris-acetate-EDTA buffer. A 123 bp DNA ladder (BRL, Burlington, Ont.) was used to size the fragments.

2.8 Southern Blotting

Southern blots were prepared according to the method of Maniatis *et al.*, (1989). Following electrophoresis of the PCR products, the gel was washed for 45 min in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) on a rotating platform. The gel was then rinsed briefly with distilled water and transferred to a dish containing neutralization buffer (1 M Tris pH 8.0, 1.5 M NaCl) for an additional 45 min. Transfer of the DNA to nylon membrane, nick translation, (pre- and post-) hybridization, and autoradiography were carried out as described in Northern blotting.

2.9 Protein Radiolabeling

Embryos and A6 cells were radiolabeled with ^{35}S-methionine (Trans ^{35}S-label, specific activity> 1,000 Ci/mmol; ICN, Montreal, Que.) during the last 2 or 4 h of treatment periods in order to detect newly synthesized proteins. Embryos prior to the tailbud stage were microinjected with 0.5 µCi of ^{35}S-methionine in a volume of 50 nl using a Nanojet microinjector (Drummond Scientific, Broomall, PA). Embryos beyond the tailbud stage were bathed in Steinberg's solution containing 100 µCi/ml of ^{35}S-methionine.

In the case of A6 cells growing in T25 flasks, their media was drained and the flasks rinsed twice with 1.5 ml of methionine-free L15 media. They were then brought to a volume of 2 ml of methionine-free media and ^{35}S-methionine was added to a final concentration of 50 µCi/ml. A6 cells that were also being exposed to various chemical inducers were replenished with fresh inducer at this point.

2.10 Protein Isolation

Embryos to be used for protein isolation were collected in 1.5 ml Eppendorf tubes, and excess Steinberg's solution was removed. The embryos were stored frozen at -80^{o}C. A6 cells were collected as described previously in Section 2.3.

For two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2-D SDS-PAGE - both IEF and NEPHGE), proteins were isolated from embryos and A6 cells as described in Smith (1986) and modified

by Winning *et al.*, (1991). A 100 µl volume of modified 2-D lysis buffer [O'Farrell, 1975; 9.5 M Urea (ICN, Montreal, Que.), 0.1% SDS, 1.6% (v/v) ampholines (BioRad, Mississauga, Ont.) pH 5-7, 0.4% (v/v) ampholines pH 3-10, 5% (w/v) ß-mercaptoethanol (Sigma, St. Louis, Mo.)] was added to the frozen samples, and they were homogenized using 10 strokes with a plastic pestle. The samples were then sonicated using a Branson Sonifier-200 (Branson Sonic Power Co., Danbury, Co.) using ten one second pulses at 20 W (output = 4, 65% duty cycle). The samples were then incubated on ice for 10 min, after which an equal volume of unmodified 2-D lysis buffer, containing 2% (w/v) Nonidet P40 (NP-40; Sigma, Mississauga, Ont.) in place of 0.1% SDS was added. The samples were centrifuged for 5 min at 12,000 rpm to remove the yolk (from embryos) and any remaining fragments. The samples were stored at -80 ^{o}C until required.

2.11 2-D SDS-PAGE

2.11.1 Isoelectric Focusing (First Dimension)

Isoelectric focusing (IEF) was used to resolve proteins with an isoelectric point (pI) of 5 to 7, and was based on the methodology of O'Farrell (1975). IEF capillary tubes were placed in a casting tube (BioRad, Mississauga, Ont.) sealed on the bottom with several layers of parafilm. A mixture of 8 M urea, 4% acrylamide, 2% (w/v) NP-40, 1.5% (v/v) ampholytes pH 5-7, and 0.5% ampholytes pH 3-10 was warmed to dissolve the urea. The gel mixture was then degassed for 5 min and freshly prepared 0.2% ammonium persulfate, and 0.14% (v/v) TEMED was added to initiate polymerization. Immediately, using a

long Pasteur pipette, the gel mixture was added near the bottom of the casting tube so the liquid filled the capillary tubes from the bottom. The tubes were filled until approximately halfway between the blue line which acts as a reference for the orientation of the capillary tube, and the top of the tube (0.25 cm from the top). The top of the casting tube was sealed with parafilm and allowed to polymerize overnight. The next day, the capillary tubes were wiped clean of excess acrylamide and a 50 µl Hamilton syringe was used to remove unpolymerized acrylamide from the top of the gels. Tube connectors and reservoirs were then attached to the capillary tubes and inserted tightly into the Mini-Protean II tube gel module using pliers. The module was placed into an electrophoresis tank containing 800 ml of degassed 0.06% (w/v) phosphoric acid. Bubbles were removed from the bottoms of the capillary tubes using a Pasteur pipette with a curved tip. Degassed 0.2 M NaOH was added to the reservoirs of the capillary tubes using a 50 µl Hamilton syringe avoiding the introduction of air bubbles. The remainder of the upper chamber of the module was filled with degassed 0.2 M NaOH (approximately 100 ml). Samples were added to the surface of the tube gels using a Hamilton syringe (again avoiding introducing bubbles). The samples were overlain with 30 µl 2-D overlay buffer [5 M urea, 1.8% (v/v) ampholytes pH 5-7, 0.2% (v/v) ampholytes pH 3-10]. Electrophoresis was carried out for 15 min at 500 V followed by 4-5 h at 750 V. Following electrophoresis, the ends of the samples were wrapped in parafilm to prevent dehydration, and stored at -20^{o}C. The second dimension (SDS-PAGE) is described following NEPHGE.

In order to determine isoelectric points, 2 blank gels were prepared as, and run alongside, the samples. Following isoelectric focusing, the blanks were

extruded, rinsed with deionized water, cut into 0.5 cm pieces and placed in 0.5 ml distilled water in 1.5 ml Eppendorf tubes. The next day, a pH meter was used to determine the pH of the water and thus the pH range over the length of the tube gels.

2.11.2 Non-equilibrium pH Gradient Electrophoresis (NEPHGE; First Dimension)

The resolution of more basic proteins (pH 7 to 9) was accomplished using the non-equilibrium pH gradient electrophoresis method of O'Farrell *et al.* (1977). NEPHGE capillary tubes were placed in a casting tube (BioRad, Mississauga, Ont.) sealed on the bottom with several layers of parafilm. A mixture of 9 M urea, 5% acrylamide, 2% (w/v) NP-40, and 1% (v/v) ampholytes pH 3-10 was warmed to dissolve the urea. The gel mixture was then degassed for 5 min and freshly prepared 0.2% ammonium persulfate, and 0.14% (v/v) TEMED was added to initiate polymerization. Immediately, using a long Pasteur pipette, the gel mixture was added near the bottom of the casting tube so the liquid would fill the capillary tubes from the bottom. The top of the casting tube was sealed with Parafilm and the gels polymerized for 1 h. The capillary tubes were wiped clean of excess acrylamide and a Hamilton syringe was used to remove unpolymerized acrylamide from the tops of the tubes. Tube connectors and reservoirs were then attached to the capillary tubes and inserted into the Mini-Protean II tube gel module. The module was placed into an electrophoresis tank containing 800 ml of degassed 20 mM NaOH. Bubbles were removed from the bottoms of the capillary tubes using a Pasteur pipette

with a curved tip. Samples were added to the surface of the tube gels using a Hamilton syringe (avoiding introducing bubbles). The samples were overlain with 10 µl NEPHGE overlay buffer [9 M urea, 0.32% (v/v) ampholytes pH 5-7, 0.08% (v/v) ampholytes pH 3-10, and methyl green]. Degassed 10 mM H_3PO_4 was added to the reservoirs and the upper chamber of the gel module, again avoiding introducing bubbles into the reservoirs. The electrodes were connected in reverse polarity, and electrophoresis was carried out at 500 V for 1 to 2 h (average time was 1 h and 25 min). Following electrophoresis, the ends of the samples were wrapped in parafilm and stored at -20^{o}C.

2.11.3 SDS-PAGE (second dimension)

The second dimensions for IEF and NEPHGE gels were identical. SDS-PAGE gels were cast in a multi-casting chamber (BioRad, Mississauga, Ont.). One millimeter thick, 12% separating gels were prepared from a 30% (w/v) acrylamide stock [29.2% (w/v) acrylamide, 0.8% (w/v) n'n'-BIS methylene acrylamide], 4 X separating buffer (1.5 M Tris pH 8.8; 0.375 M working concentration) and 1% (v/v) SDS [10% (w/v) stock solution]. The mixture was degassed and 0.2% (v/v) ammonium persulfate [10% (w/v) stock] and 0.14% (v/v) TEMED were added to initiate polymerization. The gel mixture was immediately added to one end of the gel sandwiches already prepared in the multi-casting chamber. Each gel was overlayed with 250 µl of water-saturated butanol to create a smooth surface. After 30 min, the polymerized gels were rinsed with deionized water and inserted into Mini Protean II gel rigs (BioRad, Mississauga, Ont.). A 4% stacking gel was prepared from the aforementioned

30% (w/v) acrylamide, 1% (v/v) SDS [10% (w/v) stock], and, in this case, 4 X stacking buffer (0.5 M Tris pH 6.8; 0.125 M working concentration). The gel mixture was degassed for 5 min and ammonium persulfate and TEMED were added. Using a Pasteur pipette, the stacking gel mixture was layered on to the separating gel. A 2-D comb was then quickly inserted to generate a large well for the IEF gel and a small well for a protein size standard.

Following polymerization of the stacking gel (approximately 1 h), the IEF or NEPHGE tube gels were thawed, and extruded, using a BioRad tube gel ejector, into the large well. A spatula was used to gently push the tube gel into contact with the stacking gel, avoiding bubbles. A 250 µl volume of 1 X Laemmli buffer [0.0625 M Tris pH 6.8, 10% (w/v) glycerol, 2% SDS, 5% (v/v) ß-mercaptoethanol, 0.00125% (w/v) bromophenol blue] was added to the gel, and any remaining air bubbles were removed. The gel was left for at least 5 min to equilibrate in the 1 X Laemmli buffer. The gel apparatus was then assembled and electrophoresis buffer (25 mM Tris, 0.2 M glycine, 1 mM SDS) was added to the tanks. Air bubbles were removed from below the gels. Protein size standards (BioRad, Mississauga, Ont.) were loaded into the reference well [pre-stained standards for gels to be Western blotted (see below), and unstained, diluted (1:20), and boiled (5 min) broad range size standards for those to be dried]. Electrophoresis was carried out at 200 V until the dye front reached the bottom of the gel (approximately 45 min).

Gels to be stained were bathed on a rotating shaker in 40% methanol and 10% acetic acid, containing 0.1% Coomassie Blue R-250 (BioRad, Mississauga, Ont.) for 30 min followed by destaining in 5% methanol and 10% acetic acid, overnight. The destained gels were then treated with the

fluorophore Entensify (NEN-Dupont, Boston, MA; 40 min in Entensify A, followed by 40 min in Entensify B). The gels were laid out onto 3MM Whatman paper and the surface covered with Saran Wrap. The gels were dried under vacuum at 80^{o}C for 1 h using a BioRad Slab Gel Dryer. The Saran Wrap was removed and the dried gels were exposed to Kodak XAR-5 X-ray film at -80^{o}C.

2.12 Western Blotting

Following electrophoresis, gels to be prepared for Western blot analysis were rinsed in cold transfer buffer [4^{o}C; 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol]. Similarly, nitrocellulose membrane (0.45 μm; Amersham, Oakville, Ont.), several pieces of 3MM Whatman paper, and filter pads were rinsed in cold transfer buffer. The black half of a gel holder cassette was then placed flat in a pyrex dish containing cold transfer buffer. A filter pad followed by two layers of 3MM Whatman paper were layered onto the cassette, with bubbles smoothed out after each layer. The equilibrated gel was then placed inverted onto the layers of 3MM Whatman paper, followed by a piece of equilibrated nitrocellulose membrane. Two additional layers of 3MM Whatman paper, and finally a filter pad completed the sandwich. Bubbles were smoothed out of each layer. The cassette was clamped closed and placed into a cassette holder, with the black portion of the cassette facing the black portion of the cassette holder (the gel oriented towards the cathode). The cassette holder was placed into a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, Mississauga, Ont.) and an ice pack was placed alongside it. The electrophoresis chamber was filled

with cold transfer buffer and the buffer was mixed continuously by a stir bar. The transfer took place in a cold room (4^{o}C), at 100 V for 1 h.

Following the transfer, the nitrocellulose membrane was examined for the presence of the pre-stained protein size standards, and monitored for radioactivity (in the case of radioactive protein samples), as evidence for successful transfer. The gels were stained with Coomassie blue (as described previously) as a final monitor for successful protein transfer.

2.13 Immunological Detection Using an HSP30C Polyclonal Antibody

In order to detect the presence of HSP30 protein, the aforementioned Western blots were first soaked in a blocking solution [phosphate-buffered saline pH 7.4 (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 5% (w/v) skim milk powder, 1% (v/v) normal goat serum (Cedarlane Laboratories, Hornby, Ont.), 0.05% Tween 20 (Sigma, Mississauga, Ont.)] overnight, with gentle shaking. The blocking solution was drained, followed by the addition of the primary antibody [polyclonal HSP30C antibody, at a dilution of 1:1,000; Tam and Heikkila, 1996; or, hamster anti-αB-crytallin rabbit antibody (L_4R_3; 1:1,000 dilution; gift of Dr. J. Landry); or, anti-HSP70 mouse monoclonal antibody (clone 5a5; 1:5,000 dilution, as recommended by the supplier; Affinity Bioreagents, Neshanic Station, NJ)] in PBS, 1% (w/v) skim milk powder, and 0.05% Tween 20. The blots were exposed to the primary antibody for at least 6 h. The blots were then washed 3 times with 1% skim milk powder and 0.05% Tween 20 to remove unbound primary antibody. Following the washes, the

blots were incubated with a secondary antibody [for the HSP30C and αB-crytallin antibodies, the secondary antibody was an anti-rabbit alkaline phosphatase conjugate used at a dilution of 1:10,000; for the HSP70 antibody, an anti-mouse alkaline phosphatase conjugate at a 1:1,000 dilution was employed (Sigma, Mississauga, Ont.)], in a solution of 1% (w/v) skim milk powder, and 0.05% Tween 20, for at least 6 h. The blots were then washed twice as before, and twice in pH 9.5 buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). Finally, the color reaction with the alkaline phosphatase conjugated to the secondary antibody was carried out by incubating the blots in 15 ml of pH 9.5 buffer with 66 µl of nitroblue tetrazolium chloride (NBT) and 40 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 10 min with gentle shaking. The blots were transferred to a solution of 20 mM Tris (pH 7.5) and 5 mM EDTA for 30 min to stop the colour reaction. The blots were then air dried.

2.14 Pore Exclusion Limit Electrophoresis

Proteins were isolated from control (22 ^{o}C) and heat-shocked A6 cells (T25 or T75 flasks; 4 h at 35^{o}C) which had been labeled with 50 µCi/ml of ^{35}S-methionine for the duration of the heat shock. Immediately following the treatment, the A6 cells were collected as described previously, and 75 µl of freshly prepared protein isolation buffer [10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM ß-mercaptoethanol, 2 mM dithiothreitol, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 2 mM PMSF (prepared in ethanol)] was added per sample. The cell pellet was resuspended and sonicated with 10, 1 s pulses, as described previously. Cell debris was pelleted by centrifugation in an Eppendorf

microcentrifuge at 12,000 rpm for 5 min. The supernatant was transferred to a fresh tube and an equal volume of 2 X Laemmli buffer lacking SDS and ß-mercaptoethanol was added to the sample.

The protein samples were resolved on a previously prepared 2-22.5% (0-12.5% glycerol) gradient gel. The gradient gel was prepared from degassed, chilled, light [2% acrylamide, 0.375 M Tris pH 8.8] and heavy [22.5% acrylamide, 0.375 M Tris pH 8.8, and 12.5% glycerol] acrylamide solutions which were loaded into the mixing chamber and reservoir, respectively, of a BioRad gradient maker. APS and TEMED were added to initiate polymerization, and the acrylamide solutions were mixed in the mixing chamber of the gradient maker by a stir-bar as the solution was pumped into a BioRad multicasting chamber via a peristaltic pump. The liquid filled the gel sandwiches (1.0 mm spacers; with 1.0 mm 1-D combs positioned at the top) in the chamber from the bottom-up, generating 2-22.5% (0-12.5% glycerol; top to bottom) continuous acrylamide gradient gels. The gels were left to polymerize for 1 h, after which the combs were removed and the wells were washed with deionized water and drained. The gels were then chilled at 4^{o}C.

The gels were assembled [in a cold room (4 ^{o}C)], as described previously for 2-D gels, except that the 5 X running buffer was prepared without any SDS (i.e. just Tris and glycine). Volumes of up to 40 µl of protein were loaded into the wells. A size standard (67 kDa-669 kDa; Pharmacia, Uppsala, Sweden) was dissolved in 100 µl of electrophoresis buffer and 1% bromophenol blue and 5 µl was loaded onto the gel. Electrophoresis was carried out for 48 h at 100 V to separate proteins to their pore exclusion limits (Helm *et al.*, 1993; Anderson *et al.*, 1972). Following electrophoresis the gels

were stained (as described previously for 2-D gels) for 10 min in a cold room. In some cases gels were then destained overnight, entensified and dried down for autoradiography, as described previously.

In order to examine the composition of the aggregates by one dimensional analysis, some native gels were destained for 10 min to permit visualization of the proteins. Regions of interest were then excised with a razor blade (0.5 cm x lane width) and the gel pieces frozen in Eppendorf tubes at -80^{o}C. In order to denature and extract the proteins from the gel pieces, the fragments were homogenized in 40 µl of 1 X Laemmeli buffer (containing SDS and ß-mercaptoethanol) with a plastic pestle and left to soak for 10 min on ice. The samples were subsequently boiled for 10 min, cooled on ice, and an additional 40 µl of 1 X Laemmeli buffer was added. Finally, 40 µl of the samples were loaded with a pipetteman onto a one-dimensional denaturing 12% acrylamide gel (with a 4% stacking gel). A pre-stained low molecular weight SDS size standard was loaded and the samples electrophoresed and immunoblotted, as previously described.

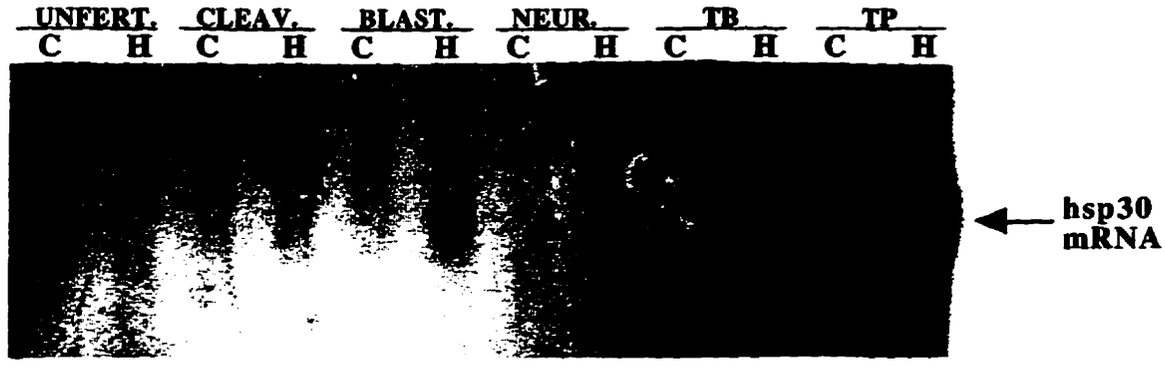
In order to examine the aggregate components by 2-D SDS-PAGE, gel pieces were excised as described above, but in this case, the pieces were homogenized in 40 µl of modifed 2-D isolation buffer (as described previously for 2-D SDS-PAGE). A 40 µl volume of unmodified 2-D isolation buffer was added and the sample was loaded onto capillary tube gels and resolved by 2-D SDS-PAGE as described previously.

3. Results

3.1 Detection of relatively low levels of hsp30 mRNA in late blastula embryos.

In previous Northern blotting studies we were unable to detect any heat-inducible hsp30 accumulation until the early tailbud stage (Krone and Heikkila, 1988). RNase protection assays and RT-PCR have specifically identified that hsp30A and hsp30C are first heat-inducible at the early tailbud stage (stage 23-24), while hsp30D is first heat-inducible approximately one day later at the mid-tailbud stage (stages 34-35; Krone and Heikkila, 1989; Ali *et al.*, 1993; Ohan and Heikkila, 1995). Current improvements to the RNA isolation protocol and the sensitivity of the Northern blot analysis methodology, including the use of ultraviolet light to cross-link RNA to the nylon blot (Nierzwicki-Bauer *et al.*, 1990) and higher specific activity probes, prompted a re-examination of hsp30 gene expression during *Xenopus* development. While hsp30 mRNA accumulation was not detected in either control or heat-shocked unfertilized eggs or cleavage stage embryos, we did detect relatively low levels of heat-inducible hsp30 mRNA at the late blastula stage using a radioactively labeled hsp30C genomic probe (Figure 3). The relative levels of this heat-inducible pre-tailbud (PTB) hsp30 mRNA increased slightly at the neurula stage and then increased dramatically at the early tailbud and tadpole stages. It should be noted that the autoradiogram displayed in Figure 3 was exposed for a sufficient period of time to allow for the detection of hsp30 mRNA in blastula and neurula samples. These conditions resulted in an overexposure of the tailbud and tadpole

FIGURE 3. Detection of low levels of hsp30 mRNA accumulation in heat-shocked late blastula stage embryos. Total RNA was isolated from control (C) and heat-shocked (H; 1 h at 33°C) unfertilized eggs (UNFERT.), cleavage (CLEAV.), late blastula (BLAST.), neurula (NEUR.), tailbud (TB; stage 24-25), and tadpole (TP) *Xenopus* embryos. Northern blot analysis was carried out employing a [^{32}P]-labeled hsp30C clone. The arrow indicates the position of the 1.1 kb hsp30 mRNA. This autoradiogram is representative of five experiments.


UNFERT.
CLEAV.
BLAST.
NEUR.
TB
TP
C H C H C H C H C H C H
hsp30
mRNA

signals. Densitometric measurements on appropriately exposed autoradiograms indicated that the level of hsp30 mRNA in tailbud embryos was at least 20 times greater than found in late blastula embryos (Figure 4). This ratio was even greater (> 40-fold) when comparing the tadpole and blastula samples. These results suggest that selected hsp30 genes (other than hsp30A, hsp30C, and hsp30D which have been previously shown to be first heat-inducible at the tailbud stages using RNase protection assays and RT-PCR; Ohan and Heikkila, 1995; Ali *et al.*, 1993; Krone and Heikkila, 1989) which can cross-hybridize with the hsp30C clone are heat-inducible at relatively low levels before the tailbud stage of development.

3.2 Stabilization of PTB hsp30 mRNA at high heat shock temperatures and by cycloheximide treatment

In order to further characterize PTB hsp30 gene expression in gastrula embryos, the effect of various heat shock temperatures was examined (Figure 5). Surprisingly, incubation of embryos at 37°C resulted in a dramatic accumulation of PTB hsp30 mRNA (Figure 5A). Slightly enhanced accumulation of hsp30 mRNA was also found at 36°C (data not shown). While PTB hsp30 mRNA was not detectable in 30°C samples, hsp30 mRNA was observed in 33°C and 34°C samples only after much longer film exposure times (data not shown). In contrast to the above results, hsp70 mRNA was first detected at 30°C, peaked at 34°C and declined thereafter (Figure 5B). These trends are clear from densitometric analysis of the autoradiograms (Figure 6). Both hsp30

FIGURE 4. Densitometric analysis of heat-induced hsp30 mRNA accumulation during *Xenopus* development. The representative autoradiogram shown in Figure 3 was scanned using the Macintosh OneScanner. Densitometric analysis of the resultant computer image was carried out using NIH Image 1.55. The obtained values were expressed as a percentage of the maximum mRNA accumulation (in this case the tadpole stage). The shaded areas represent the heat shock samples from unfertilized eggs (unfert.), cleavage (cleav.), blastula (blast.), neurula (neur.), tailbud (TB), and tadpole (TP) stages.

Hsp30 mRNA accumulation

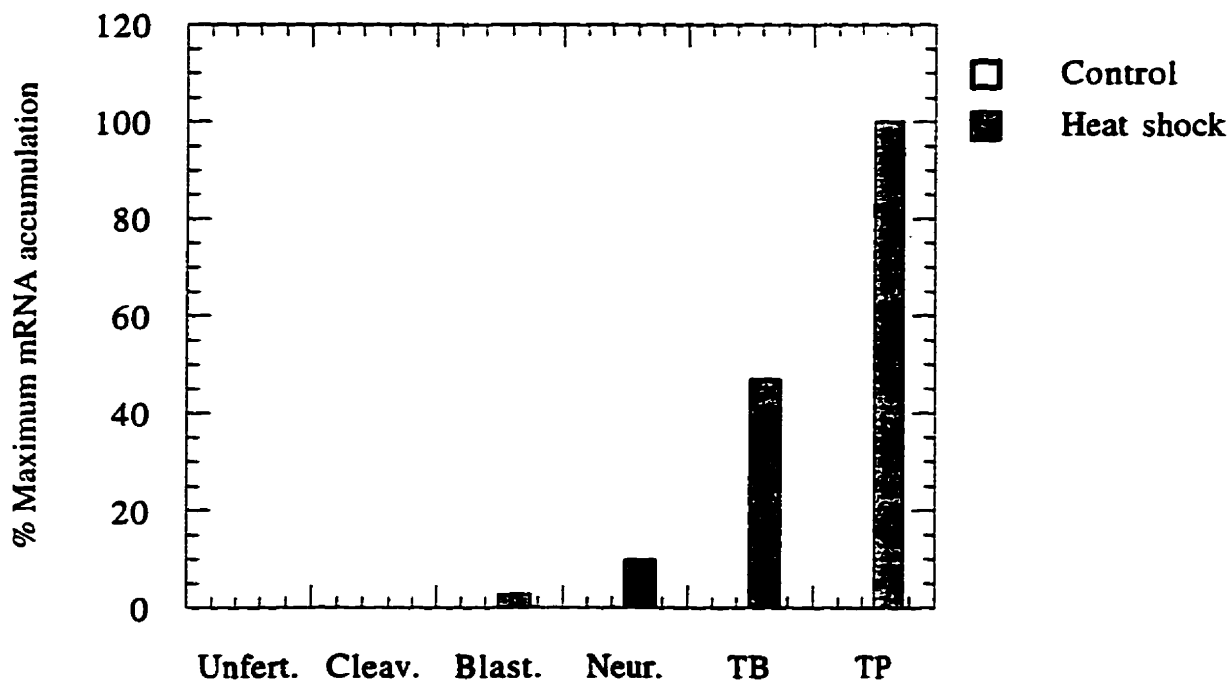


FIGURE 5. Effect of heat shock temperature on the accumulation of hsp30 and hsp70 mRNA in *Xenopus* gastrula stage embryos. Total RNA was isolated from gastrula stage embryos exposed to a range of temperatures (22°C to 37°C) for 1 h. Northern blot analyses were carried out employing [^{32}P]-labeled hsp30 (Panel A) or hsp70 (Panel B) clones. The sizes of the hsp30 and hsp70 mRNAs are 1.1 kb and 2.7 kb, respectively. These autoradiograms are representative of two experiments.

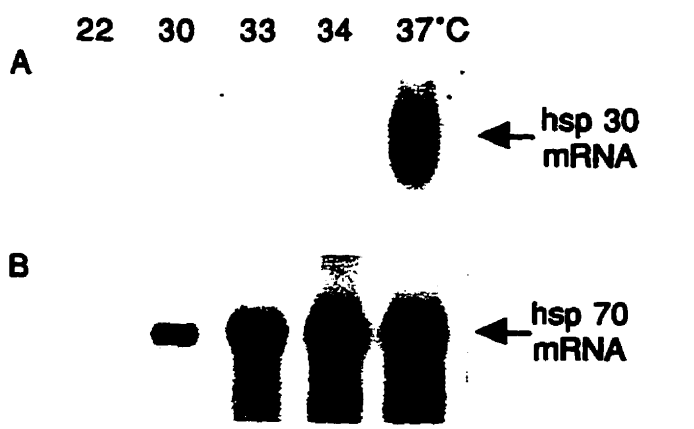
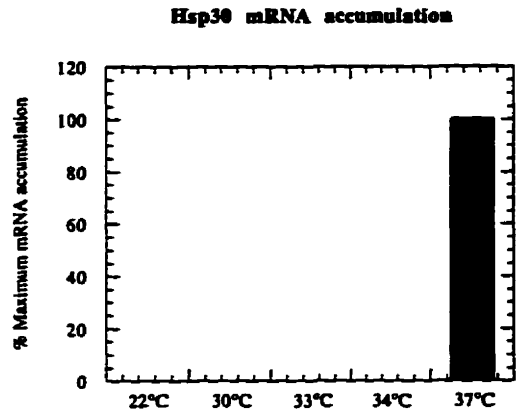
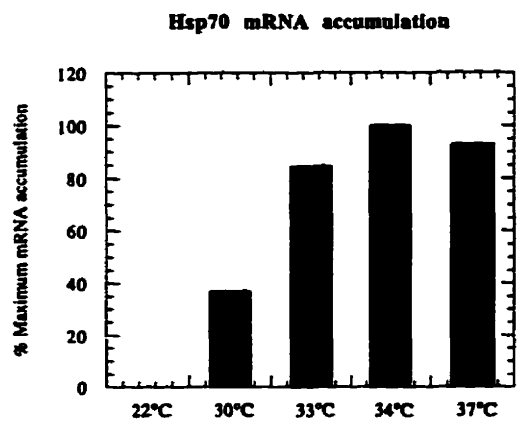

22
30
33
34
37°C
A
hsp 30
mRNA
B
hsp 70
mRNA

FIGURE 6. Densitometric analysis of heat-induced hsp30 and hsp70 mRNA accumulation in *Xenopus* gastrula stage embryos. The representative autoradiograms shown in Figure 5 were scanned using the Macintosh OneScanner. Densitometric analysis of the resultant computer images were carried out using NIH Image 1.55. The obtained values were expressed as a percentage of the maximum mRNA accumulation found in each autoradiogram. The shaded areas represent the samples at temperatures ranging from control (22°C) to heat shock temperatures of 37°C.


Hsp30 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
22°C
30°C
33°C
34°C
37°C


Hsp70 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
22°C
30°C
33°C
34°C
37°C

and hsp70 mRNA levels were found to decrease significantly at 39°C (data not shown).

Previous studies have shown that exposure of *Xenopus* embryos to high incubation temperatures such as 37°C for 1 h results in an inhibition of protein synthesis by at least 85% (Heikkila *et al.*, 1985). The inhibition of protein synthesis by cycloheximide has been shown to stabilize unstable mRNAs in a wide range of cell types (Kelly *et al.*, 1987; De *et al.*, 1991; Ooi *et al.*, 1993; Nanbu *et al.*, 1994; Sheu *et al.*, 1994) including *Eg2* mRNA in *Xenopus* embryos (Duval *et al.*, 1990). Thus, it was possible that the relatively low levels of heat-inducible hsp30 mRNA in late blastula embryos was due, at least in part, to hsp30 mRNA instability. It is likely that incubation of gastrula embryos at 37°C, which inhibits protein synthesis, resulted in an increased stability of these PTB hsp30 mRNAs. In order to examine the effect of protein synthesis inhibition on hsp30 mRNA accumulation, *Xenopus* embryos were dissociated and treated with cycloheximide (10 and 25 µg/ml) (Duval *et al.*, 1990). Dissociation of the embryos should facilitate the entry of cycloheximide into early *Xenopus* embryos by permitting equal access of the agent to all cells (Dawid, 1991). Dissociated gastrula stage embryos which were treated with cycloheximide and subsequently heat-shocked exhibited a greater accumulation of PTB hsp30 mRNA than untreated heat-shocked embryos (Figure 7A). Dissociated control embryos treated with cycloheximide did not display any detectable hsp30 mRNA accumulation. A similar analysis of hsp70 and hsp87 mRNA accumulation in dissociated gastrula embryos indicated that these other hsp mRNAs were not stabilized by cycloheximide treatment (Figure 7B and C). In fact, densitometric analysis of the autoradiograms indicated that the presence of

cycloheximide actually resulted in slightly lower levels of heat-inducible hsp70 and hsp87 mRNA accumulation (Figure 8). Constitutive hsp87 mRNA accumulation increased slightly in the presence of cycloheximide. Therefore, the stabilization of PTB hsp30 mRNA by cycloheximide may reflect a specific effect, and not a general phenomenon associated with hsp mRNAs at this stage of development.

The effect of cycloheximide on PTB hsp30 mRNA stability was also examined in intact, non-dissociated *Xenopus* embryos in order to verify the above finding and also to compare the response of different developmental stages. The resultant Northern blot shown in Figure 9A, demonstrated that treatment of blastula embryos with either 25 or 75 µg/ml cycloheximide for 1 h prior to heat shock resulted in a marked accumulation of hsp30 mRNA compared to embryos heat-shocked in the absence of cycloheximide treatment. A similar result with respect to cycloheximide-induced PTB hsp30 mRNA stability was found with heat-shocked neurula stage embryos. Comparable results were observed in embryos treated with up to 200 µg/ml of cycloheximide (data not shown). In other experiments, cycloheximide treatment was not found to significantly enhance hsp30 mRNA accumulation in cultured *Xenopus* A6 kidney epithelial cells (data not shown). The effect of cycloheximide on the stability of hsp70, hsp87 and actin mRNA stability in intact embryos was also examined (Figure 9B, C, D). In both blastula and neurula RNA samples the relative levels of these mRNAs isolated from heat-shocked embryos either did not change or decreased slightly after treatment with cycloheximide, as compared to embryos not treated with cycloheximide (Figure 10).

FIGURE 7. Effect of cycloheximide on the accumulation of heat-induced hsp30, hsp70, and hsp87 mRNA in dissociated *Xenopus* gastrula stage embryos. Late blastula embryos were dissociated as described in Materials and Methods. At the end of the dissociation procedure the embryos were at the equivalent of the early gastrula stage of development. Total RNA was isolated from control (C) and heat-shocked (H; 33°C for 1 h) embryos which had been incubated in the presence or absence of cycloheximide (CHX). The Northern blots were hybridized against [^{32}P]-labeled hsp30 (Panel A), hsp70 (Panel B) or hsp87 (Panel C) clones. The sizes of the hsp30, hsp70 and hsp87 mRNAs are 1.1, 2.7 and 3.3 kb, respectively. These autoradiograms are representative of four experiments.

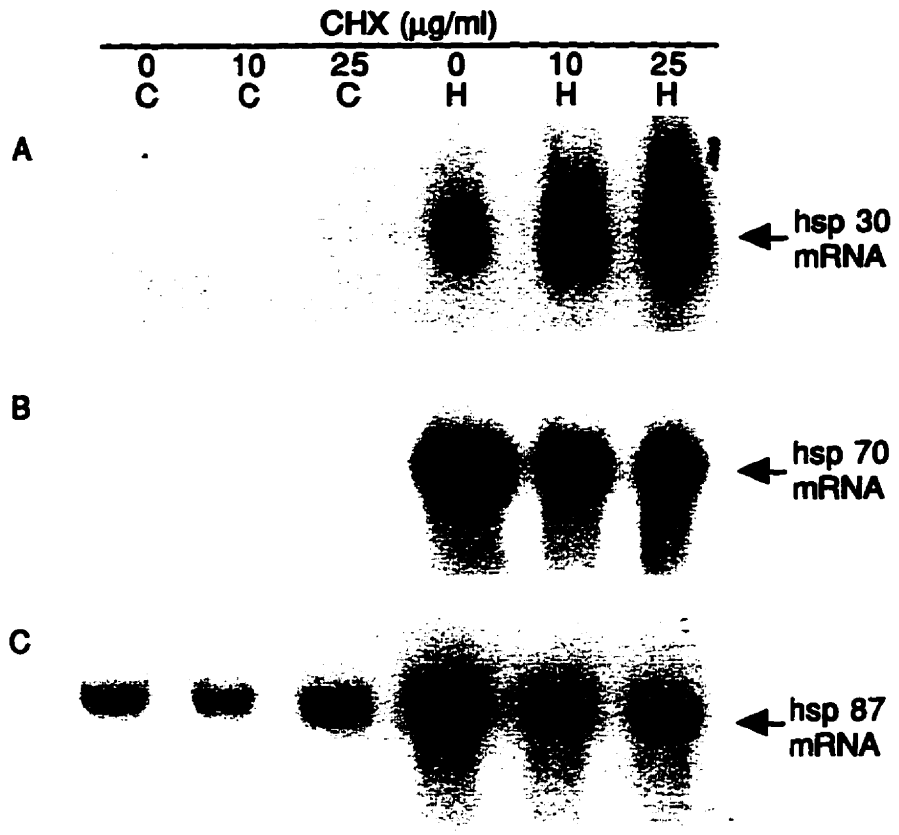

CHX (µg/ml)
0 C
10 C
25 C
0 H
10 H
25 H
A
hsp 30 mRNA
B
hsp 70 mRNA
C
hsp 87 mRNA

FIGURE 8. Densitometric analysis of heat-induced hsp30, hsp70, and hsp87 mRNA accumulation in dissociated *Xenopus* gastrula stage embryos. The representative autoradiograms shown in Figure 7 were scanned using the Macintosh OneScanner. Densitometric analysis of the resultant computer images were carried out using NIH Image 1.55. The obtained values were expressed as a percentage of the maximum mRNA accumulation found in each autoradiogram. The shaded areas represent the control (C) and heat-shocked (HS) samples incubated in the presence or absence of cycloheximide (CHX).

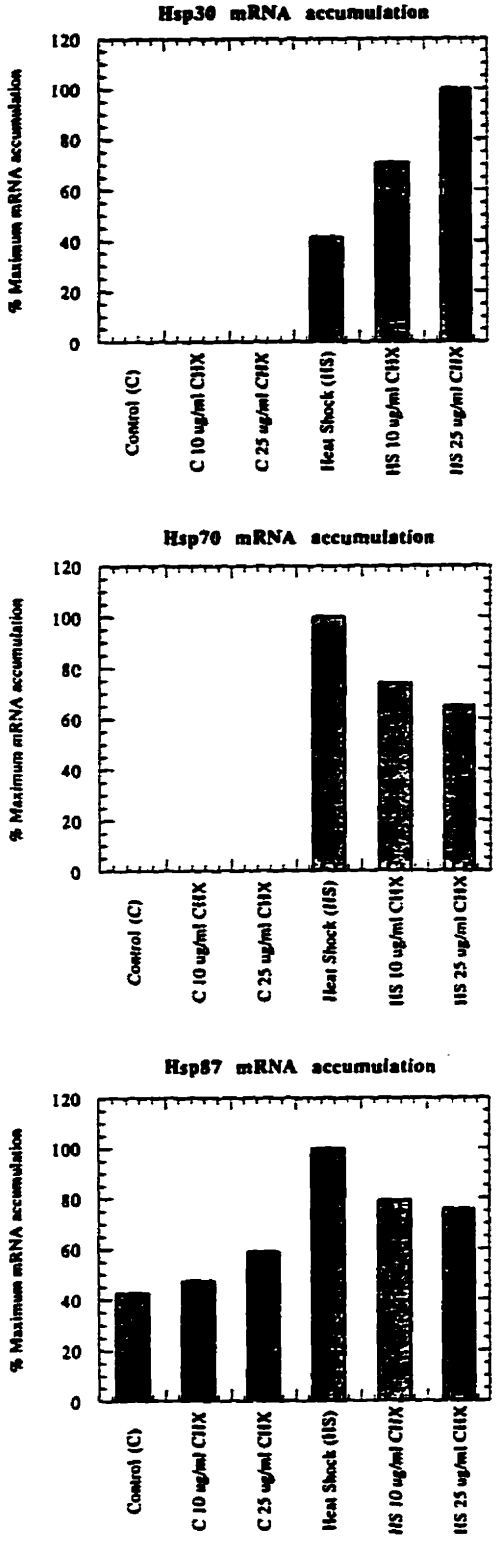

Hsp30 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control (C)
C 10 ug/ml CHX
C 25 ug/ml CHX
Heat Shock (HS)
HS 10 ug/ml CHX
HS 25 ug/ml CHX
Hsp70 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control (C)
C 10 ug/ml CHX
C 25 ug/ml CHX
Heat Shock (HS)
HS 10 ug/ml CHX
HS 25 ug/ml CHX
Hsp87 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control (C)
C 10 ug/ml CHX
C 25 ug/ml CHX
Heat Shock (HS)
HS 10 ug/ml CHX
HS 25 ug/ml CHX

FIGURE 9. Effect of cycloheximide on the accumulation of hsp30, hsp70, hsp87, and actin mRNA in intact *Xenopus* embryos during early development. Total RNA was isolated from control (C) and heat shocked (H) embryos which had been incubated in the presence or absence of cycloheximide (CHX). The Northern blots were hybridized against either a [^{32}P]-labeled hsp30 (Panel A), hsp70 (Panel B), hsp87 (Panel C) or cytoskeletal actin (Panel D) clone. The transcript sizes for cytoskeletal actin mRNA are 1.8 and 2.2 kb. These autoradiograms are representative of two experiments.

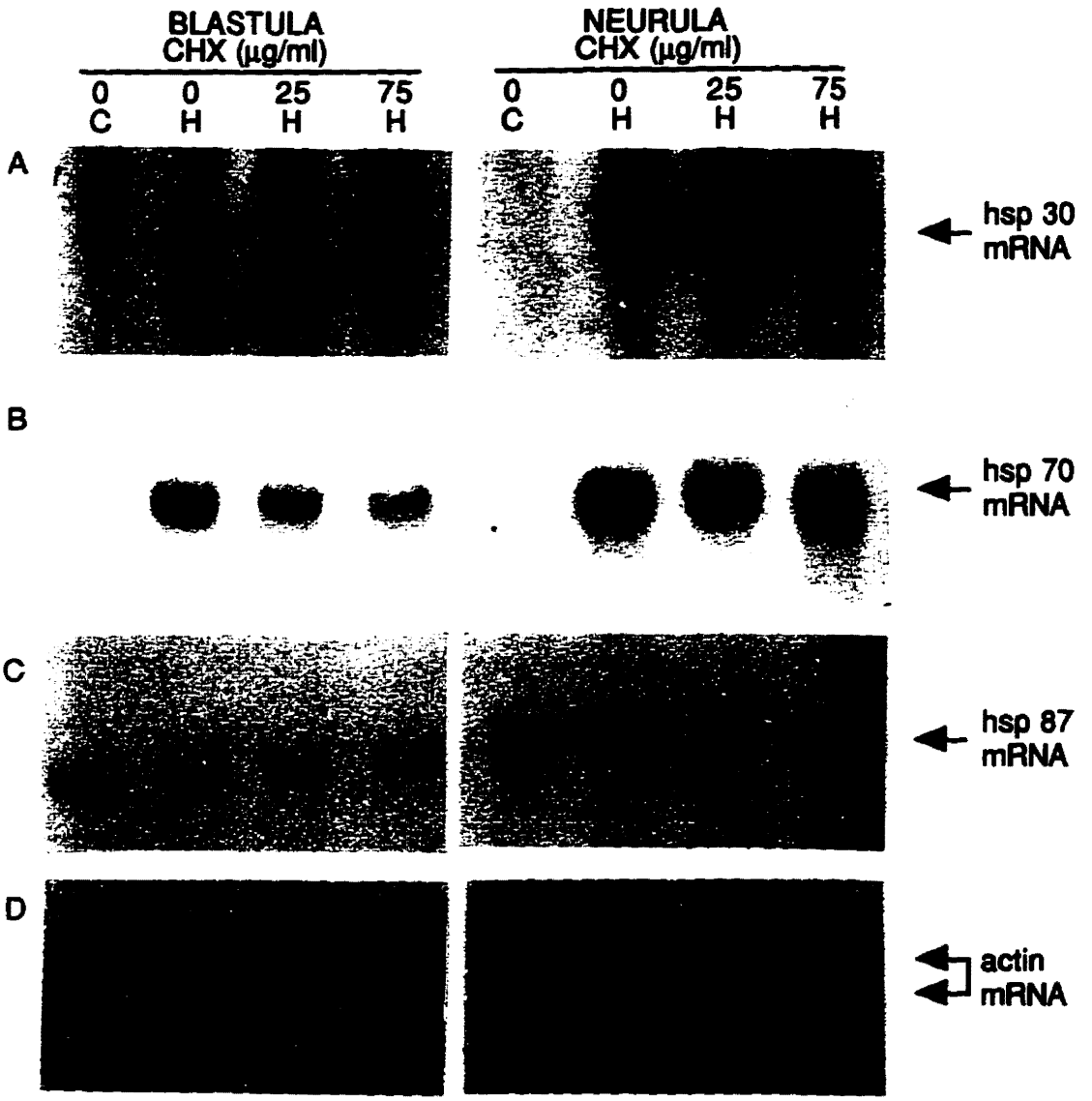
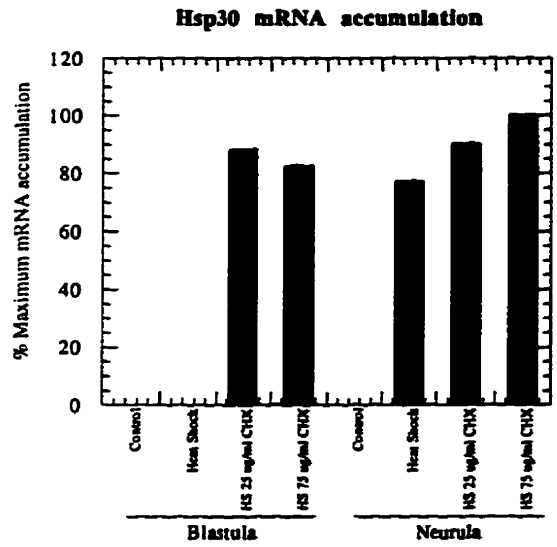
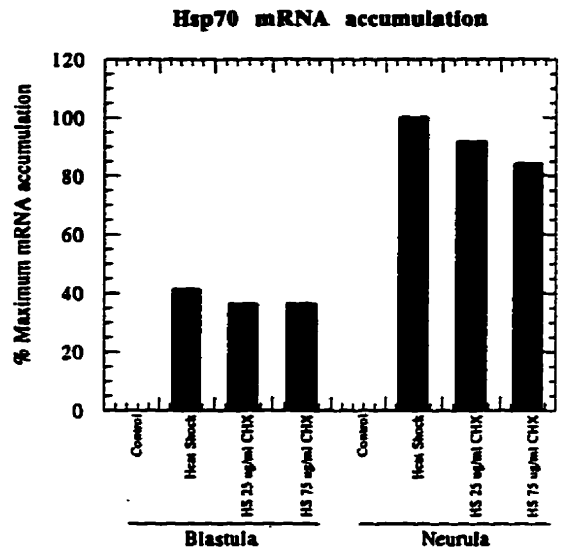
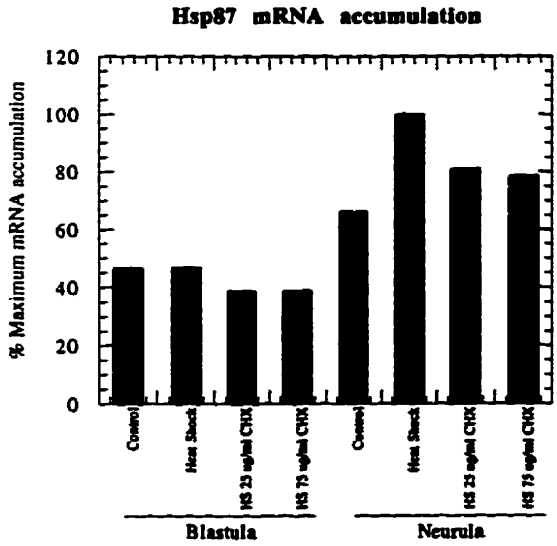
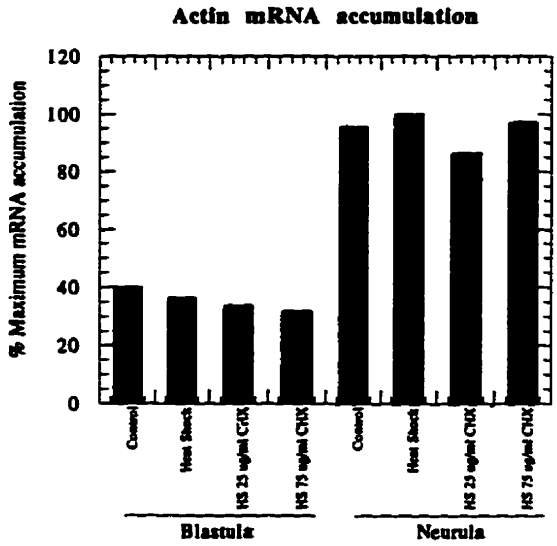

BLASTULA
CHX (µg/ml)
0 C
0 H
25 H
75 H
NEURULA
CHX (µg/ml)
0 C
0 H
25 H
75 H
A
hsp 30 mRNA
B
hsp 70 mRNA
C
hsp 87 mRNA
D
actin mRNA

FIGURE 10. Densitometric analysis of hsp30, hsp70, hsp87, and actin mRNA accumulation in *Xenopus* embryos during early development. The representative autoradiograms shown in Figure 9 were scanned using the Macintosh OneScanner. Densitometric analysis of the resultant computer images were carried out using NIH Image 1.55. The obtained values were expressed as a percentage of the maximum mRNA accumulation found in each autoradiogram. The shaded areas represent control and heat-shocked (HS) blastula and neurula samples incubated in the presence or absence of cycloheximide (CHX).


Hsp30 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control
Heat Shock
HS 25 ug/ml CHX
HS 75 ug/ml CHX
Blastula
Neurula


Hsp70 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control
Heat Shock
HS 25 ug/ml CHX
HS 75 ug/ml CHX
Blastula
Neurula


Hsp87 mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control
Heat Shock
HS 25 ug/ml CHX
HS 75 ug/ml CHX
Blastula
Neurula


Actin mRNA accumulation
% Maximum mRNA accumulation
120
100
80
60
40
20
0
Control
Heat Shock
HS 25 ug/ml CHX
HS 75 ug/ml CHX
Blastula
Neurula

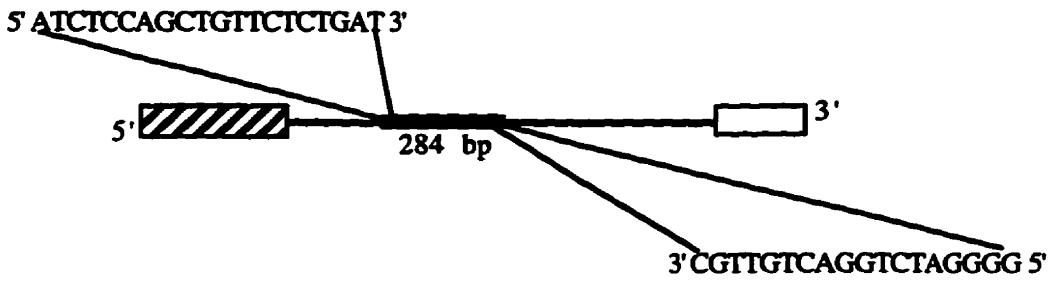
3.3 *Xenopus* hsp30D mRNA is not detected in cycloheximide-treated pre-tailbud stage embryos

Given these previous sets of experiments with cycloheximide, it was possible that the hsp30D gene, which has been previously shown to be first heat-inducible at the mid-tailbud stage using RT-PCR (Ohan and Heikkila, 1995), was in fact active in heat-shocked late blastula stage embryos but that it was highly unstable and thus not detected. In order to test this possibility, RT-PCR was carried out on RNA isolated from late blastula stage embryos treated with relatively high concentrations (200 μg/ml) of cycloheximide followed by heat shock. Primers directed against a region of the hsp30D gene were utilized (Figure 11A). As a control for genomic DNA contamination, primers directed against a region of the cytoskeletal actin gene containing an intron were also used (Figure 11B). Amplified DNA was distinguished from amplified cDNA by their different PCR product sizes. The sensitivity of the analysis was increased by carrying out Southern blotting on the resultant PCR fragments. As shown in Figure 12A, hsp30D mRNA accumulation was not detected in any of the blastula RNA samples. It is interesting to note the presence of a second, smaller hsp30D PCR fragment in the heat-shocked tadpole sample, which probably represents another, as yet unidentified, hsp30 gene transcript. The results of the actin RT-PCR assay on the same set of RNA samples demonstrated the lack of genomic DNA contamination (Figure 12B).

FIGURE 11. Hsp30D and actin PCR primers. The primer sequences and expected PCR products are indicated. A. Hsp30D PCR primers were designed with the aid of Amplify 1.0 and OSP (Hillier and Green, 1991). The expected PCR product was 284 bp. B. Cytokeletal actin primers were designed to span a 79 bp intron in order to distinguish amplified DNA from cDNA. A 290 bp fragment was expected from amplified actin cDNA, whereas amplified actin DNA would yield a 369 bp product.

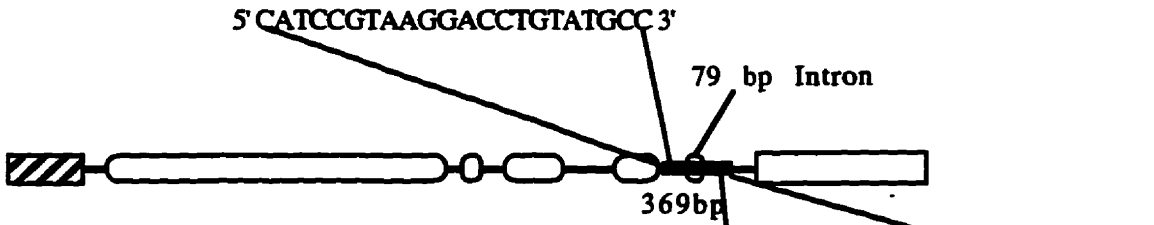
A. Hsp 30D

Scale: 1.2 cm= 200 bp



B. Cytoskeletal Actin Type 5

Scale: 1.2 cm = 600 bp



In the absence of the 79 bp intron (i.e. mRNA), the PCR product from the cDNA will be 369 bp -79 bp = 290 bp.

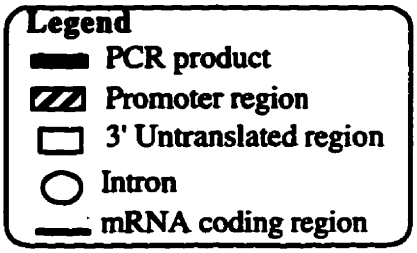
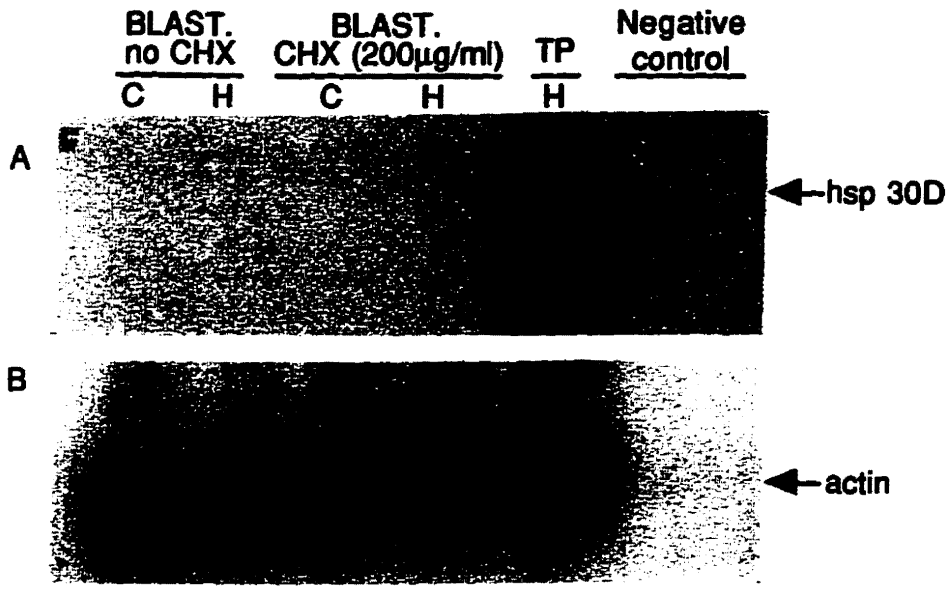


FIGURE 12. The effect of cycloheximide on the detection of hsp30D mRNA in late blastula stage embryos. One microgram of total RNA isolated from control (C) and heat-shocked (H) embryos in the presence or absence of cycloheximide (CHX) was reverse transcribed into cDNA. The cDNA was subjected to PCR with hsp30D and actin primers. Heat-shocked tadpole (TP) mRNA was used as a positive control. The negative control contained all PCR reagents except cDNA. The RT-PCR products were Southern blotted and probed with the [^{32}P]-labeled hsp30D (Panel A) or actin (Panel B) clone. The size of the hsp30D PCR fragment is 284 bp while the actin fragment is 290 bp.

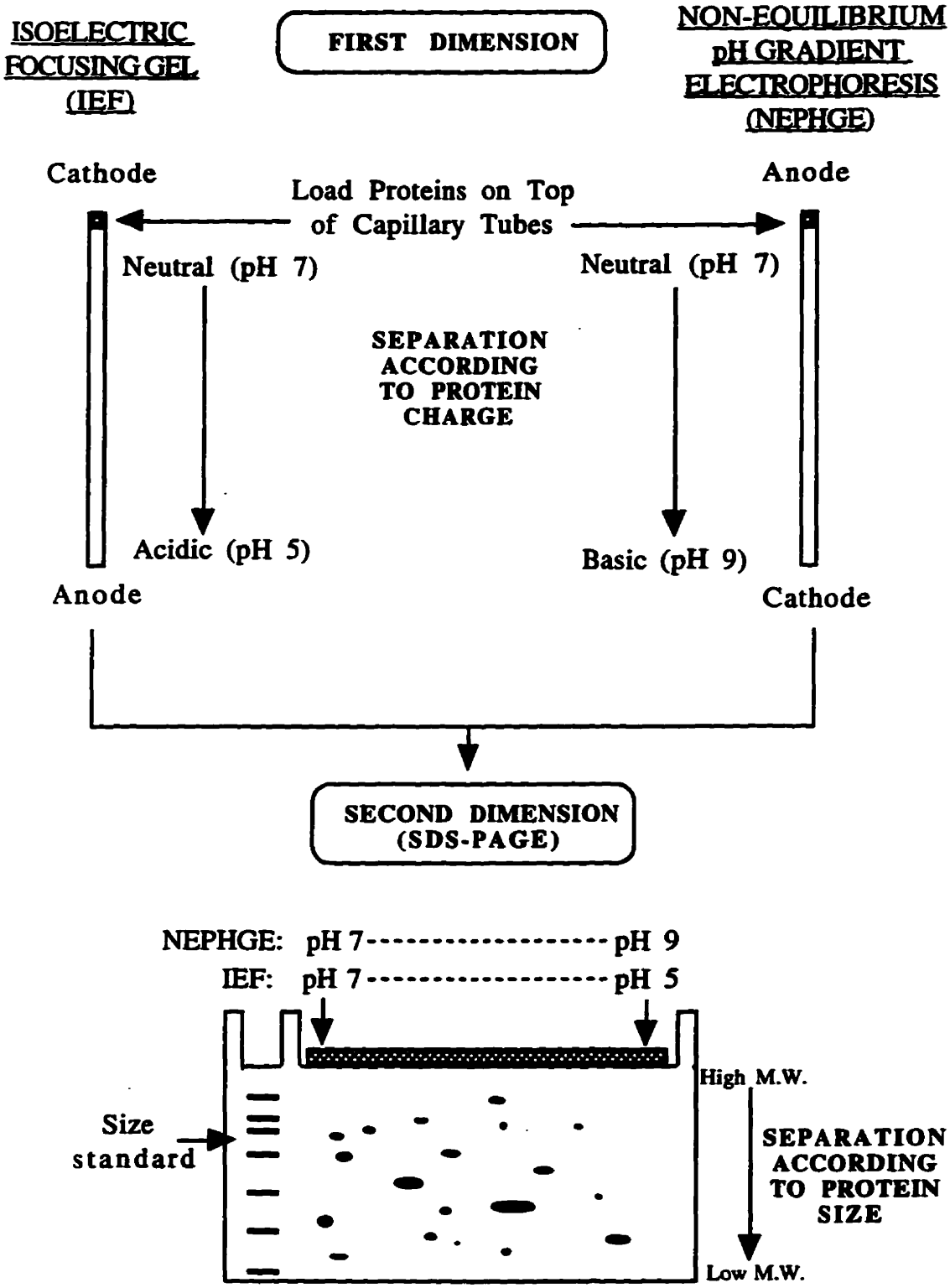

BLAST. no CHX
BLAST. CHX (200µg/ml)
TP
Negative control
C
H
C
H
H
A
hsp 30D
B
actin

3.4 Detection of Basic Small HSPs in *Xenopus* A6 Cells

Recently, a group of basic small HSPs were identified in the organism *Poeciliopsis lucida* using non-equilibrium pH gradient electrophoresis (NEPHGE) 2-D SDS-PAGE (L. Hightower, University of Connecticut, unpublished data). The conventional method of isoelectric focusing (IEF) 2-D SDS-PAGE allows the resolution of moderately acidic proteins (typically in a pH range of 5-7; O'Farrell, 1975). NEPHGE 2-D SDS-PAGE permits the resolution of moderately basic proteins, typically in a pH range of 7 to 9 (O'Farrell *et al.*, 1977). A comparison of the two techniques is presented in Figure 13. The main differences lie in the first dimension; specifically, in the charge of the ampholytes used to generate the pH gradient, as well as the polarity of the applied current. The second dimension (SDS-PAGE), in which the proteins are separated according to their size, is identical for both techniques. The only disadvantage of NEPHGE vs. IEF is that the NEPHGE-resolved proteins do not reach their isoelectric points (pI; thus 'non-equilibrium') and pI values cannot be accurately determined. However, it does permit the resolution of proteins which cannot be readily resolved by isoelectric focusing.

In preliminary experiments, a group of heat-inducible small HSPs were identified in *Xenopus* A6 cells using NEPHGE 2-D SDS-PAGE (Tam, 1995). The following section will involve the characterization of the expression of the basic small HSPs in the presence of a variety of inducing conditions, with comparisons made with the already identified acidic small HSPs in A6 kidney epithelial cells (Darasch *et al.*, 1988; Tam and Heikkila, 1995).

FIGURE 13. A comparison of isoelectric focusing (IEF) and non-equilibrium pH gradient electrophoresis (NEPHGE) 2-D SDS-PAGE. In the first dimension proteins are separated according to charge. In IEF tube gels a pH range from 7 to 5 is produced by appropriately charged ampholytes, permitting the resolution of acidic proteins. In NEPHGE tube gels a pH range of 7 to 9 is produced, permitting the resolution of basic proteins. The polarity of the applied charge is the opposite for NEPHGE gels as compared to IEF gels. The procedure for the second dimension is identical for both IEF and NEPHGE gels. It involves extruding the tube gel onto an SDS-PAGE gel and separating the proteins according to size. The charge orientation of the NEPHGE and IEF tube gels is noted.


ISOELECTRIC FOCUSING GEL (IEF)
FIRST DIMENSION
NON-EQUILIBRIUM pH GRADIENT ELECTROPHORESIS (NEPHGE)
Cathode
Load Proteins on Top of Capillary Tubes
Anode
Neutral (pH 7)
Neutral (pH 7)
SEPARATION ACCORDING TO PROTEIN CHARGE
Acidic (pH 5)
Basic (pH 9)
Anode
Cathode
SECOND DIMENSION (SDS-PAGE)
NEPHGE: pH 7 ---------- pH 9
IEF: pH 7 ---------- pH 5
High M.W.
Size standard
SEPARATION ACCORDING TO PROTEIN SIZE
Low M.W.

To begin, the pattern of acidic HSP synthesis in heat-shocked *Xenopus* A6 cells is presented in Figure 14. ^{35}S-methionine labeled proteins from control (22°C) and heat-shocked (35°C for 4 h) A6 cells were resolved by IEF 2-D SDS-PAGE and subjected to fluorography. A number of proteins were observed to be heat-inducible, including the HSP70 family members, which have previously been identified with a human HSP70 antibody (demarcated by the upper brackets; Tam, 1995) and the small HSPs denoted by the lower brackets. Other heat-induced proteins included 33 (pI 5.0), 35 (5.0), 48 (pI 5.1), 56 (pI 6.3), 69 (pI 5.9), 70 (6.3, 6.4, 6.5, 6.6), and 87 kDa proteins.

^{35}S-methionine labeled proteins isolated from control (22°C), heat-shocked (35°C for 4 h), and sodium arsenite-treated (50 µM for 4 h) A6 cells were then resolved by NEPHGE 2-D SDS-PAGE (Figure 15). A 62 kDa basic heat and sodium arsenite-inducible protein was observed, as were 36, 38, and 56 kDa heat-inducible proteins. Seven basic small HSPs were also present. In order to more readily view the basic small HSPs, the region outlined by brackets in Figure 15 was enlarged and the seven basic, approximately 30 kDa, small HSPs were labeled (Figure 16). Proteins 1 thru 5 and protein 7 were present constitutively, with proteins 1 and 2 exhibiting relatively high levels of synthesis relative to the other basic small HSPs. The levels of synthesis of these basic small HSPs increased in the presence of heat shock or sodium arsenite. In addition, a basic small HSP (protein 6) which was not present constitutively, was also induced by the stresses. Both sodium arsenite and heat shock induced similar patterns of basic small HSP synthesis.

In order to determine if the basic small HSPs are related to their acidic counterparts, an HSP30C antibody, which has been shown to recognize up to 8

FIGURE 14. The 2-D SDS-PAGE pattern of heat shock protein synthesis in heat-shocked *Xenopus* A6 kidney epithelial cells. ^{35}S-methionine labeled proteins isolated from control (22°C) and heat-shocked (35°C for 4 h) A6 cells were resolved by isolelectric focusing (IEF) 2-D SDS-PAGE and subjected to fluorography. The basic portion of the gel is oriented to the left, and the acidic to the right. Molecular weight standards are shown on the left. The upper brackets delimit the strongly induced HSP70 proteins which have been previously identified using a human HSP70 antibody. The lower brackets denote the region containing the heat-inducible small heat shock proteins. Arrows indicate other heat-inducible proteins, including 33 (pI 5.0), 35 (pI 5.0), 48 (pI 5.1), 56 (pI 6.3), 69 (pI 5.9), 70 (pI 6.3, 6.4, 6.5, 6.6), and 87 (pI 6.6, 6.65) kDa proteins. These autoradiograms are representative of three experiments.

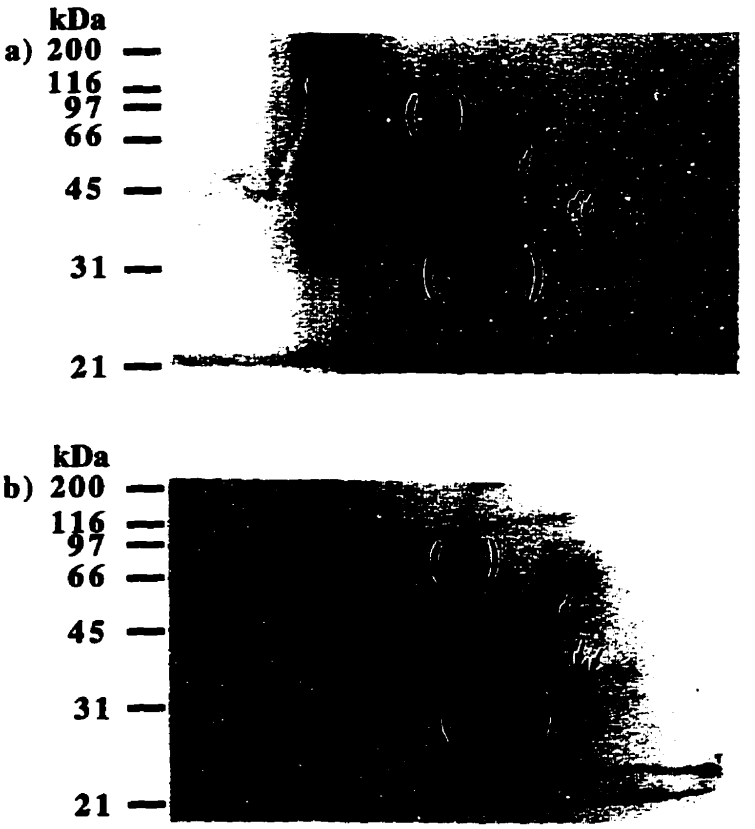

kDa
a) 200
116
97
66
45
31
21
CONTROL
kDa
b) 200
116
97
66
45
31
21
HEAT SHOCK
(35°C, 4 h)

FIGURE 15. The 2-D NEPHGE pattern of heat shock proteins in heat-shocked and sodium arsenite-treated A6 cells. ^{35}S-methionine labeled proteins were isolated from control (22°C), heat-shocked (35°C for 4 h), and sodium arsenite-treated (50 μM for 4 h) A6 cells. The proteins were resolved by non-equilibrium pH gradient electrophoresis (NEPHGE) 2-D SDS-PAGE and subjected to fluorography. The acidic portion of the gel is oriented to the left, and the basic to the right. Molecular weight standards are shown on the left. The arrows indicate the positions of inducible proteins, including 7 basic small heat shock proteins (delimited by brackets) and a 62 kDa heat and sodium arsenite-inducible protein, as well as 36, 38 and 56 kDa heat-inducible proteins. These autoradiograms are representative of three experiments.

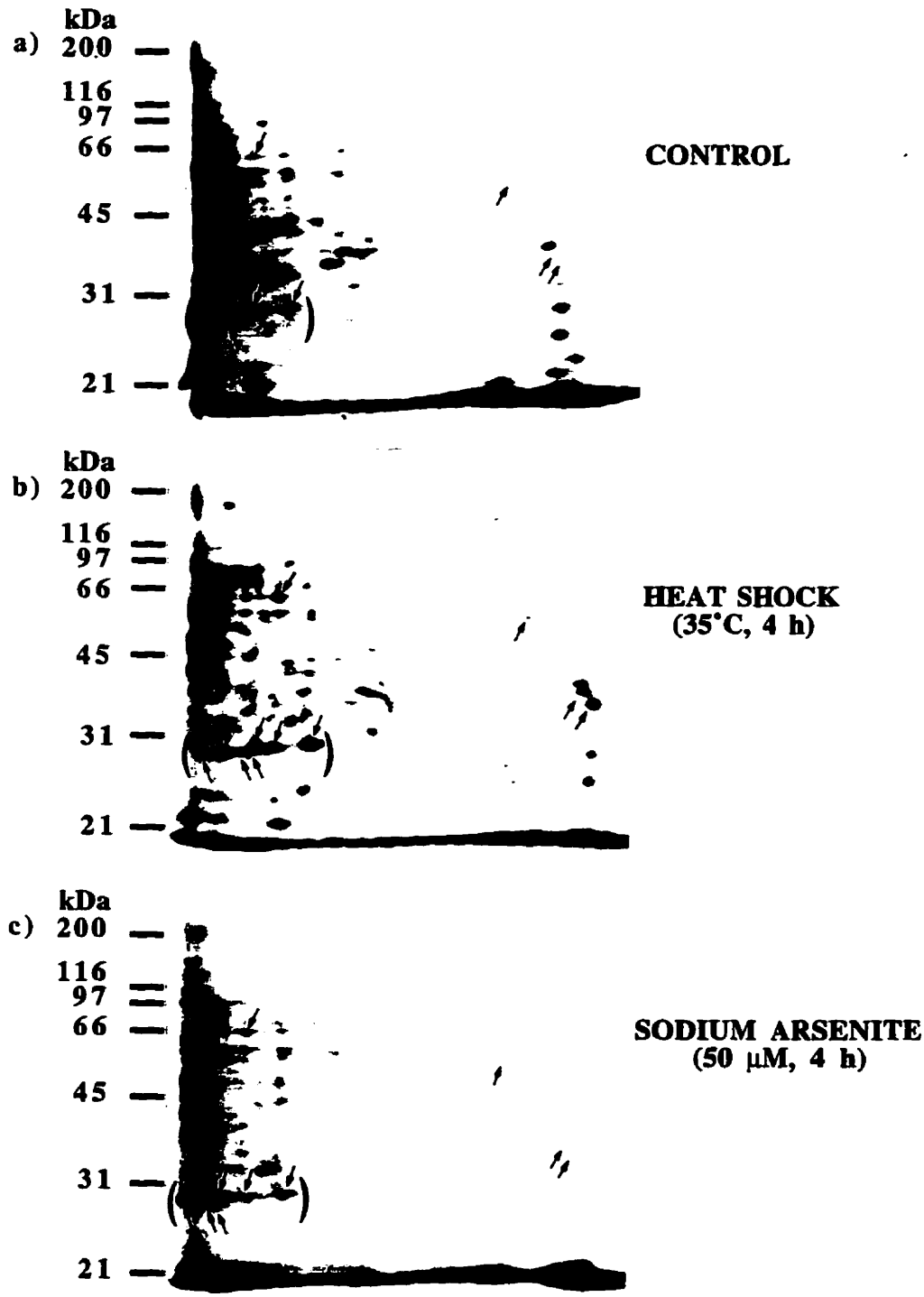
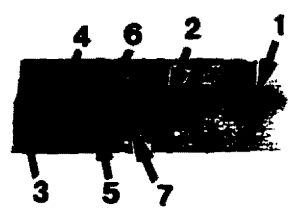
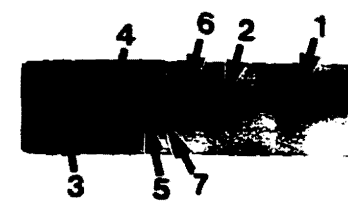

a)
kDa
200
116
97
66
45
31
21
CONTROL
b)
kDa
200
116
97
66
45
31
21
HEAT SHOCK
(35°C, 4 h)
c)
kDa
200
116
97
66
45
31
21
SODIUM ARSENITE
(50 µM, 4 h)

FIGURE 16. Basic small HSP synthesis in heat-shocked and sodium arsenite-treated A6 cells. The region outlining the basic small HSPs (demarcated by brackets in Figure 15) was enlarged to more readily view the basic small HSPs. The acidic portion of the gel is oriented to the left, and the basic to the right. Subsequent figures will present this region to specifically examine basic small HSP synthesis. Arrows indicate the basic small HSPs. 1 - 30 kDa; 2 - 30 kDa; 3 - 30 kDa; 4 - 30 kDa; 5 - 29.5 kDa; 6 - 30 kDa; 7 - 29.5 kDa. Isoelectric points cannot be accurately determined for 2-D NEPHGE resolved proteins.

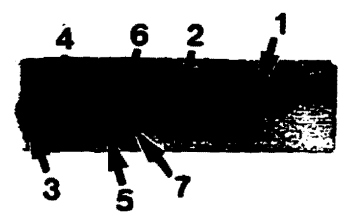
a) **CONTROL**



b) **HEAT SHOCK (35°C, 4 h)**



c) **SODIUM ARSENITE (50 µM, 4 h)**



heat-inducible acidic small HSPs in A6 cells, was employed to see if it would cross-react with the basic small HSPs. The HSP30C antibody was generated from a synthetic peptide comprised of amino acid residues 199-213 in the carboxyl end of the HSP30C protein, which had been injected into New Zealand white rabbits (Tam and Heikkila, 1995). ^{35}S-methionine labeled proteins isolated from control and heat-shocked A6 cells were resolved using IEF and NEPHGE 2-D SDS-PAGE and Western blotted. Autoradiograms were made from the Western blots, after which the blots were reacted with the HSP30C antibody. The HSP30 regions are presented in Figure 17. From the autoradiograms, the 7 heat-inducible basic small HSPs were clearly visible, as were proteins 1 and 3 from the control sample (proteins 2, 4, 5 and 7 were present faintly; Figure 17a, b). From the Western blots, it can be seen that the HSP30C antibody did not react with any of the basic small HSPs, while it did react with five of the acidic small HSPs (proteins c and e were too faint to be photographed; Figure 17d, f). This is in agreement with a similar previous preliminary study, and suggests that the basic small HSPs are distinct from the acidic small HSPs (Tam, 1995). Previous analysis at the mRNA level using an hsp30 genomic probe failed to detect the accumulation of constitutive small hsp mRNA, further suggesting that the genes encoding the basic small hsps are distinct from those encoding the acidic small hsps (Krone and Heikkila, 1988, 1989; Ali *et al.*, 1993; This study, Figures 3, 5, 7, 9).

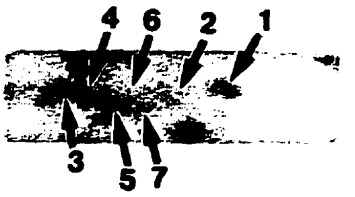
This is the first time that constitutively expressed small HSPs have been detected. The similarity between small HSPs and α-crystallin proteins, as well as their shared molecular chaperone properties, has been reviewed extensively in the literature (Boelens and de Jong, 1995; Sax and Piatigorsky, 1994; de

FIGURE 17. The HSP30C antibody recognizes acidic small HSPs but does not cross-react with basic small HSPs in heat-shocked A6 cells. ^{35}S-methionine labeled proteins were isolated from control (22°C) and heat-shocked (35°C for 4 h) A6 cells. The proteins were resolved using IEF and NEPHGE 2-D SDS-PAGE, after which they were Western blotted onto nitrocellulose paper. Autoradiograms were produced from the NEPHGE Western blots (HSP30 region shown in panels a and b). The blots were then subjected to immunoblot analysis with the HSP30C antibody and the results are presented in Panels c, d, e, and f (HSP30 region). The NEPHGE gels are oriented from acidic to basic (left to right) while the IEF gels are oriented from basic to acidic (left to right). Arrows indicate the positions of small HSPs. Acidic small HSPs: a - 30.0 kDa, pI 5.65; b - 30.5 kDa, pI 5.4; c - 30.5 kDa, pI 5.25; d - 29.5 kDa, pI 5.95; e - 29.5 kDa, pI 5.6. In Panel f, proteins c and e were too faint to be photographed. These results are representative of two experiments.

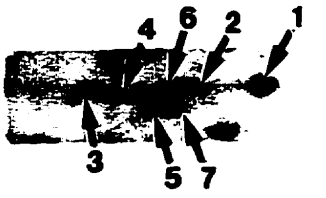
NEPHGE 2-D SDS-PAGE

Autoradiograms

a) **CONTROL**



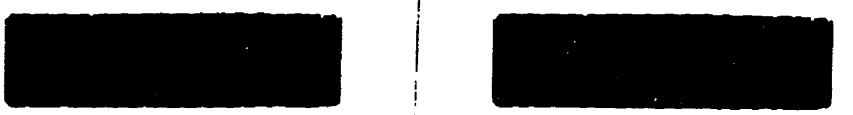
b) **HEAT SHOCK (35°C, 4 h)**



Westerns

c) **CONTROL**

d) **HEAT SHOCK (35°C, 4 h)**



IEF 2-D SDS-PAGE

Westerns

e) **CONTROL**

f) **HEAT SHOCK (35°C, 4 h)**

Jong *et al.*, 1993). It is possible that the constitutive basic small HSPs detected here may be related to the α-crystallin protein family. While α-crystallin proteins are predominantly expressed in the eye lens, they have also been detected in other tissues (Sax and Piatigorsky, 1994; Kato *et al.*, 1991). In order to test this possibility, a hamster αB-crystallin antibody was used to probe a Western blot of the NEPHGE-resolved proteins from control and heat-shocked A6 cells. No cross-reaction was observed (data not shown).

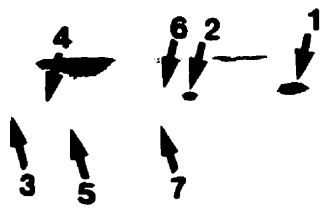
3.5 Heat Shock Induction of Basic Small HSP Synthesis

In order to characterize the expression of the basic small HSPs, A6 cells were exposed to a range of heat shock temperatures (27 to 35°C for 4 h). Proteins were radiolabeled with ^{35}S-methionine during the last 2 h of the treatment period. The proteins were then resolved by NEPHGE 2-D SDS-PAGE and subjected to fluorography (Figure 18). Proteins 1 and 2 were clearly present constitutively (Figure 18a). A 27°C heat shock induced the enhanced synthesis of proteins 3 thru 6. A heat shock temperature of 35°C produced the strongest response by the basic small HSPs. A heat shock at 37°C for 4 h resulted in the general inhibition of protein synthesis (data not shown).

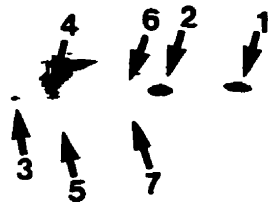
A time course of basic small HSP synthesis in A6 cells exposed to a heat shock temperature of 35°C over a 6 h period is shown in Figure 19. Proteins 1 and 3 were clearly present constitutively, while proteins 2, 4, 5, and 7 were present weakly (Figure 19a). Heat-inducible expression of proteins 1 thru 6 were observed after a 2 h heat shock. At 4 h, synthesis of the 7 basic small HSPs were induced. The levels peaked at 4 h and declined slightly after a 6 h

FIGURE 18. The effect of heat shock temperature on basic small HSP synthesis in A6 cells. ^{35}S-methionine labeled proteins were isolated from control (22°C) and heat-shocked (27-35°C for 4 h) A6 cells. The proteins were then resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. The positions of the basic small HSPs are denoted by arrows. These autoradiograms are representative of three experiments.

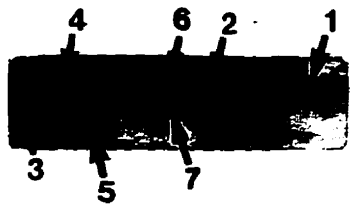
a) CONTROL



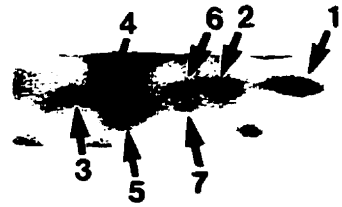
b) HEAT SHOCK (27°C, 4 h)



c) HEAT SHOCK (30°C, 4 h)



d) HEAT SHOCK (33°C, 4 h)



e) HEAT SHOCK (35°C, 4 h)

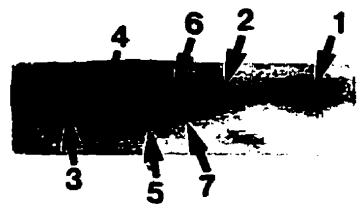
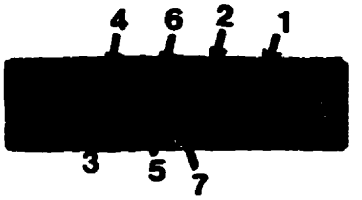
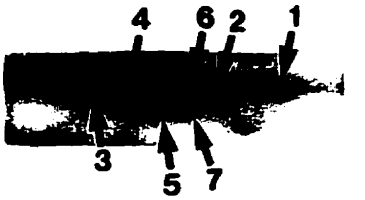


FIGURE 19. Time course of basic small HSP synthesis in A6 cells exposed to a 35°C heat shock. ^{35}S-methionine labeled proteins were isolated from control (22°C) and heat-shocked (35°C for 2 to 6 h) A6 cells. The proteins were then resolved by NEPHGE 2-D SDS-PAGE and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. The positions of the basic small HSPs are denoted by arrows. These autoradiograms are representative of two experiments.

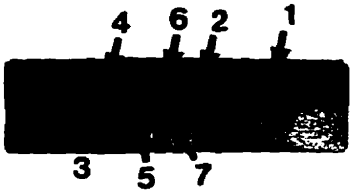
a) CONTROL



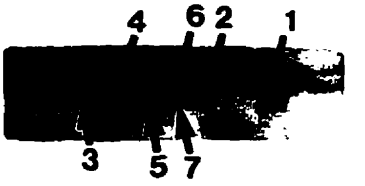
b) HEAT SHOCK (35°C, 2 h)



c) HEAT SHOCK (35°C, 4 h)



d) HEAT SHOCK (35°C, 6 h)



heat shock. A 12 h heat shock resulted in the general reduction of protein synthesis, including the basic small HSPs (data not shown).

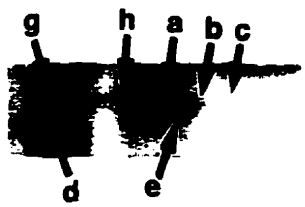
3.6 Sodium Arsenite Induction of Small HSP Synthesis

Previous time-course studies of sodium arsenite induction of HSPs in *Xenopus* A6 cells have been limited to one-dimensional analysis (Darasch *et al.*, 1987). Presented in Figure 20 is a 2-D SDS-PAGE time course of acidic small HSP synthesis in A6 cells exposed to 50 μM sodium arsenite. Three small HSPs (a, b, g) were first sodium arsenite-inducible after a 2 h treatment. By 4 h, all of the acidic small HSPs were present. Their levels peaked at 12 h and declined by 24 h.

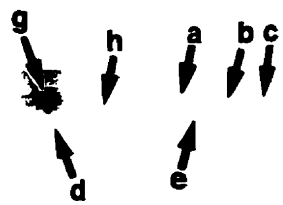
Faint smears appeared in the control sample (Figure 20a). While it is possible that they may represent the constitutive synthesis of low levels of acidic small HSPs, it is not likely for a number of reasons: 1) constitutive acidic small HSP synthesis has not been detected before using high resolution 2-D SDS-PAGE of ^{35}S-methionine labelled proteins (Darasch *et al.*, 1988); 2) the HSP30C antibody does not cross-react with any proteins in control samples, (Tam and Heikkila, 1995; this study, Figure 17; although it is possible that the carboxyl region against which the HSP30C protein was generated may differ in the proteins detected here); 3) no constitutive small hsp mRNA has been detected using Northern blotting, RNase protection assays, or RT-PCR (Krone and Heikkila, 1988, 1989; Ali *et al.*, 1993; Ohan and Heikkila, 1995). Future studies employing more sensitive Western blotting techniques, such as enhanced chemiluminescence, may more definitively resolve this question.

FIGURE 20. Time course of acidic small HSP synthesis in A6 cells exposed to 50 μM sodium arsenite. ^{35}S-methionine labeled proteins were isolated from control and sodium arsenite-treated (50 μM for 2 to 24 h) A6 cells. The proteins were resolved by IEF 2-D SDS-PAGE and subjected to fluorography. The gels are oriented from basic (left) to acidic (right) portions of the gel. The positions of the acidic small HSPs are denoted by arrows. These autoradiograms are representative of two experiments.

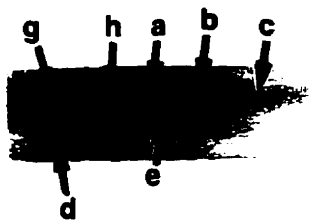
a) **CONTROL**



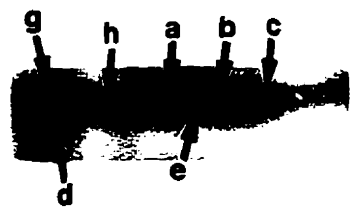
b) **SODIUM ARSENITE (50 µM, 2 h)**



c) **SODIUM ARSENITE (50 µM, 4 h)**



d) **SODIUM ARSENITE (50 µM, 12 h)**



e) **SODIUM ARSENITE (50 µM, 24 h)**

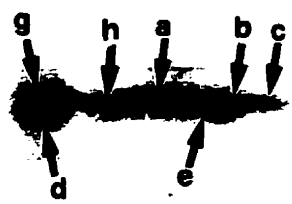
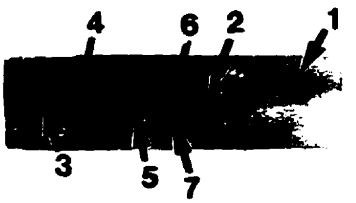
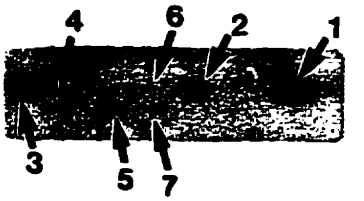


FIGURE 21. Time course of basic small HSP synthesis in A6 cells exposed to 50 μM sodium arsenite. ^{35}S-methionine labeled proteins were isolated from control and sodium arsenite-treated (50 μM for 2 to 24 h) A6 cells. The proteins were then resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. The positions of the basic small HSPs are denoted by arrows. These autoradiograms are representative of two experiments.

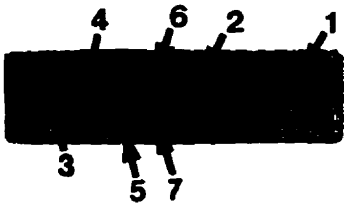
a) CONTROL



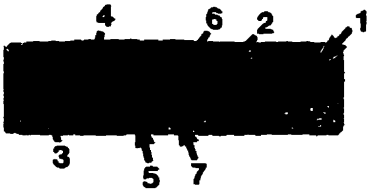
b) SODIUM ARSENITE
(50 μM, 2 h)



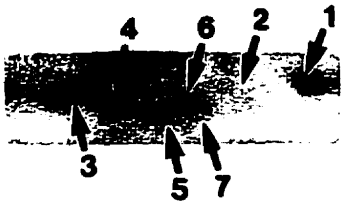
c) SODIUM ARSENITE
(50 μM, 4 h)



d) SODIUM ARSENITE
(50 μM, 12 h)



e) SODIUM ARSENITE
(50 μM, 24 h)



In the case of basic small HSP synthesis, proteins 1 thru 7 were first sodium arsenite-inducible after a 4 h treatment (Figure 21b). By 12 hours all of the basic small HSPs were observed. As with their acidic counterparts, levels of basic small HSP synthesis peaked after a 12 h treatment, and declined by 24 h. Proteins 1-5, and 7 were present constitutively (Figure 21a).

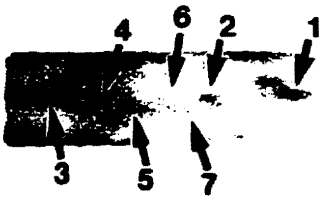
The synergistic induction of HSP synthesis by concurrent mild heat shock and sodium arsenite has been demonstrated in A6 cells using 1-D SDS-PAGE (Heikkila *et al.*, 1986). In order to examine if the basic small HSPs respond synergistically to heat shock and sodium arsenite, ^{35}S-methionine labeled proteins were isolated from control (22°C), sodium arsenite-treated (10 µM for 2 h), heat-shocked (30°C for 2 h), and both sodium arsenite and heat-shocked A6 cells. As can be seen in Figure 22, the mild sodium arsenite treatment weakly induced the enhanced synthesis of the basic small HSPs (proteins 2-5, 7; Figure 22b). Similarly, the mild heat shock temperature also weakly induced the synthesis of the basic small HSPs (Figure 22c). The combined heat shock and sodium arsenite treatment resulted in a very strong, synergistic induction of all 7 of the basic small HSPs (Figure 22d).

3.7 Herbimycin A Induction of Small HSP Synthesis

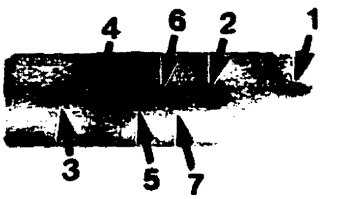
Herbimycin A, a tyrosine-kinase inhibitor, has recently been found to induce heat shock protein expression in cell culture (Murakami *et al.*, 1991; Hedge *et al.*, 1994). One-dimensional SDS-PAGE studies performed in our laboratory have revealed that herbimycin A also induces HSP synthesis in *Xenopus* A6 cells (Briant, 1995). In Figure 23 the IEF 2-D SDS-PAGE

FIGURE 22. The synergistic effect of mild heat shock and sodium arsenite on the synthesis of basic small HSPs in A6 cells. ^{35}S-methionine labeled proteins were isolated from control (22°C), sodium arsenite-treated (10 µM for 2 h), heat-shocked (30°C for 2 h), and both sodium arsenite and heat-shocked, A6 cells. The proteins were resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. The positions of the basic small HSPs are denoted by arrows. These autoradiograms are representative of two experiments.

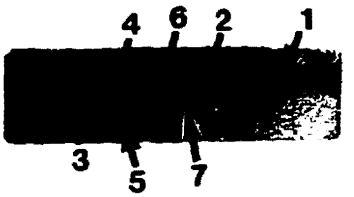
a) CONTROL



b) SODIUM ARSENITE (10 μM, 2 h)



c) HEAT SHOCK (30°C, 2 h)



d) SODIUM ARSENITE & HEAT SHOCK

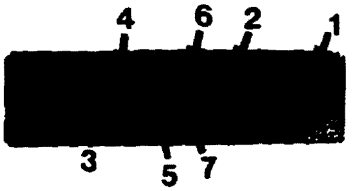
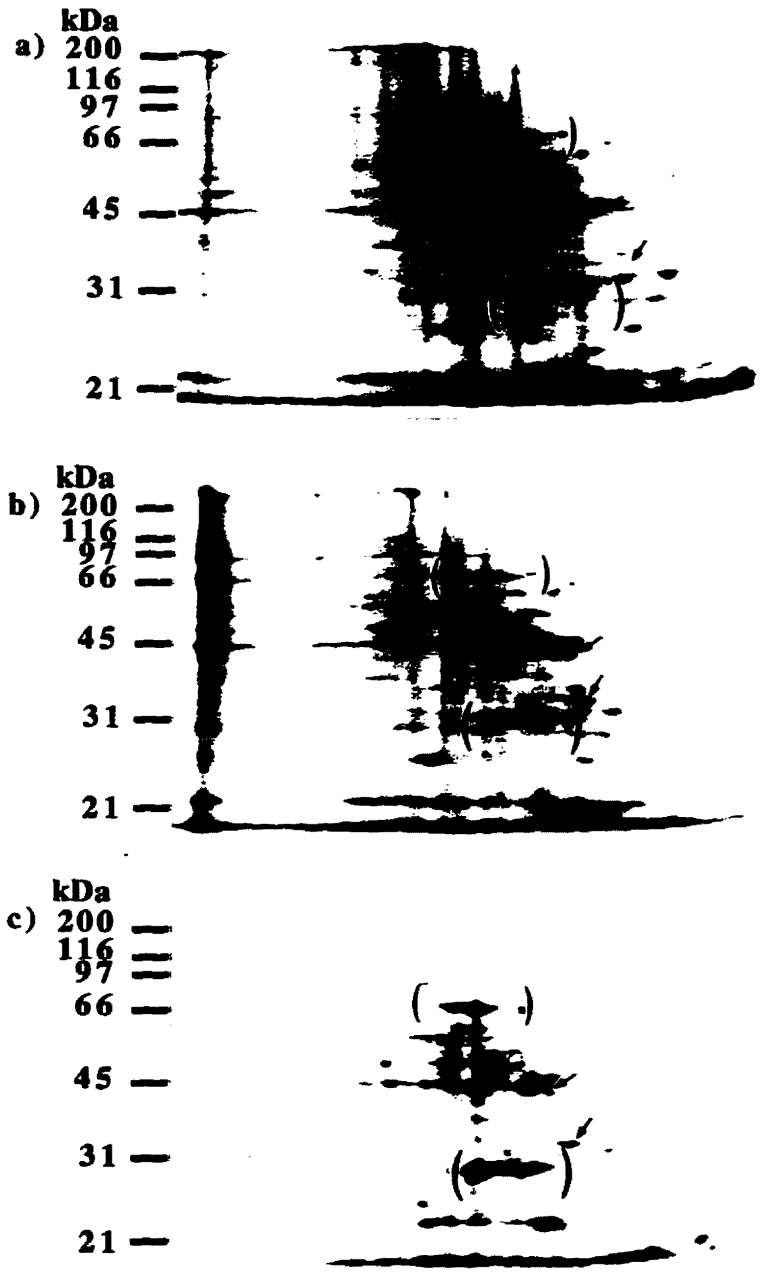


FIGURE 23. The pattern of acidic HSP synthesis in A6 cells exposed to herbimycin A. ^{35}S-methionine labeled proteins were isolated from control, herbimycin A-treated (1 μg/ml for 6 h), and heat-shocked (35°C for 4 h) A6 cells. The proteins were resolved by IEF 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from basic (left) to acidic (right) portions of the gel. Size standards are denoted on the left. The upper brackets delimit the HSP70 proteins, lower brackets delimit the small HSPs, and arrows indicates a 45 kDa herbimycin A-induced protein (pI 5.2) and a 33 kDa (pI 5.0) herbimycin A and heat-induced protein. These autoradiograms are representative of two experiments. (Note: the horizontal bracket below two 66 kDa proteins in panel c is present to mark these proteins as reference points for the HSP70 proteins in Figure 36)


a)
kDa
200
116
97
66
45
31
21
CONTROL
b)
kDa
200
116
97
66
45
31
21
HERBIMYCIN A
(1 µg/mL, 6 h)
c)
kDa
200
116
97
66
45
31
21
HEAT SHOCK
(35°C, 4 h)

pattern of HSP synthesis in the presence of herbimycin A (1 µg/ml for 6 h) is presented. Herbimycin A was dissolved in dimethylsulfoxide (DMSO) and thus the control was treated with an equivalent concentration of DMSO as the herbimycin A sample. For comparison, 2-D SDS-PAGE resolved proteins from heat-shocked A6 cells (35°C for 4 h) are also presented (Figure 23c). Herbimycin A induced the synthesis of the HSP70 proteins (denoted by the upper brackets), as well as the acidic small HSPs (denoted by the lower brackets) in a comparable manner as the heat shock sample. In addition a 45 kDa protein (pI 5.2) was induced by herbimycin A, and a 33 kDa protein (pI 5.0) was induced by both herbimycin A and heat shock.

A comparison of basic HSP synthesis in the presence of herbimycin A and heat shock also revealed a similar pattern of induction (Figure 24). Both herbimycin A and heat shock induced the synthesis of the basic small HSPs. Both inducers also resulted in the increased synthesis of a 62 kDa protein. In addition, heat shock induced the synthesis of 36 and 38 kDa proteins.

The synergistic effect of mild heat shock and herbimycin A treatment has been demonstrated in A6 cells by 1-D SDS-PAGE analysis (Briant, 1995). A 2-D SDS-PAGE examination of synergistic induction of small HSP synthesis is presented in Figure 25. A6 cells were exposed to herbimycin A (0.5 µg/ml for 2 h), heat shock (27°C for 2 h), and both herbimycin A and heat shock. As can be seen, herbimycin A or mild heat shock alone resulted in the synthesis of low levels of some of the acidic small HSPs (Figure 25b and c). The combined treatment resulted in a strong, synergistic induction of the full set of the acidic small HSPs (Figure 25d). Similarly, the HSP 70 proteins were synergistically

FIGURE 24. The pattern of basic HSP synthesis in A6 cells exposed to herbimycin A. ^{35}S-methionine labeled proteins were isolated from control, herbimycin A-treated (1 μg/ml for 6 h), and heat-shocked (35°C for 4 h) A6 cells. The proteins were resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. Size standards are denoted on the left. Arrows indicate the positions of the basic small HSPs in the 30 kDa range, a 62 kDa herbimycin A and heat-inducible protein, as well as 36 and 38 kDa heat-inducible proteins. These autoradiograms are representative of two experiments.

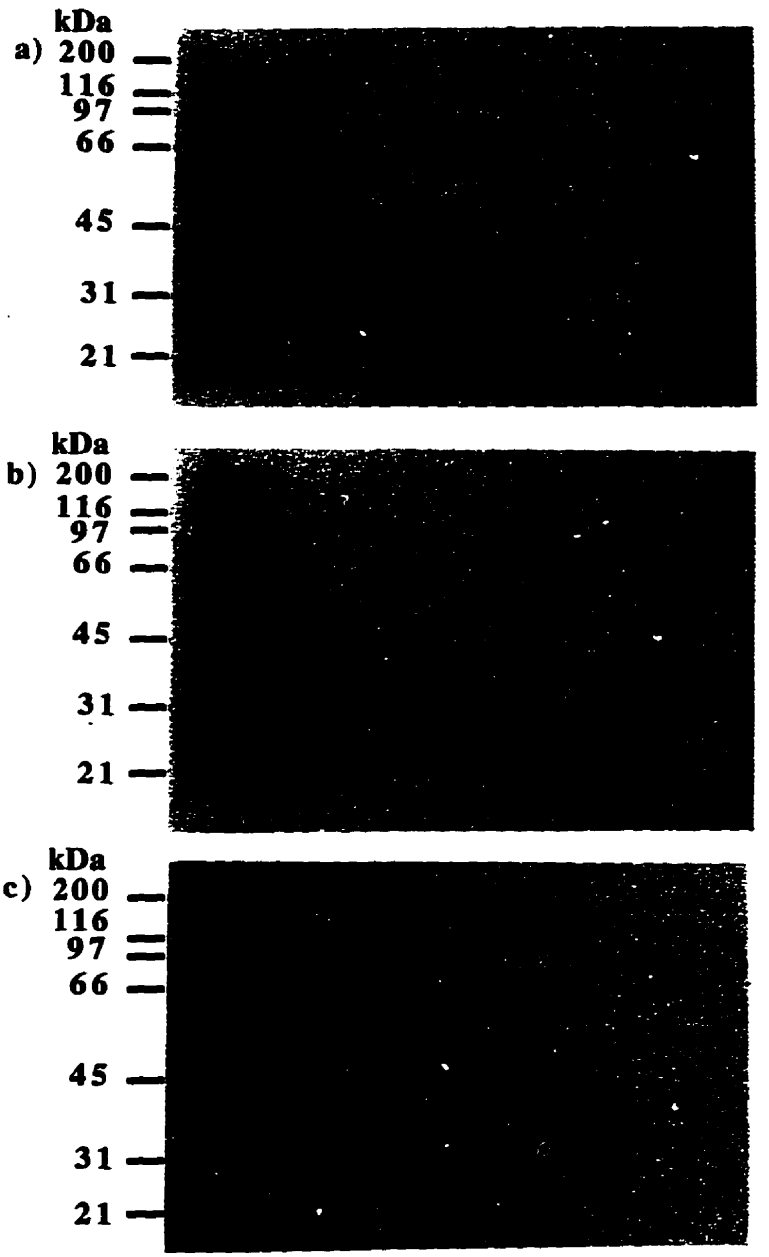
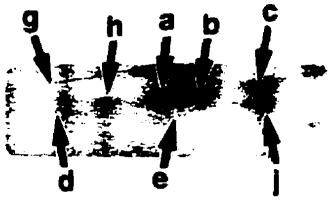
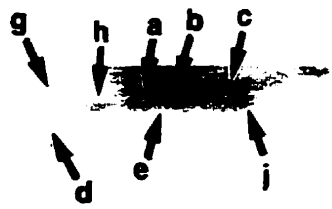

kDa
a) 200
116
97
66
45
31
21
CONTROL
kDa
b) 200
116
97
66
45
31
21
HERBIMYCIN A
(1 μg/mL, 6 h)
kDa
c) 200
116
97
66
45
31
21
HEAT SHOCK
(35°C, 4 h)

FIGURE 25. The synergistic effect of mild heat shock and herbimycin A on the synthesis of acidic small HSPs in A6 cells. ^{35}S-methionine labeled proteins were isolated from control (DMSO), herbimycin A-treated (0.5 μg/ml for 2 h), heat-shocked (27°C for 2 h), and both herbimycin A and heat-shocked, A6 cells. The proteins were resolved by IEF 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from basic (left) to acidic (right) portions of the gel. Arrows indicate the positions of acidic small HSPs. These autoradiograms are representative of two experiments.

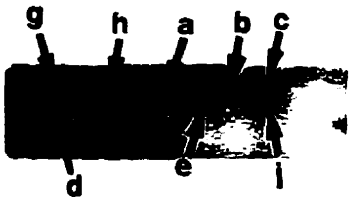
a) **DMSO CONTROL**



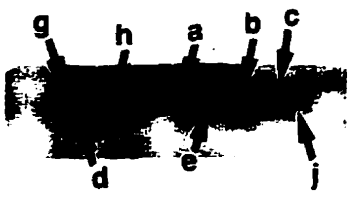
b) **HERBIMYCIN A (0.5 µg/mL, 2 h)**



c) **HEAT SHOCK (27°C, 2 h)**



d) **HERBIMYCIN A & HEAT SHOCK**



induced (data not shown). Proteins a, b, c, and j were weakly induced by DMSO (Figure 25a).

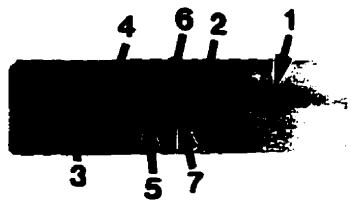
Analysis of the effect of mild heat shock and herbimycin A on the induction of basic small HSPs also revealed a synergistic effect (Figure 26). Mild heat shock or herbimycin A concentrations alone resulted in the weakly induced synthesis of proteins 1 thru 5, and 7 (Figure 26b, c). The combination of the two inducers resulted in a dramatic synergistic induction of all 7 of the basic small HSPs (Figure 26d). It is interesting to note that in all of the examples of synergistic acidic or basic small HSP induction that were just presented, the combination of the two inducers (whether it was heat and sodium arsenite, or heat and herbimycin A) not only resulted in a greater synthesis of individual proteins, it also resulted in the induction of the full set of small HSPs.

3.8 Hydrogen Peroxide and Heavy Metals Do Not Induce Basic Small HSP Synthesis

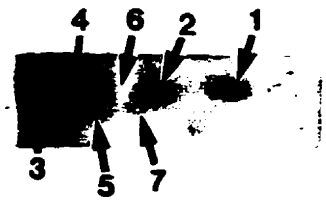
Hydrogen peroxide and heavy metals such as cadmium and zinc have been found to induce HSP synthesis in other organisms (Abe *et al.*, 1994; Gedamu and Zaffarullah, 1993; Heikkila *et al.*, 1982). Previous studies in *Xenopus* however, have failed to detect HSP synthesis in the presence of these inducers (N. Ovsenek, A. Ali, and J. Heikkila, unpublished results). In order to determine if the basic small HSPs are induced by these chemicals, A6 cells were exposed to hydrogen peroxide (100 µM for 4 h), cadmium chloride (10 µM for 4 h), or zinc chloride (10 µM for 4 h), conditions previously shown to induce HSP synthesis in fish tissue culture cells (Heikkila *et al.*, 1982). Resolution of

FIGURE 26. The synergistic effect of mild heat shock and herbimycin A on the synthesis of basic small HSPs in A6 cells. ^{35}S-methionine labeled proteins were isolated from control (DMSO), herbimycin A-treated (0.5 μg/ml for 2 h), heat-shocked (27°C for 2 h), and both herbimycin A and heat-shocked, A6 cells. The proteins were resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. Arrows indicate the positions of basic small HSPs. These autoradiograms are representative of two experiments.

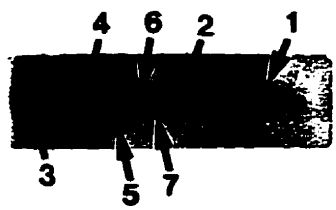
a) DMSO CONTROL



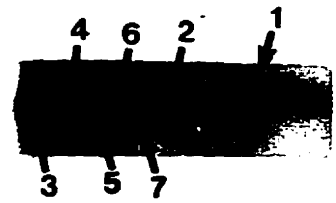
b) HERBIMYCIN A (0.5 μg/ml, 2 h)



c) HEAT SHOCK (27°C, 2 h)



d) HERBIMYCIN A & HEAT SHOCK



the radiolabeled proteins by NEPHGE 2-D SDS-PAGE revealed that mainly constitutive levels of basic small HSPS were present, with a slight induction of protein 2 being observed in the hydrogen peroxide treated cells (Figure 27). Exposures to higher concentrations (100 μM) of the heavy metals also failed to induce the enhanced synthesis of the basic small HSPs (data not shown).

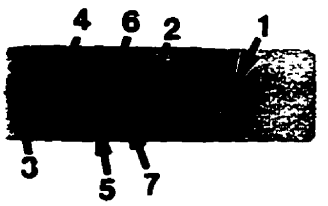
3.9 Two-Dimensional Patterns of Stress Proteins in the Presence of BiP Inducing Agents: Tunicamycin and Dithiothreitol (DTT)

BiP protein synthesis is induced by sulfhydryl reducing agents such as dithiothreitol (DTT), and inhibitors of glycosylation such as tunicamycin (Lee, 1992; Nover, 1991). Figure 28 presents the 2-D SDS-PAGE pattern of protein synthesis in A6 cells treated with tunicamycin (1 μg/ml for 24 h) or DTT (0.1 mM, 24 h; proteins were labeled with ^{35}S-methionine during the last 4 h of the treatments). As expected, both tunicamycin and DTT resulted in the induction of two 78 kDa BiP proteins (pI 5.2, 5.3), as well as a 98 kDa BiP proteins (pI of 5.1; Figure 28b, c; Winning *et al.*, 1989, 1991)

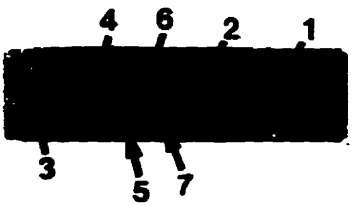
In order to determine if there are any basic BiP proteins induced by these agents, radiolabeled proteins isolated from tunicamycin and DTT-treated A6 cells were resolved by NEPHGE 2-D SDS-PAGE (Figure 29). A constitutively present 45 kDa protein was found to be induced by tunicamycin (Figure 29b). However, the synthesis of the basic small HSPs were not induced under these conditions.

FIGURE 27. The effect of hydrogen peroxide, cadmium chloride, and zinc chloride on basic small HSP synthesis in A6 cells. ^{35}S-methionine labeled proteins were isolated from control, hydrogen peroxide (100 μM for 4 h), cadmium chloride (10 μM for 4 h), or zinc chloride (10 μM for 4 h) treated A6 cells. The proteins were resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. Arrows indicate the positions of basic small HSPs. These autoradiograms are representative of two experiments.

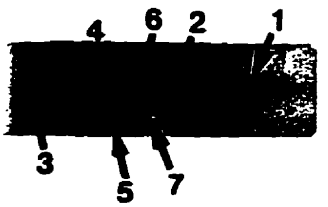
a) CONTROL



b) HYDROGEN PEROXIDE
(100 μM, 4 h)



c) CADMIUM CHLORIDE
(10 μM, 4 h)



d) ZINC CHLORIDE
(10 μM, 4 h)

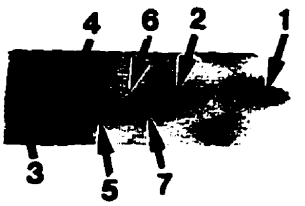


FIGURE 28. The pattern of acidic BiP synthesis in A6 cells exposed to tunicamycin and dithiothreitol (DTT). ^{35}S-methionine labeled proteins were isolated from control, tunicamycin-treated (1 µg/ml for 24 h), and DTT-treated (0.1 mM for 24 h) A6 cells. The proteins were resolved by IEF 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from basic (left) to acidic (right) portions of the gel. Molecular weight standards are indicated on the left. Arrows facing upwards indicate two induced 78 kDa BiP proteins (pI 5.2, 5.3) and an arrow facing downwards indicates a 97 kDa Bip protein (pI 5.1). These autoradiograms are representative of two experiments.

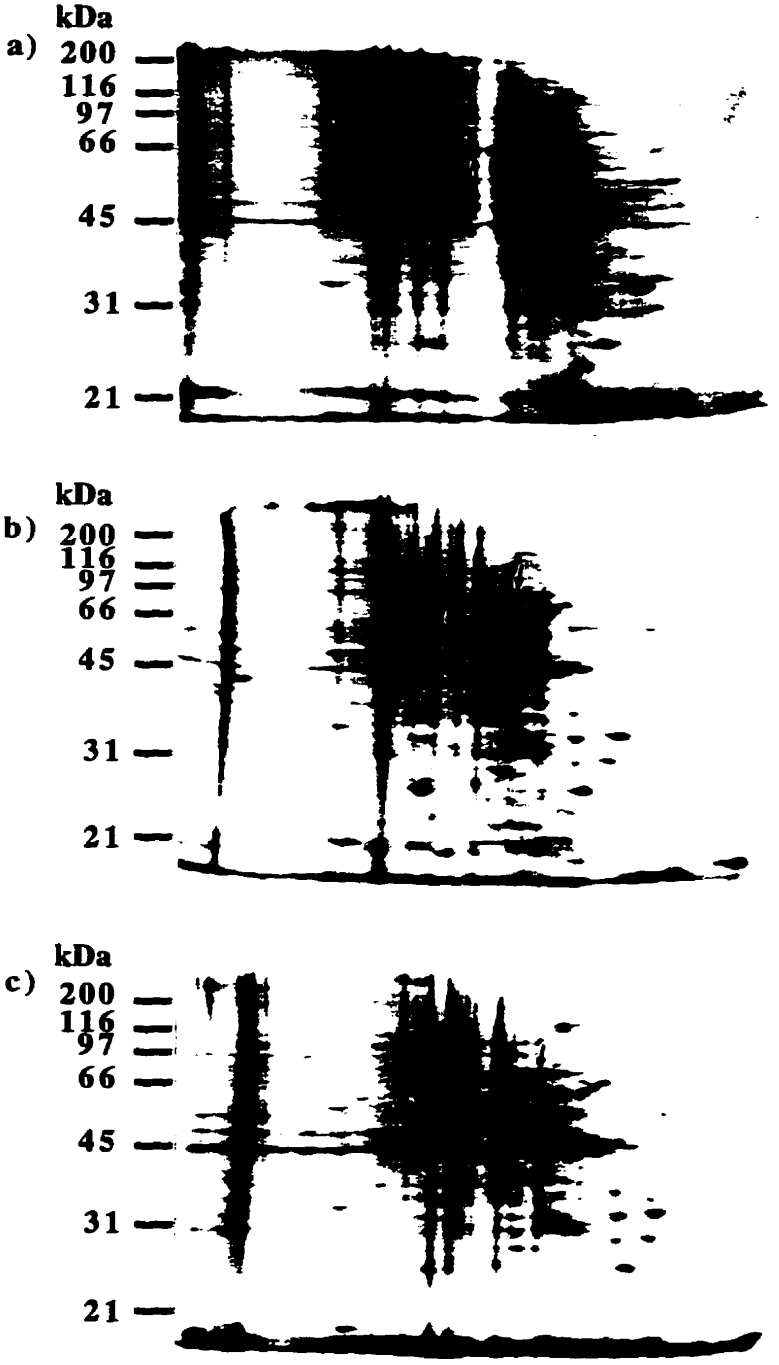
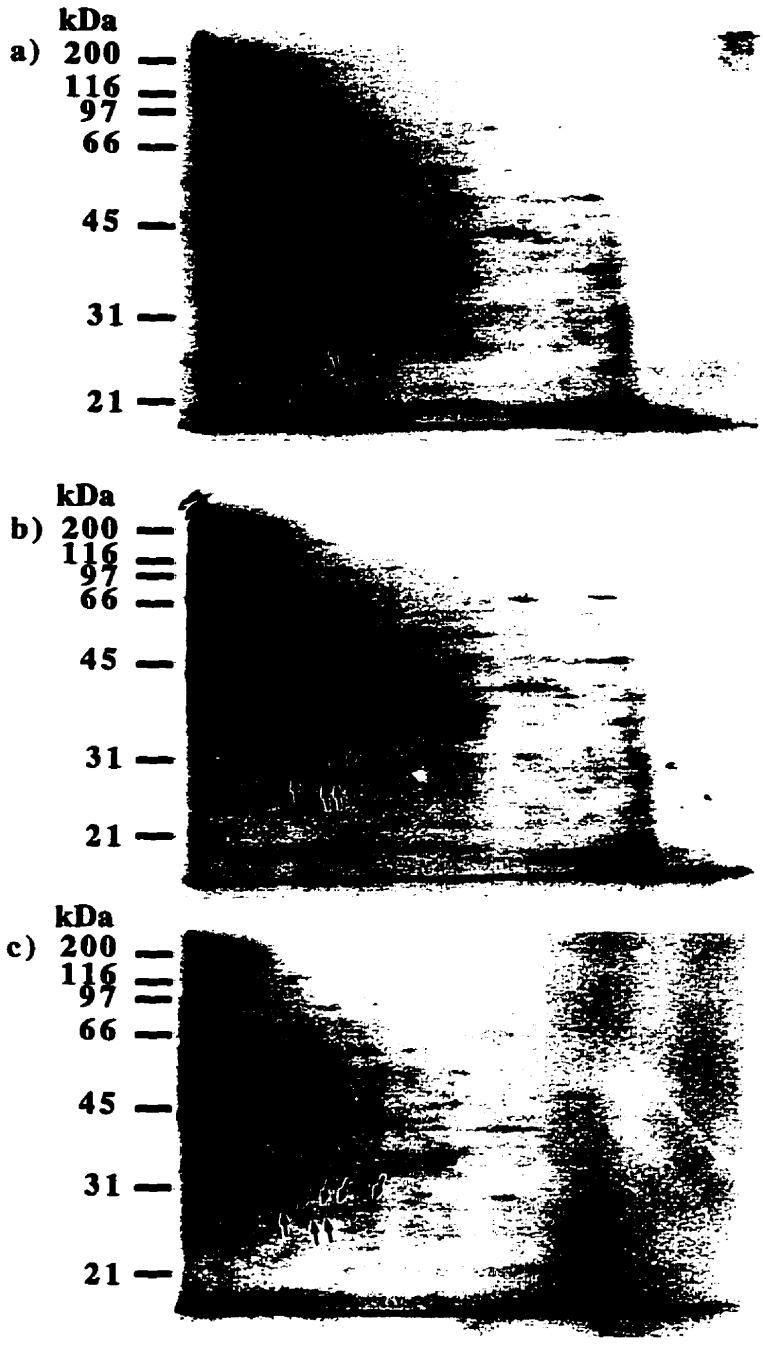

a)
kDa
200
116
97
66
45
31
21
CONTROL
b)
kDa
200
116
97
66
45
31
21
TUNICAMYCIN
(1 µg/mL, 24 h)
c)
kDa
200
116
97
66
45
31
21
DITHIOTHREITOL
(0.1 mM, 24 h)

FIGURE 29. The effect of tunicamycin and dithiothreitol (DTT) on basic BiP synthesis in A6 cells. ^{35}S-methionine labeled proteins were isolated from control, tunicamycin-treated (1 µg/ml for 24 h), and DTT-treated (0.1 mM for 24 h) A6 cells. The proteins were resolved by NEPHGE 2-D SDS-PAGE, and subjected to fluorography. The gels are oriented from acidic (left) to basic (right) portions of the gel. Molecular weight standards are indicated on the left. The positions of basic small HSPs are labeled with arrows. An arrow with an asterisk indicates a tunicamycin-induced 45 kDa basic protein. These autoradiograms are representative of two experiments.


kDa
a) 200
116
97
66
45
31
21
CONTROL
kDa
b) 200
116
97
66
45
31
21
TUNICAMYCIN
(1 μg/mL, 24 h)
kDa
c) 200
116
97
66
45
31
21
DITHIOTHREITOL
(0.1 mM, 24 h)

3.10 The Pattern of Basic HSP Synthesis in Gastrula Embryos

Finally, the pattern of basic protein synthesis in control (22°C) and heat-shocked (33°C for 6 h) gastrula stage embryos was examined using NEPHGE 2-D SDS-PAGE (Figure 30). Two 50 kDa heat-inducible basic proteins were detected (Figure 30b). These proteins were present at very low levels constitutively. No basic small HSP synthesis was detected.

3.11 A Basic Small Hsp27 cDNA From *Poeciliopsis lucida* Does Not Cross-React with *Xenopus* Basic Small Hsp mRNA

As mentioned previously, Dr. Hightower's laboratory was the first to identify the presence of basic small HSPs (unpublished data). They have succeeded in isolating an hsp27 cDNA from *Poeciliopsis lucida* which they generously made available in order to see if it might cross-react with the *Xenopus* basic hsp mRNA (Figure 31). Total RNA was isolated from control (22°C) and heat-shocked (33°C for 1 h) *Xenopus* tadpoles and A6 cells. As a positive control Dr. Hightower also made available 2 µg of oligo(dT)-selected RNA samples from control and heat-shocked *P. lucida.* Hybridization with the [^{32}P]-labeled hsp27 cDNA clone resulted in the expected 1.8 kb signal from heat-shocked *P. lucida* samples, however no signal was observed in the *Xenopus* samples (note that the faint background smudge in the heat shock tadpole lane should not be interpreted as a positive signal, based on repetitions of this experiment). Hybridization with the hsp27 cDNA followed by post-hybridization washes under lowered stringency also failed to produce a positive

FIGURE 30. The pattern of basic heat shock protein synthesis in *Xenopus* gastrula stage embryos. ^{35}S-methionine labeled proteins isolated from control (22°C) and heat-shocked (33°C for 6 h) gastrula stage embryos were resolved by NEPHGE 2-D SDS-PAGE and subjected to fluorography. The acidic portion of the gel is oriented to the left, and the basic to the right. Molecular weight standards are indicated on the left. Arrows indicate two 50 kDa basic heat-inducible proteins. These autoradiograms are representative of three experiments.

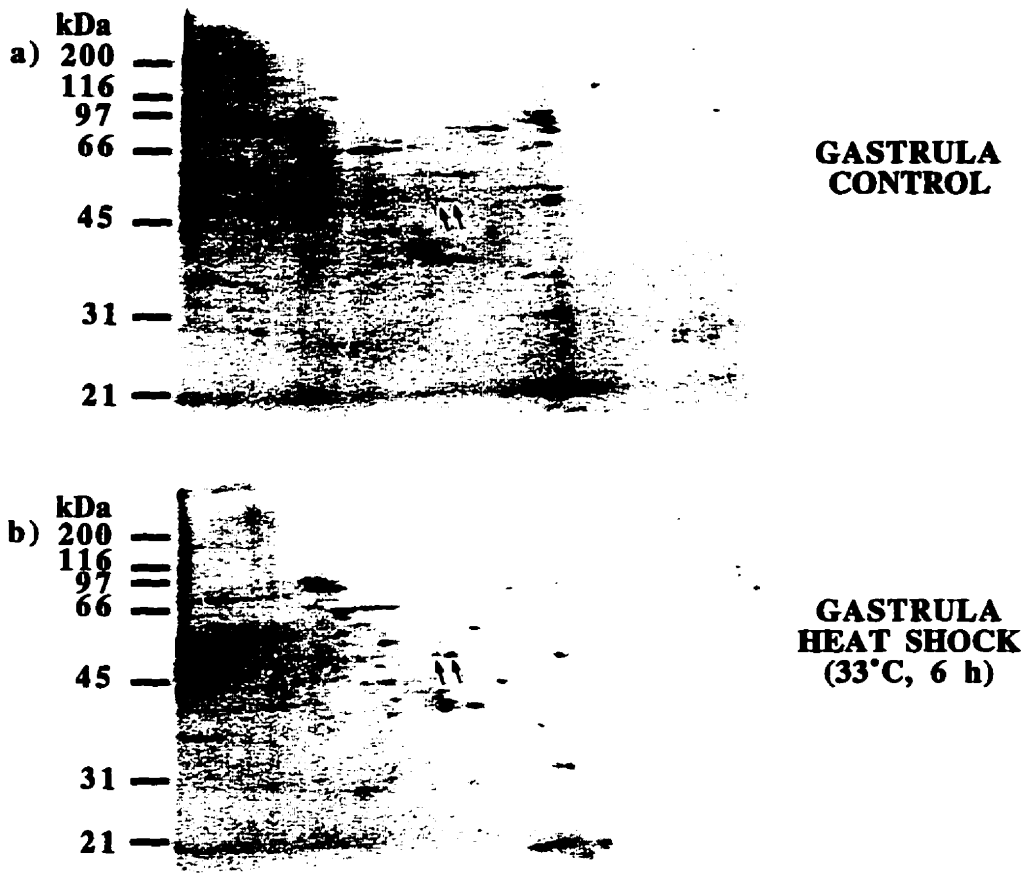
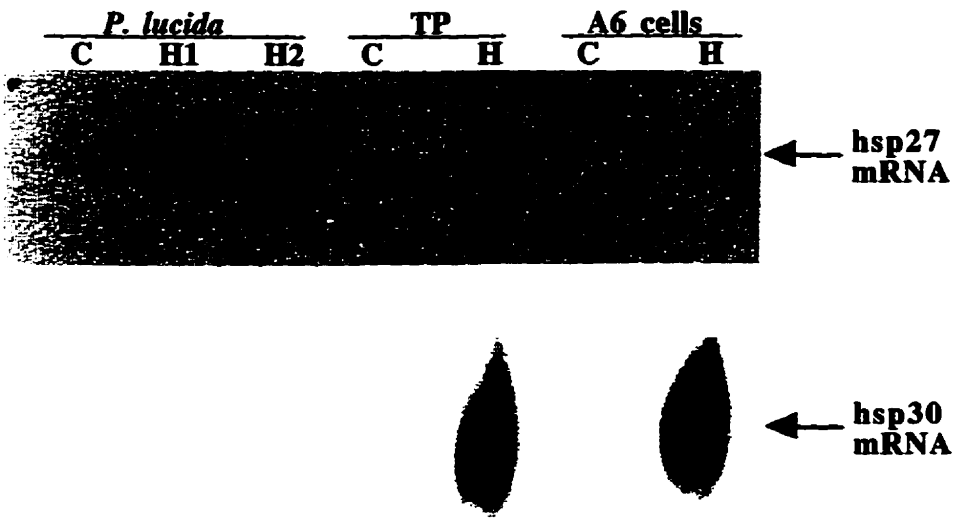

kDa
a) 200
116
97
66
45
31
21
GASTRULA
CONTROL
kDa
b) 200
116
97
66
45
31
21
GASTRULA
HEAT SHOCK
(33°C, 6 h)

FIGURE 31. *P. lucida* hsp27 cDNA encoding a basic small HSP does not cross-react with *Xenopus* small hsp mRNA. Total RNA was isolated from control (22°C) and heat-shocked (33°C for 1 h) tadpoles (TP) and A6 cells. Two micrograms each of oligo(dT)-selected *P. lucida* RNA from control and heat-shocked (H1 = 1.5 h, H2 = 10 h) samples were a generous gift from Dr. Larry Hightower. The Northern blot was hybridized against [^{32}P]-labeled hsp27 (generous gift of Dr. Larry Hightower) or hsp30 clones. The hsp27 and hsp30 mRNAs are 1.8 kb and 1.1 kb, respectively. An artifact is present in the heat-shocked tadpole sample. These autoradiograms are representative of three experiments.


P. lucida
TP
A6 cells
C
H1
H2
C
H
C
H
hsp27 mRNA
hsp30 mRNA

signal from *Xenopus* heat-shocked samples (including cycloheximide-treated heat-shocked embryo RNA samples; data not shown). Re-hybridization of the same blot with the *Xenopus* [^{32}P]-labeled hsp30 clone identified the 1.1 kb hsp30 mRNA in the heat-shocked tadpole and A6 cell samples, and did not cross-react with the *P. lucida* mRNA.

3.12 Examination of Heat Shock Aggregate Formation

One of the characteristic properties of small heat shock proteins is their ability to form large aggregates (200-800 kDa) upon heat-shock (Waters *et al.*, 1996; Arrigo and Landry, 1994). In order to examine *Xenopus* small HSP aggregation pore exclusion limit electrophoresis was employed (Helm *et al.*, 1993). ^{35}S-methionine labeled proteins were isolated in their native state (see Materials and Methods) from control (22°C) and heat-shocked (35°C for 4 h) A6 cells labeled for the duration of their treatments. The proteins were resolved under non-denaturing conditions on a 2-22.5% (0-12% glycerol) polyacrylamide gel for 48 h, long enough that all proteins should migrate until they reach their pore exclusion limits (Anderson *et al.*, 1972; Figure 32). Four heat-induced aggregates were identified: 'a' - 510 kDa; 'b' - 350 kDa; 'c' - 290 kDa; 'd' - 240 kDa, with aggregate 'a' being the most prominent, and aggregates 'b' and 'd' quite faint.

In order to determine if HSP30 proteins were present in any of these aggregates, the native gel proteins were immunoblotted with the HSP30C antibody (Figure 33). The HSP30C antibody cross-reacted with heat-induced aggregates 'a' and 'b' (Figure 33b). Pre-immune serum failed to produce a

FIGURE 32. Analysis of HSP aggregation in heat-shocked A6 cells using pore exclusion limit electrophoresis. ^{35}S-methionine labeled native proteins were isolated from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells. The proteins were separated on a 2-22.5% acrylamide gradient gel until they reached their pore exclusion limits, following which the gels were subjected to fluorography. Size standards are denoted along the left. Arrows indicate heat-inducible aggregates: 'a' - 510 kDa; 'b' - 350 kDa; 'c' - 290 kDa; 'd' - 240 kDa. This autoradiogram is representative of three experiments.

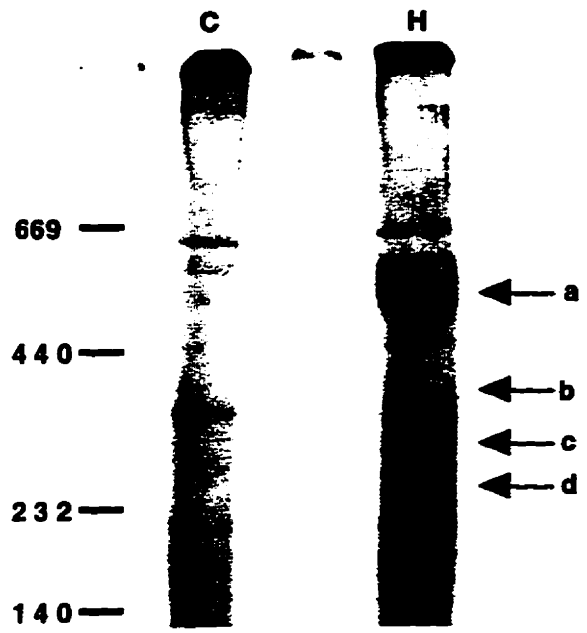
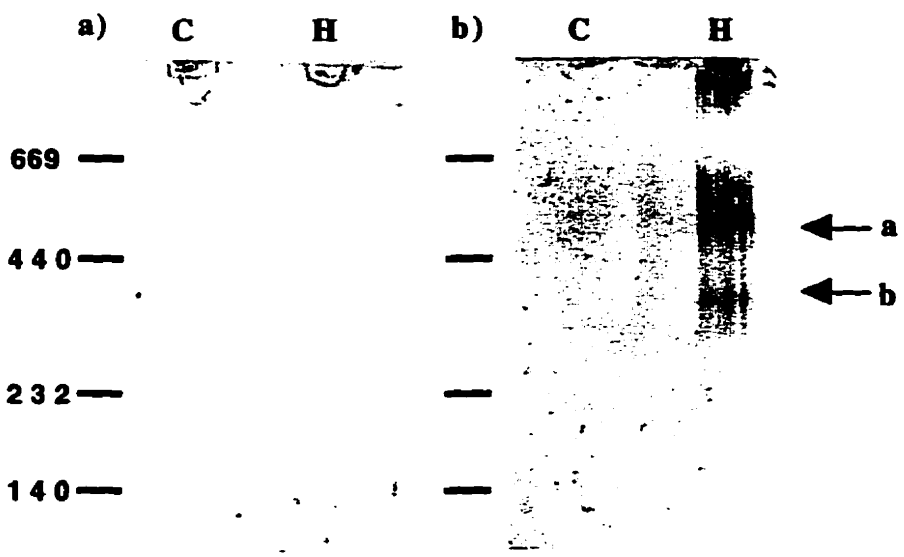

C
H
669
440
232
140
a
b
c
d

FIGURE 33. The HSP30C antibody cross-reacts with heat-induced aggregates identified by pore exclusion limit electrophoresis. ^{35}S-methionine labeled native proteins were isolated from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells. The proteins were separated on a 2-22.5% acrylamide gradient gel until they reached their pore exclusion limits, and subsequently Western blotted onto nitrocellulose paper. The blots were then probed with a) pre-immune serum, or b) the HSP30C antibody. The aggregates which reacted with the HSP30C antibody are denoted by the arrows: 'a' - 510 kDa; 'b' - 350 kDa. These Western blots are representative of three experiments.


a)
C
H
b)
C
H
669
440
232
140
a
b

signal on the Western blotted aggregates (Figure 33a), thus ensuring that the observed signals arose from the HSP30C antibody alone. Probing of theWestern blotted aggregates with an HSP70 antibody also failed to produce a distinct signal, although a smear above background levels was present in the heat-shocked sample, suggesting that HSP70 protein may be present, but was not localized to the HSP30 aggregate (Figure 34).

In order to confirm the presence of the HSP30 proteins, the region containing aggregate 'a' was excised from the gel, along with its corresponding region from the control sample. Furthermore, regions 0.5 and 1.0 cm above and below the aggregate region were also excised from control and heat-shocked samples. The gel fragments were homogenized in 1-D protein isolation buffer and boiled to denature the proteins. They were then resolved using 1-D SDS-PAGE. For comparison, radioactively labeled samples from control and heat-shocked A6 cells were electrophoresed on the same gel. As can be seen in Figure 35, a 30 kDa protein was present in the heat-shocked aggregate fraction, which was absent in the control sample. In addition there were two smaller 24 and 20 kDa proteins present in the heat shock aggregate sample. There were traces of HSP30 protein present in the other heat-shocked fractions, especially fractions -1 and -2 taken from below the aggregate, possibly arising from smaller aggregates that were still forming.

Finally, the region containing aggregate 'a' was excised from control and heat-shocked samples and the gel fragments were homogenized in 2-D protein isolation buffers and resolved using 2-D SDS-PAGE (Figure 36). It can be seen that heat shock aggregate 'a' was resolved into the characteristic pattern of small HSPs, and that small HSPs were not present in the control sample. As

FIGURE 34. The HSP70 antibody does not cross-react with the heat-induced aggregate identified by pore exclusion limit electrophoresis. ^{35}S-methionine labeled native proteins were isolated from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells. The proteins were separated on a 2-22.5% acrylamide gradient gel until they reached their pore exclusion limits, and subsequently Western blotted onto nitrocellulose paper. The blot was then probed with an HSP70 antibody. No distinct signals were observed. This Western blot is representative of two experiments.

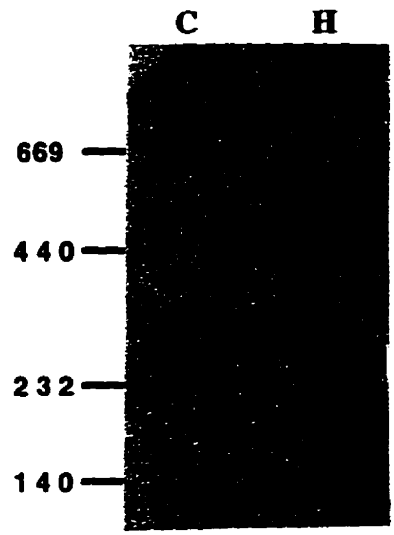

C
H
669
440
232
140

FIGURE 35. One dimensional analysis of proteins comprising the heat-induced aggregate. ^{35}S-methionine labeled proteins were isolated from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells in a native state. The proteins were separated on a 2-22.5% acrylamide gradient gel until they reached their pore exclusion limits, and were subsequently stained with Coomassie blue. The region of the gel containing aggregate 'a' (shown in Figure 32) and the corresponding control region were excised and proteins were isolated by the crush and soak method, in 1-D protein isolation buffer (•; see Materials and Methods). Similarily, proteins from the regions 0.5 cm and 1.0 cm above (+1 and +2) and below (-1 and -2) the aggregate region were also isolated. The proteins were then resolved on a 1-D SDS-PAGE gel and subjected to fluorography. For comparison, radioactively labeled proteins from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells were electrophoresed on the same gels. Size standards (kDa) are denoted on the left. Arrows indicate the 30, 24, and 20 kDa proteins present in the heat-shocked aggregate fraction (•). Heat-inducible proteins found in the total protein samples are also indicated. These autoradiograms are representative of three experiments.

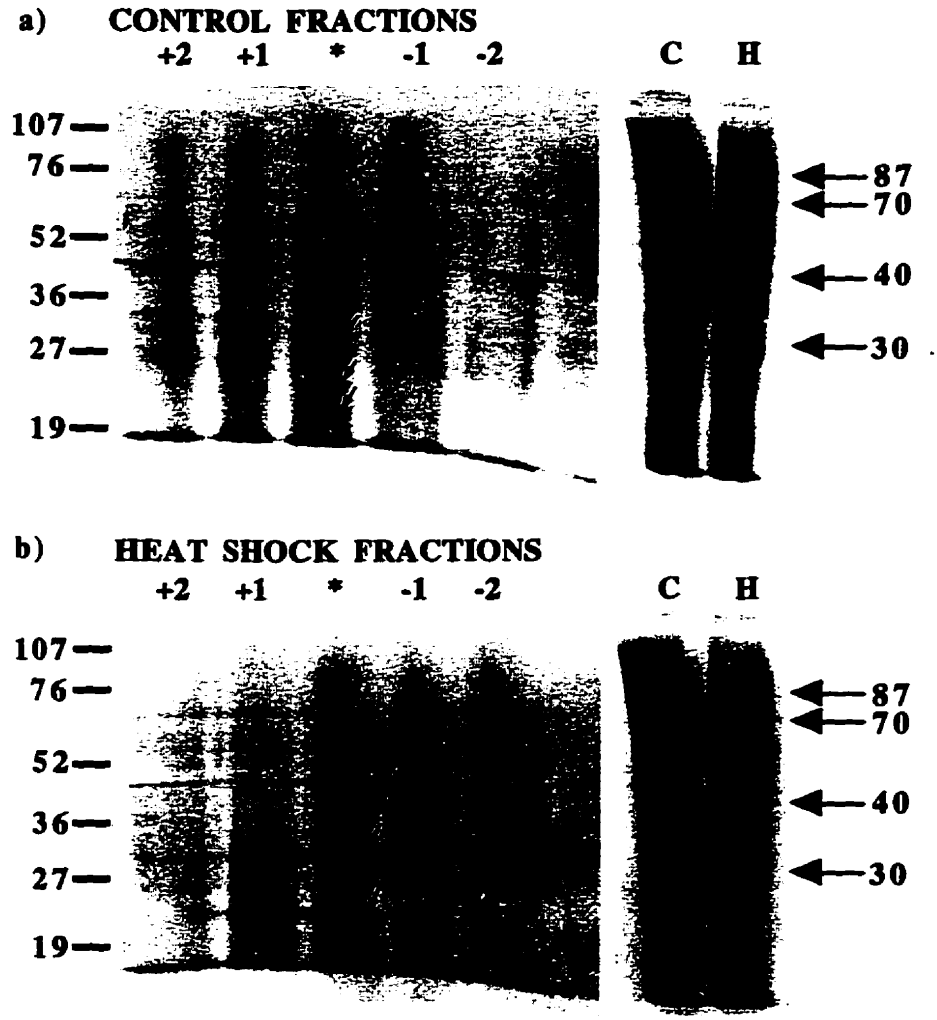
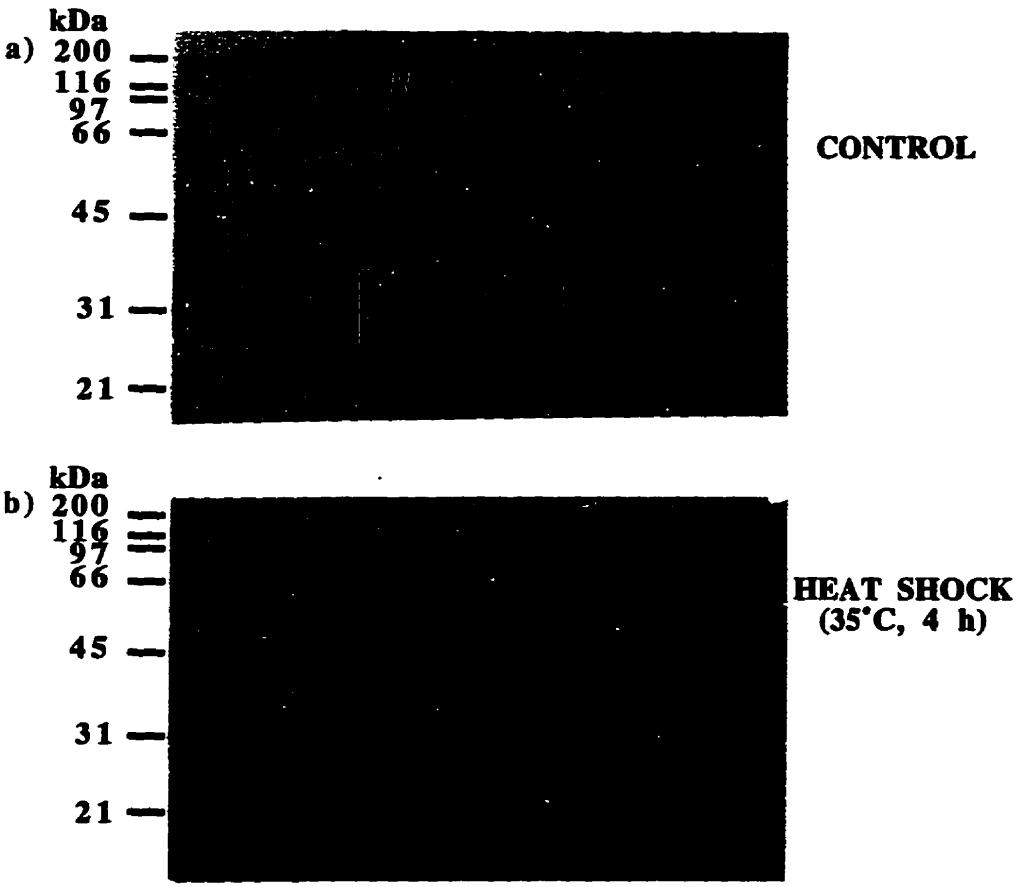

a) CONTROL FRACTIONS
+2 +1 * -1 -2
C H
107
76
52
36
27
19
87
70
40
30
b) HEAT SHOCK FRACTIONS
+2 +1 * -1 -2
C H
107
76
52
36
27
19
87
70
40
30

FIGURE 36. IEF 2-D SDS-PAGE analysis of the proteins comprising the heat-induced aggregate. ^{35}S-methionine labeled native proteins were isolated from control (C; 22°C) and heat-shocked (H; 35°C for 4 h) A6 cells. The proteins were separated on a 2-22.5% acrylamide gradient gel until they reached their pore exclusion limits, and were subsequently stained with Coomassie blue. The region of the gel containing aggregate 'a' (shown in Figure 32) and the corresponding control region were excised and proteins were isolated by the crush and soak method, in 2-D protein isolation buffers (see Materials and Methods). The proteins were then resolved by IEF 2-D SDS-PAGE and subjected to fluorography. Brackets delimit the small HSP region and the individual small HSPS are labeled. Arrows indicate two HSP70 proteins [for a frame of reference, compare the position of the two 66 kDa proteins (underlined with a horizontal bracket in panels a and b) with the similarly labeled 66 kDa proteins in Figure 23c and the HSP70 proteins].


kDa
a) 200
116
97
66
45
31
21
CONTROL
kDa
b) 200
116
97
66
45
31
21
HEAT SHOCK
(35°C, 4 h)

well, using the labeled protein doublet as a frame of reference (compare with Figure 23c), very low levels of heat-inducible HSP70 proteins (small arrows) were also found in the heat-shocked sample (they are absent in the control sample). Additional proteins in the 45 and 66 kDa size range were present in both control and heat-shocked samples.

4. Discussion

Members of the small molecular weight hsp gene family, hsp30, are differentially expressed in a heat-inducible fashion during early development of *Xenopus laevis* (Table I). In previous studies, RNase protection assays and RT-PCR experiments have specifically shown that hsp30A and hsp30C mRNAs are not heat-inducible until the early tailbud stage (stage 23-24) of development (Ali *et al.*, 1993; Krone and Heikkila, 1989). RT-PCR analysis has also specifically shown that hsp30D mRNA is not heat-inducible until the mid-tailbud stage (stage 34-35) of development, which is approximately one day later than hsp30A and hsp30C (Ohan and Heikkila, 1995). In this study I have detected low levels of heat-inducible hsp30 mRNA at the late blastula, gastrula, and neurula stages of development using an hsp30C genomic clone as a general probe. The membership of these PTB mRNAs in the hsp30 family is based on their heat shock-inducible detection by the hsp30C genomic probe and their size (approx. 1.1 kb) which is similar to other members of the family. The exact identity and relationship of the PTB hsp30 mRNAs with other members of the hsp30 family will have to await the cloning of their cDNAs. Nevertheless, it is likely that these PTB hsp30 mRNAs are transcribed from some as yet uncharacterized members of the hsp30 gene family. It should be mentioned that Northern blotting did not allow us to differentiate between PTB hsp30 mRNA and those mRNAs (hsp30A and hsp30C) which are first heat-inducible at the tailbud stage. Thus, it is possible, although unlikely, that PTB hsp30 mRNAs are not present in the population of heat-induced mRNAs in post-tailbud embryos. In addition, cycloheximide-treated blastula embryos, which have enhanced

TABLE 1. Differential expression of the hsp 30 gene family during *Xenopus* development

Hsp 30 Family Member	Developmental stages (1 hour heat shock at 33°C)						
	Cleav.	Blast.	Gast.	Neur.	EarlyTB	Mid-TB	TP
PTB Hsp 30[a]	-	+	+	+	?	?	?
Hsp 30A[b]	-	-	-	-	+	+	+
Hsp 30C[b,c]	-	-	-	-	+	+	+
Hsp 30D[c]	-	-	-	-	-	+	+

[a] Determined via Northern blotting
[b] Determined via RNase protection assay
[c] Determined via RT-PCR

levels of PTB hsp30 mRNAs (discussed below) were examined for the presence of hsp30D transcripts using RT-PCR. The inability to detect hsp30D mRNA in these embryos supports the previous results as to the stage-dependent expression of this particular small hsp gene. While the exact number of hsp30 genes is not known, Southern blotting experiments have suggested at least seven different members of the hsp30 gene family (Krone *et al.*, 1992). A number of other examples of differential expression of gene families during *Xenopus* development have been reported, including actin (Mohun *et al.*, 1983), activin (Thomsen *et al.*, 1990), insulin (Shuldiner *et al.*, 1991), bone morphogenetic protein (Suzuki *et al.*, 1993), and integrin (Whittaker and DeSimone, 1993). However, we have not detected differential gene expression in other hsp gene families such as with hsp70, whose heat-inducible members, hsp70A and hsp70B are both detected after the midblastula stage (Krone and Heikkila, 1989). In other systems, differential gene activation of small hsps has been reported during *Drosophila melanogaster* development and aging (Marin *et al.*, 1993), as well as in *Caenorhabditis elegans* (Dixon *et al.*, 1990; Stringham *et al.*, 1992), and during maize microsporogenesis (Atkinson *et al.*, 1993).

The mechanism(s) involved in the differential expression of the *Xenopus* small hsps is not known. Site-specific methylation of key genomic DNA regions has been suggested as a possible mechanism for repression of gene expression (Adams, 1990). An examination of the 5'-flanking DNA sequences of the hsp30C gene indicated the presence of potential methylation sites. However, I have been unable to prematurely induce the expression of the

hsp30D gene in embryos treated with 5-azacytidine prior to the tailbud stage (N. Ohan, M.Sc. Thesis). Thus, DNA methylation may not be involved in the regulatory mechanism associated with *Xenopus* small hsp expression.

It is also possible that the differential expression of the hsp30 gene family may be controlled at the level of chromatin. In fact, differences in chromatin structure have been correlated with differential expression of the small heat shock protein genes in *Caenorhabditis elegans* (Dixon *et al.*, 1990). Furthermore, as mentioned previously, the hsp30 genes occur as clusters, which is a feature common with globin genes. Interestingly, studies of human globin gene expression have indicated that they are regulated at the level of chromatin conformation by a globin locus element located 10-20 kb upstream (Lowrey *et al.*, 1992). Indeed, regulation by chromatin structure would be consistent with previous hsp30 microinjection studies in which it was found that microinjected hsp30A and hsp30C clones were heat-inducible, but prematurely expressed at the heat-shocked midblastula stage (Krone and Heikkila, 1989; Ali *et al.*, 1993). It is possible that the microinjected constructs did not attain the proper chromatin conformation, probably due to insufficient 5' or 3' flanking sequences, and thus were not correctly regulated during development. One could speculate that sequential changes in chromatin structure might result in a sequential expression of the genes within a single cluster. This set of events would account for the later expression of the hsp30D gene as compared to hsp30C, for example.

In the present study I have also found that treatment of late blastula and gastrula embryos with cycloheximide results in an increase in the levels of PTB hsp30 mRNAs after heat shock. As mentioned previously, cycloheximide has been shown to stabilize unstable mRNAs in numerous studies with a variety of systems (Kelly *et al.*, 1987; De *et al.*, 1991; Ooi *et al.*, 1993; Nanbu *et al.*, 1994; Sheu *et al.*, 1994). In *Xenopus* embryos, Duval *et al.* (1990) found that the *Eg2* mRNA was stabilized in the presence of cycloheximide. The mechanism for cycloheximide-induced stability of unstable mRNAs appears to involve the inhibition of protein synthesis which may reduce the levels of a factor(s) involved in mRNA degradation. Consistent with this is the finding of enhanced hsp30 mRNA accumulation at high (37°C) heat-shock temperatures, as compared to the decline of hsp70 mRNA levels. The reduction of protein synthesis in general at these temperatures could prevent the continued expression of an mRNA degradation factor, or it is also possible that translation of the message is required before the message can be degraded. As well, the results with cycloheximide are supported by the finding of a potential mRNA instability element in the 3' untranslated region of the hsp30C gene (Krone *et al.*, 1992). In contrast to the PTB hsp30 mRNAs, the levels of hsp70, hsp87, and actin mRNA were not stabilized by cycloheximide treatment under heat shock conditions.

Messenger RNA stability has been shown to play a role in hsp gene regulation in other organisms. For example, *Drosophila* hsp70 mRNA was reported to be unstable under control conditions, and that enhanced accumulation during heat shock was due to the stabilization of the message as

well as increased transcription (Petersen and Lindquist, 1989). Interestingly, *Xenopus* PTB hsp30 mRNAs are unstable under moderate heat shock conditions which have been shown to stabilize other hsp mRNAs (Heikkila *et al.*, 1987; Krone and Heikkila, 1988). Messenger RNA stability has also been implicated as one mechanism of regulating the chicken hsp23 gene (Edington and Hightower, 1990). While transcription rates of the chicken hsp23 mRNA remained fairly constant between control and heat shock conditions, the levels of mRNA were found to increase 4-fold upon heat shock, suggesting some sort of post-transcriptional stabilizing event. The mechanisms associated with the instability of the PTB hsp30 mRNA are not known, but future experiments analyzing the 3' end of these mRNAs may shed some light on this phenomenon.

To summarize this section, I have shown that the expression of the hsp30 gene family is differentially regulated and that at least some of its members are also regulated at the level of mRNA stability. The PTB hsp30 genes are heat inducible and their relatively low mRNA levels in early embryos appears to be a result of their instability, even at heat shock temperatures. Then, at the tailbud stage, either via changes in chromatin structure or some other mechanism, additional hsp30 family members are sequentially expressed resulting in a dramatic increase in heat-inducible hsp30 mRNA at the tailbud and tadpole stages as observed in Northern blotting experiments. It is also possible that the large increase (at least 20-fold) in heat-induced hsp30 mRNA accumulation at the tailbud stage relative to blastula may be due, in part, to stabilization of PTB hsp30 mRNAs. It is interesting to note that immunoblotting and protein labeling

experiments have failed to detect hsp30 protein production prior to the tailbud stage of development (Tam and Heikkila, 1995). Thus, it appears that while the PTB hsp30 genes are active in blastula embryos their mRNAs may not be translated very efficiently.

In the future, the cDNA(s) encoding the PTB hsp30 mRNAs may be isolated by producing a cDNA library from mRNA isolated from blastula stage *Xenopus* embryos heat-shocked in the presence of cycloheximide, in order to enrich for the presence of the PTB hsp30 mRNA. Sequencing and subsequent deletion analysis of the PTB cDNA would permit further studies of regions important in the regulation of its mRNA stability.

In this study, a new group of seven basic heat-inducible small HSPs detected in A6 kidney epithelial cells has been described. These basic proteins are distinct from the previously characterized acidic small HSPs (Darasch *et al.*, 1988; Heikkila *et al.*, 1987; Tam and Heikkila, 1995) based on their resolution on NEPHGE 2-D SDS-PAGE gels as compared with the acidic small HSPs which are resolved on IEF SDS-PAGE gels. The NEPHGE gels permit the resolution of basic proteins in a pH range of 7 to 10 which would not be resolved by typical IEF gels having a pH range of 5-7. As well, an HSP30C antibody which reacts with a number of the acidic small HSPs (Tam and Heikkila, 1995) failed to cross-react with the basic small HSPs. Finally, 6 of the 7 basic small HSPs were present constitutively, whereas none of the acidic small HSPs have been observed to exhibit constitutive expression. Interestingly, *Xenopus* small hsp mRNA has not been detected constitutively

using hsp30A, C, or D genomic clones as probes, also suggesting that the genes encoding the basic small hsps are different from those encoding the acidic small HSPs. Thus it is not likely that the aforementioned PTB hsp30 mRNAs encode the basic small HSPs as they were only observed upon heat-shock and were detected using an hsp30C clone.

The presence of 7 basic small HSPs (this study) as well as up to 16 acidic small HSPs (Darasch *et al.*, 1988) in heat-shocked A6 cells, reveals that *Xenopus laevis* probably possesses one of the most diverse collections of small HSPs in eukaryotes. A total of 7 small hsp genes/cDNAs have already been isolated in *Xenopus* (Bienz, 1984a; Krone *et al.*, 1992) and this study suggests that there are at least two more genes, encoding the PTB hsp30 mRNA and the basic small HSPs. By way of comparison, mammalian cells and yeast only possess a single small HSP. *Drosophila* has 4-7 small HSPs, while *C. elegans* has 13 isoforms of HSP16 and HSP18 (Nover and Scharf, 1991). Plants still possess the greatest number of small HSPs, in which, for example, four families of small HSPs have been characterized in pea plants (Helm *et al.*, 1993).

This study shows for the first time, constitutive expression of small HSPs (basic) in *Xenopus* A6 cells. Proteins 1 thru 5, and 7 were observed constitutively. Protein 1 was consistently present at relatively high levels, as were proteins 2 and 3. Relatively lower levels of synthesis were observed for proteins 4, 5, and 7. Constitutive expression of small HSPs has been observed in other organisms, including *Drosophila* (Sirotkin and Davidson, 1982; Cheney and Shearn, 1983; Arrigo and Pauli, 1988; Arrigo and Tanguay, 1991; Marin *et*

al., 1993), mouse (Walsh *et al.*, 1991; Gaestel *et al.*, 1993), and yeast (Kurtz *et al.*, 1986), as well as in tissue cultures such as human HeLa cells and monkey COS cells (Arrigo and Landry, 1994). Perhaps the most extensive analysis of constitutive small HSP expression has been carried out in *Drosophila*, in which differential developmental and tissue-specific expression of different small HSPs has been characterized. For example, immunological analysis of the expression of the levels of HSP23 and HSP27 in head, thorax, and abdomen regions during *Drosophila* development revealed that in the late pupae stage the levels of HSP23 and HSP27 are highest (as compared to other stages of development), with HSP23 distributed mainly in the abdomen region and HSP27 present in the head region in double the quantity present in each of the thorax and abdomen regions (Arrigo and Pauli, 1988; Arrigo, 1987). The presence of constitutive levels of small HSPs in *Xenopus* A6 cells provides evidence that they may play a role in normal cellular activity, and elucidation of this role may give insights into their function during stress.

A recently isolated basic hsp27 cDNA from the fish species *P. lucida* (Hightower, University of Connecticut, unpublished data), failed to cross-react with RNA isolated from control and heat-shocked *Xenopus* embryos and A6 cells which induced hsp30 mRNA accumulation, suggesting that the genes encoding the basic small hsps may not be highly conserved between species. This is not surprising given the relatively low degree of DNA and protein sequence homology between small hsps from different organisms (Arrigo and Landry, 1994). As a possible means of isolating the gene(s) encoding the basic small HSPs, the proteins could be purified from the gel and antibodies

produced against them. Subsequent screening of an expression library with the resultant antibodies would permit the isolation of the cDNAs. Alternatively, the purified protein could be sequenced and degenerate probes produced based on their sequence could be used to screen cDNA or genomic libraries.

The possibility that the basic small HSPs are α-crystallin-like proteins was tested using a hamster αB-crystallin antibody which failed to cross-react with the *Xenopus* basic small HSPs. However, since the DNA and amino acid sequences of small HSPs are quite diverse between species, these results are not conclusive. While the majority of α-crystallin expression occurs in the eye lens, a number of researchers have discovered the presence of α-crystallins in other tissues, including kidney (Kato *et al.*, 1991, 1993; de Jong *et al.*, 1995). As well, the α-crystallin proteins are present constitutively, consistent with the expression of the basic small HSPs detected here. To date, the α-crystallins examined are generally relatively acidic, with pIs ranging around 5.0 (de Jong *et al.*, 1995). It is still possible that the basic small HSPs are indeed α-crystallin-like proteins, and it awaits the isolation of the *Xenopus* α-crystallin genes, or the generation of a *Xenopus* α-crystallin antibody to conclusively determine it one way or the other.

In this study, the expression of the basic small HSPs was extensively characterized in A6 cells using NEPHGE two-dimensional analysis. For example, heat shock temperatures ranging from 27°C to 35°C induced the synthesis of small HSPs, with maximal induction occurring at 35°C. A 37°C heat shock resulted in the general inhibition of basic small HSP synthesis. A 35°C

time course revealed maximal induction of basic small HSP synthesis between 4-6 h, declining by 12 h. These results parallel previous 1-D SDS-PAGE studies in *Xenopus* A6 cells which revealed similar coordinate and transient patterns of acidic small HSP synthesis (Darasch *et al.*, 1987).

This study also involved the examination of sodium arsenite on the expression of basic as well as acidic small HSP synthesis. A time course of A6 cells exposed to a 50 μM concentration of sodium arsenite revealed that the synthesis of both basic and acidic small HSPs peaked at 12 h, and declined by 24 h. This temporal pattern differs from the previously described induction of small HSP synthesis under heat shock conditions in which maximal levels of protein synthesis occurred much more rapidly, after only a few hours. This result is consistent with previous one-dimensional analyses, as well as RNA dot-blot analysis which revealed a similar pattern at the mRNA level in *Xenopus* A6 cells (Darasch *et al.*, 1987). The opposite situation occurs in chinese hamster ovary cells, in which heat shock induces the synthesis of the HSP70 and HSP28 families (except HSP28c) maximally after 6-9 hours, whereas with sodium arsenite maximal synthesis (HSP70b, HSP28b,c) occurs after only 0-2 hours (Lee *et al.*, 1991). Why this occurs is not yet known. It is possible that sodium arsenite induction involves a different mechanism than heat shock induction. This may occur through the existence of more that one type of heat shock factor (HSF), whereby one HSF responds to the presence of sodium arsenite and another responds to heat shock. The presence of multiple HSFs which respond to different types of stresses has been documented (reviewed by Morimoto *et al.*, 1994; refer to discussion of synergism below). Interestingly, different types of

HSFs within a species have been found to possess different properties. For example, it has been noted that equivalent levels of vertebrate HSF1 and HSF2 DNA binding activity do not equally activate hsp70 gene transcription, and thus multiple HSFs could possibly account for the differential response time of small hsp induction in heat-shocked versus sodium arsenite-treated cells (Sistonen *et al.*, 1992).

Herbimycin A, a tyrosine kinase-inhibitor, has been found to induce the expression of heat shock proteins (Murakami *et al.*, 1991; Hedge *et al.*, 1995). Previous one-dimensional studies indicated that the synthesis of a number of heat shock proteins, including the HSP70 proteins were induced in *Xenopus* A6 kidney epithelial cells in the presence of herbimycin A (Briant, 1995). In the present study similar conditions were employed in order to examine HSP synthesis in the presence of herbimycin A using IEF and NEPHGE 2-D SDS-PAGE. As expected, 1 µg/ml herbimycin A induced the synthesis of the HSP70 proteins. As well, the full sets of both acidic and basic small HSPs were also induced. Interestingly, the aforementioned 1-D SDS-PAGE studies, as well as the studies by Murakami *et al.* (1991) and Hegde *et al.* (1995) failed to detect the presence of small HSP synthesis. This may have related to the labeling regime employed, in which the cells were incubated with ^{35}S-methionine during a recovery period following the treatment. In this study, cells were labeled during the last 2 h of the treatment period. This suggests that small HSP synthesis decreases rapidly following exposure to herbimycin A. Small hsp mRNA accumulation was observed to increase upon exposure to herbimycin A at the end of the treatment period (Briant, 1995). Thus, it is possible that some

form of translational regulation is involved in the case of herbimycin A induction that results in the rapid termination of small HSP protein synthesis. In addition, a 45 kDa acidic protein (pI 5.2), as well as a basic 62 kDa protein was observed to be induced by herbimycin A. Hedge *et al.* (1995) noted that herbimycin A-induction of constitutive HSPs in animal cells appeared to occur in the absence of the typical effects of a stress, such as increased protein denaturation found with heat shock, on a cell. As well, the authors observed that HSF1 activation occurred in herbimycin A-treated primate cells, but not in similarly treated rodent cells. It would be interesting to examine *Xenopus* HSF activity in herbimycin A-treated A6 cells to see if it is activated by the inducer.

The mechanism of stress-induced basic small HSP synthesis remains to be determined. It could be due to an increased rate of transcription, as occurs with the acidic small HSPs, or to increased mRNA stability. Alternatively, increased translation of basic small HSP mRNA may be involved. Studies employing NEPHGE 2-D SDS-PAGE analysis of proteins isolated from A6 cells treated with a transcription inhibitor such as actinomycin D followed by heat shock could be used to determine if increased rates of transcription are involved in basic small HSP stress induction.

The synergistic synthesis of small HSPs was also examined. *Xenopus* A6 cells were exposed to mild heat shock conditions in conjunction with either mild sodium arsenite treatments or herbimycin A treatment. These conditions resulted in the synthesis of both acidic and basic small HSPs at levels that were greater than that of individual induction conditions alone. The synergistic

induction of hsp synthesis is well documented in numerous cases (for example, HSP28 and αB-crystallin proteins in a human glioblastoma line by heat and sodium arsenite, Kato *et al.*, 1993; HSPs in mouse lymphocytes in the presence of heat shock and ethanol, Rodenheiser *et al.*, 1986). Indeed, one dimensional studies as well as Northern blot analysis have previously demonstrated the synergistic induction of HSP synthesis as well as mRNA accumulation in *Xenopus* A6 cells in the presence of heat shock and sodium arsenite or heat shock and herbimycin A (Heikkila *et al.*, 1987; Darasch *et al.*, 1988; Briant, 1995 - it should be noted that Briant did not detect the synergistic induction of small HSPs in the presence of herbimycin A via 1-D SDS-PAGE, however this probably again relates to the labeling regime employed). Thus, the synergism occurs at the mRNA level as well as the protein level (in the case of basic small hsp mRNA, this has yet to be determined). The two-dimensional analysis presented in this study reveals that the synergism arises not only from an increase in individual protein's synthesis, but also from an increase in the number of heat-inducible proteins (i.e. the presence of two inducers resulted in the synthesis of the full sets of both acidic and basic small HSPs, whereas individual inducers only induced a subset of the small HSPs). In all likelihood, this is paralleled at the mRNA level.

As an explanation of a possible mechanism for synergistic induction of HSPs, Sistonen *et al.* (1994) proposed a model in which two different heat shock factors are involved. In their research they employed a human erythroleukemia cell line (K562) in which it has been found that HSF1 is activated by heat shock, while HSF2 is activated by treating the cells with hemin

(Baler *et al.*, 1993; Sistonen *et al.*, 1992). They found that K562 cells induced by hemin treatment followed by heat shock resulted in a synergistic effect on hsp70 transcription. In order to further investigate this occurrence, they employed *in vivo* genomic footprinting. This revealed that under heat shock conditions alone, two HSF1 trimers are present on the heat shock elements (HSEs) of the hsp70 promoter. During hemin treatment, two HSF2 trimers are present on the HSEs. Hemin treated, heat-shocked K562 cells produced a genomic footprint similar to that found during heat shock alone, suggesting that under these conditions, the HSF2 trimer is replaced by an HSF1 trimer. The authors postulate that synergism may result from the binding of an HSF1 trimer and an HSF2 trimer possibly resulting in a stronger transcriptional activation of the hsp70 gene than with two identical trimers (Sistonen *et al.*, 1994). To date, only one *Xenopus* HSF cDNA has been isolated (Stump *et al.*, 1995). It is quite likely that more than one heat shock factor is present in *Xenopus* given the finding of multiple HSFs in many higher eukaryotes. For example, three HSFs have been detected in humans (Rabindran *et al.*, 1991; Schuetz *et al.*, 1991), two in mouse (Sarge *et al.*, 1991), and three in tomato (Scharf *et al.*, 1990). The isolation of additional *Xenopus* HSF genes would facilitate further analysis of synergistic gene expression.

In this study, it was also shown that 10 μM and 100 μM concentrations of zinc chloride and cadmium chloride (conditions which have been found to induce HSP synthesis in fish cells; Heikkila *et al.*, 1982), failed to induce the synthesis of basic small HSPs. Previously, it has been shown that *Xenopus* HSPs do not generally seem to be induced by heavy metals (Ali *et al.*, 1996).

This contrasts with *E. coli*, *Drosophila*, and chicken and tomato cell cultures, for example whose HSPs are induced by heavy metals, including cadmium (Nover, 1991). In fact, cadmium has been referred to as a universal inducer (Nover, 1991). However, not all heavy metals induce HSPs in all organisms, for example, in chicken, only some heavy metals are inducers (e.g. cadmium, zinc, and copper) while others such as cobalt, nickel, and iron, are not (Levinson *et al.*, 1980). As well, in a human glioblastoma cell line cadmium, zinc, and aluminum did not induce HSP28 or αB-crystallin (Kato *et al.*, 1993), and in *Neurospora crassa*, no heavy metals seem to induce HSP production (Kapoor, 1986). Thus, there is a wide variability in the response of different organisms to different heavy metals.

In this study, the effect of tunicamycin and dithiothreitol (DTT), two agents known to induce immunoglobulin binding protein (BiP) stress proteins, was examined using NEPHGE 2-D SDS-PAGE in order to detect any previously unidentified basic BiP proteins. Tunicamycin is an antibiotic which inhibits protein glycosylation, whereas DTT is a sulfhydryl-reducing compound. IEF 2-D SDS-PAGE revealed the two expected BiP 78 kDa proteins as well as a BiP 94 kDa protein showing that the tunicamycin and DTT were indeed affecting the cell's protein synthesis (Winning *et al.*, 1989; Miskovic *et al.*, in press; this study). NEPHGE 2-D SDS-PAGE revealed a constitutively present basic 45 kDa protein whose synthesis was induced in the presence of tunicamycin, but not in the presence of DTT. While most BiP proteins belong to the 78 and 94 kDa families, a smaller BiP, Bip58 (pI 5.0) has been detected in chicken cells, and identified as protein disulfide isomerase (Whelan and Hightower, 1985). A more

basic BiP58 (pI 5.8) was also identified in hamster cells (Lee, 1981). The detection of a basic *Xenopus* BiP45 protein in this study will act as a basis for future examination of BiP proteins in *Xenopus* A6 cells and embryos. The basic small HSPs were not induced by either of the BiP-inducing agents.

Finally, this study also employed NEPHGE 2-D SDS-PAGE to examine the pattern of expression of basic HSPs in heat-shocked gastrula stage embryos. Two heat-inducible basic HSP50 proteins were detected. These proteins were not detected in the A6 kidney epithelial cells, and thus are either expressed in tissues other than the kidney epithelium, or possibly are only expressed during development. Attempts at examining basic HSP synthesis at later developmental stages were hampered by poor radioactive labeling of the proteins.

No basic small HSP synthesis was observed in either the control or heat-shocked gastrula stage embryos. This suggests that even if the aforementioned PTB hsp30 mRNA does encode the basic small HSPs, they are not translated at the gastrula stage. Considering that the pattern of basic small HSP expression under a variety of inducing conditions parallels their acidic counterparts, it will be interesting to see if they are similarly developmentally regulated. Perhaps the isolation of the genes encoding the basic small HSPs will reveal common motifs with the acidic small hsps that could be involved in their regulation. This may provide further insights into their mechanism of developmental regulation.

One of the characteristic features of the small heat shock proteins is their ability to form aggregates upon heat shock which have been found to range from 300-800 kDa, with sedimentation coefficients ranging from 15-20S. For example, Behlke *et al.* (1991) have found that a recombinant murine hsp25 forms a 730 kDa aggregate comprised of 32 monomers, in a spherical structure 15-18 nm in diameter. Arrigo and Welch (1987) determined that the mammalian HSP28 protein forms a 500 kDa aggregate with a sedimentation coefficient of 10-18S. Helm *et al.* (1993) have found that the pea plant *Pisum sativum* HSP22.7 protein aggregates into 80-240 kDa high molecular weight structures. The single yeast HSP26 protein forms a 550 kDa aggregate comprised of an estimated 20 subunits during heat shock, as well as in cells over-expressing the protein constitutively (Bentley *et al.*, 1992). In *C. elegans*, in which two-dimensional analysis has identified 13 isoforms of the HSP16 and HSP18 proteins which are only present during heat shock, 4-500 kDa aggregates have been described (Hockertz *et al.*, 1991). One common feature of the aforementioned proteins is a conserved 72 amino acid domain which is also found in α-crystallin proteins, which are known to form highly structured high molecular weight aggregates in the lens, and it is thought that this domain is involved in aggregate formation of the small HSPs (Lindquist and Craig, 1988; de Jong *et al.*, 1995).

The first evidence of small HSP aggregation in *Xenopus laevis* came from rate-zonal centrifugation of proteins isolated from control and heat-shocked A6 cells on a linear sucrose gradient followed by 1-D immunoblot analysis of the gradient fractions with the *Xenopus* HSP30C antibody. This

revealed an estimated 10-16S heat-induced HSP30 aggregate (Tam, 1995). Part of the present study involved examining small HSP aggregate formation using pore exclusion limit electrophoresis. The proteins were resolved under non-denaturing conditions in a polyacrylamide/glycerol gradient gel that would allow them to separate until reaching their pore exclusion limits, thereby facilitating a more accurate estimation of the apparent molecular mass of the aggregate. This technique has been used previously to identify small HSP aggregation in pea plants (Helm *et al.*, 1993). Using this technique on proteins isolated from control and heat-shocked *Xenopus* A6 cells, 4 heat-induced aggregates were detected, including 510, 350, 290, and 240 kDa aggregates, with the 510 kDa aggregate being the most prominent. The sizes of these heat-induced aggregates are comparable to the previously mentioned sizes of aggregates in other organisms. Subsequent immunoblot analysis with the HSP30C antibody revealed that two of these heat-inducible aggregates, (510 and 350 kDa), possessed acidic HSP30 proteins as part of their structure. It is possible that the other two aggregates, which were not recognized by the HSP30C antibody, were made up of small HSPs not recognizable by the HSP30C antibody, or that the recognition sequence in the carboxy-terminal (from which the HSP30C antibody was generated) was not accessible to the antibody. Finally, they may be comprised of other heat-inducible proteins.

To further characterize the HSP30 aggregates, the region containing the 510 kDa aggregate was excised from the native gel and resolved on a standard 1-D SDS-PAGE gel, which should reduce the aggregate to its individual components. This revealed the presence of a 30 kDa heat-induced proteins as

well as two more prominent proteins (24 and 20 kDa) which were absent in the corresponding control fractions. It seems likely that the 24 and 20 kDa proteins probably represent degradation products of the small HSPs which occurred during the isolation of the proteins from the gel. Future immunoblot analysis of the proteins with the HSP30C antibody may clarify this. As well, gel fractions above and below the 510 kDa aggregate were similarly examined by 1-D SDS-PAGE. A faint 30 kDa band was present in the heat-shocked fractions above and below the aggregate, suggesting that intermediate structures may have been present. These could either be smaller aggregates which are growing to form the 510 kDa aggregate, while the fractions from above the aggregate may be forming an aggregate greater than 510 kDa, perhaps a heat shock granule (HSG) which can attain sizes greater than 1 MDa (for example, in tomato tissue culture cells; Nover *et al.*, 1983)

Finally, 2-D SDS-PAGE of proteins similarly isolated from the 510 kDa aggregate conclusively showed that acidic small HSPs are present in the heat-induced aggregate, displaying their characteristic pattern on a two-dimensional gel. All of the acidic small HSPs were present, revealing that they are all involved in the formation of the aggregate structure. Interestingly, HSP70 proteins (identified based upon their size, pI and relative migration) were also detected in the heat-induced aggregate region even though immunoblot analysis with an HSP70 protein of the heat-induced aggregate separated on a native gel only produced an indistinct smear. It is possible that the HSP70 proteins are present at levels too low to be distinctly detected by the antibody, or that the structure of the aggregate conceals the epitope from the HSP70

antibody. Nover *et al.* (1989) have found that the heat shock granules present in tomato cell culture accounted for approximately 50-80% of the small HSPs, and about 5% of HSP70 proteins in the cells. Other researchers have found that the heat shock aggregates appear to only be comprised of small HSPs (Collier *et al.*, 1988). At this point it cannot be determined if the HSP70 proteins are associated with the small HSP aggregate, or if they are part of another aggregate which co-migrates with it.

Two 66 kDa proteins as well as two 45 kDa proteins were also present in both the control and heat-shocked fractions of the 2-D resolved proteins. The levels of protein synthesis of these proteins did not appear to increase with heat shock. It is possible that these proteins are simply part of another aggregate structure which co-migrates at a similar size as the heat-induced aggregate. Alternatively, these proteins may indeed be associated with the heat-inducible aggregate, although other studies have failed to detect the presence of any other major proteins in the aggregates. Perhaps the analysis of the 350 kDa aggregate will reveal whether these proteins are also present, in which case it would be safe to assume that the 66 kDa and/or 45 kDa proteins are part of the small HSP high molecular weight structure.

The phosphorylation state of the small HSPs has been associated with aggregate formation. For example, Mehlen and Arrigo (1994) have found that in HeLa cells, HSP27 which is present constitutively is dephosphorylated in serum-starved cells and exists as small structures with a molecular mass less than 200 kDa. The addition of serum results in a rapid phosphorylation of

HSP27 and concommitant increases in the mass of the aggregate resulting in up to 700 kDa structures. However, Kato *et al.* (1994) have found that phosphorylation of HSP27 in human glioma cells actually results in the dissociation of 3-400 kDa heat-induced aggregates to structures less than 70 kDa, suggesting that the effect of phosphorylation may vary. Some *Xenopus* small HSPs are phosphorylated upon heat shock, as determined by ^{32}P-orthophosphate labeling/1-D SDS-PAGE studies, as well as a phosphoserine antibody which reacted with small HSPs in 1-D and 2-D immunoblot analyses (Tam, 1995). It is possible that the phosphorylation of the small HSPs may play a role in their aggregation, however further studies are required to determine this.

It will be interesting to determine whether the basic small HSPs are also present in the aggregates, or perhaps they give rise to the other detected heat-inducible aggregates? As well, since acidic small HSP expression is developmentally regulated, with a single acidic small HSP present at the early tailbud stage, followed by increasing numbers of acidic small HSPs at subsequent stages (Tam and Heikkila, 1995), it will be interesting to see at what developmental stage aggregation can occur. The capability of other inducers such as sodium arsenite or herbimycin A may also provide insights into small HSP aggregate formation. There is growing evidence that the small HSP aggregates associate with cytoskeletal elements and it has been postulated that they may play a role in protecting actin filament integrity during stress (Lavoie *et al.*, 1995).

As mentioned in the Introduction, a number of possible roles for the rather enigmatic small HSPs have been described. These predominantly include: acting as molecular chaperones (Jakob *et al.*, 1993), conferring thermotolerance (Berger and Woodward, 1983; Landry *et al.*, 1989), and playing a role in cell division and differentiation of cells, specifically through interactions with actin filaments (Lavoie *et al.*, 1995). In all likelihood, small HSPs play different roles in different organisms (Arrigo and Landry, 1994). The function of the acidic small HSPs in *Xenopus* A6 cells seems to be directly related to thermoprotection, considering that they are only stress-induced. They may also play a role in thermotolerance. The function(s) of the basic small HSPs are likely more complex, considering that they are synthesized constitutively. They may play a role in cell division in A6 cells, possibly accounting for the varying constitutive levels of individual basic small HSPs observed between different experimental groups. This could relate to the state of the cells at the times that they were collected, possibly reflecting different requirements by the cell for different basic small HSPs. Their stress-inducibility, which at the protein level parallels that of their acidic counterparts, is also indicative of a protective role. Finally, it is possible that because they are moderately basic proteins, they could interact directly with DNA (as other basic proteins such as histones do, for example) or RNA, although what this role may be remains to be determined. The identification of where the small HSPs are located within the cell (in the nucleus vs. the cytoplasm, for example) may be a good place to begin a more detailed examination of the functions of the small HSPs in *Xenopus laevis*.

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