A CHARACTERIZATION OF BiP GENE EXPRESSION IN *XENOPUS LAEVIS* EMBRYOS AND A6 KIDNEY EPITHELIAL CELLS

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ABSTRACT

We have characterized a full length cDNA clone encoding a *Xenopus laevis* immunoglobulin binding protein, BiP. The BiP cDNA sequence includes an open reading frame of 1,965 bp encoding a 655 amino acid protein with an N-terminal hydrophobic leader sequence and a C- terminal KDEL tetrapeptide which has been found in other lumenal proteins of the endoplasmic reticulum. The 3' untranslated region contains a polyadenylation and an adenylation control element (ACE) as well as a putative mRNA instability sequence. Although the *Xenopus* BiP amino acid sequence displayed high identity with BiP from other vertebrates including chicken (91.3%), rat (90.7%), and human (89.9%), the identity with *Xenopus* hsp70 (57%) and hsc70.I (55.2%) was much lower. Northern hybridization analysis demonstrated that BiP mRNA was present constitutively in the *Xenopus* A6 kidney epithelial cell line and that BiP mRNA levels could be enhanced by treatment of the cells with galactose-free media, 2-deoxyglucose, 2-deoxygalactose, glucosamine, tunicamycin, heat shock, dithiothreitol, and the calcium ionophore, A23187. Although BiP mRNA was detected in all of the adult tissues examined, the relative level of BiP mRNA differed dramatically between organs. Relatively high levels of BiP mRNA were detected in liver with moderate levels in testis, ovary and heart and reduced levels in eye and muscle tissue. Constitutively expressed BiP mRNA was detected in all stages of *Xenopus* early development from unfertilized egg to 4-day-old tadpole. Relative BiP mRNA levels rose slightly at the early neurula

stage and then increased dramatically in early and late tailbud stage embryos. Whole mount *in situ* hybridization employing a DIG-labeled BiP antisense RNA probe revealed that BiP mRNA was present constitutively in the animal pole of the unfertilized egg, cleavage and blastula stage embryos. At the gastrula stage, BiP mRNA was present throughout the embryo with slightly less accumulation in the yolk plug region. At the neurula stage, BiP mRNA levels were enriched in the neural plate and around the blastopore as well as along the neural folds. In the early and late tailbud stage embryos BiP mRNA was distributed primarily along the dorsal region of the embryo, in the somitic region, spinal cord, cranial nerves, otic vesicle and in the forebrain. BiP mRNA was also present in the heart, gills, liver diverticulum, pronephros, pronephric duct and around the anus. BiP mRNA was first tunicamycin and A23187 inducible at the neurula stage. A23187 treatment of neurula stage embryos increased BiP mRNA levels throughout the embryo with enhanced accumulation in the neural plate, along the neural folds, around the blastopore and in the ectoderm. At the tailbud stage, ionophore treatment increased accumulation of BiP mRNA primarily in the head region as well as along the spinal cord, in the somites, tail, pronephros, pronephric duct, heart and liver. In heat shock studies, the incubation of embryos for 1 h at 33°C resulted in an enhanced accumulation of BiP mRNA immediately after MBT at the gastrula stage. In gastrulae, heat shock enhanced the accumulation of BiP mRNA throughout the embryo. In neurula embryos BiP mRNA levels were enhanced by heat shock in the neural plate, along the neural folds, around the blastopore and in portions of the

epidermis. At the early tailbud stage heat shock increased the accumulation of BiP mRNA along the spinal cord as well as in the somites, the forebrain, the tail and around the anus. At the late tailbud stage heat-induced accumulation of BiP mRNA increased dramatically in a global fashion throughout the embryo.

Since the BiP gene is a member of hsp70 family we were interested in comparing its pattern of expression in embryos with the expression of cytosolic hsc70 and hsp70. *In situ* hybridization experiments employing DIG-labeled hsc70 antisense RNA probe demonstrated that hsc70 mRNA was constitutively present during development in a global fashion and did not appear to increase upon heat shock. *In situ* hybridization analysis of *Xenopus* embryos with DIG-labeled hsp70 antisense RNA probe confirmed that hsp70 mRNA was not constitutively present but was heat-shock inducible in post-midblastula stages of *Xenopus* development including late blastula, gastrula, neurula and tailbud stages. Given the strong induction of hsp70 gene expression at 33°C we examined the effect of a range of temperatures from 22°C to 35°C on hsp70 mRNA accumulation in early and late tailbud stage embryos. This study revealed a preferential induction of hsp70 gene expression in selected tissues of tailbud embryos at lower heat shock temperatures. Placement of early tailbud embryos at 30°C resulted in the accumulation of hsp70 mRNA in the olfactory placode, lens vesicle, optic cup, cranial nerves, otic vesicle, spinal cord and in the somatic region along the spinal cord. Hsp70 mRNA accumulation in late tailbud embryos was first induced at 26°C in the somitic region of the growing tail. At 30°C hsp70 mRNA

was more pronounced in the somitic region and was also present in the fore brain, cranial nerves, otic vesicle, heart and liver. Hsp70 mRNA accumulation in the late tailbud stage embryos exposed to 33 and 35°C was strongly induced and displayed a global pattern of distribution.

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1. INTRODUCTION

1.1. Heat shock proteins

Almost 35 years ago it was shown that after a brief exposure of *Drosophila* larvae to high temperatures specific puffs were formed on salivary gland chromosomes (Ritossa, 1964). A few years later, it was shown that these puffs represented transcriptionally active sites (Berendes, 1968). In following years it was revealed that elevated temperatures induced the synthesis of heat shock proteins in a variety of systems (reviewed by Atkinson and Walden, 1985; Nover *et al.*, 1991; Morimoto *et al.*, 1994, Feige *et al.*, 1996). Heat shock protein families account for 5-10% of the total protein content in all cell types under normal growth conditions (Jindal, 1996). Their levels significantly increase not only after heat shock but also when the cell is under different types of stress such as heavy metals (e.g., zinc, cadmium and arsenite, reviewed by Nover, 1991), hydrogen peroxide (Compton and McCarthy, 1978), glucose deprivation (Lin and Lee, 1984), viral infection (Sarnow, 1989), tissue injuries, free radicals (Patierno *et al.*, 1987) and some chemical reagents (Lindquist and Craig, 1988).

It has been shown that heat shock genes are present in organisms as diverse as bacteria, plants, and human (reviewed by Morimoto *et al.*, 1990). Some members of these families are expressed under normal physiological conditions and are termed heat shock cognate (hsc) genes. In general, sequence analysis of hsp genes has revealed that they have a very high degree of similarity at the nucleotide and amino acid sequence level

suggesting that these genes have been highly conserved during the course of evolution and that their protein function(s) might also be conserved among different species.

1.1.1. Families of heat shock proteins

In essentially all organisms there are three major families of hsps, categorized according to their size. These are the hsp90 family (80-90 kDa), hsp70 family (68-74 kDa) and the small hsp family (12-40 kDa). In higher eukaryotes some of the family members may have functional homologues in different cellular compartments (Table 1; Jindal, 1996). Genes encoding hsp90 family members are highly conserved and exhibit constitutive as well as heat-inducible expression (reviewed by Morimoto *et al.*, 1994; Jakob and Buchner, 1994). Most organisms synthesize at least one member of this family. The hsp70 family is a highly conserved family of hsps and includes the stress-inducible hsp70 and constitutively expressed members including hsc70, mitochondrial p75 and the immunoglobulin heavy chain binding protein (BiP), a resident protein of the ER. The third major family, which comprises the least conserved group of heat shock proteins, consists of low molecular weight heat shock proteins which range in size between 12 to 40 kDa (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Jakob and Buchner, 1994).

Table 1. Families of heat shock proteins.

Family (alternative designation in prokaryotes)	Members in eukaryotes (location)	Major functions/features
Hsp100 (Clp)	Hsp104 (cytoplasm)	Thermotolerance
Hsp90 (HtpG)	Hsp90 (cytoplasm) GRP94 (ER)	Stabilize inactive forms of certain hormone receptors until hormone is present; interact with certain protein kinases; prevent aggregation of denatured proteins
Hsp70 (DnaK)	p75 (mitochondria) Hsc70 (cytoplasm) Hsp70 (cytoplasm) BiP (ER)	Stabilize prefolded/unfolded structures towards translocation/folding; assembly of different proteins; target old proteins to lysosomes for degradation; help in protein secretion; antigen presentation and thermotolerance
Hsp60 (GroEL)	Hsp60 (mitochondria) Rubisco-binding protein (chloroplast)	Stabilize prefolded structures towards folding/assembly; re-export precursors to intermembrane space
Hsp28	Hsp28 (cytoplasm)	Prevent polypeptide aggregation; help in thermotolerance

1.1.2. Cytosolic members of hsp70 family

In most organisms studied, hsp70 genes are present in multiple copies which include heat-inducible hsp70, constitutively expressed hsc70 and other hsp70-like genes such as BiP and mitochondrial p75 protein (reviewed by Nover and Scharf, 1991; Rensing and Maier 1994). It has been proposed that the presence of multiple copies of these genes is the result of an early duplication event during evolution (Lindquist and Craig, 1988). The high identity between different hsp70 genes at the DNA as well as amino acid level has allowed the isolation of a number of hsp70 genes from different organisms and the analysis of their expression under normal conditions as well as during stress. Nine members of this gene family are present in yeast, whereas 4 hsp70 genes are found in *Caenorhabditis elegans* and 8 in mammals (Nover and Scharf, 1991). In contrast to hsc70 genes, human and mouse hsp70 genes do not contain introns (Dworniczk and Mirault, 1987). There is a high degree of amino acid identity among the members of the hsp70 family within a single species and between different species, even if they are evolutionary distant. For example, *Escherichia coli* DnaK and human hsp70 are 50% identical at the amino acid level, whereas, *Drosophila* hsp70 homologue and human hsp70 proteins are 73% identical (Hunt and Morimoto, 1985). Human BiP and hsp70 genes have been shown to have an identity of approximately 75-85%. Hsp70 proteins can be divided into two functional domains. The N-terminal region which is necessary for ATP binding and the carboxy-terminal which is responsible for intracellular localization and substrate interactions

(Morimoto and Milarski, 1990). The polypeptide binding domain has been shown to lie within a 160 amino acid region of the C-terminus (Sadis, *et al.*, 1990). The transport of the karyophilic proteins into nuclei requires a specific nuclear localization signal (NLS) which is found in mammalian hsp70 and hsc70 (Imamato, *et al.*, 1994, Morimoto and Milarski, 1994).

High constitutive levels of hsc70 protein have been reported in mammals, reptiles, amphibians, and fish (Sorger and Pelham, 1987; Yu *et al.*, 1994; Ali *et al.*, 1996; Santacruz *et al.*, 1997). The constitutive expression of hsc70 in the absence of stress suggests that it is essential for cell viability under normal growth conditions (Moreau *et al.*, 1998). Accumulation of hsc70 mRNA was 5-fold higher in tissue culture cells growing under normal conditions in comparison to the cells arrested in growth by serum starvation (Sorger and Pelham, 1987). In this latter study, hsc70 gene expression was thought to be triggered by the increasing amount of nascent polypeptides. An increase in hsc70 gene expression can be triggered by a variety of stimuli. For example, partial hepatectomy induced the expression of the hsc70 gene in rats (Krawczyk *et al.*, 1989). Also ischemia has been shown to induce the expression of hsc70 gene in the brains of rats and gerbils, (Abe *et al.*, 1993; Kaneko *et al.*, 1993). An increase in hsc70 mRNA accumulation has also been reported in mouse splenocytes after exposure to low dose ionizing radiation (Nogami *et al.*, 1993).

1.1.2.1. Functions of hsp70 proteins

Hsp70 proteins are the most extensively studied chaperones (Craig *et al.*, 1993; Hartl *et al.*, 1994). They are important to the cells under normal as well as stress conditions: hsc70 protein binds to nascent polypeptide chains coming from ribosomes and assists protein transport into the ER and mitochondria by maintaining the translocation-competent state of precursor proteins (reviewed in Becker and Craig, 1994; Wynn *et al.*, 1994; Zimmerman, 1998). In *in vitro* studies with HeLa cells it was revealed that hsp70 and hsc70 proteins were required for the transport of the nucleoplasmin protein into the nucleus (Shi and Thomas, 1992). Also the introduction of hsp70 and hsc70 specific antibodies into the HeLa cells blocked the transport of nucleoplasmin proteins into the nucleus. Hsp70 proteins assist in the formation of cytoskeleton by influencing the organizations and functions of microtubule, microfilaments and intermediate filaments (Liang and MacRae, 1997). As a result of hsp70 protein, organisms can acquire thermotolerance (Parsell and Lindquist, 1993). Also, heat-induced apoptosis was blocked in the human acute lymphoblastic leukemia T-cells expressing either constitutively elevated hsc70 or transiently elevated hsp70 (Mosser *et al.*, 1997). When *Drosophila* cells have been pretreated with mild temperatures that induce hsp70 protein synthesis, splicing of precursor mRNAs occured under otherwise restricted temperatures (Yost and Lindquist, 1986). In the fly, *Chironomus thummi*, the transport of the existing hsc70 protein from the cytoplasm towards the nucleus during heat shock as well as its association

with heterogeneous RNPs suggested that hsc70 might be involved in protecting the splicing complex against degradation during heat shock (Vazquez *et al.*, 1992). It has also been shown that in the heat shocked T-cells hsc70 interacts with topoisomerase-1, an enzyme involved in DNA synthesis and transcription (Ciavarra *et al.*, 1994). This interaction may not only protect the enzyme from heat inactivation but may also reactivate it.

1.1.2.2. Transcriptional activation of hsp70 genes

The inducibility of heat shock protein genes by a mild heat shock has made them a useful model system for the examination of the regulation of gene expression. The hsp genes have been studied extensively with respect to the *cis*- and *trans*-acting factors directing their expression (reviewed by Morimoto *et al.*, 1990; 1994). The transcriptional regulation of heat shock protein genes in response to elevated temperatures is mediated by a *cis*-acting enhancer sequence, the heat shock element (HSE). The analysis of the promoter of a number of heat shock genes has shown that the HSE is actually a contiguous repeat of a 5 bp sequence (5'-nGAAn-3') arranged in alternating orientations (Lis and Wu, 1994). The consensus sequence for the heat shock element is defined as 5'-nGAAnnTTCnnGAAn-3'.

Upon heat shock a specific *trans*-acting transcription factor called the heat shock factor (HSF) binds to the HSE. The HSF gene has now been identified in a number of species including tomato, yeast, *Xenopus*, mouse, and human (Morimoto, 1993; Morimoto *et al.*, 1994). Genes encoding HSF

have been isolated in a number of species. While there is a high amino acid identity between heat shock proteins from different speces, this does not appear to be the case for HSFs. It has been found that a single gene encodes HSF in yeast, *Drosophila* and *Arabidopsis* (reviewed by Fernandes *et al.*, 1994). However some eukaryotes possess multiple HSF genes including tomatoes, chickens and humans which have three HSFs, while mice have two (reviewed by Fernandes *et al.*, 1994). In human also, both HSF1 and HSF2 are involved in the transcriptional activation of heat shock genes (Sistonen *et al.*, 1994). It has been proposed that HSF1 is responsible for the stress response while, HSF2 regulates heat shock protein gene expression during differentiation and development.

Structural analysis of HSF protein has revealed the presence of only a few conserved domains (Morimoto, 1993; Lis and Wu, 1994) including the helix-turn-helix motif DNA-binding domain in the amino terminus, which recognizes the HSE, and an adjacent hydrophobic oligomerization domain (leucine zipper). The carboxyl terminus also contains a leucine zipper domain, required to maintain the monomeric state (Wu, 1995). It was shown that HSF binding occurs independently of new protein synthesis (Zimarino and Wu, 1987). This result indicated that a pool of stable HSF is present in an inactive form during non-stress conditions and that it is activated for DNA binding by heat shock (Zimarino and Wu, 1987; Zimarino *et al.*, 1990; Karn *et al.*, 1992). In some organisms such as *Drosophila* and human, HSF must be activated by heat shock prior to its binding to HSE. However, in some systems the binding of HSF to the HSE may not be

sufficient for the activation of transcription. In yeast and HeLa cells, it was shown that HSF was phosphorylated under heat shock conditions, but it is not known whether this post-translational modification affects transcriptional activation (Jakobsen and Pelham, 1988; Larson *et al.*, 1988: Elia *et al.*, 1996). It has been shown that the inactive HSF exists as a monomer, whereas, it binds to HSE as a trimer. Studies have shown that trimerization is necessary but not sufficient for the transcriptional activation of heat shock genes. There are two different explanations for the inhibition of trimerization under non-stress conditions. One model suggests that under normal conditions the N-terminal oligomerization domain binds with the C-terminal leucine zipper domain of the HSF protein (Rabindran *et al.*, 1993). Stress promotes the unfolding of the HSF and its interaction with two other monomers to form a trimeric complex which is able to bind to the DNA. The alternative model proposes that under non-stress conditions HSF monomers interact with other cellular proteins and that these interactions suppress trimerization (Morimoto, 1993). It has been suggested that one of the cellular proteins involved in these interactions with HSF might be hsp70 (Abravaya *et al.*, 1992). Heat shock would promote the release of hsp70 protein from HSF, which would allow the binding of released hsp70 to unfolded proteins as well as trimerization of HSF and subsequent hsp gene expression. This type of regulation implies a possible role of hsp70 protein in a negative feed back mechanism of the hsp gene expression under prolonged exposure to stress.

1.1.2.3. Developmental regulation of hsp70 genes

Hsp genes have been shown to be regulated during development under non-stress conditions. A number of hsps including hsc70 are detectable throughout *Xenopus* embryogenesis (Krone and Heikkila, 1988). However, the relative level hsc70 mRNA significantly increased after the midblastula transition (MBT) due to new transcription which correlated with the activation of the embryonic genome (Ali *et al.*, 1996). Also, at this stage *Xenopus* hsp70 gene is first heat inducible (reviewed by Hightower and Nover, 1991; Heikkila 1993a; 1993b). Developmental stage-dependent heat inducibility of hsp70 gene expression has been reported in number of other organisms. For example, in *Drosophila*, heat-inducible synthesis of heat shock proteins was not detectable until the blastoderm stage (Dura, 1981). Similarly, heat shock-induced hsp synthesis occurred at the blastula stage in sea urchin and at the blastocyst stage in mouse and rabbit embryos (Heikkila and Schultz, 1984; Heikkila *et al.*, 1985b). In all of these organisms hsp gene induction by heat shock occurs after the activation of embryonic genome at a comparable stage to *Xenopus* MBT. It is possible that this timing with respect to inducibility of hsp70 gene expression by heat shock has been conserved during evolution (Heikkila *et al.*, 1997). DNA mobility shift experiments with oligonucleotides corresponding to the proximal HSE of the *Xenopus* Hsp70 gene revealed that heat-shock induced binding of HSF occured at all stages of *Xenopus* development including unfertilized eggs and cleavage stages and that the properties of HSF binding in pre- and post-MBT were similar (Ovsenek and Heikkila, 1990). These results

suggested that HSF was maternal in origin in cleavage stage embryos and that the lack of a heat shock response was not due to the absence of activatable HSF. It has been proposed that the rapid cell cycles during cleavage and blastula stages prevent RNA transcription. The lengthening of the cell cycle at MBT allows the zygotic gene expression and subsequent RNA transcription to proceed (Kimelman *et al.*, 1987).

The constitutive expression of hsc70 mRNA has been found in the embryonic chicken lens and was correlated with the differentiation process (Dash *et al.*, 1994). Also it has been suggested that the hsp70 family members are involved in the nuclear transport of proteins involved in the transcriptional processes during early embryonic development of *Pleurodeles waltl* (Moreau *et al.*, 1994). The transfer of hsc70 occured in the early S phase of the *Pleurodeles waltl* embryonic cell cycle which indicated that it might be involved in either replication or transcription (Moreau *et al.*,1998). The tissue-specific distribution of hsc70 mRNA during early development of zebrafish embryo suggested their role in neurogenesis and somitogenesis (Santacruz *et al.*, 1997). During testis development in mice hsc70 gene expression exhibited discrete developmental specificity (Zakeri *et al.*, 1987; 1988). The transcripts for this gene were not detectable in day 7 in developing testes whereas hsc70 was expressed in this tissue in day 17 embryos as well as in the mature testes. The expression of testis specific hst70 gene, which contains a consensus sequence of an estrogen response element (ERE) in the promoter region, was first detectable in 21 days old rats (Wisniewski, *et al.*, 1990). It was demonstrated that the small

amounts of estrogens produced by Leydig cells were involved in the regulation of the cell-type and/or developmental-stage specific expression of this gene (Wisniewski *et al*, 1993).

1.1.3. BiP, the hsp70 family member of the endoplasmic reticulum

Almost 25 years ago it was reported that transformation of chicken cells with avian RNA tumor viruses increased the accumulation of two proteins, 73 kDa and 95 kDa in size (Stone *et al.*,1974). A few years later it was shown that retroviral infection led to acute glucose deficiency in the host cell due to rapid cell proliferation and that two induced proteins, 78 kDa and 94 kDa in size, were not involved in the viral transformation process but in the transport or metabolism of glucose (Pouyssegur *et al.*, 1977). This was supported by the finding that glucose starvation of cultured fibroblasts increased the accumulation of the same two proteins which were named glucose regulated proteins, GRP78 and GRP94 (Shiu *et al.*, 1977). Induction of GRP78 also occurred after treatment with the antibiotic tunicamycin, which inhibited N-glycosylation of newly synthesized proteins (Olden *et al.*, 1979). Zala *et al.* (1980) showed that the most abundant GRP, GRP78, was a resident protein of the ER. cDNA clones encoding GRP78 and GRP94 were isolated from hamster (Lee *et al.*, 1981) and these probes were used to show that GRP78 was upregulated at the transcriptional level under Ca^{2+} ionophore treatment in HeLa cells and in a temperature-sensitive (ts) hamster cell line K12, in which protein glycosylation was blocked at 40°C (Lee *et al.*, 1983, 1986; Lin and Lee, 1984; Kim and Lee, 1986). The cloning and function of the rat GRP78 promoter were

described in 1984 (Attenello and Lee). In 1983, a separate series of investigations determined that a 78 kDa protein called immunoglobulin heavy chain binding protein, BiP, was non-covalently bound to the free immunoglobulin heavy chain and that binding was abolished when the light chains associated with the heavy chains (Haas and Wabl, 1983). In 1986, Munro and Pelham showed by sequence analysis that BiP and GRP78 were the same protein and that it was related to the hsp70 family of molecular chaperones involved in protein folding, assembly and translocation. In the past 25 years BiP has been found in a variety of organisms ranging from yeast to man.

1.1.3.1. Secretory proteins and the role of BiP

The ER is the first site of the biosynthetic membrane flow in the secretory pathway of eukaryotic cells. Through co-translational or post-translational translocation, proteins destined for export or for locations at certain sites along the secretory pathway are transported into the lumen of the ER. Correct *in vivo* folding and assembly of newly synthesized proteins appears to involve two classes of ER proteins, namely, foldases (e.g. protein disulfide isomerase or proline isomerase) which catalyze slow chemical steps such as disulfide formation or isomerization and chaperones (e.g. BiP and GRP94) which bind to non-native proteins to inhibit non-productive aggregation and misfolding (Puig and Gilbert, 1994). Chaperones may function as holding/stabilizing proteins, rather then as catalysts of productive folding. This suggests a co-

operative, sequential pathway during protein synthesis, assembly and transportation.

Proteins destined for secretion have a signal sequence, 16-30 amino acids at the N-terminus, that directs ribosomes towards the ER membrane (Munro and Pelham, 1986; von Heijne, 1990; Landry and Gierach, 1991). The signal sequence is recognized by a signal recognition particle (SRP) in the cytoplasm. The receptor for SRP is localized in the ER membrane at the translocon which is a structurally multi-layered aqueous pore that spans the entire ER membrane and consists of at least 6 proteins including signal peptidase and gate proteins (Andrews and Johnson, 1996). The newly synthesized polypeptide is targeted to the ER through SRP-SRP receptor interaction and once in the ER the signal sequence is removed by signal peptidase (Rapoport, 1992). BiP binds to short sequences of unfolded polypeptides that have just been translocated into the ER lumen to prevent their degradation or nonspecific aggregation before they reach the final folded state (Sanders and Schelman, 1992; Gilmore, 1993). In the next step, BiP binds and hydrolyzes ATP causing the release of the bound polypeptide. In the absence of BiP parts of nascent proteins aggregate within the ER lumen which inhibits further elongation of the protein through the ER membrane and blocks protein translocation channels. Some improperly folded proteins are halted by BiP, selectively degraded in the ER or translocated back into cytoplasm for degradation (Sanders *et al.*, 1992; Gilmore, 1993).

The C-terminal amino acid sequence of BiP and other ER resident proteins is KDEL (Lys-Asp-Glu-Leu; Munro and Pelham, 1986). This sequence

is necessary for retention of these proteins in the ER. The receptor for KDEL is found in ER membranes, membranes of ER-to-Golgi transport vesicles, and *cis*-Golgi reticulum (Ceriotti and Colman, 1988). These receptors serve to retrieve any proteins with KDEL that escape to the *cis*-Golgi reticulum. Experiments in yeast showed that blocking ER-to-Golgi transport caused a concentration of BiP in a distinct subregion of the ER to form dense bodies with a size of 200-500nm, termed BiP bodies (Nishikama *et al.*, 1994). When the KDEL signal was deleted, BiP was secreted but the rate of secretion was unexpectedly slow. They suggested that BiP bodies may represent the supportive mechanism for the slowdown of BiP exit from the ER. It was proposed that they might operate in the event of an ER-to-Golgi transport block by trying to keep BiP away from the accumulating secretory proteins ready to exit the ER.

1.1.3.2. BiP protein structure

A comparison of BiP cDNAs from different species revealed the presence of a signal sequence that directs the protein to the ER which was not present in the mature protein (Munro and Pelham, 1986). The signal sequence had a positive charge at the N-terminus followed by a hydrophobic stretch and two small non-polar amino acids at positions -1 and -3 relative to the signal sequence cleavage site. In the first 44 kDa from the N-terminal end of the mature protein there is an ATP binding site and ATPase activity, which are conserved among hsp70 family members. The peptide binding domain of BiP is in the last 30 kDa of the C-terminal end. It is likely that there is significant

interaction between ATP and the protein binding domains, because ATP hydrolysis which is catalyzed by the N-domain causes the dissociation of polypeptides bound to the C-terminal domain. BiP preferentially recognizes heptameric motif of preferably hydrophobic amino acids, which would be located in the interior of the polypeptide chain after folding (Flynn *et al.*, 1991). Blond-Elguindi *et al* (1993) reported that the BiP binding site could help selected amino acid residues from the newly synthesized polypeptide to arrange into a structure that was energetically equivalent to the interior of the mature, folded protein. At the very C-terminus of BiP is the previously described KDEL sequence, important for the retention of BiP in the ER.

1.1.3.3. Post-translational modifications of BiP

BiP can exist in the cell as a free unmodified monomer, complexed with other proteins or as a free modified oligomer (aggregates). Both stressed and unstressed cells contain similar amounts of free BiP, but in unstressed cells BiP exists as a modified oligomer and in stressed cells it exists as an unmodified monomer. Post-translational modifications of BiP occur on free BiP, and are restricted to its oligomeric form (Freiden *et al.*, 1992).

It has been reported that BiP possesses Ca^{2+} stimulated autophosphorylation activity, high binding affinity to ATP, and a weak Mg^{2+}-dependent ATPase activity (Leushek *et al.*, 1991; Carlino *et al.*, 1992; Wei and Hendershot, 1995). *In vivo* phosphorylated mouse BiP, isolated by immunoprecipitation, was modified almost entirely on threonine residues located in the peptide binding domain (Gaut, 1997). It seems unlikely that

phosphorylation occured through autophosphorylation since *in vitro* autophosphorylation occurs on the threonine which is inside the ATP binding cleft. Given that the molecular interactions between the ATP binding domain and ATP are very precise it is not likely that this cleft could simultaneously accomodate a site within the peptide binding domain to permit autophosphorylation (Gaut, 1997). Post-translational modifications of BiP, namely phosphorylation and ADP-ribosylation are associated only with the oligomeric form (Hendershot *et al.*, 1988; Freiden *et al.*, 1992; Gaut and Hendershot, 1993). These post-translationally modified oligomers represent an inactive form of BiP. Wei *et al.* (1995) characterized three classes of BiP mutants with respect to the ATP-binding domain specifically, ATP binding mutants, ATP hydrolysis mutants and conformational change mutants. Their results suggested that all three phases, namely ATP binding, autophosphorylation and subsequent conformational change of BiP represent very important steps in its function(s) and mechanism(s) of action. Other post-translational modifications of BiP which have been reported include ADP-ribosylation in mouse, N-glycosylation in yeast BiP and methylation in avian and mammalian BiP (Leno and Ledford, 1989, 1990, 1994; Pridoux and Armstrong,1992; Wang *et al.* 1981, 1982). The physiological significance of these modifications remains unclear.

1.1.3.4. BiP functions

1.1.3.4.1. Protein folding, assembly and quality control

The ER is a specialized folding compartment with a unique oxidizing environment. Detailed mechanisms of chaperone action are still poorly defined, but it is well documented that BiP, as a chaperone protein, has a very important role in the folding and assembly of newly synthesized polypeptides including proinsulin (Kjeldsen *et al.* 1997), collagen type IV (Ferriera *et al.* 1996), α subunit of prolyl 4-hydroxylase (John and Bulleid, 1996), α and β subunites of Na, K-ATPase (Beggah *et al.*, 1996), keratin (Lao *et al.*, 1997) and placental alkaline phosphatase (Oda *et al.*, 1996). BiP is involved in the recognition and degradation of aberrant proteins as well. This step represents a level of quality control in the ER. It was shown that under-expression of BiP in CHO cells induced by BiP antisense RNA led to improved secretion of aberrant exogenous tissue plasminogen activator (tPA) with a mutated N-glycosylation site (Dorner *et al.*, 1988). Also in experiments employing the transient expression of influenza hemagglutinin (HA) glycoproteins in African green monkey kidney (COS) cells, it was shown that BiP was bound strongly and irreversibly to the mutant HA glycoproteins, and poorly and transiently to the wild type (wt) influenza HA glycoproteins (Murray *et al.*, 1995). Finally, Sagt *et al.* (1998) reported that an increase in the expression of wt cutinase in *S.cerevisiae* did not result in higher levels of BiP but that BiP levels were raised after expression of mutant hydrophobic cutinase. Immunoprecipitation studies showed that mutant cutinase interacts with BiP in contrast to the wt.

Blond-Elguindi *et al.* (1993b) proposed a model of BiP action. They suggest that in unstressed cells post-translationally phosphorylated and/or ADP-ribosylated BiP was present in oligomeric form. Stressful conditions would lead to an increase in the population of new, unfolded proteins, which could bind to BiP. Protein binding then induced the reversal of BiP modifications, the dissociation of BiP into its more active monomeric form, and the stimulation of BiP's ATPase activity. BiP hydrolysis of ATP, presumably by change in BiP conformation, promoted the release of the polypeptide from the complex. This was confirmed by Brot *et al.* (1994) and the model was expanded later by the same group (Weissbach *et al.*, 1995; Vidal *et al*, 1996). They found a difference in the action of BiP on normal and abnormal proteins. Their results suggested that abnormal proteins could bind to both BiP oligomers and monomers, and did not cause the dissociation of BiP oligomers to monomers. Normal proteins bound to BiP were able to dissociate from BiP after ATP hydrolysis and to release the BiP-ADP complex while aberrant proteins stayed stably bound with the BiP-ADP complex and were released only when very high concentrations of ATP was added. They suggested that the role of hydrolysis was not to release the peptides but to change BiP conformation from BiP-ATP to BiP-ADP by an exchange reaction in order to enable BiP to become available for another interaction with a protein substrate. This change in conformation was probably enough to release a normal peptide from BiP. However, Hendershot *et al.* (1996) constructed an ATPase mutant incapable of peptide release and they showed that peptides are incapable of proper folding without release from BiP.

1.1.3.4.2. Translocation to ER

Protein translocaton into the ER can occur either during or after polypeptide synthesis. The lumenal proteins, including BiP, likely accomplish two functions through their interactions with nascent peptide chains. They support unidirectional transport of the nascent chain early in translocation and suppress irreversible aggregation by enhancing the efficiency of protein folding (Lyman and Schekman, 1995; Haynes *et al.,* 1997). In yeast, cytosolic hsc70 and ER localized BiP are asymmetrically oriented to the opposing surfaces of the ER membrane and both are required to recruit protein (Brodsky *et al.,* 1993, 1995; Dierks *et al.* 1996). A constitutively expressed hsc70 is required to maintain the newly synthesized polypeptide, or a portion of a polypeptide, in a translocation competent form, because newly synthesized and partly folded proteins are unable to cross the ER membrane. Experiments with different yeast mutants have shown that in the ER BiP binds the transiting polypeptide in order to facilitate its passage through a membrane protein Sec63p (Nguyen *et al.,* 1991; Lyman and Schekman, 1995; Brodsky *et al.,* 1995). However, it also needs the help of the integral membrane protein. Stimulation of BiP ATPase activity in conjuction with Sec63p probably has multiple roles including ATP-mediated conformational changes of BiP in the lumen of the ER. This not only regulates the release of BiP-bound polypeptide but might also trigger the pore in the ER membrane to open and let in awaiting precursor at the beginning of its translocation into the ER (Lyman and Schekman, 1995).

1.1.3.4.3.The preservation of the Ca^{2+} concentration gradient

Hamman *et al.*, (1998) suggested that BiP maintains the permeability of the ER membrane by sealing the lumenal end of the translocon pore. According to their hypothesis, BiP is the gating protein which binds to the aqueous translocon pore when the translocon is unoccupied and seals its lumenal end. At the beginning of translation, the newly targeted ribosome binds and tightly seals the cytosolic end of the pore. After the translation of approximately 70 amino acids of nascent chain this peptide interacts with BiP or other translocon protein(s) which triggers the release of BiP from the lumenal end of the pore. This causes the expansion of the pore from 9 - 15 Å to 40 - 60 Å. The ribosome is dissociated from the ER at the end of translation only after the newly synthesized secretory protein leaves the translocon. Ribosome release is stimulated by the binding of BiP-ATP to the translocon which results in ATP hydrolysis and the simultaneous narrowing of the pore. Thus, BiP appears to be responsible for the maintaining of ER membrane permeability which is important for the preservation of the Ca^{2+} concentration gradient. Lievremont *et al.*, (1997) has suggested a more direct role of BiP in the storage of the rapidly exchanging pool of Ca^{2+}. According to their results, BiP is a Ca^{2+} storage protein which binds approximately 25% of the ion store with low affinity due to its abundance of acidic amino acids.

1.1.3.4.4. Role in degradation of aberrant proteins

Protein is allowed to exit the ER when it reaches a conformation that does not offer binding sites for chaperones (Zhang *et al.*, 1997). Most misfolded and incompletely assembled proteins are retained in the ER and degraded without transport to the Golgi complex. Shmitz *et al.* (1995) have proposed a model for BiP binding to malfolded proteins and its role in their degradation in the ER. Their model suggests that both signals for BiP binding and degradation are localized in the internal domains of the protein which are normally buried in the interior of the correctly folded protein. BiP is an abundant protein in the ER so the degradation apparatus usually has no chance to reach folding intermediates. However if protein is unable to achieve the correct conformation, the probability of degradation increases with time. Eventually, binding of the misfolded protein to the degradation apparatus occurs. Further cycles of binding to and release from BiP are interrupted and the protein is degraded.

Recently it was suggested that many aberrant proteins may be transported from the ER into cytoplasm for the degradation by the cytoplasmic proteasome (Plemper *et al.*,1997; Pilon *et al.*, 1997). It is possible that proteins could not be exported in the globular state due to the diameter of translocon pore and that the role of BiP would be in binding to proteins to help them unfold prior to export.

1.1.3.5. Inducers

1.1.3.5.1. Accumulation of unfolded or malfolded proteins in the ER

The induction of BiP gene expression in response to a variety of stress conditions occurs at the transcriptional level probably as a result of an accumulation of unfolded proteins in the ER. Eukaryotic cells have an intracellular signaling pathway which enables them to supply additional amounts of molecular chaperones including BiP and folding enzymes to the ER in order to cope with the harmful effects of unfolded proteins. This is achieved by monitoring the events in the ER and transducing appropriate signals accros the lipid bilayer to the nucleus. This pathway is termed the unfolded protein response (UPR; Cox *et al.*, 1996, 1997; Mori *et al.*, 1996, 1998; Kawahara *et al.*, 1997, 1998). Conditions which result in the accumulation of unfolded and/or misfolded proteins and the activation of the UPR are listed below.

1.1.3.5.1.1. Impaired glycosylation

Most plasma membrane and secretory proteins are glycosylated containing one or more carbohydrate chains. The carbohydrate groups have important roles in biological recognition as signals for protein targeting and cell-cell interactions. Furthermore, they provide important physical properties to many glycoproteins such as stabilization of conformation, protease resistance, correct charge and water binding capacity (Paulson, 1989; Ruddon and Bedows, 1997). Some glycosylation reactions occur in the lumen of the ER while others occur in the lumen of the *cis, medial* or *trans*-Golgi vesicles. The

intermediates used in the biosynthesis of oligosaccharides are nucleoside-diphosphate or nucleoside-monophosphate sugars. The oligosaccharide chain, because of its size, can mask directly some of the hydrophobic residues or can cause a steric impediment that forces the protein to acquire a certain conformation. If glycosylation is impaired the protein will misfold and in some cases aggregate (Paulson, 1989). Agents such as tunicamycin, 2-deoxyglucose, 2-deoxygalactose or glucosamine, which inhibit the N-glycosylation of newly synthesized proteins have been shown to induce BiP gene expression (Lee *et al.*, 1986; Ting and Lee, 1988; Lee, 1987). Also, Chen *et al* (1997) reported BiP induction in 9L rat brain tumor cells after treatment with a protein phosphatase inhibitor okadaic acid which affects protein glycosylation and/or glycoprotein trafficking.

1.1.2.5.1.2. Calcium ion deprivation

Most of the Ca^{2+} within a cell is sequestered in intracellular organelles, where the Ca^{2+} concentration can reach the millimolar range. The concentration of free Ca^{2+} in the cytoplasm is extremely low and is estimated to be in the micromolar range (Roy and Lee, 1995). Changes in the cytoplasmic Ca^{2+} concentration have been shown to affect growth rate, intermediary metabolism, electrical activities and cell movements (Resendez *et al.*, 1985). Calcium deprivation in the ER has been shown to induce BiP gene expression. For example, treatment of cells with the Ca^{2+} chelating agent, EGTA or the Ca^{2+} ionophore A23187 can induce BiP synthesis (Lamarshe *et al.*, 1985; Lee, 1987). In these studies ER Ca^{2+} deprivation leads to a block in protein

glycosylation and BiP gene activation (Kim and Lee, 1986; Chang *et al.*, 1989; Li and Lee, 1991). The Ca^{2+} level in the ER is maintained by ER-specific Ca^{2+}-ATPase (Kang *et al.*, 1995). Inhibitors of this enzyme, such as thapsigagin, can cause an increased level of BiP synthesis by decreasing the level of ER Ca^{2+} and a concomitant increase in the cytosolic Ca^{2+}. The results of Kang *et al.* (1995) with BAPTA-AM, a membrane permeant Ca^{2+} chelator, which reduces the cytosolic Ca^{2+} level, demonstrated that BiP gene expression was related only to the changes in the level of ER Ca^{2+}.

1.1.3.5.1.3. Sulfhydryl reducing agents

The oxidizing environment in the ER allows the formation of disulfide bonds. Sulfhydryl reducing agents such as β-mercaptoethanol (β-ME) and dithiothreitol (DTT) induce BiP synthesis by interfering with the formation of disulfide bonds. BiP gene induction by a low dosage of β-ME requires *de novo* protein synthesis and is preceded by a drop in the rate of protein glycosylation (Kim *et al.*, 1987; Kim and Lee, 1987). Whelan and Hightower (1985) in experiments with cycloheximide have shown that β-ME has two mechanisms of BiP induction. The first mechanism which occurs at low concentrations of β-ME involves *de novo* protein synthesis and affects nascent polypeptides by interfering with disulfide bond formation. The second one occurs at higher β-ME concentrations and does not depend on protein synthesis to generate the induction signal for BiP. It was suggested that high β-ME might disrupt disulfide bonds in mature proteins as well.

1.1.3.5.1.4. Miscellaneous treatments which cause UPR

A predominant metabolic response to hypoxia is an increased rate of glucose consumption with a corresponding decrease in oxygen consumption. This effect arises from a shift in oxidative phosphorylation to the glycolytic pathway for energy production (Roll *et al.*, 1991). Also, hypoxia has been shown to induce BiP in most mammalian cells and tissues studied to date including CHO, rat embryo cells, rat fibroblasts, cardiac and brain tissue (Graven *et al.*, 1993). Ischemia can also induce the accumulation of BiP protein in rat cerebral cortex (Wang *et al.* 1993). In these studies the accumulation was proportional to the duration of ischemia. Furthermore, Kuznetsov *et al.* (1996) reported that BiP gene expression was increased *in vivo* after acute kidney ischemia caused by renal arterial ligation. In their cellular model they produced ischemia by treating cells in culture with antimycin A, an inhibitor of oxidative phosphorylation. It was proposed that ichemia affected ATP levels, cellular redox state, Na/K ratio, intracellular pH and Ca^{2+} homeostasis which ultimately leads to the preturbation in the folding and assembly of the secretory and membrane proteins (Kuznetsov *et al.* 1996). Ethanol (Miles *et al.*, 1994, Hsieh *et al.*, 1996) and cyclopentenone prostaglandins (Odani *et al.*, 1996) should be mentioned in addition to the listed BiP inducers. Treatment of cells with either of these two agents result in proteins which are incapable of folding properly.

1.1.3.5.2. Increased traffic through the ER

A number of studies have shown that increased protein trafficking through the ER can result in an increase in BiP gene expression. For example, Brefeldin A, a chemical isolated from the fungus *Eupenicillium brefeldianum*, inhibits protein transport from the ER to the Golgi complex in hamster K12 cells (Liu *et al.*, 1992). This causes the resorption of Golgi membrane into the ER and the subsequent accumulation of non-ER resident proteins in the lumen of the ER. Under these conditions BiP is induced and its binding to these proteins within the ER probably protects them from degradation by proteinases. Also butyrate treatment of CHO cells expressing stably integrated heterologous genes such as human factor VIII, von Wilebrant factor (vWF) and erythropoetin (EPO) under the control of a butyrate responsive SV40 promoter, lead to an increase in synthesis of these three proteins and consequently to an increase in BiP gene expression (Dorner *et al.*, 1989, 1992). BiP was found in complexes with factor VIII and vWF, but not with EPO. Finally, Umebayashi *et al.* (1997) found that accumulation of a mutant fungal protein in the ER of yeast led to an increase in BiP gene expression and the formation of aggregates associated with BiP.

1.1.3.5.3. Viral infection

It has been reported that the levels of BiP mRNA and BiP protein are increased in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (CEF; Stoeckle *et al.*, 1988). Also, in poliovirus-infected cells one of the early events is the increased translation of BiP mRNA (Sarnow, 1989). This

occurs at a time when cap-dependent translation of other host mRNA is greatly inhibited due to the proteolysis of eIF-4G protein component of eIF-4F complex, which is one of the consequences of the infection. The translation of BiP mRNA is mediated by an internal ribosome binding site, localized in the 5' region of the BiP mRNA (Macejak and Sarnow, 1991; Yang and Sarnow, 1997). The increased level of BiP in virally infected cells is probably required to deal with the influx of viral proteins into the ER.

1.1.3.5.4. Glucose deprivation and nutritional shock

It has been shown that hamster fibroblast cells which are starved of glucose adapt to this situation by shutting off certain genes, including genes for membrane glycoproteins and turning on new sets of genes, such as BiP and GRP94 (Lin and Lee, 1984). Restoration of the glucose concentration of the medium can repress the expression of these two genes. In the canine kidney epithelial cell line MDCK, a cell culture system with the ability to differentiate *in vitro*, glucose starvation induces differentiation which occurs with a similar time course as levels of BiP induction (Taub *et al.*, 1989). The mechanism by which loss of glucose stimulates MDCK cell differentiation may be unique because glucose deprivation does not affect the Na^{+}/K^{+} ATPase activity of MDCK monolayers, as do other inducers of cell differentiation including DMSO and prostaglandins.

1.1.3.5.6. Hormones

Hormones can also influence BiP gene expression. For example, Day and Lee (1989) reported that testosterone induced BiP in Sertoli cells as a result of increased protein synthesis and secretion. It is probable that the transient association of BiP with newly synthesized proteins promotes their correct structural maturation. Insulin can also induce an increase in BiP gene expression in cultured cells (Kasambalides and Lanks, 1983, 1985) and in the brain and liver of non-obese diabetic mice (Parfett *et al.*, 1989, 1990). Furthermore, it has been reported that TSH-induced expression of BiP in FRTL thyroid cell lines. Increased BiP levels were required to assist in the folding and transport of the newly synthesized glycoproteins (Endo *et al.*, 1991).

1.1.3.6. BiP gene structure and regulation

Since only yeast, plant and mammalian BiP genes have been isolated, information regarding copy number and regulatory regions across species is limited. In yeast, BiP is encoded by a single essential gene (Mori *et al.*, 1992). Spinach and tomato have only one BiP gene while additional BiP genes are present in tobacco and soybean (Wrobel *et al.*, 1997). In hamster and rat BiP appears to be encoded by a single copy gene. In rat, while two BiP genes were detected during the screening of a rat genomic library only one was functional (Attenello and Lee, 1984). Also, when hamster BiP cDNA was used to screen a human fetal liver genomic library, both a 5 kb functional gene consisting of 8 exons and a processed BiP pseudogene were detected (Ting and Lee, 1988).

As mentioned previously, the accumulation of unfolded proteins in the ER as a result of different physiological or enviromental stresses (Lee, 1987) can trigger an intracellular signaling pathway from the ER to the nucleus, called the unfolded protein response (UPR; Cox *et al.*, 1996, 1997; Nikawa *et al.*, 1996). The UPR results in an increase in the synthesis of ER proteins, which allows eukaryotic cells to respond to changing conditions in the ER. The UPR is best explained in *S. cerevisiae*. Mori *et al.* (1992) reported a 22 bp cis-acting element (UPRE) in the promoter of KAR2/BiP gene, necessary and sufficient for the induction of BiP by unfolded proteins. In addition to the UPRE, they found a functional HSE as well as a GC-rich region which contributed to the high level of constitutive expression of BiP. To date three components of the UPR pathway have been identified. The first component is ire1 (or ern1p), an ER transmembrane kinase (Cox *et al.*, 1993; Mori *et al.*, 1996). Its glycosylated N-terminal domain is located in the lumen of the ER while the C-terminal domain has protein kinase activity and is localized in the cytoplasm or nucleus. Upon accumulation of unfolded proteins in the ER, the protein kinase domain of ire-1 is activated and induces the unconventional splicing of HAC1 precursor mRNA by tRNA ligase (Sidrauski *et al.*, 1996; Kawahara *et al.*, 1997, 1998). The translation of the mRNA gives rise to the third component of the UPR pathway, hac1p (ern4p or ire2) protein, a basic leucine zipper transcription factor, which binds* to the UPRE (Nikawa *et al.*, 1996; Mori *et al.*, 1996, 1998; Kawahara *et al.*, 1997, 1998).

This situation is more complicated and still not completely clear in higher eukaryotes. The mammalian UPR includes the induction of a GADD153,

growth arrest and DNA damage protein, which always accompanies BiP induction (Haleck *et al.*, 1997a, 1997b; Brewer *et al.*, 1997). This protein is not constitutively present and its role in the UPR is still unknown, although it may be involved in the initiation of apoptosis in response to ER stress. However, Brewer *et al.*, (1997) reported that BiP expression was stimulated by growth factors without coordinated induction of GADD153. They suggested that growth factors were able to regulate basal expression of BiP by a pathway distinct from the stress induced UPR. This pathway allows cells to modulate expression of BiP in correlation with need during growth and differentiation and without activating the other genes that may be harmful under normal conditions.

Deletion analysis of the BiP promoter in hamster K12 cells showed that the region from -85 to -480 nucleotides upstream of the major transcription initiation site contained overlapping regulatory elements and was important for induction by different inducers (Chang *et al.*, 1987). Promoter sequences of the chicken, hamster, rat and human share a high level of nucleotide sequence identity (Ting *et al.*,1987; Resendez *et al.*, 1988). BiP promoters contain few CCAAT and CCAAT-like elements, with GC-rich flanking regions. These motifs are termed C1-C5, C1 being the most proximal to the TATA box (Chang *et al.*, 1989).

Experiments employing site-directing mutagenesis have revealed that one part of the BiP promoter sequence was highly conserved across different species (Wooden *et al.*, 1991). This part, termed the BiP core element, contained the motifs C5 and C4. Together with the C1 element it represented the most critical region for both basal and stress-induced expression. It has

been reported that during stress induction of BiP, the BiP core element was occupied by a 70 kDa transcriptional factor termed p70CORE (Liu *et al.*, 1992; Li *et al.*,1993; Li *et al.*, 1994). In an experiment employing an *in vivo* transfection assay, it was demonstrated that a BiP subfragment, containing only C1 was sufficient to confer a BrefeldinA (BFA)-induced Ca^{2+} stress response. The C1 region contains a 34 bp sequence with the CCAAT element most proximal to the TATA box. The mutation or deletion of C1 led to a decrease in the activity of an upstream regulatory complex. It could not act optimally which resulted in a lower basal level of BiP gene expression and impaired expression under stress conditions. The transcription factor that interacts with C1 during BiP induction has been identified as CCAAT-binding factor, CBF/NF-Y. Binding of CBF to the C1 element was higher at low Ca^{2+} concentrations. A GGAGG sequence which flanks the CCAAT motif is very important for high affinity binding of CBF to C1 (Roy and Lee, 1995). In this study an increase in Ca^{2+} concentration in the ER corresponded to an increase in Ca^{2+} in the nucleus and did not affect the kinetics of formation of the CBF complex but increased the dissociation rate of CBF binding. A drop in Ca^{2+} concentration in the nucleus lead to the increased stability of CBF binding to the C1 motif in BiP promoter which increased BiP transcription. Recently, it was reported that another transcription factor, YY1, an abundant zinc finger DNA binding protein binds to the C1 element under less stringent conditions (Roy *et al.*, 1996). The binding sites for both YY1 and CBF factors were found in the BiP core as well. It seems that transcriptional activation of BiP in mammals is conveyed through two important regulatory regions in the BiP promoter, C1 and the BiP core which

share common factors but with different transcription factors affinities under different conditions (Roy *et al.*, 1996).

1.1.3.7. BiP gene expression during development

BiP gene expression has been reported during development in a wide range of organisms and embryonic systems. For example, BiP gene expression occurs during the differentiation of a parasitic protozoan, *Giardia lamblia,* which represents the earliest branch of the eukaryotic lineage (Lujan *et al.*, 1996). Also, high constitutive amounts of BiP were present during exponential growth and during the late phase of conidiation in the fungus, *Neurospora crassa* (Hafker *et al.*, 1998). BiP gene expression has been reported in plants where the relative level of BiP mRNA accumulation varied with tissue type and stage of development in *Arabidopsis thaliana* (Koizumi, 1996), soybean (Fontes *et al.*, 1996), pumpkin (Hatano *et al.*, 1997) and maize (Wrobel *et al.*, 1997). In mammalian systems it was reported that undifferentiated and unstimulated F9 cells (teratoma stem cell line in mouse), which resemble early embryonic cells, expressed basal levels of BiP (Kim *et al.*, 1990). Experiments with mouse embryos showed that BiP was present at low levels up to the morula stage, and that the level increased in the blastocyst stage (Kim *et al.*, 1990). Also, synthesis of BiP preceded the synthesis of the structural proteins and enzymes characteristic for differentiated macrophages. This differentiation led to the accumulation of a phosphorylated oligomer form of BiP, which probably served as a pool from which unphosphorylated active monomers could be recruited (Nakai *et al.*, 1995).

In amphibians, constitutive levels of BiP have been documented in *Xenopus laevis* embryos throughout early development (Winning *et al.* 1991). Two dimensional-PAGE analysis revealed that BiP existed in 3 isoforms (Winning *et al.*, 1989; Winning *et al.*, 1991). One isoform was detected in embryos and in adult A6 and white blood cells while the second was detected only in the embryos and the third only in the adult cells. These results suggested that BiP gene expression might undergo a developmental switch from an embryonic to an adult pattern. Further examination of the developmental regulation of *Xenopus* BiP gene expression has been performed by analyzing the expression of a microinjected chimeric gene, consisting of a rat BiP promoter fused to a chloramphenicol acetyl transferase (CAT) reporter gene (Winning *et al.*, 1992; Vezina *et al.*,1994). This chimeric gene was first expressed in a constitutive manner in late blastula stage *Xenopus* embryos. Its expression increased with development to the gastrula and neurula stage (Vezina *et al.*, 1994). Tunicamycin-induced expression of the chimeric gene during *Xenopus* development was first detected after the midblastula stage. Deletion analysis showed that *cis*-acting CCAAT box and CCAAT-like elements between -154 and -90 nucleotides upstream of the transcription initiation site were necessary for both constitutive and tunicamycin-inducible expression of the fusion gene (Winning *et al.*,1992; Vezina *et al.*,1994). These results suggested conservation of the regulatory elements in the BiP promoter region between mammals and *Xenopus*. Interestingly, deletion or alteration of sequences between -130 and -149 nucleotides upstream of the transcription initiation site had a dramatic

stimulatory effect on basal promoter activity, which was not observed in rat cells (Vezina *et al.*, 1994). One possibility was the presence of additional *cis*-acting element(s) further upstream in the promoter region that could compensate for the mutated or deleted sequences. The other possibility was the presence of a negative regulatory element in this region, that was recognized by a *Xenopus* *trans*-acting factor (Vezina *et al.*, 1994).

1.2. *Xenopus laevis* development

The African clawed frog, *Xenopus* laevis, has been used extensively for the examination of the molecular mechanisms involved in early vertebrate embryogenesis. There are several advantages to using *Xenopus* as an experimental animal. They are commercialy available, easy to maintain and able to produce hundreds-to-thousands of eggs. Also, the relatively large eggs (1-1.2 mm in diameter) are easy to manipulate, can be fertilized externally, and are amenable for microinjection studies. Furthermore, the stages of *Xenopus* embryonic development are well characterized (Nieuwkoop and Faber, 1967).

In *Xenopus*, the egg is responsible for initiating and directing early development. Near the end of the oogenesis the oocyte is arrested in the diplotene stage of the meiotic prophase I. The cytoplasm of the oocyte contains an abundance of energy sources, enzymes and precursors for DNA, RNA and protein synthesis, stored tRNAs, mRNAs, structural proteins and morphogenetic regulatory factors. This material will be used by the early embryo until the activation of the zygotic genome at the midblastula stage. Resumption of meiosis and germinal vesicle breakdown are controlled by

progesterone secreted by surrounding follicle cells in response to gonadotropic hormones secreted by the pituitary gland. The mature ovulated egg which is in second meiotic metaphase, is released from the ovary coated with jelly.

Animal-vegetal polarity of the unfertilized egg is characterized by a pigmented animal half and unpigmented vegetal half. The sperm entry point during fertilization occurs at the animal pole and determines the dorso-ventral polarity of the embryo. The dorsal region forms opposite the sperm entry point. The first cleavage occurs about 100 minutes after fertilization (at 22-23°C). This is followed by subsequent cleavages which occur at aproximately 30 min intervals. The cleavages are rapid and synchronous with alternating phases of DNA synthesis and mitosis until cycle 13. At this point inner cell material is segregated into presumptive ectodermal (animal), mesodermal (equatorial, marginal) and endodermal (vegetal) areas.

Gastrulation is initiated in the dorsal part of the marginal zone between the animal and vegetal hemisphere. The prospective local endodermal cells change shape and become bottle-like and then invaginate into the embryo forming dorsal blastopore lip. These cells are presumptive pharyngeal cells of the foregut. Their constriction during the change of shape pushes up the involuting marginal zone (IMZ) cells. IMZ cells are prospective head mesoderm and the prospective chordamesoderm and lead the movement of the mesoderm into the blastocoel. The mesoderm moves toward the animal pole pulling along the superficial marginal cells. Mesodermal movement displaces and then entirely eliminates the blastocoel by forming the archenteron

(primitive gut). The superficial marginal cells will form the endodermal roof of archenteron. As the cells reaching the blastopore change shape and involute into the embryo, the dorsal blastopore lip expands laterally and then ventrally. The large endodermal cells which form the yolk plug on the vegetal surface are progressively internalized. During the whole process, animal pole cells which are the ectodermal precursors undergo epiboly and converge at the blastopore covering the entire embryo. After the internalization of the yolk plug, presumptive endoderm is in the interior of the embryo while the ectoderm is completely on the surface, and the mesoderm is between them. Ectoderm will form skin, brain, spinal cord and represents the origin of neural crest cells while endoderm will give rise to the linings of digestive and respiratory tubes. Mesoderm will ultimately form muscle, cartlidge, bone, heart, the urogenital system, blood cells and parts of most of the internal organs.

The interaction between the dorsal mesoderm and its overlying ectoderm initiates organogenesis, the creation of specific tissues and organs. The chordamesoderm starts neurulation by inducing overlying ectoderm to form the neural tube. Neurulation in the anterior (head) region of the embryo is well advanced while the posterior (tail) region is still undergoing gastrulation. The neural crest cells originate at the dorsal most region of the neural tube and their segregation from the neural plate begins at the early neural fold stage (stage 15). They start to migrate extensively to generate different cell types including neurons and glial cells of the sensory, sympathetic and parasympathetic nervous system, medullar cells of the adrenal gland,

melanocytes, skeletal and connective tissue components of the head and musculo-connective tissue wall of the large arteries.

The regional segregation of the brain into archencephalon and deuterencephalon is visible first after the fusing of the neural folds into the neural tube in the anterior part of the embryo at stage 20. This is followed by further segregation of the neural tube in fore-, mid- and the hind- brain at stage 22. The primary eye vesicle extends laterally from each side of the developing forebrain at stage 21. The ear placodes develop as dorso-lateral thickening of the sensorial layer of the ectoderm at the level of the hindbrain at the same stage. The olfactory placodes develop at stage 23 from the same layer, but latero-dorsal to the forebrain.

At the late neural fold stage (stage 17) mesodermal cells which are not involved in notochord formation start to migrate laterally along each side of the notochord and neural tube to group into blocks called somites. They are transient structures which will give rise to the cells that form vertebrae and ribs, dermis of the dorsal skin, skeletal muscles of the back and skeletal muscles of the limbs. The first somites appear at the anterior part of the embryo and are a good indicator of the stage of development. The somitic cells which are closest to the neural tube (sclerotome cells) will become vertebral chondrocytes and the cells which are farthest from the neural tube are precursors of the limb muscles (myotome cells) and dermis (dermatome cells).

Laterally adjacent to the somitic mesoderm is the intermediate mesodermal region. After approximately 23 h of *Xenopus* development (stage

21), they will develop into the pronephric tubule, the precursor of the kidney and genital ducts. Laterally adjacent to the intermediate mesoderm on each side of the embryo is lateral plate mesoderm, split horizontally into somatic mesoderm which underlies the ectoderm and ventral splanchic mesoderm which underlies endoderm. Between these layers is the body cavity or coelom. Later during development the left and right coelom fuse and folds of somatic mesoderm divide the coelom into separate cavities. Two presumptive heart forming regions in *Xenopus* start to develop in the anterior part of the splanchnic mesoderm at the early neural fold stage (stage 15). During neurulation they come together in the ventral region of the embryo to form a single pericardial cavity.

The digestive tube is formed from the primitive gut archenteron. Buds from this endodermal tube form the liver, gall bladder and pancreas. The hepatic diverticulum which will form the liver, extends out from the foregut into the surrounding mesenchyme at the initial neural plate stage (stage 13 1/2). Later during development, the mesenchyme induces the endoderm to proliferate, branch and form the glandular epithelium of the liver. The respiratory tube and pharynx form as an outgrowth of the digestive tube.

By the fourth day the embryo is a free-swimming tadpole. The yolk sac has been slowly used up and the tadpole now depends on external sources of food. Limb development begins during the fourth day of *Xenopus* development (stage 43) when mesenchymal cells are released from the somatic layer of the limb fields lateral plate mesoderm (limb skeletal precursors) and from the somites (limb muscle precursor). The cells migrate from the somites laterally

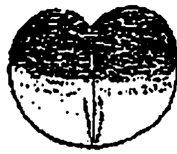
and accumulate under the epidermal tissue of the neurula. The circular bulge on the surface of the embryo is called the limb bud and is visible at stage 45. Growth and differentiation of the limb bud is made possible by a series of interactions between the limb bud mesoderm and overlying ectoderm. The forelimb bud is first visible after 7 1/2 days of *Xenopus* development (stage 48). Metamorphosis starts roughly around stage 50, after emerging of the fore limbs. Certain developmental processes are reactivated by secretion of specific hormones as the entirely aquatic tadpole prepares for adulthood. These changes occur in all systems, including respiratory, circulatory, nervous, excretory, locomotory and integumental. Some of the stages of *Xenopus* development are depicted in Figure 1.

1.2.1. Developmental regulation of gene expression in *Xenopus*

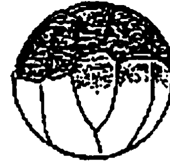
The development of an organism proceeds through a precisely timed set of cellular migrations, cellular differentiations and cell-cell interactions. The regulation of these events is determined by the correct temporal and spatial expression of specific genes. The timing of developmental events, the developmental stage at which the embryonic genome is first turned on and the longevity of functional maternal message are species-specific and differ greatly among different species. The repression of transcription of the embryonic genes during cleavage stages is not clearly understood. *Xenopus* nuclei have almost eliminated both G1 (prereplication gap) and G2 (premitosis gap) phases during the first 12 cleavages such that the cells

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

St. 1

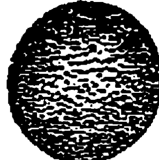


2 cell stage



St. 5 blastula

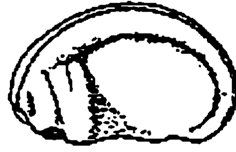
St.7 blastula



St.9 blastula

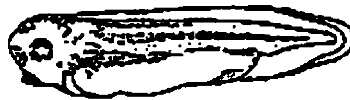
gastrula

neurula



St. 23

St. 25



St. 35/36

divide synchronically in a biphasic (S to M and M to S) cell cycle (Newport *et al.*, 1985). It has been suggested that rapid cell cycling during cleavage might be regulated by maternal factors in the cytoplasm, such as mitosis initiation factor (MIF; Newport *et al.*, 1985). The cell cycle pattern returns to normal when this factor is titrated by an increase in cell number at the midblastula stage. At this stage the embryo has approximately 4000 cells and passes through a developmental transition characterized by slower and asynchronous cell divisions, cell motility and transcriptional activation of the zygotic genome. This stage is referred to as the midblastula transition (MBT). It has been suggested that at MBT a specific DNA to cytoplasm ratio may be one of the factors which determines the onset of zygotic gene expression (Newport *et al.*, 1985). This was supported by experiments in which the DNA to cytoplasm ratio was increased by microinjecting DNA into *Xenopus* fertilized eggs or by induction of polyspermy (Newport and Kirschner, 1982a, 1982b; Newport *et al.*, 1985). This increase caused the premature initiation of transcription during cleavage stages. It is also possible that the embryonic genes which are first expressed may be activated by maternal factors in the oocyte which are inactive until some other factors activates it (Ovsenek *et al.*, 1992). Such a factor would determine the onset of zygotic transcription. On the other hand it was shown that the presence of large amounts of histones can repress the interaction of TATA-binding protein with DNA, suppressing gene activity during early cleavage stages (Prioleau *et al.*, 1994). Titration of histones which occurs

during subsequent cell divisions allows the formation of the transcriptional complex assembly and results in the transcriptional activation of genes.

At the midblastula stage, mostly 5S rRNA, tRNA, small nuclear RNA and a specific set of mRNAs are synthesized (Newport and Kirschner, 1982a, 1982b; Shiokawa *et al.*, 1981). Among the genes expressed at this stage are EF1a, heat-inducible hsp70 and ubiquitin (Krieg *et al.*, 1989; Bienz, 1984a; Heikkila *et al*, 1987; Ovsenek and Heikkila, 1988). Most of the other genes are expressed at later stages of *Xenopus* development. For example, muscle-specific actin genes (α-skeletal and α-cardiac) and a gene encoding *Xenopus* ribosomal protein L8 are not transcribed until gastrulation (Mohun *et al.*, 1984, Shi and Liang, 1994). Similarly, βT1-globin and tadpole β–globin genes are first transcribed at the tailbud stage, while, α-globin genes are not transcribed until metamorphosis (Bendig and Williams 1983). The exact mechanisms associated with the developmental regulation of these genes is still unknown. However, DNA methylation, chromatin structure, interactions of the *cis*-acting DNA sequences with the specific transcription factors and mRNA stability could all be responsible for the temporal and spatial regulation of these genes (for review see Gilbert, 1994).

OBJECTIVES

As shown in the Introduction, there is a very little information regarding the expression of BiP genes during early animal development. Therefore, the present study was carried out in order to characterize *Xenopus* BiP cDNA and analyze *Xenopus* BiP gene expression in cultured cells and embryos. The main objectives of this study are listed below:

1. To characterize the nucleotide sequence of a *Xenopus* cDNA clone encoding BiP protein.

2. To examine the constitutive and stress-inducible accumulation of BiP mRNA in *Xenopus* kidney epithelial A6 cells and adult tissues.

3. To examine constitutive and stress-inducible BiP mRNA accumulation and spatial distribution in *Xenopus* embryos.

4. To compare constitutive and heat-inducible spatial distribution of BiP mRNA with hsc70 and hsp70 mRNA in *Xenopus* embryos.

2. MATERIAL AND METHODS

2.1. Characterization of BiP cDNA

2.1.1 Isolation of a BiP cDNA

The *Xenopus laevis* cDNA library screening was performed in Dr. Martin Flajnik's laboratory (University of Miami). The cDNA library was prepared from liver, spleen and thymus RNA obtained from young adult *Xenopus* using the Uni-Zap system (Stratagene; Flajnik *et al.*, 1991). The library was screened with a rat BiP cDNA clone (kindly provided by M. J. Gething, University of Texas) as described by Sato *et al.* (1993) except that the hybridization was carried out under low stringency conditions: 40% formamide, 6 X SSC (1 X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% sodium dodecyl sulfate (SDS), 5 X Denhardt's solution (1 X Denhardt's is 0.02% bovine serum albumin, 0.02% ficoll, and 0.02% polyvinylpyrolidone) and 200 µg/ml of sheared and denatured salmon sperm DNA. The filters were washed in 2 X SSC, 0.2% SDS at 55 °C.

2.1.2. Isolation of plasmid DNA

Plasmid DNA isolation was performed using the modified alkaline lysis method originally described by Sambrook *et al.* (1989). Five ml of YT broth [1% tryptone (w/v), 0.5% (w/v) yeast, 0.5% (w/v) sodium chloride] with ampicillin at a concentration of 75 µg/ml was inoculated with the *Escherichia coli* DH-5α cells carrying the desired plasmid. The cells were

grown overnight at 37°C with shaking. Cells from overnight cultures were transferred to 1.5 ml microcentrifuge tube and pelleted by centrifugation at 10,000 rpm in a Hermle model Z 320 K refrigerated microcentrifuge for 5 min. The cell pellet was resuspended by vortexing in 200 µl of ice cold solution containing 10 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 7.5), and 400 µg/ml RNase I (Boehringer Mannheim), and 200 µl of a freshly prepared solution of 0.2 N NaOH and 1% SDS (w/v) was added and mixed by inverting. After incubation at room temperature for 5 min, 200 µl of 3 M potassium acetate and 5 M glacial acetic acid was added to the tube which was then incubated on ice for 5 min. The precipitated material was pelleted at 12,500 rpm for 10 min (Hermle microcentrifuge) and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. The plasmid DNA was precipitated at room temperature for 15 min with 420 µl of isopropanol and pelleted by centrifugation at 12,500 rpm for 30 min (Hermle microcentrifuge). The resultant pellet was rinsed two times with 70% ethanol, dried in air for 10-15 min and dissolved in 100 µl of sterile distilled water. DNA quantity was determined by taking 5 µl of DNA in 1 ml of water and then measuring the UV absorbance at 260 nm as well as 260/280 ratio in a Beckman DU7 spectrophotometer (1 O.D. A_{260} unit = 50 µg DNA/ml). DNA preparations were considered to be pure if the ratio of the value of optical density at 260 to the value at 280 was 1.7-1.8 (Sambrook *et al.*, 1989).

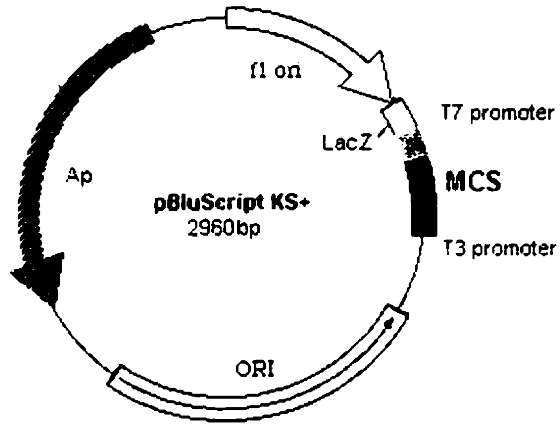
2.1.3. Restriction enzyme analysis

Restriction enzyme digests of recombinant plasmid DNA were performed according to Sambrook *et al.*, (1989). High (1 M sodium chloride, 500 mM Tris-HCl, 10 mM magnesium chloride, 1 mM DTT, pH 7.5), medium (50 mM sodium chloride, 10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM DTT, pH 7.5) and low (10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM DTT, pH 7.5) salt buffers were obtained as 10X stock. One µl of the appropriate 10X salt buffer for each 10 µl of final reaction mixture was added to one µg of DNA and 1-5 units of restriction endonuclease. The final volume was brought up to the desired volume with sterile distilled water and the reaction was allowed to proceed at 37°C for a period of at least 6 h. The digested DNA was mixed with the loading buffer (50% v/v glycerol, 0.1% v/v bromophenol blue) and loaded onto a 1% agarose gel containing ethidium bromide at a concentration of 0.4 µg/ml which was added for DNA staining. Approximately 250-500 ng of λ–Hind III marker DNA (BRL) was loaded beside the restriction digests for the size determinations of DNA fragments. The electrophoresis was performed in 1X TAE buffer [40 mM Tris acetate, 1 mM EDTA (pH 8.0)] at 80-90 volts for 2-3 h.

2.1.4. Purification of DNA fragments for subcloning

BiP cDNA, originally cloned in pBluescript plasmid DNA (Fig. 2), was isolated by the alkaline lysis method and digested with EcoRI, KpnI and PstI restriction endonucleases as described above, which generated four fragments, 0.7 kb, 0.5 kb, 0.35 kb and 0.85 kb in size (Fig. 3 and Fig. 4). Individual restriction fragments of DNA were isolated by electroelution using DEAE paper [NA-45 DEAE membrane, 0.45 mm; Schleicher and Schuell (Sambrook *et al.*, 1989)]. The DEAE paper (0.5 cm x 3-4 cm) was activated by washing in 10 mM EDTA (pH 8.0) for 10 minutes followed by a 10 min wash in 0.5 N NaOH and three 10 min washes in sterile distilled water, and used immediately as described below. The plasmid DNA digested with the appropriate restriction endonuclease as described earlier and the λ–Hind III marker DNA were loaded onto a 1% agarose gel containing ethidium bromide (0.4 μg/ml). Electrophoresis was carried out in 1X TAE running buffer at 80V. The migration of the DNA was visualized by a hand held UV lamp. A cut was made in the gel with a clean razor blade just 0.5 cm ahead of the desired band and a small piece of activated DEAE paper was inserted into the cut by spreading the gel with forceps. The DNA was electrophoresed onto the paper at 200 V for 2-3 min. After the transfer was complete, DEAE paper was given a quick rinse in a low salt solution containing 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), and 150 mM NaCl. The paper was cut into small pieces with a razor blade and transferred to a 1.5 ml microcentrifuge tube containing 500 μl

Figure 2. BiP cDNA. BiP cDNA was originally cloned into XhoI and EcoRI sites in pBluescript KS+ plasmid vector, generating plasmid pBlue-BiP.


f1 ori
T7 promoter
LacZ
MCS
Ap
pBluScript KS+
2960bp
T3 promoter
ORI

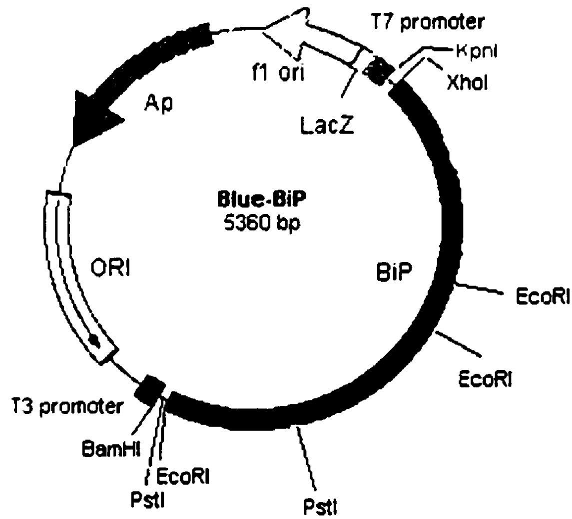
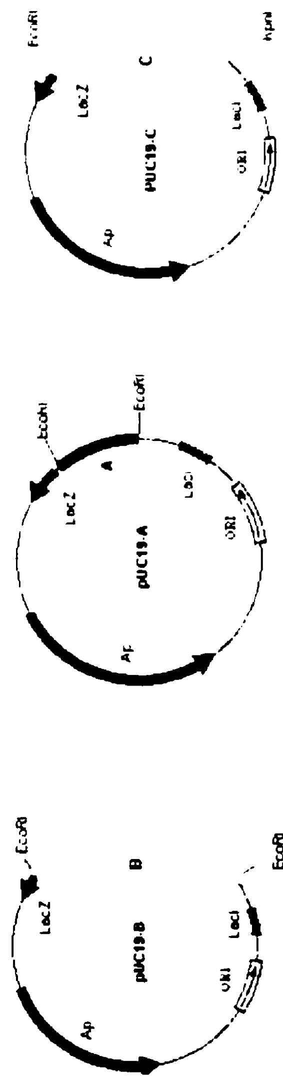
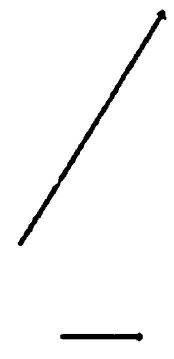

T7 promoter
KpnI
XhoI
f1 ori
Ap
LacZ
Blue-BiP
5360 bp
ORI
BiP
EcoRI
EcoRI
T3 promoter
BamHI
EcoRI
PstI
PstI

Figure 3. Subcloning strategy. Plasmid pBlue-BiP was digested with EcoRI, KpnI and PstI restriction endonucleases which generated four fragments, namely B2 (0.5 kb), B1 (0.7 kb), A (0.35 kb) and C (0.85 kb).The fragments were subcloned into the plasmid vectors pUC18 and pUC19, suitable for sequencing due to the presence of standard forward and reverse M13 primers.


pUC19-C
C
LacZ
LacI
ORI
Ap
EcoRI
pUC19-A
A
LacZ
LacI
ORI
Ap
EcoRI
EcoRI
pUC19-B
B
LacZ
LacI
ORI
Ap
EcoRI
EcoRI



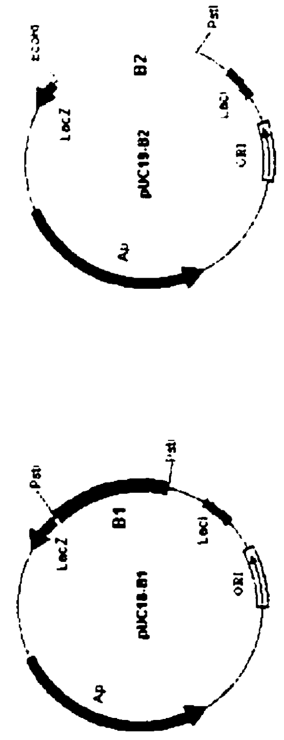
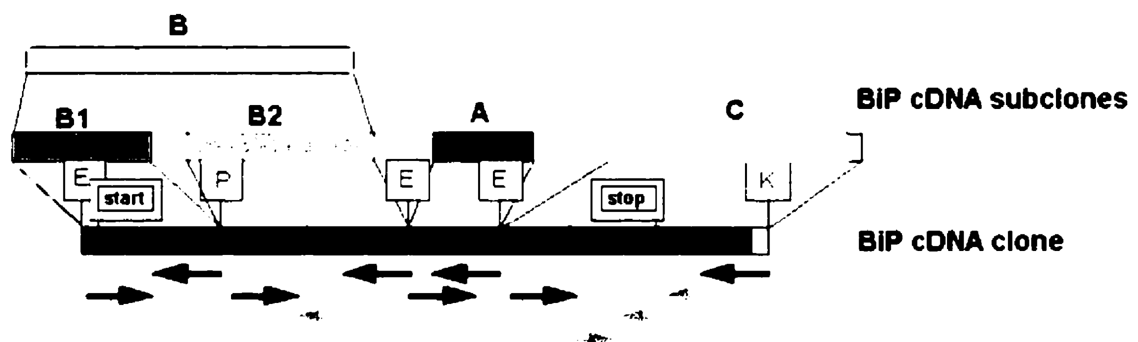

pUC19-B2
B2
LacZ
LacI
ORI
Ap
PstI
pUC19-B1
B1
LacZ
LacI
ORI
Ap
PstI

Figure 4. The DNA sequencing strategy of the *Xenopus* BiP cDNA clone. The boxes A, B, B1, B2 and C represent the subclones used for DNA sequencing. The shaded regions represent the isolated BiP cDNA clone and subclones while the open boxes refer to the vector sequence. Arrows indicate the direction of the different sequencing reactions. Darker arrows represent standard sequencing primers, and lighter arrows represent custom synthesized primers. The translation start and stop site are labeled. E, EcoRI; P, PstI; K, KpnI.


B
B1
B2
A
C
BiP cDNA subclones
E
start
P
E
E
stop
K
BiP cDNA clone

of high salt buffer (20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), and 1.5M NaCl). DNA from the DEAE paper was eluted in this buffer by incubating the tube at 65°C, and vortexing it every 15 min. After 1 h of incubation the supernatant was transferred to a fresh tube, another 500 µl of high salt buffer was added and then incubated for an additional 30 min with occasional vortexing. The elutant was then extracted with phenol/chloroform and chloroform and the DNA was precipitated in 0.3M sodium acetate (pH 5.2) and two volumes of ethanol at -20°C overnight. After centrifugation at 12,500rpm for 30 min, the pellet was air dried for 10-15 min and dissolved in 50 µl of sterile distilled water. The concentration of the eluted DNA was determined by UV spectrophotometry. The size and the concentration of the DNA fragments was further confirmed by electrophoresis. Some fragments (cDNA for the large ribosomal protein L8 and cDNA for the cytoskeletal actin) were purified using a Spin-X Centrifuge Tube Filter (Corning Costar Corporation), according to the protocol provided by the manufacturer.

2.1.5. DNA subcloning

Approximately 15 µg of pUC19, pUC 18 or pSP64 (polyA) plasmid DNA was digested with the appropriate restriction endonucleases as mentioned above and purified by one phenol/chloroform and one chloroform extraction. The linearized DNA was precipitated with sodium acetate and cold ethanol as described earlier. After centrifugation and air drying, plasmid DNA was

dissolved in 20-40 μl of sterile distilled water and 5 μl was loaded onto a 1% agarose gel containing ethidium bromide (0.4 μg/ml). This linearized DNA was subjected to dephosphorylation as described by Sambrook *et al.* (1989). Two μg of linearized vector was combined with 10 mM Tris-HCl (pH 8.0), 10X calf intestinal phosphatase (CIP) buffer [0.5 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine (Sigma)], and 1 U of CIP (1U/μl; Boehringer Mannheim). Dephosphorylation was carried out at 37°C for 30 min after which an additional 1 U of CIP was added to the reaction mixture and incubated at 37°C for an additional 30 min. To inactivate the enzyme, 10 X STE buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] and 1 μl of 20% SDS were added to the mixture and heated up to 65°C for 15 min. One phenol and one chloroform extraction were performed and the DNA was precipitated with sodium acetate and cold ethanol at -20°C overnight. After centrifugation, washing with 70% ethanol and air drying, the DNA pellet was dissolved in 40 μl of sterile distilled water. After quantification by UV spectrophotometry, it was diluted to a final concentration of 50 ng/μl.

In ligation reactions, 50 ng of dephosphorylated digested pUC19, pUC18 or pSP64 (polyA) vector DNA was combined with the linearized DNA fragments in an insert to vector ratio of 1:1, 2:1 and 1:2. To the reaction mixture, 5X DNA ligase buffer (BRL) and 1 U of T4 DNA ligase (1 U/μl; BRL) were added and the final volume was brought up to the desired value with

sterile distilled water. Ligations were carried out at 14°C for 16-20 h after which a double volume of TCM buffer [10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$] was added. This ligation mixture was then used to transform *E.coli* strain DH-5α. Five ml of YT broth was inoculated with 100 ml of the overnight culture of DH-5α cells and incubated at 37°C with shaking until the optical density at 600 nm reached 0.5 (approximately 4-5 h). The cells were pelleted at 2,000 g for 10 min in a clinical table top centrifuge and resuspended in 2.5 ml of 50 mM $CaCl_2$. After incubating on ice for 1 h, the cells were pelleted at 2,000 g for 10 min, and resuspended on ice in 500 µl of 50 mM $CaCl_2$. One hundred µl of this competent cell suspension was added to the DNA+TCM ligation mixture in a 0.5 ml microcentrifuge tube and incubated on ice for 45 min. The tube was then transferred to 42°C for 2 min and after equilibration to room temperature for 10 min, 300 µl of pre-warmed (37°C) sterile YT broth was added to the tube and incubated at 37°C for 30 min without shaking. One hundred and fifty µl of the transformation mixture was spread directly onto YT-agar plates [1% (w/v) Tryptone, 0.5% (w/v) yeast, 0.5% (w/v) NaCl and 1.5% (w/v) agar] containing 75 µg/ml ampicillin (GIBCO, BRL), 53 µg/ml X-gal (5-bromo,4-chloro,3-indoyl, b-D galactopyranoside; Boehringer Mannheim) and 67 µM IPTG (isopropyl-b-D- thiogalactopyranoside; Boehringer Mannheim). The plates were incubated at 37°C for 12-20 h and then stored overnight at 4°C.

The screening for the transformants was carried out by growing the white colonies in 5 ml of YT broth with ampicillin overnight. Rapid mini-preparation of plasmid DNA (as described above) was performed for this quick screening. The plasmid DNA digested with the appropriate restriction endonuclease was analyzed by agarose gel electrophoresis as described earlier.

2.1.6. BiP cDNA subclones

In order to obtain complete sequence information, BiP cDNA, which was originally cloned into pBluescript (blue-BiP) plasmid, was subcloned into pUC19 and pUC18 vectors (Fig. 3). The blue-BiP plasmid was digested with EcoRI and XhoI restriction endonucleases (double digest) to generate three fragments (B:1.2 kb, EcoRI-EcoRI fragment; A: 0.35 kb, EcoRI-EcoRI fragment; and C: 0.85 kb, EcoRI-KpnI fragment). Fragments were isolated by electroelution using DEAE-cellulose membrane (Sambrook *et al.*, 1989) as described above. Fragments A and B were then subcloned into the plasmid vector pUC19 previously digested with EcoRI and dephosphorylated. This generated subclones pUC19-A and pUC19-B (Fig. 3). Fragment C was subcloned into the plasmid vector pUC19 which was double digested with EcoRI-KpnI restriction endonucleases, producing subclone pUC19-C (Fig. 3). Subclone pUC19-B was digested with restriction endonuclease PstI, releasing a 0.5 kb long PstI-PstI fragment (B1), which was then subcloned into PstI site in a pUC18 vector, generating

pUC18-B1 (Fig. 2). The remaining pUC19 with 0.7 kb long EcoRI-PstI fragment (B2) was religated and named pUC19-B2 subclone (Fig. 2).

2.1.7. DNA sequencing

The DNA sequences of the different subclones were determined using Sanger's dideoxy chain-termination method (Sanger *et al.*, 1977) except that the sequenase kit including the cloned version of T7 DNA polymerase (Sequenase^{TM} version 2.0; Amersham Life Science; Tabor and Richardson, 1987, 1989) was used, and sequencing reactions were performed according to the directions by United State Biochemical Corp. (Step-by-Step protocol for DNA sequencing with sequenase^{TM} version 2.0, 2nd edition).The final sequence was confirmed by automated sequencing at MOBIX (Mc Master University, Hamilton, Ontario).

Two µg of DNA obtained in double-stranded form by the miniprep procedure described above, was combined with 2 µl of denaturing solution [2 M NaOH, 0.1 mM EDTA (pH 8.0)] in a 0.5 ml microcentrifuge tube. The final volume was brought up to 20 µl with sterile distilled water and the tube was incubated at 85°C for 5 min. The mixture was neutralized with 2 µl of 3 M sodium acetate (pH 5.2) and DNA was precipitated in 2 volumes of ethanol at -20°C for 4-5 h or at -80°C for 1-2 h. The DNA was pelleted by centrifugation at 12,500 rpm for 30 min (Hermal centrifuge). After two

consecutive 70% ethanol washes the pellet was dried under vacuum and dissolved in 5 µl of sterile distilled water.

Sequencing primers which were used in different sequencing reactions were purchased from Amersham. The internal primers were custom synthesized (The Department of Genetics, University of Guelph) and the DNA synthesis facility at MOBIX, McMaster University, Hamilton, Ont. Canada). The sequence of the primers are listed below (Fig. 4).

Standard sequencing primers (present in pUC18 and pUC19):

- M13 (-40) primer: 5'-GTTTTCCCAGTCACGAC-3'
- M13 reverse primer: 5'-TTCACACAGGAAACAG-3'

Custom synthesized primers:

- C1 reverse primer 5'-GCTGAGCGAAGACTGC-3'
- C1 forward primer 5'-GGATGGTGACTGATGC-3'
- C2 reverse primer 5'-CGTAATACCAGACCCACC-3'
- B reverse primer 5'-TACTCCTGTGTTGGAGTG-3'

Five µl of denatured plasmid DNA (2 µg) was mixed with 2 µl of 5 X Rx buffer (sequenase reaction buffer: 400 mM Tris-HCl, pH 7.5, 200 mM magnesium chloride, 500 mM sodium chloride, 50 mM DTT; Amersham) and 3 µl (50 pmol) of DNA sequencing primer. For annealing of the primer

to the DNA templates, the tubes were kept at 37°C for 30 min followed by incubation at room temperature for 15 min. The labeling mix (for dGTP reaction; Amersham) was diluted in sterile distilled water (1:4). The labeling reaction mixture was produced by mixing the template-primer construct with 1 µl of 100 mM DTT, 2 µl of diluted labeling mix, 1 µl of deoxyadenosine 5'-[α-^{35}S] triphosphate (sp. act. >1,000 Ci/µmol; Amersham) and 2 µl of sequenase^{TM} (diluted 1:7 with enzyme dilution buffer, according to the suggestions of the manufacturer), and 0.5 µl of SSB (single stranded binding protein; Amersham) in order to overcome the problem of compression due to the secondary structure of the DNA. The labeling reaction was allowed to proceed at room temperature for 5 min.

A volume of 3.7 µl of the labeling reaction mixture was then added to each of 4 tubes prepared in advance, containing 2.5 µl of prewarmed (at 37°C) dGTP termination mixtures (ddATP, ddGTP, ddCTP, or ddTTP termination nucleotides). The tubes were incubated at 37°C for 5 min. To stop the reaction, 4 µl of stop solution [95% (w/v) formamide, 20 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] was added. The SSB protein that would otherwise interfere during the electrophoresis, was inactivated with 1 µl of proteinase-K (0.1 mg/ml) and incubated at 65°C for 20 min. The reactions were briefly centrifuged and stored at -20°C overnight.

Electrophoresis was performed on 40 cm X 30 cm, 8% (w/v) polyacrylamide (acrylamide to bis ratio of 19:1)/50% urea denaturing gel with a 96 well sharks-tooth comb (BRL) in 1X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), using model S2 electrophoretic apparatus (BRL). Glass plates were washed with RAIN-X (Unelco Corporation), glass conditioner designed for automobile windshields, to facilitate the pouring of the gel and to prevent sticking of the gel to the plates (Barnett and Davidson, 1989). The wedge sequencing gels with the side spacers of 0.4 mm, and the bottom spacer of 0.8 mm were prepared in order to increase resolution. The gels were pre-run for 30 min to bring the temperature up to 45°C prior to loading. The tubes were heated at 85°C for 5 min and 2.5 μl of the samples were loaded onto the gel. The electrophoresis was performed at 45-50°C at a constant power of 45-47 watts. In order to further increase the resolution the second and third loadings were done at intervals of 30-45 min after the bromophenol blue dye front from the preceding loading had ran off from the bottom of the gel. After electrophoresis, the glass plates were gently separated. The gel which adhered to one of the glass plate was placed for 30 min in a plastic tray with 1X fixing solution [5% (v/v) glacial acetic acid, 5% (v/v) methanol]. After the fixing solution was removed and the gel was transferred to 3MM Whatman filter paper. The gel was covered with plastic wrap and dried at 80°C for 45 min in a vacuum gel dryer (Slab dryer, model 483; Bio-Rad) followed by exposure to XAR-5 film (14" X 17" size, M & S X-ray services) overnight at room temperature.

Computer analysis of DNA sequences was performed with DNA strider 1.1 on an Apple Macintosh computer. The DNA sequences which were obtained were used to search the DNA sequence databases at Genbank.

2.2. Maintenance of Xenopus A6 cells and embryos

2.2.1. Culturing and treatment of *Xenopus* A6 kidney epithelial cells

The *Xenopus laevis* A6 kidney epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 22°C in a medium consisting of 55% (v/v) L-15 medium, 10% (v/v) fetal bovine serum (both from Canadian Life Technologies, Inc.) and 35% (v/v) sterile distilled water. Penicillin and streptomycin (both from Flow Laboratories) were added to give 100 I. U./ml and 100 µg/ml respectively.

When the cells reached 90% confluence, the medium was removed, and fresh medium was added to ensure that cells were not starved of glucose at the time of treatment. The cells were then treated with various potential BiP inducers for a periods up to 24 h. The final concentrations used for the inducers were as follows: calcium ionophore A23187 (7 µM for 4, 6 and 24 h); tunicamycin (1 µg/ml for 24 h); DTT (0.1 mM for 24 h); β-mercaptoethanol (0.25 % (v/v) for 24 h) and homocysteine (1-5 mM for 24 h). To examine the effect of heat shock on BiP induction, A6 cells were incubated in the complete 55% L15 medium, until they reached 90% confluence. The old media was replaced with

fresh media, and the cells were incubated at 33°C for 1 h. To obtain glucose-starved cells, A6 cells were incubated in complete 55% L-15 medium until they reached about 80-90% confluence. At that point the medium was replaced with galactose-free 55 % L-15 medium, and the cells were treated with glucosamine (final concentration 10 mM), 2-deoxyglucose (10 mM) or 2-deoxygalactose (10 mM) for additional 24 h. Finally some cells were grown in galactose-free media, without any treatment, for 24, 48 and 72 h.

Following the treatments, cells were rinsed with 65% Hank's balanced salt solution (HBSS; Flow Laboratories, Mississauga, Ont.). One and a half ml of 100% HBSS was added to the flask and the cells were removed using a plastic spatula and Pasteur pipette. They were collected in a 1.5 ml Eppendorf tube and pelleted by centrifugation for 5 min at 10,000 rpm (Eppendorf Microcentrifuge, Model 5414). The supernatant was drained and the pelleted cells were stored at -80°C until RNA isolation.

2.2.2. *Xenopus* embryo maintenance

Adult *Xenopus laevis* were purchased from Boreal, St. Catherine's, Ontario. The females were induced to ovulate by injecting 900 I. U. of human chorionic gonadotropin (hCG; resuspended in 0.65% NaCl; Sigma, Oakville, ON.) into the dorsal lymph sac. Eggs were collected in 55x15 mm petri dishes containing approximately 10 ml of 27% DeBoer's solution [0.11 M NaCl, 1.3 mM

$CaCl_2$, 0.44 mM KCl, (pH 7.2); Grey *et al.*, 1982,] approximately 10-12 h after injection by the application of gentle pressure to the abdomen. Testes were isolated and minced using a razor blade and forceps in 2 ml of 100% DeBoer's solution to release the sperm. The sperm suspension was then mixed with the eggs at a ratio of 0.5 ml of sperm suspension to 500 eggs in 10 ml of 27% DeBoer's solution. The petri dish was sealed with parafilm and rotated for 25 min on a rotating shaker (70 rpm; American Rotator V, Canlab, Toronto, Ont.) at room temperature. After fertilization, the embryos were washed twice with 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], and transferred to a petri dish containing 2% (w/v) cysteine (pH 7.8) dissolved in Steinberg's solution, for approximately 3 min to remove their jelly coating (Dawid, 1965). The dejell solution was washed away with 8-10 rinses of fresh Steinberg's solution. The embryos were maintained in a 22°C incubator, with periodic changes of fresh Steinberg's solution and the removal of any dead or deformed embryos. Developmental stages were determined according to the external criteria developed by Nieuwkoop and Faber (1967).

2.2.3. Heat shock and chemical treatments of embryos

Embryos to be heat shocked were placed into a 35 x 10 mm petri dish containing Steinberg's solution. The petri dish was then sealed with parafilm and immersed in a heated water bath at 33 °C for 1 h. Control embryos were

maintained in 35 x 10 mm petri dishes in a 22°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°C until required for RNA isolation. Some tadpole stage embryos were also treated with the Ca^{2+} ionophore A23187 at concentrations of 4 µM and 7 µM for 1, 2, 3 or 4 h. The tadpoles were then rinsed in fresh Steinberg's solution after which the samples were frozen at -80°C. In a following experiments, embryos were collected in different stages and bathed in Steinberg's solution with 4 µM A23187 for 3 h, after which they were rinsed in fresh Steinberg's solution and kept at -80°C. To examine BiP mRNA induction by tunicamycin, embryos were treated with 2 µg/ml of tunicamycin in Steinberg's solution for 12 h, after which they were washed with fresh Steinberg's solution and kept at -80°C.

2.3. RNA isolation and northern blotting

2.3.1. RNA isolation

All the glassware used during the RNA isolation was baked at 180°C overnight while all plasticware was washed with RNase-ZAP (Sigma, Oakville, ON), and rinsed with 0.1 % diethyl pyrocarbonate (DEPC, ICN pharmaceuticals Canada Inc.) treated water (Sambrook *et al.*, 1989) to reduce the effect of any possible contaminating RNases. All of the solutions used in the RNA isolation procedure were prepared in 0.1 %

DEPC. Total RNA was isolated from cultured A6 cells, embryos and adult tissues by the GIT/CsCl centrifugation method as described by Chirgwin *et al.*, (1979) with some modifications. A6 cells, embryos and/or adult tissues were homogenized in 10 ml of GIT solution [4 M guanidine isothiocyanate (Bio Shop), 0.025 M sodium acetate (pH 6.0), 0.835% (v/v) β-mercaptoethanol] in a Potter-Elvehjem tissue grinder with teflon pestle (Fisher Scientific). The homogenate was layered on top of the 3.3 ml of the cesium chloride solution [5.7 M cesium chloride (ICN), 0.025 M sodium acetate (pH 6.0)] in 14 X 89 mm ultra-clear ultracentrifuge tube (Beckman). Samples were then centrifuged for 16-23 h at 30K rpm in SW-41 Ti rotor (Beckman) at 21°C. After centrifugation the RNA pellets were washed with the 70 % ethanol and dissolved in 360 μl of TES buffer [0.01 M Tris-HCl (pH 7.4), 0.005 M EDTA (pH 8.0), 1 % (w/v) SDS]. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M. After adding 1 ml of 100 % ethanol the samples were incubated on dry ice or -80°C for 30 min followed by a centrifugation at 13,500 rpm for 10 min in a Hermle model Z320K centrifuge (Mandel scientific, Guelph, Ontario). The pellet was dissolved in 360 μl of 0.1 % DEPC water followed by another ethanol precipitation and subsequent centrifugation. The supernatant was discarded and the RNA pellet was dissolved in 60-80 μl of 0.1 % DEPC water. RNA quantity was determined by taking 4 μl of RNA in 1 ml of 0.1 % DEPC water and then measuring the UV absorbance at 260 nm as well as 260/280 ratio in a

Beckman DU7 spectrophotometer (1 O.D. A_{260} unit = 40 µg RNA/ml). RNA preparations were considered to be pure if the ratio of the optical density values obtained at 260 and 280 was 1.7-1.8 (Sambrook *et al.*, 1989). The integrity and concentration was further confirmed by formaldehyde agarose gel electrophoresis as described below. In order to prepare an RNA sample for electrophoresis 5 µg of RNA was dried in a Speedvac for 10 min and supplemented with 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°C for 5 min followed immediately by 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 3-4 h at 80 V (approximately 0.5 V/cm^2). The 1 X MOPS running buffer was rotated between the electrodes every 30 min to avoid pH changes. Intact RNA was indicated by strong 28S and 18S rRNA staining with ethidium bromide.

2.3.2. Northern blotting

Fifteen µg of each RNA sample or 5 µg of RNA ladder (GIBCO, BRL, BRL, Burlington, Ont.) was dried in a Speedvac for 15 min. The RNA samples

were then subjected to formaldehyde agarose gel electrophoresis as described in the previous section except that ethidium bromide was omitted from the gel. After electrophoresis, the RNA ladder lane was cut from the gel and stained in ethidium bromide (1 µg/ml) for 5-10 min. The ladder was destained in water overnight, and photographed together with a ruler for size determination. The remainder of the gel with RNA samples was rinsed in 0.1% (v/v) DEPC-treated water for 10 min, and in 10 mM sodium phosphate buffer (pH 7.0) for 10 min. The gel was inverted onto a presoaked 3MM Whatman filter paper wick lying on a glass plate positioned over a dish filled with 20 X SSC buffer [3 M sodium chloride, 300 mM sodium citrate (pH 7.0)]. A piece of nylon membrane (Amersham, Oakville, Ont.) cut to the exact size of the gel, was layered onto it. Bubbles were removed by rolling a Pasteur pipette along the membrane. Two pieces of 3MM Whatman paper were layered onto the nylon membrane (bubbles rolled out from each layer), followed by a 5 cm stack of paper towels. Everything was wrapped in Saran Wrap to prevent evaporation and a weight was placed on top. The transfer was allowed to proceed overnight. In the morning the layers of paper towels and 3 MM Whatman paper were discarded. The nylon membrane was placed RNA-side up on a piece of Whatman paper, and the RNA was cross-linked to the membrane in an ultraviolet cross-linker (GS Gene Linker, program 'C3' 150 mJ; BioRad, Mississauga, Ont.). The Northern blots were stored between two pieces of Whatman paper at room temperature, until hybridization. In some cases the blots were subjected to rapid reversible staining with 0.02% methylene blue prior to hybridization to

check for equal sample loading (Herrin and Shmidt, 1988). The Northern blot was placed in a sealable bag with 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; Boehringer Mannheim, Laval, Que.). Air bubbles were removed and the bag was sealed using a Seal-a-Meal bag sealer. The blot was incubated overnight at 42°C in a water-filled container pre-equilibrated at 42°C, with occasional shaking.

2.3.3. Northern blot detection using nick translated probes

The nick translated DNA probes used for northern hybridization analysis were the *Xenopus* BiP cDNA (Blue-BiP), a *Xenopus* cytoskeletal actin cDNA clone (pX1cA1; present from T. Mohun, Department of Zoology, University of Cambrige, UK) and a *Xenopus* L8 cDNA (pBlue-L8; present from Y.-B. Shi, Laboratory of Molecular Embryology, National Institutes of Health, Bethesda, Maryland).

For nick translation, DNA probe (100-300 ng) 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; SA 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; Boehringer Mannheim, Laval, Que). The reaction was incubated at 14°C for 90 min. 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 nucleotides by loading it onto a 1 ml Sephadex G-50 column (ICN, Montreal, Que.) prepared in a 1 cc syringe, and centrifuging at 3000 rpm in an IEC clinical centrifuge. The labeled probe was mixed with 0.5 ml herring sperm DNA (250 μg/ml final concentration) 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 sulfate]. The pre-hybridization buffer was removed from the bag and replaced with hybridization buffer. Bubbles were removed, and the bag was resealed. The reaction was incubated at 42°C for 48 h with occasional shaking. The hybridization buffer was removed and the blot was given two 8 min washes in 1 X SSC and 0.1% (w/v) SDS at room temperature. 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, a 5 min wash was carried out at 42°C in 0.1 X SSC and 0.1% (w/v) SDS. The blot was placed onto a glass plate, wrapped in Saran Wrap and exposed to Kodak BioMax film in conjunction with Cronex intensifying screen (VWR, Toronto, Ont.) at -80°C. After exposure the film was developed in Kodak developer for 4 min, rinsed in deionized water and fixed for 4 min in Kodak fixer. The autoradiogram was rinsed in deionized water and air dried. The autoradiograms were

scanned using Image Master (Pharmacia Biotech). In some cases, a blot was stripped for re-hybridization with additional DNA probes. Stripping of the blot involved soaking it in boiling 0.1% (w/v) SDS and leaving it until the solution cooled to room temperature. The blot was pre-hybridized and hybridized as described above.

2.3.4. Northern blot detection using Digoxygenin-labeled probes

DIG-labeled riboprobes were generated from the different templates indicated below:

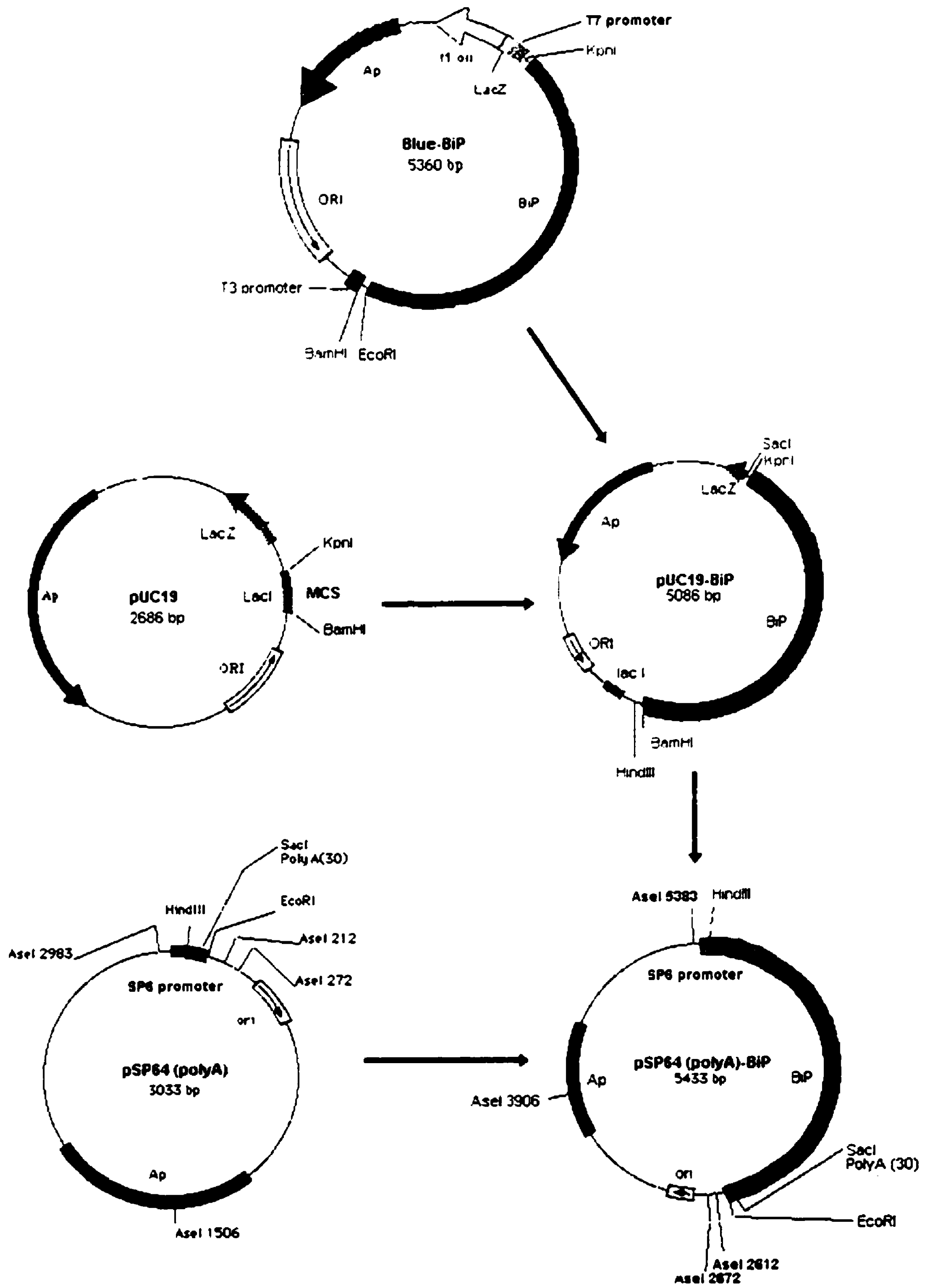
BiP probes:

- antisense probe: blueBiP DNA was linearized with BamHI, and served as a template for T7 RNA polymerase (Fig. 5).
- sense probe: blueBiP was digested with Kpn I and Bam HI to release BiP cDNA. This BiP cDNA was then subcloned into the pUC19 vector (pUC-BiP) to create more restriction enzyme sites. From pUC-BiP, BiP cDNA was cut out with Sac I and Hind III, and subcloned into pSP64 (polyA). This plasmid was linearized with Ase I restriction enzyme, to generate three fragments, one of which contained the full length BiP cDNA with a polyA tail at the 3' end and the site for SP6 RNA polymerase at the 5' end (Fig. 5).

L8 probes:

L8 was originally cloned into pBluescript (blueL8, present from Dr. Yun-Bo Shi). Sense probe was generated by linearization of blueL8 with EcoRI

Figure 5. BiP templates for *in vitro* transcription. Antisense RNA probe was generated by linearization of pBlue-BiP DNA with BamHI which served as a template for T7 RNA polymerase. For the generation of the sense RNA probe, Blue-BiP DNA was digested with KpnI and BamHI to release BiP cDNA which was then subcloned into pUC19 vector (pUC19-BiP). BiP cDNA was cut out from pUC19-BiP, with SacI and HindIII, and subcloned into pSP64-polyA (pSP64-BiP), and linearized with restriction enzyme AseI, forming the template for SP6 RNA polymerase.


T7 promoter
KpnI
f1 ori
Ap
LacZ
Blue-BiP
5360 bp
ORI
BiP
T3 promoter
BamHI
EcoRI
SacI
KpnI
LacZ
Ap
LacZ
KpnI
Ap
pUC19
2686 bp
LacI
MCS
BamHI
ORI
pUC19-BiP
5086 bp
BiP
ORI
lac I
BamHI
HindIII
SacI
PolyA(30)
HindIII
EcoRI
AseI 2983
AseI 212
AseI 272
SP6 promoter
ori
pSP64 (polyA)
3033 bp
Ap
AseI 1506
AseI 5383
HindIII
SP6 promoter
pSP64 (polyA)-BiP
5433 bp
Ap
BiP
AseI 3906
ori
SacI
PolyA (30)
EcoRI
AseI 2612
AseI 2672

and transcription with T7 RNA polymerase. Antisense probe was generated by linearization with XhoI, and transcription with T3 RNA polymerase (Fig. 6).

Hsp70 and hsc70 probes:

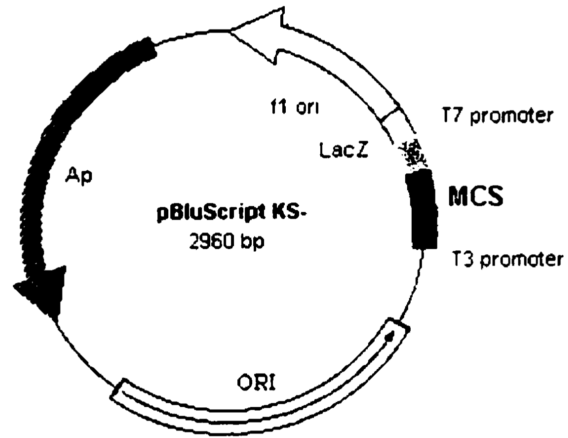
The coding region of both hsp70 genomic DNA and hsc70 cDNA were cloned into pSP72 (present from Dr. Adnan Ali). Hsp70 sense probe was generated by linearization of pSP72 with XhoI, and transcription with T7 RNA polymerase. Hsp70 antisense probe was generated by linearization with Bal I, and transcription with SP6 RNA polymerase (Fig. 7). Hsc70 sense probe was generated by linearization of pSP72 with XhoI, and transcription with T7 RNA polymerase. Hsc70 antisense probe was generated by linearization with XbaI and transcription with SP6 RNA polymerase (Fig. 7).

Actin probes:

Full length type 8 cytoskeletal actin cDNA was originally cloned in Okayama/Berg SV40/pBR322 cloning vector pX1cA1 (present from Dr. Tim Mohun). A 2000 bp fragment was cut from the pX1cA1 with HindIII restriction enzyme, and subcloned into the HindIII site of pBluescript KS. The orientation of the subcloned DNA was checked by restriction digestion analysis with KpnI (Fig. 8). Actin antisense RNA probe was generated by a linearization of the template DNA with EcoRI and transcription with T3 RNA polymerase. The actin sense RNA probe was generated by a linearization of the same template with XhoI followed by transcription with T7 RNA polymerase.

The ingredients for RNA transcription included 2 μg of DNA template plus the NTP mix [2.5 mM rCTP, 2.5 mM rGTP, 2.5 mM rATP, 1.625 mM UTP

Figure 6. L8 templates for *in vitro* transcription. L8 cDNA was originally cloned into pBluescript KS- (pBlue-L8). Sense RNA probe was generated by linearization of pBlue-L8 with EcoRI and transcription with T7 RNA polymerase. Antisense probe was generated by linearization with XhoI and transcription with T3 RNA polymerase.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T7 promoter
LacZ
Ap
MCS
pBluScript KS-
2960 bp
T3 promoter
ORI

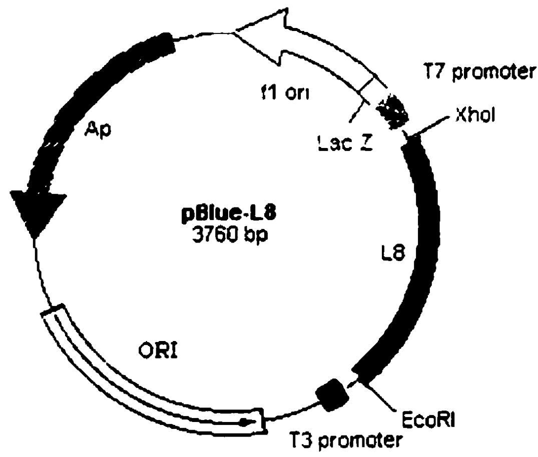
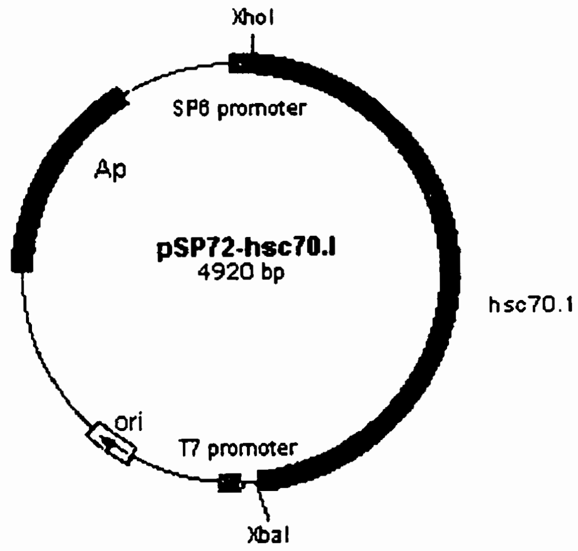

T7 promoter
f1 ori
XhoI
Ap
Lac Z
pBlue-L8
3760 bp
L8
ORI
EcoRI
T3 promoter

Figure 7. hsp70 and hsc70 templates *for in vitro* transcription. Both hsp70 and hsc70 were cloned into pSP72 plasmid vector (pSP72-hsc70 and psp72-hsp70 respectively). Hsp70 sense RNA probe was generated by linearization of pSP72 with XhoI and transcription with T7 RNA polymerase. Hsp70 antisense RNA probe was generated by linearization with Bal I and transcription with SP6 polymerase. Hsc70 sense RNA probe was generated by linearization of pSP72 with XhoI and transcription with T7 RNA polymerase. Hsc70 antisense RNA probe was generated by linearization with XbaI and transcription with SP6 RNA polymerase.


XhoI
SP6 promoter
Ap
pSP72-hsc70.I
4920 bp
hsc70.1
ori
T7 promoter
XbaI

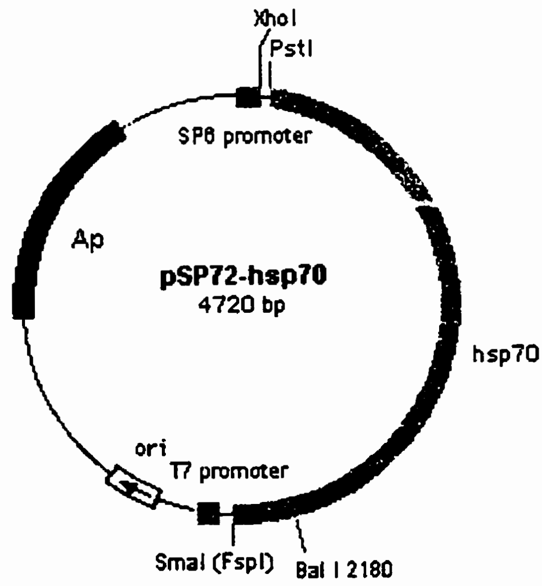
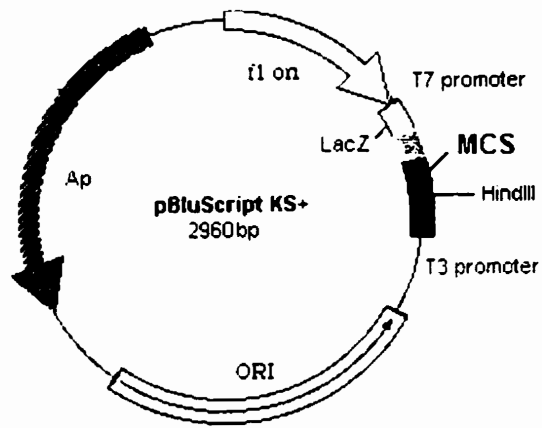
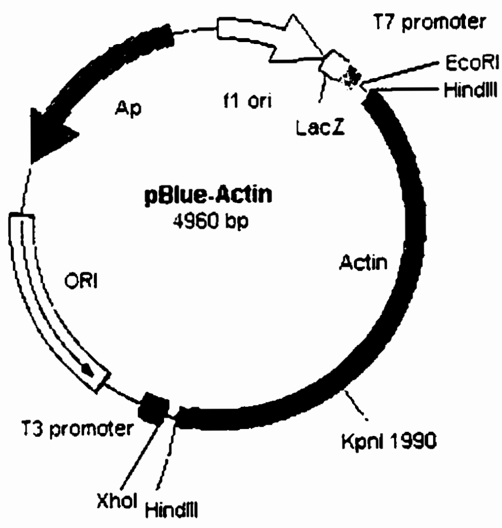

XhoI
PstI
SP6 promoter
Ap
pSP72-hsp70
4720 bp
hsp70
ori
T7 promoter
SmaI (FspI)
Bal I 2180

Figure 8. Actin templates for *in vitro* transcription. Type 8 cytoskeletal actin cDNA was originally cloned into pX1cA1 cloning vector. An actin fragment 2000 bp in length was cut from the pX1cA1 with HindIII, and subcloned into HindIII site of pBluescriptKS+ (pBlue-Actin). Actin antisense RNA probe was generated by a linearization of the template DNA with EcoRI and transcription with T3 RNA polymerase, and sense RNA probe was generated by a linearization of the template with XhoI and transcription with T7 RNA polymerase.


f1 ori
T7 promoter
LacZ
MCS
HindIII
Ap
pBluScript KS+
2960bp
T3 promoter
ORI


T7 promoter
EcoRI
HindIII
Ap
f1 ori
LacZ
pBlue-Actin
4960 bp
Actin
ORI
T3 promoter
KpnI 1990
XhoI
HindIII

(Promega) and 0.875 mM Dig-11 UTP (Boehringer Mannheim, Laval, Que.)], 20 mM DTT (Promega), 1U / 1 µg template DNA of RNase Inhibitor (Promega) and 1X RNA polymerase buffer [10X buffer: 400 mM Tris-HCl pH 7.5; 60 mM MgCl2 and 20 mM spermidine-HCl (Promega)] maintained at room temperature. The appropriate RNA polymerase [SP6 RNA polymerase (Promega), T3 and T7 RNA polymerases (Boehringer Mannheim)] was added (at least 20 IU/1 µg of DNA template) and incubated for 2 h at 37°C. The DNA template was then digested for 10 min at 37°C with RNase-free Dnase (1 IU/1 µg of DNA template; Promega). After DNase digestion of the template DNA, 1 µl of DIG-labeled riboprobe was removed for electrophoretic analysis while SDS in TE buffer (0.1% final concentration) and sodium acetate (0.3M final concentration) were added to the reminder. Finally, DIG-labeled riboprobe was precipitated with two volumes of cold absolute ethanol for 1 h at -80°C. The RNA was pelleted by centrifugation for 30 min at 12.500 rpm in Hermle centrifuge (Mandel scientific, Guelph, ON). The yield was usually 5-10 µg of a riboprobe from 1 µg of template DNA. The probe was resuspended in 5 ml of hybridization buffer [(50% formamide, 5XSSC, 1 mg/ml Torula RNA (Boehringer Mannheim, Laval, Que.), 1X Denhart's solution, 0.1% Tween20 (Sigma, Oakville, ON) and 5 mM EDTA].

The northern blot was placed in a plastic bag containing 20 ml of prehybridization buffer and incubated for at least 6 h at 68°C on a shaker. The

prehybridization buffer was replaced with 20 ml of hybridization buffer containing 2 ml of DIG-labeled antisense RNA probe and incubated overnight at 68°C on a shaker.

The blot was washed twice for 5 min each wash in 2 X SSC and 0.1% (w/v) SDS at room temperature. This was followed by two 15 min washes in 0.5 X SSC and 0.1% (w/v) SDS at 68°C. The nylon membrane was equilibrated in washing buffer [100 mM maleic acid, 150 mM NaCl pH 7.5, 0. 3% (v/v) Tween 20] for 1 min at room temperature followed by incubation in blocking buffer (1:10 blocking reagent [10% (w/v) blocking reagent powder in maleic acid] to maleic acid) for 30 min at room temperature. The membrane was then incubated for 30 min at room temperature in an anti-DIG-alkaline phosphatase antibody diluted 1:5000 in blocking buffer. To remove unbound antibody, the membrane was washed twice in washing buffer for 15 min followed by equilibration in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl) for 2 min at room temperature. The color substrate solution which contained nitroblue tetrazolium chloride (NBT, 4.5 μl/ml) and bromo-4-chloro-3-indolyl phosphate (BCIP, 3.5 μl/ml) in detection buffer was added to the blot in a sealable bag. After air bubbles were removed and the bag was sealed and quickly wrapped in aluminum foil followed by incubation at room temperature without shaking for 10 min. The membrane was then removed from the plastic bag and placed in TE buffer to stop the reaction. A picture of the blot was taken by Image Master.

In some cases, the blot was stripped for rehybridization with other RNA probes. The membrane was incubated with dimethyl formamide (DMF, Calbiochem) at 55°C on a shaker to remove the color substrate. DMF was replaced every 30 min to speed up the stripping process. When all of the color substrate was completely removed the membrane was placed in boiling 0.1% (w/v) SDS for 30 min at room temperature to remove the RNA probe. The membrane was then placed in a sealed plastic bag containing TE buffer until further use.

2.4. *In Situ* hybridization

Whole mount *in situ* hybridization was carried out in *Xenopus* embryos (product of albino female and normal male) using a protocol developed by Harland (1991),incorporating the modifications of Dr. Tom Drysdale (Lawson Institute, London, ON). Unless otherwise indicated all of the procedures were carried out using a nutator (VWR, Mississauga, ON). *Xenopus* embryos were transferred into 6 ml plastic screw-cap vials containing MEMFA (0.1 M MOPS pH 7.4, 2 mM EDTA, 1 mM MgSO4 and 4% paraformaldehyde) and fixed for 2 h at room temperature or overnight at 4°C. The embryos were then washed in cold methanol (two rinses) and stored at -20°C until needed. *In situ* hybridization was performed in Sigmacoated (Sigma, Oakville, ON,) 6 ml glass vials. The embryos were first rehydrated with decreasing concentrations of methanol [75% and 50% methanol in sterile distilled water, and 25% methanol in TTw

buffer (200 mM NaCl, 50 mM Tris pH 7.4 and 0.1% Tween 20)] at room temperature. The samples were then digested with proteinase K (5 μg/ml) for 20 min at room temperature, washed for 10 min in TTw buffer ana treated twice for 5 min with 0.1 M triethanolamine (Sigma, Oakville, ON,), pH 7-8. Two ml of fresh triethanolamine plus 5 μl of acetic anhydride (Sigma, Oakville, ON,) was added for 5 min followed by adding an additional 5 μl of acetic anhydride for another 5 min. Following two 5 min washes with TTw, embryos were refixed in MEMFA for 20 min and washed several times in a large volume of TTw. Prehybridization took place in hybridization buffer [(50% formamide, 5XSSC, 1 mg/ml Torula RNA (Boehringer Mannheim, Laval, Que.), 1X Denhart's solution, 0.1% Tween20 (Sigma, Oakville, ON) and 5 mM EDTA] at 60°C for 2 h. The probe was added and incubated overnight at 60°C for L8 probes and at 65°C for BiP, Hsp70, Hsc70 and actin probes. The following day, the probe was removed and the embryos were washed with decreasing concentrations of hybridization buffer in 2 X SSC (10 min in 100% hybridization buffer at 65°C, 10 min in 50% hybridization buffer at 65°C, 10 min in 25% hybridization buffer at room temperature, and two washes in 2 X SSC at 37°C). RNase A treatment (1 μl/ml) was performed at 37°C, followed by two 1 h washes at 60°C or 65° depending on the probe. In preparation for antibody detection the embryos were washed for 10 min at room temperature first with TTw buffer and then with TBT buffer [200 mM NaCl, 50 mM Tris pH 7.4, 2 mg/ml BSA (ICN) and 0.1%

Triton X-100 (ICN)] followed by incubation with TBT buffer containing 20% heat-treated (30 min at 55°C) lamb serum (GIBCO, BRL) at room temperature. Alkaline phosphatase-conjugated anti-DIG antibody (Fab fragments; Boehringer Mannheim) was added at a 1:4000 dilution in TBT with 20% heat-treated lamb serum for BiP and actin probe, 1:8000 dilution for L8 and 1:12000 dilution for hsp70 and hsc70 probes and incubated overnight at 14°C. The embryos were then washed with a large volume of TBT (twelve times for 30 min), followed by treatment with alkaline phosphatase (AP) buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20 and 2mM Levamisol). The embryos were then incubated overnight at 4°C with nitroblue tetrazolium chloride (NBT; 1.375 µl/ml of buffer) and bromo-4-chloro-3-indolyl phosphate (BCIP; 0.875 µl/ml of buffer). Colorimetric reaction was stopped with increasing concentrations of methanol (25%, 50% and 75% methanol in sterile distilled water). The embryos were maintained in cold 100% methanol until the background staining was sufficiently reduced. The embryos were then rehydrated with decreasing gradations of methanol and counterstained with Bouin's Fixative (VWR, Mississauga, ON) overnight at room temperature. After washing with increasing concentrations of methanol the embryos were cleared for viewing in Benzyl alcohol/Benzyl benzoate (BABB; one part benzyl alcohol and two parts benzyl benzoate). Photos of embryos were taken using a Nikon AFX-II camera attached to a Nikon dissecting microscope employing EPT160T Kodak film. For long term storage the embryos were washed with

100% methanol and stored at -20°C in Sigmacoated screw-cap glass 6 ml vials. The resultant slides were scanned using a Polaroid SprintScan 35. The final images were prepared with Adobe Photoshop (version 4.0.1).

3. RESULTS

3.1. Characterization of BiP cDNA clone

3.1.1. Nucleotide and amino acid sequence of a *Xenopus* BiP cDNA clone

A rat BiP cDNA was used to screen a *Xenopus* cDNA library (performed in the laboratory of Dr. M. Flajnik, University of Miami, as outlined in Material and Methods). In their preliminary DNA sequence analysis, DNA isolated from one of several weakly hybridizing plaques showed sequence similarity at the 5' and 3' ends with BiP genes from other organisms, and was selected for further analysis by Dr. Heikkila's laboratory and subcloned into EcoRI-XhoI sites of pBluecriptKS+ plasmid vector DNA (Fig. 2). The sequencing strategy and the restriction map utilized for this BiP clone are shown in Figure 3 and 4. The entire 2.4 kb cDNA was digested with EcoRI and KpnI restriction endonucleases which resulted in 3 fragments A, B and C, which were in turn subcloned into a pUC19 sequencing plasmid (Fig. 3). Subsequently, the B fragment was digested with PstI, and the two resulting fragments, B1 and B2, were subcloned into pUC18 and pUC19, respectively. The results of the DNA sequence analysis are shown in Figure 9. In the search for the open reading frame, we localized an ATG codon 9 bp upstream from the AUG initiator codon, determined by the Kozak rule (Kozak, 1991a, 1991b). The protein coding region is 1965 bp long, and the final BiP protein consists of 655 amino acids with a

Figure 9. Nucleotide and amino acid sequence of *Xenopus* BiP cDNA clone. The encoded amino acids are listed below the cDNA sequence. The stop codon is labeled with asterisk. The polyadenylation consensus sequence (AATAAA), adenylation control element (TTTTTAT) and mRNA instability element (TATTTA) are underlined. The C-terminal KDEL sequence is shown in bold.

```
1     TGGCGCTCGTTTCTTATCTAGTTGTTGTGACCAACAAATCTCATGGTGACC                         51
                                                           ↓
52    ATG AAG CTG TTT GCC TTG GTG TTG ATG GTG TCT GCC AGC GTA TTT GCC TCT GAT GAT GAT   111
1     M   K   L   F   A   L   V   L   M   V   S   A   S   V   F   A   S   D   D   D     20

112   GAC AAA AAA GAT GAC ATT GGG ACA GTG GTT GGA ATA GAT TTG GGC ACA ACA TAC TCC TGT   171
21    D   K   K   D   D   I   G   T   V   V   G   I   D   L   G   T   T   Y   S   C     40

172   GTT GGA GTG TTT AAA AAC GGA CGT GTA GAA ATT ATT GCC AAT GAC CAG GGT AAC CGT ATC   231
41    V   G   V   F   K   N   G   R   V   E   I   I   A   N   D   Q   G   N   R   I     60

232   ACA CCC TCG TAT GTG GCA TTT ACC CCA GAA GGT GAG CGT CTC ATT GGA GAT GCT GCA AAA   291
61    T   P   S   Y   V   A   F   T   P   E   G   E   R   L   I   G   D   A   A   K     80

292   AAC CAG CTT ACA TCT AAT CCA GAG AAC ACC GTG TTT GAT GCC AAA CGT CGT ATT GGC CGC   351
82    N   Q   L   T   S   N   P   E   N   T   V   F   D   A   K   R   R   I   G   R     100

352   ACA TGG AAT GAC CCT TCT GTT CAG CAG GAC ATC AAG TAC CTG CCA TTT AAG GTA ATT GAG   411
101   T   W   N   D   P   S   V   Q   Q   D   I   K   Y   L   P   F   K   V   I   E     120

412   AAG AAA ACT AAA CCT TAC ATT GTA GTC GAT GTT GGA GAT CAG ATG AAA ACT TTT GCC CCA   471
121   K   K   T   K   P   Y   I   V   V   D   V   G   D   Q   M   K   T   F   A   P     140

472   GAA GAG ATT TCT GCT ATG GTG CTA GTG AAG ATG AAG GAA ACT GCA GAG ACT TAC TTG GGC   531
141   E   E   I   S   A   M   V   L   V   K   M   K   E   T   A   E   T   Y   L   G     160

532   AGA AAG GTT ACT CAT GCT GTT GTC ACT GTA CCT GCT TAT TTC AAT GAT GCA CAG CGT CAA   591
161   R   K   V   T   H   A   V   V   T   V   P   A   Y   F   N   D   A   Q   R   Q     180

592   GCC ACC AAG GAT GCA GGA ACT ATT GCA GGG CTG AAT GTA ATG AGG ATC ATC AAT GAA CCC   651
181   A   T   K   D   A   G   T   I   A   G   L   N   V   M   R   I   I   N   E   P     200

652   ACT GCT GCG GCC ATT GCT TAT GGT CTG GAC AAG AAA GAA GGA GAG AAG AAC ATC CTT GTG   711
201   T   A   A   A   I   A   Y   G   L   D   K   K   E   G   E   K   N   I   L   V     220

712   TTT GAC TTG GGT GGT GGT ACT TTT GAT GTT TCC CTA CTT ACC ATC GAT AAT GGT GTC TTT   771
221   F   D   L   G   G   G   T   F   D   V   S   L   L   T   I   D   N   G   V   F     240

772   GAA GTG GTG GCT ACT AAT GGA GAT ACC CAT CTT GGT GGA GAA GAC TTT GAC CAG AGA GTA   831
241   E   V   V   A   T   N   G   D   T   H   L   G   G   E   D   F   D   Q   R   V     260

832   ATG GAG CAT TTC ATC AAG CTC TAC AAG AAG AAA ACC GGC AAG GAT GTT AGA GCG GAT AAA   891
261   M   E   H   F   I   K   L   Y   K   K   K   T   G   K   D   V   R   A   D   K     280

892   AGA GCT GTA CAG AAA CTT CGC AGA GAG GTG GAG AAA GCA AAG AGG GCA TTG TCG GCC CAG   951
281   R   A   V   Q   K   L   R   R   E   V   E   K   A   K   R   A   L   S   A   Q     300

952   CAT CAA TCC AGA ATT GAA ATC GAA TCT TTC TTT GAA GGG GAG GAT TTC TCT GAA ACT TTG   1011
301   H   Q   S   R   I   E   I   E   S   F   F   E   G   E   D   F   S   E   T   L     320

1012  ACA AGA GCC AAG TTT GAG GAG TTG AAT ATG GAC CTT TTC CGT TCC ACT ATG AAG CCA GTA   1071
321   T   R   A   K   F   E   E   L   N   M   D   L   F   R   S   T   M   K   P   V     340

1072  CAG AAA GTC CTT GAT GAC TCT GAC CTA AAA AAG TCT GAT ATT GAT GAA ATT GTG CTT GTC   1131
341   Q   K   V   L   D   D   S   D   L   K   K   S   D   I   D   E   I   V   L   V     360

1132  GGG GGC TCT ACT CGT ATC CCA AAA ATC CAG CAA TTG GTT AAA GAA TTC TTT AAT GGC AAG   1191
361   G   G   S   T   R   I   P   K   I   Q   Q   L   V   K   E   F   F   N   G   K     380
```

1192 GAG CCT TCC CGT GGT ATC AAT CCT GAT GAG GCC GTT GCA TAT GGT GCA GCT GTT CAA GCT 1251
381 E P S R G I N P D E A V A Y G A A V Q A 400

1252 GGA GTG CTT TCT GGT GAC CAA GAT ACT GGT GAT TTG GTT CTC CTT GAT GTG TGC CCA CTT 1311
401 G V L S G D Q D T G D L V L L D V C P L 420

1312 ACT CTT GGT ATT GAG ACT GTT GGA GGT GTT ATG ACA AAA CTC ATT CCC AGA AAC ACT GTT 1371
421 T L G I E T V G G V M T K L I P R N T V 440

1372 GTA CCC ACC AAA AAG TCA CAG ATC TTC TCT ACA GCA TCC GAC AAC CAG CCC ACT GTC ACT 1431
441 V P T K K S Q I F S T A S D N Q P T V T 460

1432 ATC AAA GTT TAT GAA GGT GAG CGT CCA CTG ACA AAG GAC AAC CAA CTC CTG GGC ACT TTT 1491
461 I K V Y E G E R P L T K D N Q L L G T F 480

1492 GAC TTG ACC GGA ATT CCT CCT GCA CCA CGT GGA GTT CCC CAG ATT GAA GTT ACT TTT GAA 1551
481 D L T G I P P A P R G V P Q I E V T F E 500

1552 ATT GAC GTG AAT GGT ATT CTG CGA GTT ACT GCA GAG GAC AAG GGC ACT GGC AAC AAG AAC 1611
501 I D V N G I L R V T A E D K G T G N K N 520

1612 AAG ATT ACC ATT ACA AAT GAT CAG AAC CGT CTG ACA CCT GAG GAA ATT GAG AGG ATG GTG 1671
521 K I T I T N D Q N R L T P E E I E R M V 540

1672 ACT GAT GCA GAG AAA TTT GCA GAA GAG GAC AAA AAA TTG AAG GAG CGC ATT GAC ACT CGG 1731
541 T D A E K F A E E D K K L K E R I D T R 560

1732 AAC GAA TTG GAG AGC TAT GCT TAC TCC TTA CCG AAC CAG ATT GGC GAT ACG GCG AAA CTT 1791
561 N E L E S Y A Y S L P N Q I G D T A K L 580

1792 GGG GGA AAA CTC TCT CCT GAA GAT AAG GCG ACC ATT GAG AAG GCT GTG ACC GAG AAG ATT 1851
581 G G K L S P E D K A T I E K A V T E K I 600

1852 GAG TGG CCA GCT CGC CAT CAG GAT GCT GAT ATA GAA GAC TTC AAG GAA AAA AAG AAG GAA 1911
601 E W P A R H Q D A D I E D F K E K K K E 620

1912 TTG GAA GAA ATC GTT CAA CCA ATT GTA GGC AAA CTA TAT GGT GGA GCT GGT GCA CCT CCT 1971
621 L E E I V Q P I V G K L Y G G A G A P P 640

1972 CCA GAA GGT GCA GAA GGA GCA GAA GAG ACT GAG AAG GAT GAA TTG TAG 2019
641 P E G A E G A E E T E K D E L * 655

2020 GCAATCCGTGACCCTCTGGCTGCAGTAATATTGTAAATACTGGACTCAGAACTCAGACCATTTTGTTGAAAGAAAATATT 2099

2100 GCGGGGGAAAAAAAACTTTAATATACATGTGGATTCTTCACCTCCTGGTGGAGACATGTTAACCCGTAATGACTCTACTG 2179

2180 CTTTTTATTAGCAGTCTTCTCGCTCAGCTCATTATTTTTGGTCCACTGTTTTCCAGGGGGGGAGGTGGGTCTGGTATTA 2259

2259 CGGAGGGTTCGGTCAAACTTTTGTTTACAAGTTGCTCTAAGATGTCTTATTTATTTGCAAATGGTCCTGCATTCTAAGTG 2339

2340 GAACTTACCATCATTGACCATAATAAATGTTTGATAATTA$_n$ 2379

predicted molecular weight of 72,192. Examination of the 3' untranslated region (UTR) revealed the presence of the consensus sequence for polyadenyation, AATAAA, as well as the cytoplasmic polyadenylation element (CPE), TTTTTAT, 275 nucleotides and 160 nucleotides, respectively, downstream from the stop codon. We also detected the consensus element, TATTTA, which is thought to confer instability to a number of mRNAs (Brawerman, 1987). Examination of the amino acid sequence showed the presence of a hydrophobic N-terminal leader sequence necessary for the translocation of the newly synthesized protein into the endoplasmic reticulum (ER), and the carboxy - terminal signal sequence consisting of 4 amino acids, lys-asp-glu-leu (KDEL), important for the sequestering of proteins in the ER.

3.1.2. Amino acid sequence comparison of two *Xenopus* BiP cDNAs

Recently, another BiP cDNA has been isolated from a *Xenopus* gastric cDNA library (Beggah *et al.*, 1996). The open reading frame of this cDNA encoded a *Xenopus* BiP protein having 97.6% amino acid sequence identity with XBiP (Fig. 10). We observed a total of 16 amino acid substitutions. These results suggest that *Xenopus* contains at least 2 copies of the BiP gene.

Figure 10. A comparison of the amino acid sequences of two *Xenopus* BiP proteins. *Xenopus* (XBiP) amino acid sequence predicted from the cDNA sequenced in our laboratory and *Xenopus* BiP amino acid sequence reported by Beggah *et al.*, (XBiP2; 1996) were aligned and the amino acid identities are shown as dashes.

```
XBiP    1   MVTMKLFALVLMVSASVFASDDDDKKDDIGTVVGIDLGTTYSCVGVFKNGRVEIIANDQG 60
XBiP2   1   -----------L------------------------------------------------ 60

XBiP   61   NRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRLIGRTWNDPSVQQDIKYLPFK 120
XBiP2  61   ------------------------------------------------------------ 120

XBiP  121   VIEKKTKPYIVVDVGDQMKTFAPEEISAMVLVKMKETAETYLGRKVTHAVVTVPAYFNDA 180
XBiP2 121   ----------E--I-------------------------A-------------------- 180

XBiP  181   QRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDKKEGEKNILVFDLGGGTFDVSLLTIDN 240
XBiP2 181   ------------------------------------------------------------ 240

XBiP  241   GVFEVVATNGDTHLGGEDFDQRVMEHFIKLYKKKTGKDVRADKRAVQKLRREVEKAKRAL 300
XBiP2 241   ------------------------------------------------------------ 300

XBiP  301   SAQHQSRIEIESFFEGEDFSETLTRAKFEELNMDLFRSTMKPVQKVLDDSDLKKSDIDEI 360
XBiP2 301   ------------------------------------------------------------ 360

XBiP  361   VLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQAGVLSGDQDTGDLVLLDV 420
XBiP2 361   ------------------L----------------------------------------- 420

XBiP  421   CPLTLGIETVGGVMTKLIPRNTVVPTKKSQIFSTASDNQPTVTIKVYEGERPLTKDNQLL 480
XBiP2 421   ---------------------------------------------------------H-- 480

XBiP  481   GTFDLTGIPPAPRGVPQIEVTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIER 540
XBiP2 481   ------------------------------------------------------------ 540

XBiP  541   MVTDAEKFAEEDKKLKERIDTRNELESYAYSLPNQIGDTAKLGGKLSPEDKATIEKAVTEK 600
XBiP2 541   -------------------------------K-----KA-------S---E------E-- 600

XBiP  601   IEWPARHQDADIEDFKEKKKELEEIVQPIVGKLYGGAGAPPPEGAEGAEETEKDEL"   655
XBiP2 601   ---LES----------A--------------------------------------     655
```

3.1.3. A comparison of the amino acid sequence of *Xenopus* BiP with mammalian, avian, yeast and nematode BiP

A survey of the Genbank protein database revealed that the highest degree of identity of the *Xenopus* BiP amino acid sequence occurred with chicken, rat and human BiP proteins. A detailed comparison of the entire predicted amino acid sequence of *Xenopus* BiP with chicken (Stoeckle, *et al.*, 1988), rat (Munro and Pelham, 1986), human (Ting and Lee, 1988), *C. Elegans* (Heschl and Baillie, 1989) and yeast BiP (Nicholson and al. 1990) is shown in Figure 11. The *Xenopus* BiP amino acid sequence exhibited 91.3% identity with chicken, 90.7% with rat, 89.9% with human, 74% with *C. Elegans* and 61.8% with yeast BiP (Table 2). Most of the differences between *Xenopus* and other organisms occurs in the amino and carboxyl terminal regions of the proteins. Compared to chicken, *Xenopus* BiP has 51 amino acid substitutions, 4 deletions and 6 additions. Furthermore, all 6 BiP proteins have a hydrophobic N-terminal leader sequence and share the carboxyl terminal ER-retention sequence, KDEL, which is found in other lumenal ER proteins.

3.1.4. A comparison of the amino acid sequence of *Xenopus* BiP with other members of the *Xenopus* hsp70 family

Figure 12 compares the *Xenopus* BiP amino acid sequence with the amino acid sequences of the two cytosolic members of the *Xenopus* hsp70 family, namely hsp70 (Bienz, 1984) and hsc70.I (Ali *et al.*, 1996). The identity of

Figure 11. A comparison of the amino acid sequence of *Xenopus* BiP with mammalian, avian, yeast and nematode BiP. *Xenopus* (XBiP), yeast (YBiP), nematode (NBiP), chicken (CBiP), rat (RBiP) and human (HBiP) BiP were aligned and the amino acid identities are shown as dashes. Asterisks represent amino acid deletions.

```
N BiP    1   MKTLF                                                          5
Y BiP    1   MFFNRLSAGKLLVPLSVVLYAL                                        22

X BiP    1   MKLFALVLMVSASVFAS*DDDDKKDDIGTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNR  59
H BiP    1   ---SLVAA-LLLLSA-RAEEE---E-V---------------------------------  60
R BiP    1   --FTVVAAALLLLCAVRAEEE---E-V---------------------------------  60
C BiP    1   -RHLL-A-*LLLGG*-RA--EE--E-V---------------------------------  58
N BiP    7   LLGMIAITAVSIYCKEEEKTEKKETKYE-II-------------Y---------------  65
Y BiP   23   FVVILPLQNSFHSSNVLVRGADVEN*Y---I-----------A-M---KT--L--E----  81

X BiP   60   I*TPSYVAFTPE*GERLIGDAAKNQLTSNPENTVFDAKRLIGRTWNDPSVQQDIKYLPFK 117
H BiP   61   -*----------*-------------------------------------------F---- 118
R BiP   61   -*----------*-------------------------------------------F---- 118
C BiP   59   -*---------------------------------------------------------- 117
N BiP   66   -*-------SGDQ-D------------I-----I---------DY--KT--A---HW--- 124
Y BiP   82   -D--------DD**-----------VAA--Q--I--I-----LKY--R---K---H---N 139

X BiP  118   VIEKKTKPYIVVDVG*DQMKTFAPEEISAMVLVKMKETAETYLGRKVTHAVVTVPAYFND 176
H BiP  119   -V--------Q--I-GG-T-------------T-------A---K--------------- 178
R BiP  119   -V--------Q--I-GG-T-------------T-------A---K--------------- 178
C BiP  118   -*---A--H-Q----GG-T-------------T-------A---K--------------- 176
N BiP  125   --DKSN--SVE-K--S-NKQ*-T---V----------I--S---KE-KN----------- 183
Y BiP  140   -VN-DG--AVE-S-KGEKKV*-T-----G-I-G---QI--D---T--------------- 198

X BiP  177   AQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDKKEGEKNILVFDLGGGTFDVSLLTID 236
H BiP  179   ----------------------------------R------------------------- 238
R BiP  179   ----------------------------------R------------------------- 238
C BiP  177   ----------------------------------R------------------------- 236
N BiP  184   -----------------V-----------------D--R---------------M---- 243
Y BiP  199   -----------------L--V--------------SDK-HQ-I-Y------------S-E 258

X BiP  237   NGVFEVVATNGDTHLGGEDFDQRVMEHFIKLYKKKTGKDVRADKRAVQKLRREVEKAKRA 296
H BiP  239   ----------------------------------------K-N---------------*- 297
R BiP  239   ----------------------------------------K-N----------------- 298
C BiP  237   ----------------------------------------K-N----------------- 296
N BiP  244   ------L-------------------Y--------S---L-K------------------ 303
Y BiP  259   ------Q--S-----------YKIVRQL--AF---H-I--SDNNK-LA--K--A------ 318

X BiP  297   LSAQHQSRIEIESFFEGEDFSETLTRAKFEELNMDLFRSTMKPVQKVLDDSDLKKSDIDE 356
H BiP  298   --S---A-------Y----------------------------------E---------- 357
R BiP  299   --S---A------------------------------------------E---------- 358
C BiP  297   --S---A------------------------------------------E---------- 356
N BiP  304   --T---TKV----L-D---------------------A-L-------E------D-VH- 363
Y BiP  319   --S-MST----D--VD-I-L-------------L---KK-L---E---Q--G-E-K-V-D 378

X BiP  357   IVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQAGVLSGDQDTG*DLVLL 415
H BiP  358   ----------------------------------*-----------------V----- 416
R BiP  359   -------------------------------------------------------*----- 417
C BiP  357   -------------------------------------------------------*----- 415
N BiP  364   ------------V---I------------------------G-----EE---*EI--- 422
Y BiP  379   ------------V---LESY-D--KA-K---------------------EEGVE*-I--- 437
```

```
X BiP  416  DVCPLTLGIETVGGVMTKLIPRNTVVPTKKSQIFSTASDNQPTVTIKVYEGERPLTKDNQ  475
H BiP  417  H--------------------S-------N-----------------------------H  476
R BiP  418  -----------------------------------------------------------H  477
C BiP  416  -----------------------------------------------------------H  475
N BiP  423  --N---M-------------G----I------V----A--------Q-F-----M----H  482
Y BiP  438  --NA-------T-----P--K---AI-----------V------M--------AMS---N  497

X BiP  476  LLGTFDLTGIPPAPRGVPQIEVTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEE  535
H BiP  477  ------------------------------------------------------------  536
R BiP  478  ------------------------------------------------------------  537
C BiP  476  ------------------------------------------------------------  535
N BiP  483  Q--K-----L----------------------H-----------------------S--D  542
Y BiP  498  ---K-E------------------AL-A----K-S-T-----KSES------KG---Q--  557

X BiP  536  IERMVTDAEKFAEEDKKLKERIDTRNELESYAYSLPNQIGDTAKLGGKLSPEDKATIEKA  595
H BiP  537  -----N-----------------------------K-----KE-------S---E-M---  596
R BiP  538  -----N-----------------------------K-----KE-----------E-M---  597
C BiP  536  -----N-----------------A-----------K-----KE-------S---E-----  595
N BiP  543  --A-IN-------D---V-DKAEA---------N-K---E-KE------DED--K---E-  602
Y BiP  558  -D---EE-----S--ASI-AKVES--K--N--H--K--VNGDL**-E--EE---E-LLD-  615

X BiP  596  VTEKIEWPARHQDADI*EDFKEKKKELEEIVQPIVGKLY*GGAGAPPPEGAEGA******  648
H BiP  597  -E-----LES------*----A------------IS---*-S--*---T-E-DT******  647
R BiP  598  -E-----LES------*----A------------IS---*-S--*---T-E-DT******  648
C BiP  596  -E-----LES------*----S-------V-----S---*-S--*---T-E-E-******  646
N BiP  603  -E-A-S-LGSNAE-*SA-EL--Q--D--SK-----S---KD*---GERRPQKRD******  654
Y BiP  616  ANDVL--LDDNFETA-A---D--FES-SKVAY--TS---*---DGSGAADYDDEDEDDDG  674

X BiP  649  EETEKDEL  655
H BiP  648  **A-----  653
R BiP  649  **S-----  654
C BiP  647  A-**----  652
N BiP  655  *LDD----  661
Y BiP  675  DYF-H---  682
```

Figure 12. A comparison of the amino acid sequence of *Xenopus* BiP with other members of the hsp70 family. *Xenopus* BiP (XBiP), hsc70.1 (Xhsc70.1) and hsp70B (Xhsc70.B) were aligned and the amino acid identities are shown as dashes. Asterisks indicate amino acid deletions.

```
XBiP        1   MKLFALVLMVSASVFASDDDDK                                         22

X BiP      23   KDDIGTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAAKNQ   82
Xhsc70.I    1   M*SK-PA---------------QH-K-----------T-T------*DT-----------   58
Xhsp70B     1   MATK-VA---------------QH-K-----------T--------*DT-----------   59

XBiP       83   LTSNPENTVFDAKRLIGRTWNDPSVQQDIKYLPFKVIEKKTKPYIVVDVGDQMKTFAPEE  142
Xhsc70.I   59   VAM--T------------RFE-AV--S-M-PG--N-VSDSGRSKGQ-EYKAET-S-Y---  118
Xhsp70B    60   VAM--Q------------KF---V--C-L-HW--Q-VSDEG--KVK-EYK-EE-S-F---  119

XBiP      143   ISAMVLVKMKETAETYLGRKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIINEPTA  202
Xhsc70.I  119   --SI-------I--A---KT--N-----------S-----------YW---L--------  178
Xhsp70B   120   --------------A---HP--N--I--------S---------VL----IL--------  179

XBiP      203   AAIAYGLDKKEG*EKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDFDQRVM  261
Xhsc70.I  179   ----------V-A-R-V-I-----------I---ED-I---KLPS-----------N-MV  238
Xhsp70B   180   ---------GARG-Q-V-I-----------I----D-I---K--A-----------N-MV  239

XBiP      262   EHFIKLYKKKTGKDVRADKRAVQKLRREVEKAKRALSAQHQSRIEIESFFEGEDFSETLT  321
Xhsc70.I  239   N--VAEF-R-QK--IIDN----R*--TAC-C--**--SST-AS---D-LY--I--YTSI-  295
Xhsp70B   240   N--VEEF-R-HK--IGQN---L-R--TACDR---T--SSS-AS---D-L---I--YTAI-  299

XBiP      322   RAKFEELNMDLFRSTMKPVQKVLDDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKE  381
Xhsc70.I  296   --R-----A---IG-LD--E-S-R-AK-D--Q-HD--------------K-LQD------  355
Xhsp70B   300   --R----CS----G-LE--E-A-R-AK-D--Q-H-------------V-K-LQD----R-  359

XBiP      382   PSRGINPDEAVAYGAAVQAGVLSGDQDTGDLVLL**DVCPLTLGIETVGGVMTKLIPRNT  439
Xhsc70.I  356   LNKS---------------AI----KSENVQD--LL--T--S-----A-----V--K---  415
Xhsp70B   360   LNKS---------------AI-M--KSENVQD--LL--A--S-----A-----V--K---  419

XBiP      440   VVPTKKSQIFSTA*SDNQPTVTIKVYEGERPLTKDNQLLGTFDLTGIPPAPRGVPQIEVT  498
Xhsc70.I  416   TISH-SQTQTF-TY-----G-L-Q----G-AM----N---K-E-----------------  475
Xhsp70B   420   TI---*QTQSF-TY-----G-L-Q-F----AM----N---K-ES----------------  478

XBiP      499   FEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVTDAEKFAEEDKKLKERID  558
Xhsc70.I  476   -D--A----N-S-V-----KE--------KG--SK-D-----QE-D-SKLKVE-QRWKVS  535
Xhsp70B   479   -D--A----N-S-VE--S-KQ--------KG--SK-D--K--QE---YKAD-DAQR--V-  538

XBiP      559   TRNELESYAYSLPNQIGDTAKLGGKLSPEDKATIEKAVTEKIEWPARHQDADIEDFKEKK  618
Xhsc70.I  536   SK-S-----SNM*KATVEDE--K--I-D---QK-LEKCN-V-A-LPKN-T-EK-E-EHQQ  594
Xhsp70B   539   AK-A-----FN-*KSMVEDENVK--I-D---R--SEKC-QV-S-LENN-L-EK-EYAFQQ  597

XBiP      619   KELEEIVQPIVGKLY*GGAGAPPPEGAEGAEETEKDEL                         655
Xhsc70.I  595   ----KVCN--IT---QSAG-M-GGG-MP-FPGAGGAPTGGASSGPTIEEVD            645
Xhsp70B   598   -D--KVC---IT---Q--VPGGV-G-MP-SSCGAQ-RQGGNS*GPTIEEVD            647
```

BiP with hsp70 and hsc70.I is only 57% and 55.2%, respectively (Table 2). Therefore, *Xenopus* BiP has greater amino acid sequence identity with BiP from other species, including evolutionary distant yeast, than with other members of the *Xenopus* hsp70 family.

3.2. Characterization of BiP mRNA accumulation in *Xenopus* A6 cells and adult tissues

3.2.1. Effect of various agents on the relative level of BiP mRNA in A6 cells

We have used northern hybridization analysis to examine the effect of various agents, shown to enhance BiP gene expression in mammalian cells, on the relative level of BiP mRNA in *Xenopus* A6 kidney epithelial cells. After hybridization of the RNA blots with labeled BiP cDNA, the blots were washed under stringent conditions to minimize any potential cross-reactivity with hsp70 or hsc70 transcripts. As shown in Fig. 13, BiP mRNA accumulation was detected constitutively in *Xenopus* A6 cells. Furthermore, the largest increase in the accumulation of BiP mRNA relative to control, as determined by densitometric analysis, occurred in A6 cells treated with 2-deoxyglucose (4.2 fold), 2-deoxygalactose (3.3 fold), and the calcium ionophore, A23187 (3.4 fold, data not shown). Moderate increases in the relative level of BiP mRNA accumulation compared to control were found in cells treated with tunicamycin (2.1 fold), heat shock (2.2 fold) and glucosamine (2.0 fold). In other studies,

Table 2. A comparison of BiP amino acid sequences*

	Percent identity with XBiP
X2 BiP	97.6%
HBiP	89.9%
R BiP	90.7%
C BiP	91.3%
N BiP	76.3%
Y BiP	60.8%
X hsp70B	57.0%
X hsc70.I	55.2%

*An amino acid sequence comparison of *Xenopus* BiP with second *Xenopus* BiP (X2BiP), human BiP (HBiP), rat BiP (RBIP), chicken BiP (CBiP), *C. elegans* (NBiP), yeast (YBiP), *Xenopus* hsp70 (Xhsp70B) and *Xenopus* hsc70 (Xhsc70.I).

treatment of A6 cells with dithiothreitol or β-mercaptoethanol also enhanced BiP mRNA accumulation (data not shown). The RNA blot was also hybridized against a *Xenopus* hsp70 genomic clone (Fig. 13). Only the heat shocked A6 RNA sample demonstrated hybridizable hsp70 mRNA which was smaller (2.4 kb) than BiP mRNA (2.7 kb). The same blot was hybridized against a *Xenopus* DIG-labeled hsc70 RNA probe. Hsc70 mRNA (2.3 kb) was detected in each of the samples studied, however, we did not detect an increase in accumulation of hsc70 mRNA in A6 cells after heat shock. While homocysteine has been shown to enhance BiP gene expression in human vascular endothelial cells (Kokame *et al.,* 1996), we were not able to detect any increase in BiP mRNA accumulation after treatment of A6 cells grown in L15 medium, rich in cysteine, with this inducer (Fig. 14).

3.2.2. Effect of glucose starvation on the relative level of BiP mRNA expression in A6 cells

Figure 15 demonstrates the effect of glucose starvation on the level of A6 cell BiP mRNA. A6 cells in galactose-free media displayed an increase in the relative level of BiP mRNA after 24 to 72 h of exposure. These RNA blots were subsequently re-probed with a *Xenopus* cytoskeletal actin cDNA and found to contain relatively constant levels of actin mRNA. These latter results together with uniform levels of ethidium bromide staining RNA in formaldehyde-agarose gels (data not shown) indicates that the changes in the relative levels of BiP mRNA were not the result of unequal RNA loading.

Figure 13. Effect of various agents on the relative level of BiP mRNA in A6 cells. A6 cells were either maintained in L-15 media (C) or treated with either tunicamycin (T), A23187 (A), heat shock (HS), galactose-free medium (Gal^-), glucosamine (Glc), 2-deoxyglucose (Dgl) and 2-deoxygalactose (Dga), as outlined in the Materials and Methods. Total RNA was isolated and 15 μg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The RNA blot in the upper panel was stripped and rehybridized against the *Xenopus* hsp70 genomic clone (middle panel) and *Xenopus* DIG-labeled hsc70 antisense RNA probe.

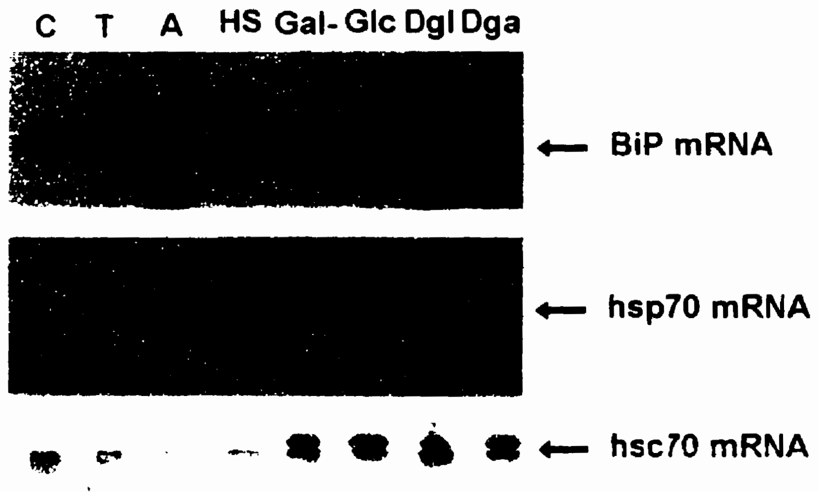

C T A HS Gal- Glc Dgl Dga
BiP mRNA
hsp70 mRNA
hsc70 mRNA

Figure 14. Effect of homocysteine on the relative level of BiP mRNA in A6 cells. A6 cells were either maintained in L-15 media (C) or treated with homocysteine (HC, 1-5 μM). Total RNA was isolated and 15 μg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The smaller panel represents the staining of the blot with methylene blue to verify equal loading of RNA.

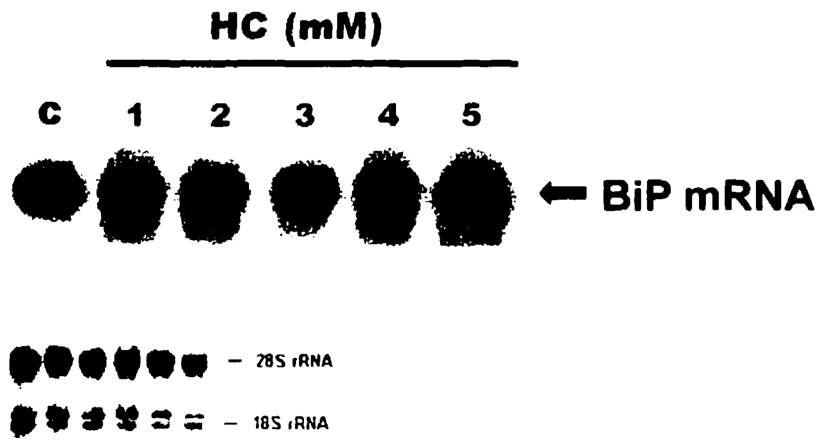
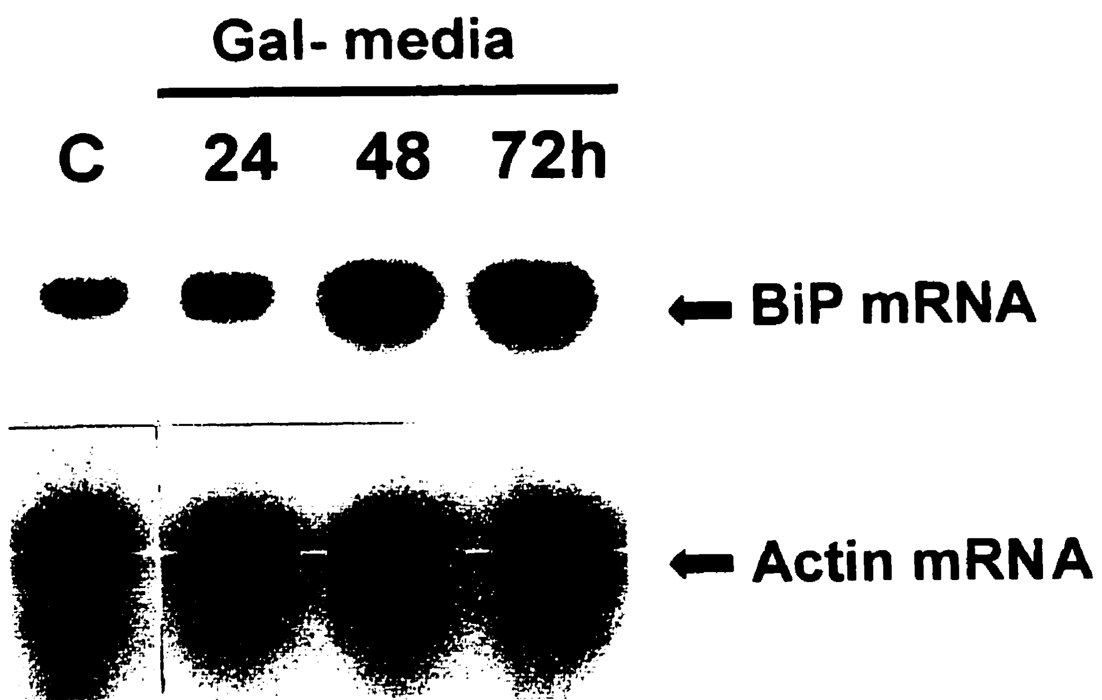

HC (mM)
C
1
2
3
4
5
BiP mRNA
28S rRNA
18S rRNA

Figure 15. Effect of glucose starvation on the relative level of BiP mRNA in A6 cells. A6 cell media was replaced with galactose free L-15 medium and maintained for 24, 48 and 72 h at 22°C. Total RNA was isolated and 15 μg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The same RNA blot was stripped of labeled BiP probe and reprobed with *Xenopus* cytoskeletal actin cDNA. C, control; Gal^-, galactose-free.


Gal- media
C
24
48
72h
BiP mRNA
Actin mRNA

3.2.3. Relative levels of BiP mRNA in different adult *Xenopus* tissues

While BiP mRNA was detected in each of the tissues studied, we observed a marked difference in the relative levels of BiP mRNA after comparing equivalent amounts of total RNA isolated from the different tissues (Fig. 16). For example, the highest relative level of BiP mRNA was detected in liver followed by testis, ovary and heart with reduced levels in eyes and muscle.

3.3. Characterization of BiP mRNA accumulation during *Xenopus* development

3.3.1. Northern blot and *in situ* hybridization analysis of BiP mRNA accumulation during early stages of *Xenopus* development

Constitutively expressed BiP mRNA was detected at all stages of *Xenopus* early development from unfertilized egg to 4-day-old tadpole. BiP mRNA was present at similar levels in unfertilized egg, cleavage, blastula and gastrula stage embryos. Relative BiP mRNA levels rose slightly at the early neurula stage and then increased further in early and late tailbud stage embryos (Fig. 17). Interestingly, the relative level of BiP mRNA in 4-day-old tadpole was lower than the amount found in the tailbud stage (see Fig. 20, 22, and 25).

In order to examine the spatial distribution of BiP gene expression during *Xenopus* early development we carried out whole mount *in situ* hybridization at selected embryonic stages employing a DIG-labeled BiP

Figure 16. Relative levels of BiP mRNA in different adult *Xenopus* tissues.

Total RNA was isolated from selected adult tissues and 10 μg of RNA was subjected to northern hybridization analysis employing the ^{32}P-labeled *Xenopus* BiP cDNA probe. L, liver; M, muscle; H, heart; E, eyes; T, testis; O, ovary.

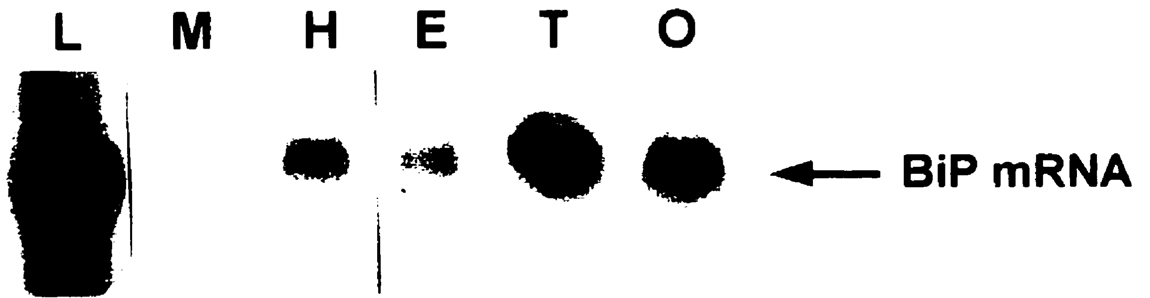
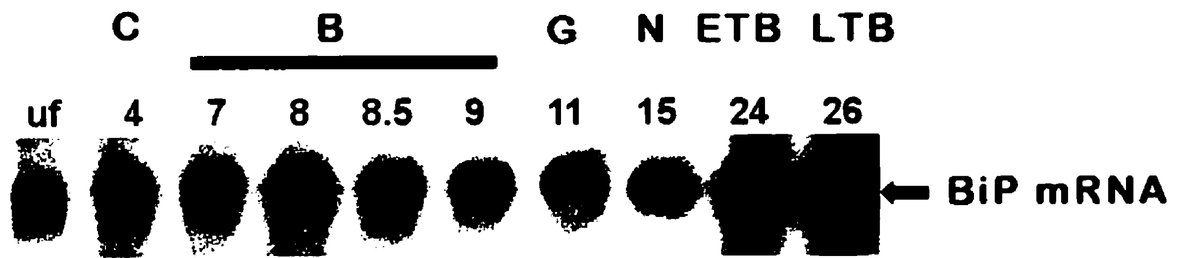

L M H E T O
BiP mRNA

Figure 17. Relative levels of BiP mRNA during early *Xenopus* development.

Total RNA was isolated from unfertilized egg (UF), cleavage stage (C), four different blastula (B) stages, neurula stage (N), and early and late tailbud stage (ETB and LTB respectively). Fifteen µg of RNA was subjected to northern hybridization analysis employing the ^{32}P- labeled *Xenopus* BiP cDNA probe. The numbers above each line indicate the embryonic stage according to Neuwkoop and Faber (1967).

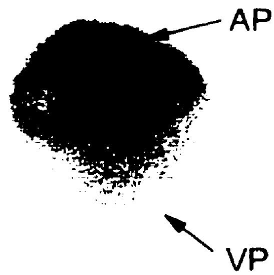

C
B
G
N
ETB
LTB
uf
4
7
8
8.5
9
11
15
24
26
BiP mRNA

antisense RNA probe. As shown in Figure 18, BiP mRNA, as indicated by the blue staining, was present constitutively in the animal pole of the unfertilized egg. This pattern was retained after fertilization in cleavage and blastula stage embryos (Fig. 18). In control experiments, we found that the DIG-labeled BiP sense RNA probe did not hybridize to RNA present in unfertilized eggs or embryos (data not shown). At the gastrula stage, BiP mRNA was present throughout the embryo with slightly less accumulation in the yolk plug region (Fig. 18). At the neurula stage, BiP mRNA levels were enriched at both anterior (in the neural plate) and posterior (around blastopore) ends of the embryo, as well as along the neural folds (Fig. 18). In the early and late tailbud stage embryos (Fig. 19), BiP mRNA was distributed primarily along the dorsal region of the embryo in the somitic region, spinal cord, cranial nerves, otic vesicle and in the forebrain. BiP mRNA was also present in the heart, gills, liver diverticulum, pronephros, pronephric duct and around the anus. In control experiments we did not observe any hybridization of the tailbud stage embryos with sense DIG-labeled BiP sense RNA probe (Fig. 19).

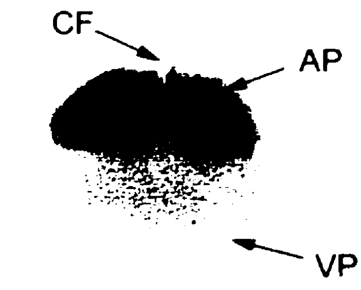
3.3.2. Effect of tunicamycin on BiP mRNA accumulation in *Xenopus* embryos

In the present study we have also examined the effect of glycosylation inhibition on BiP mRNA accumulation in *Xenopus* embryos. In a preliminary set of experiments we found that exposure of A6 cells to 2 µg/ml tunicamycin induced BiP mRNA accumulation within 12 h (data not shown). Given this

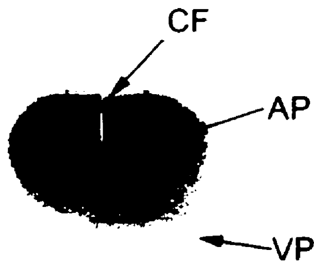
Figure 18. Spatial pattern of BiP mRNA accumulation during early *Xenopus* development. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe was carried out with *Xenopus* unfertilized egg, cleavage, blastula, gastrula and neurula embryos. AP, animal pole; VP, vegetal pole; CF, cleavage furrow; YP, yolk plug; BL, blastopore lip; NF, neural fold; BP, blastopore; A, anterior side of the embryo; P, posterior side of the embryo.



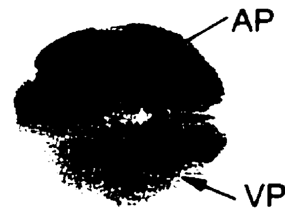
EGG



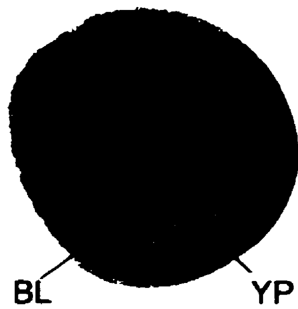
1st CLEAVAGE



2 CELL STAGE



BLASTULA

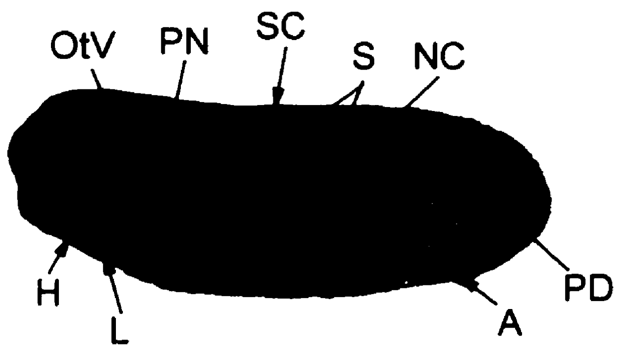


GASTRULA

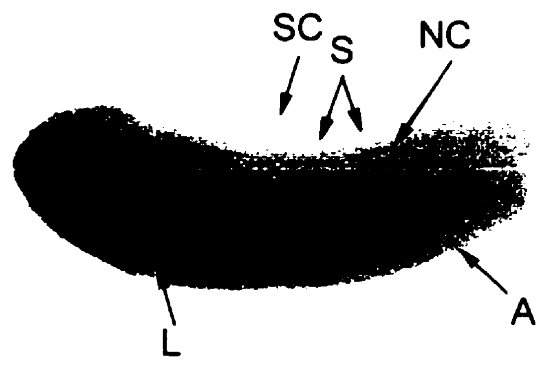
NEURULA

Figure 19. Spatial pattern of BiP mRNA accumulation during early and late tailbud stages in *Xenopus* development. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe (unless otherwise indicated) was carried out with *Xenopus* embryos at early (ETB, stage 24- 25) and late (LTB, stage 32- 33) tailbud stages of development. NC, notochord; SC, spinal cord; A, anus; S, somites; L, liver diverticulum; PN, pronephros; PD, pronephric duct; H, heart; G, gills; CN, cranial nerve; OtV, otic vesicle.

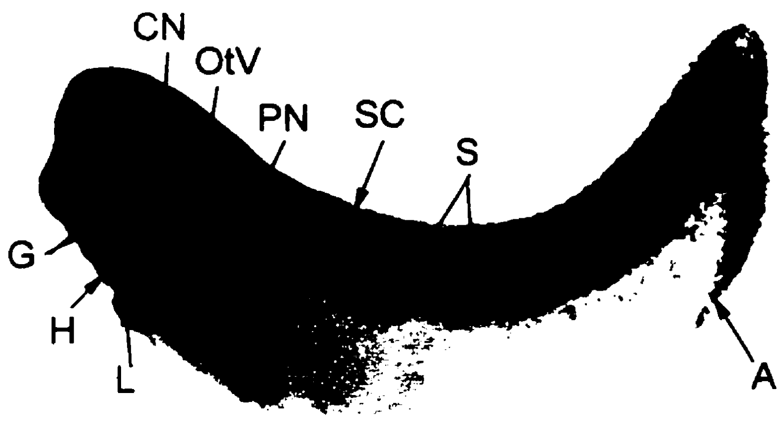
ETB



ETB (+)



LTB



regimen, the first stage we could examine was gastrula. As shown in Figure 20, BiP mRNA accumulation was tunicamycin-inducible at the neurula stage as well as at the tailbud and tadpole stages. The same blot was stripped of BiP cDNA probe and reprobed with L8 probe (lower panel) to confirm equal loading of total RNA between control and heat shock samples.

3.3.3. Effect of ionophore A23187 on BiP mRNA accumulation in *Xenopus* embryos

In order to develop a more complete stressor profile with respect to BiP mRNA accumulation in *Xenopus* embryos, we examined the effect of the calcium ionophore A23187. In initial studies, we characterized the time course of BiP mRNA accumulation in A6 cells treated with 4μM A23187, a concentration which is lower than used in other studies (Lee, 1987). BiP mRNA accumulation increased after 4h of treatment (data not shown). Given these results we treated tadpoles with 4 μM or 7 μM A23187, for 1, 2, 3 or 4 h, and found that BiP mRNA accumulation occurred within 2 h of A23187 exposure (Fig. 21).This blot was subjected to methylene-blue staining prior to hybridization with BiP cDNA (lower panel) to confirm equal loading of total mRNA. Given these preliminary studies, we characterized the effect of 4 μM A23187 for 3 h on BiP mRNA accumulation during development. As shown in Figure 22, BiP mRNA was first inducible at the neurula stage. This inducibility was also found at the tailbud and tadpole stages. This blot was stripped of BiP

Figure 20. Effect of tunicamycin on BiP mRNA accumulation in *Xenopus* embryos. Total RNA was isolated from control (C) and tunicamycin treated [(2 μg/ml for 12 h (T)] embryos at gastrula (G), neurula (N), early tailbud (TB, stage 22-23) and tadpole (TP, 4-day-old) stages. Fifteen μg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The same RNA blot was stripped of labeled BiP probe and reprobed with *Xenopus* L8 probe.

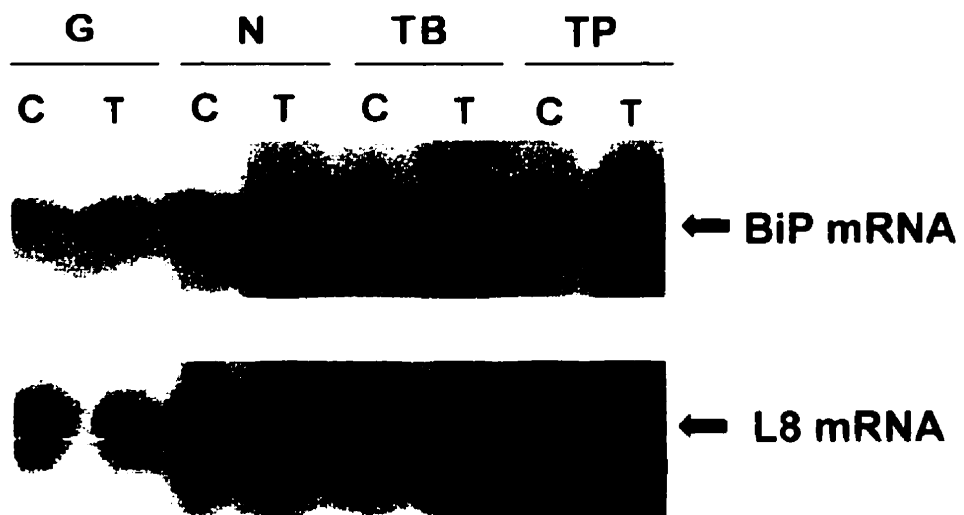
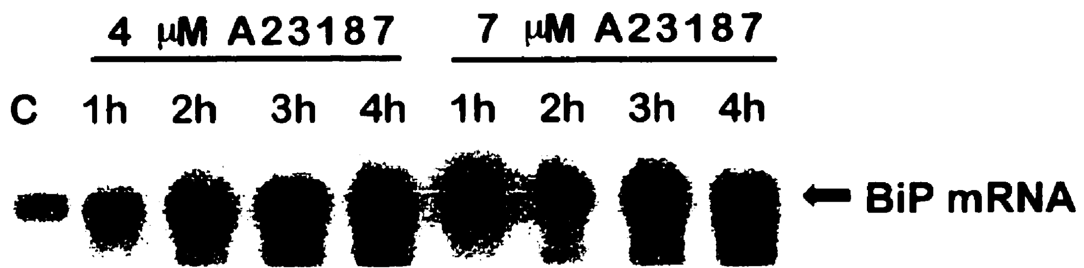
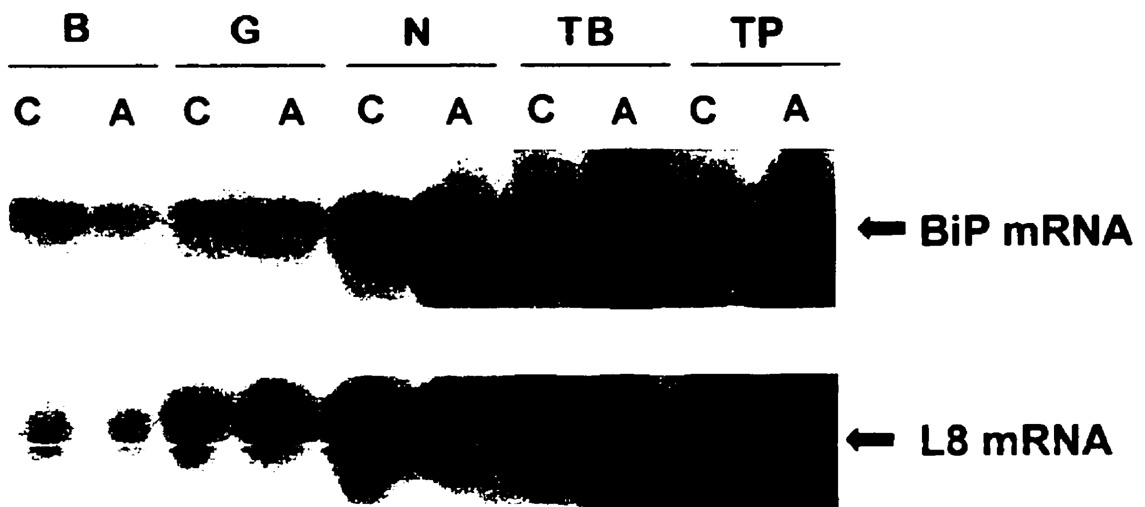

G
N
TB
TP
C
T
C
T
C
T
C
T
BiP mRNA
L8 mRNA

Figure 21. Effect of ionophore A23187 on the relative level of BiP mRNA in 4-day-old tadpoles. Tadpoles were incubated in the presence of A23187. Total RNA was isolated and 15 µg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The smaller panel represents staining of the blot with methylene-blue to check for equal loading of RNA prior to northern hybridization.


4 μM A23187
7 μM A23187
C 1h 2h 3h 4h 1h 2h 3h 4h
BiP mRNA

28S rRNA
18S rRNA

Figure 22. Effect of ionophore A23187 on the accumulation of BiP mRNA in *Xenopus* embryos. Total RNA was isolated from control (C) and A23187 treated [4 μ M for 3 h(A)] embryos at blastula (B), gastrula (G), neurula (N), tailbud (TB, stage 22-23) and tadpole (TP, 4-day-old) stages. Fifteen μg of RNA was subjected to northern hybridization analysis using a ^{32}P-labeled *Xenopus* BiP cDNA. The same RNA blot was stripped of labeled BiP probe and reprobed with *Xenopus* L8 probe.


B
G
N
TB
TP
C A C A C A C A C A
BiP mRNA
L8 mRNA

cDNA probe and reprobed with L8 probe to verify equal loading of total RNA between control and calcium ionophore-treated samples (lower panel).

The Northern blot results with A23187 were confirmed by whole mount *in situ* hybridization of embryos with DIG-labeled BiP antisense RNA probe. The spatial pattern and the relative level of BiP mRNA accumulation did not change in gastrula stage embryos treated with the calcium ionophore A23187 compared to the control (Fig. 23). However, at the neurula stage A23187 increased BiP mRNA levels throughout the embryo with enhanced accumulation in the neural plate, along the neural folds, around the blastopore and in the ectoderm. As shown in Figure 24, ionophore treatment of tailbud stage embryos resulted in increased accumulation of BiP mRNA primarily in the head region, as well as along the spinal cord, in the somites, tail, pronephros, pronephric duct, heart and liver. In control experiments, we did not observe any hybridization of DIG-labeled BiP sense RNA probe with the tailbud stage embryo. In this last panel, it should be mentioned that eye tissue undergoes a normal increase in pigmentation while darker dots along the spinal cord in a late tailbud embryo indicate the beginning of a melanocyte development.

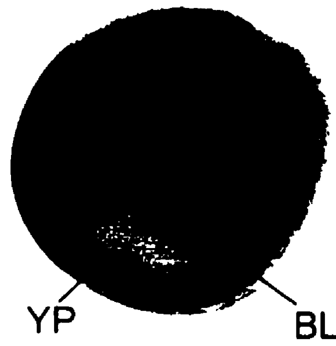
3.3.4. Effect of heat shock on BiP mRNA accumulation in *Xenopus* embryos

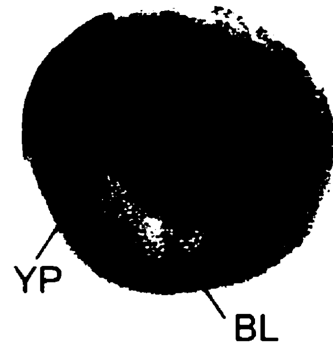
In this section, we examined the effect of heat shock on BiP mRNA accumulation during selected stages of *Xenopus* early development. While

Figure 23. Spatial pattern of BiP mRNA accumulation in gastrula and neurula embryos after treatment with ionophore A23187. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe was carried out with control (C) and A23187 treated (4 μ M for 3 h) embryos at gastrula (GAST) and neurula (NEUR) stages. YP, yolk plug; BL, blastopore lip; NF, neural fold; BP, blastopore.

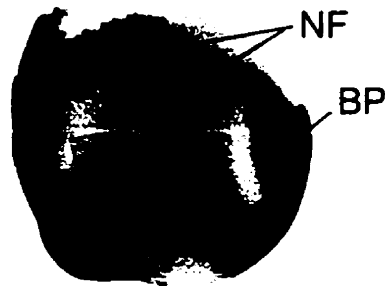
CONTROL **A23187**

GAST





NEUR



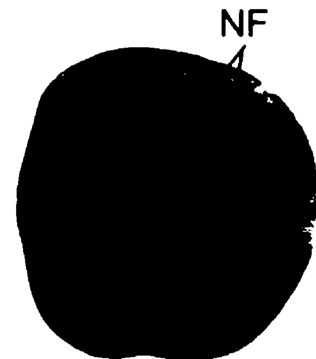
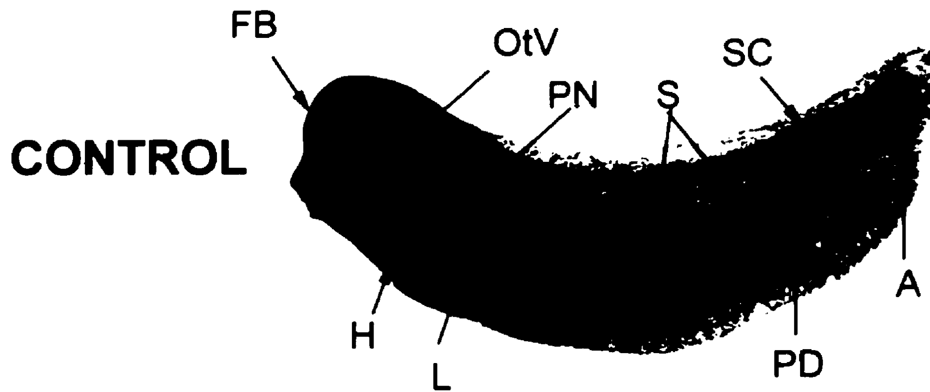
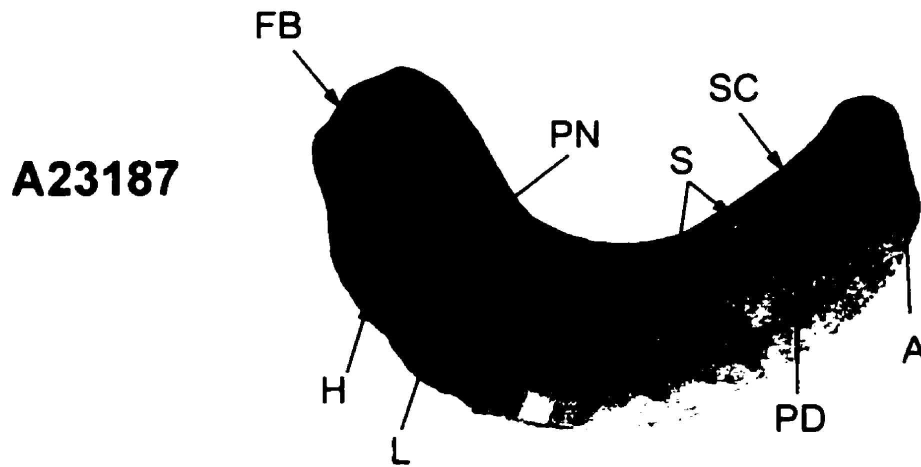
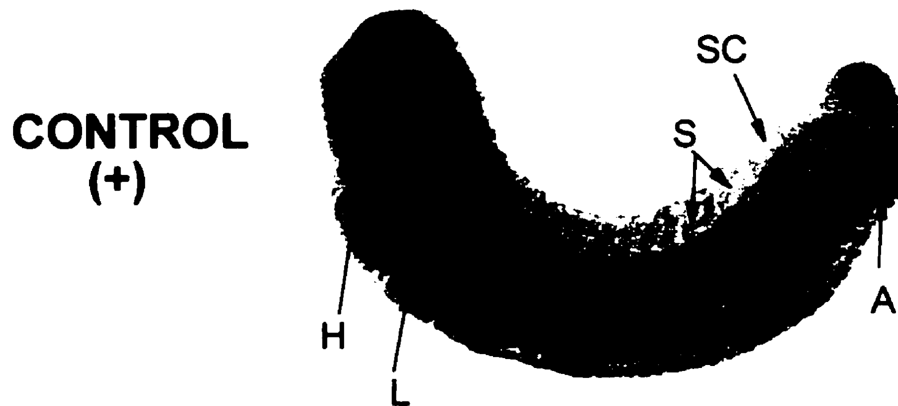


Figure 24. Spatial pattern of BiP mRNA accumulation in tailbud stage embryos after treatment with ionophore A23187. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe (unless otherwise indicated) was carried out with control (C) and A23187 treated (4 μ M for 3 h) tailbud stage embryos (stage 26-27). NC, notochord; SC, spinal cord; A, anus; S, somites; L, liver diverticulum; PN, pronephros; PD, pronephric duct; H, heart; OtV, otic vesicle.


CONTROL
FB
OtV
PN
S
SC
A
PD
H
L


A23187
FB
PN
S
SC
A
PD
H
L


CONTROL
(+)
SC
S
A
H
L

incubation of early blastula embryos for 1 h at 33°C did not affect BiP mRNA levels, treatment of gastrula stage embryos resulted in enhanced accumulation of BiP mRNA (Fig. 25). Hsp70 mRNA was not detectable in control embryos during development but was heat-inducible at all developmental stages (Fig. 25, middle panel). The cDNA for *Xenopus* large ribosomal subunit protein 8 (L8) was used as a control in these experiments. As shown in Figure 19, L8 mRNA was present at all stages of development. While the relative levels of L8 mRNA increased dramatically in gastrula stage embryos and peaked in tadpole stage, we did not observe any heat-inducible accumulation in any of the samples.

The spatial pattern of BiP mRNA accumulation in heat shocked *Xenopus* embryos is shown in Figures 26, 27 and 28. We did not detect any differences in the pattern of BiP mRNA accumulation in control and heat shocked (1h at 33°C) early blastula stage embryos (Fig. 26). However, at the gastrula stage heat shock enhanced the accumulation of BiP mRNA throughout the embryo (Fig. 26). In neurula embryos BiP mRNA levels were enhanced by heat shock in the neural plate, along the neural folds and around the blastopore (Fig. 27). In a sagittal view, heat induced BiP mRNA accumulation was evident in the notochord, spinal cord portion of the neural tube and in portions of the epidermis (Fig. 27). At the early tailbud stage heat shock increased the accumulation of BiP mRNA along the spinal cord as well as in the somites, the forebrain, the tail and around the anus (Fig. 28). At the late tailbud stage heat-

Figure 25. Effect of heat shock on the accumulation of BiP, hsp70 and L8 mRNA in *Xenopus* embryos. Total RNA was isolated from control (C) and heat-shocked (HS; 1 h at 33°C) early blastula (B), gastrula (G), neurula (N), early tailbud (TB, stage 22-23) and tadpole (TP, 4-day-old) *Xenopus* embryos. Northern blot analysis was carried out employing the ^{32}P-labeled BiP cDNA clone. The same RNA blot was stripped of labeled BiP probe and reprobed with *Xenopus* hsp70 probe (middle panel) and *Xenopus* L8 probe (lower panel).

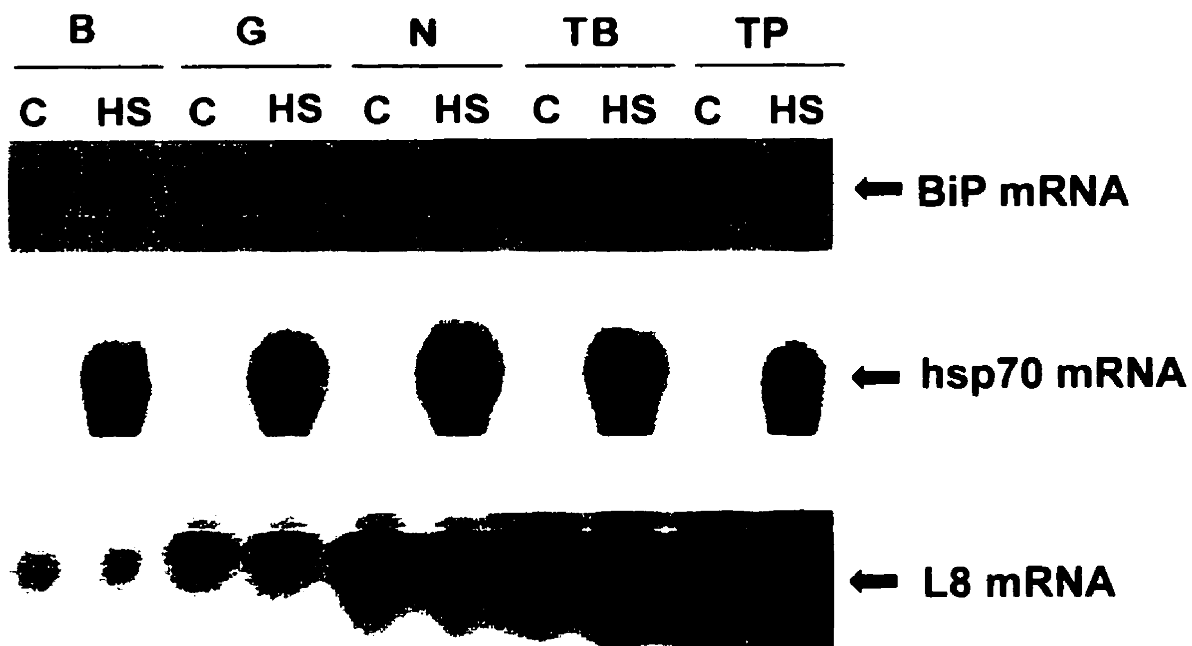

B
G
N
TB
TP
C
HS
C
HS
C
HS
C
HS
C
HS
BiP mRNA
hsp70 mRNA
L8 mRNA

Figure 26. Effect of heat shock on the spatial pattern of BiP mRNA accumulation in blastula and gastrula embryos. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at early blastula (BLAST) and gastrula (GAST) stages. AP, animal pole; VP, vegetal pole.

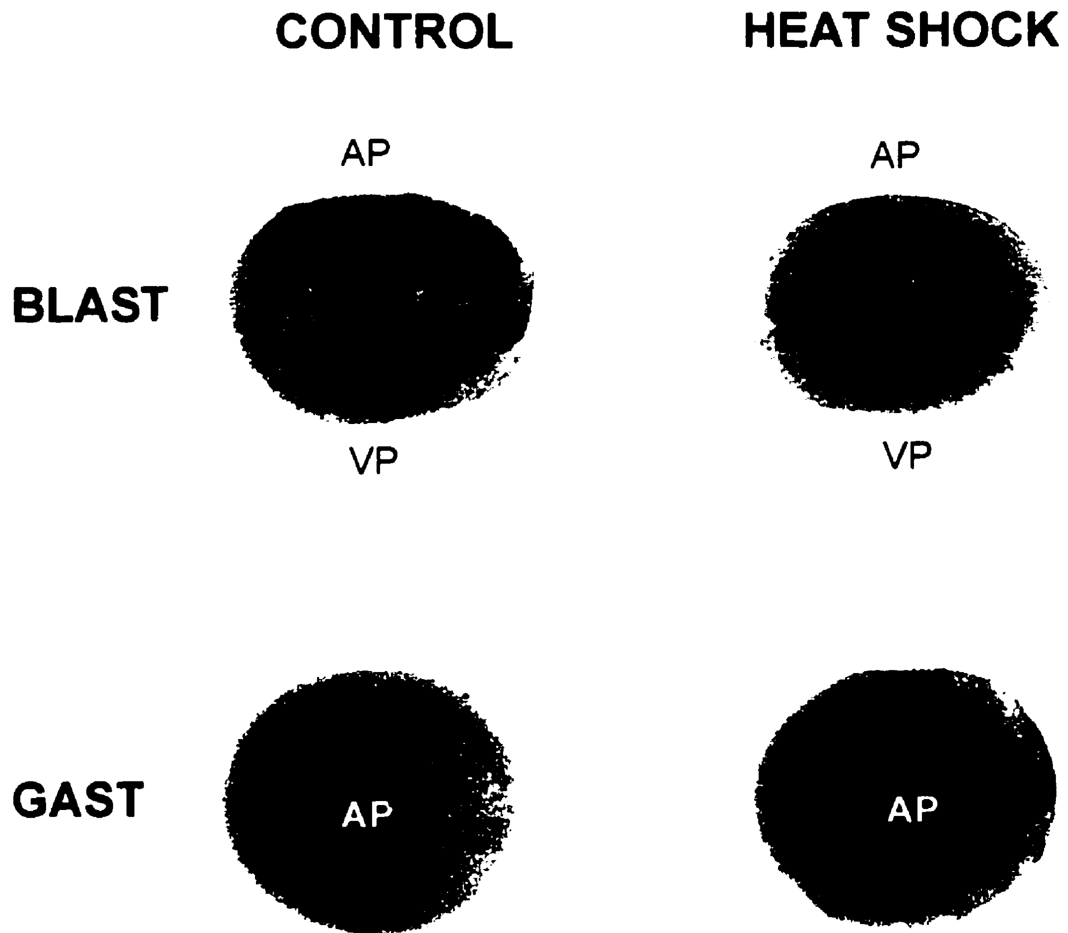
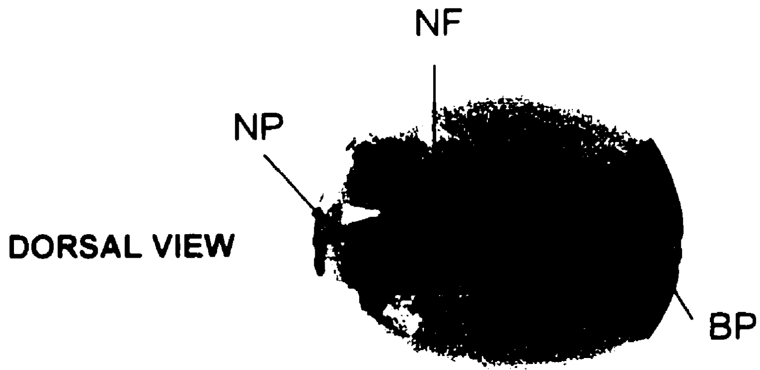
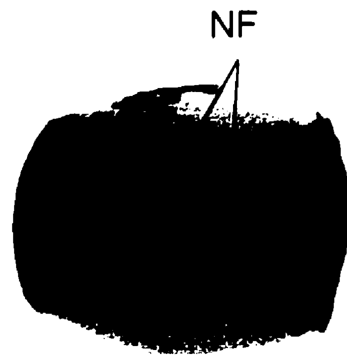

CONTROL
HEAT SHOCK
BLAST
GAST
AP
VP
AP
VP
AP
AP

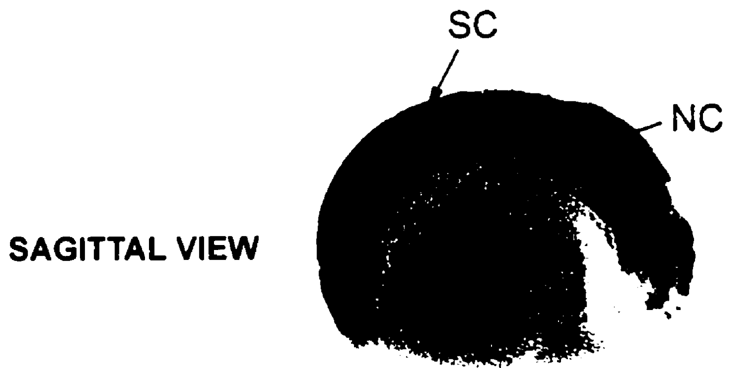
Figure 27. Effect of heat shock on the spatial pattern of BiP mRNA accumulation in neurula embryos. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* neurulae. NF, neural fold; NP, neural plate; BP, blastopore

NEURULA

CONTROL | **HEAT SHOCK**







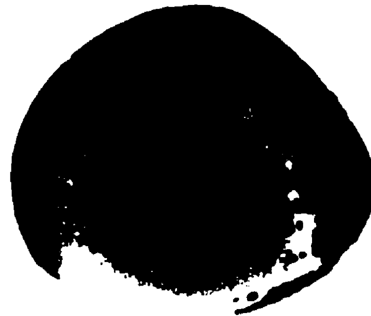
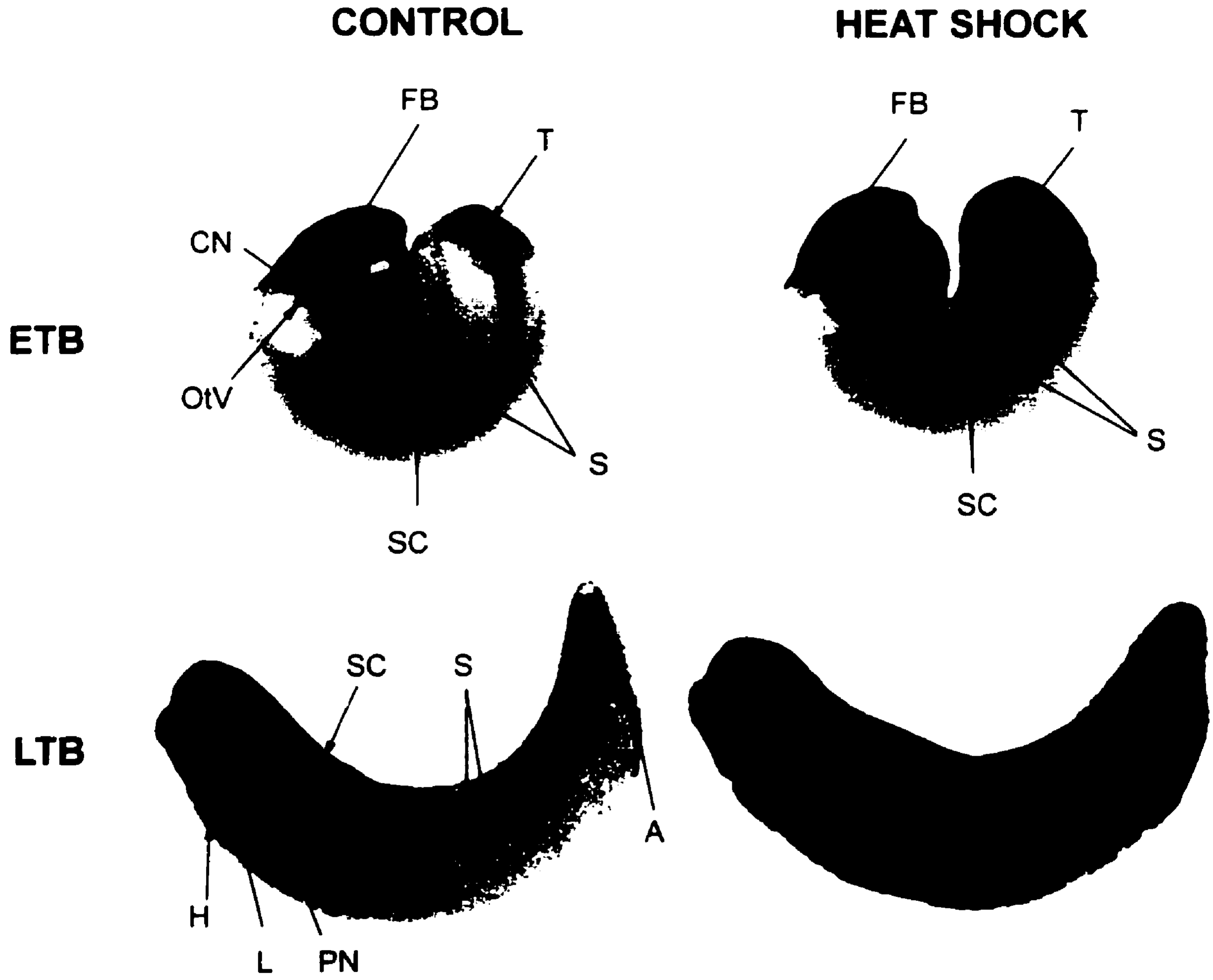


Figure 28. Effect of heat shock on the spatial pattern of BiP mRNA accumulation in early and late tailbud embryos. Whole mount *in situ* hybridization with DIG-labeled BiP antisense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at early (ETB, stage 22-23) and late (LTB, stage 32-33) tailbud stages. FB, forebrain; SC, spinal cord; A, anus; S, somites; L, liver diverticulum; H, heart; CN, cranial nerve; OtV, otic vesicle.


CONTROL
HEAT SHOCK
ETB
LTB
FB
T
CN
OtV
S
SC
FB
T
S
SC
SC
S
A
H
L
PN

induced accumulation of BiP mRNA increased dramatically in a global fashion throughout the embryo.

In these studies we have also examined the spatial pattern of L8 mRNA accumulation during development. Whole mount *in situ* hybridization with DIG-labeled L8 antisense RNA showed that L8 mRNA was constitutively present in a global fashion at all stages of *Xenopus* development (Fig. 29). Furthermore, *in situ* hybridization did not reveal any difference between control and heat shocked embryos.

3.4. Spatial pattern of hsc70 and hsp70 mRNA accumulation at selected stages of *Xenopus* development

3.4.1. Effect of heat shock on the spatial pattern of hsc70 mRNA accumulation in *Xenopus* embryos

Since the BiP gene is a member of hsp70 family we were interested in comparing its expression with the expression of cytosolic hsc70 and hsp70. Previous results from our laboratory (Ali, unpublished data) employing Northern blot analysis revealed that hsc70 message was present in all of the developmental stages studied, but its relative levels were not affected by heat shock. *In situ* hybridization experiments employing DIG-labeled hsc70 antisense RNA probe demonstrated that hsc70 mRNA was constitutively present during development in a global fashion (Fig. 30). These experiments confirmed our previous results such that the elevation of the incubation

Figure 29. Effect of heat shock on the spatial pattern of L8 mRNA accumulation in*Xenopus* embryos. Whole mount *in situ* hybridization with DIG-labeled L8 antisense and sense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at gastrula (GAST), neurula (NEUR) and early tailbud (ETB) stages.

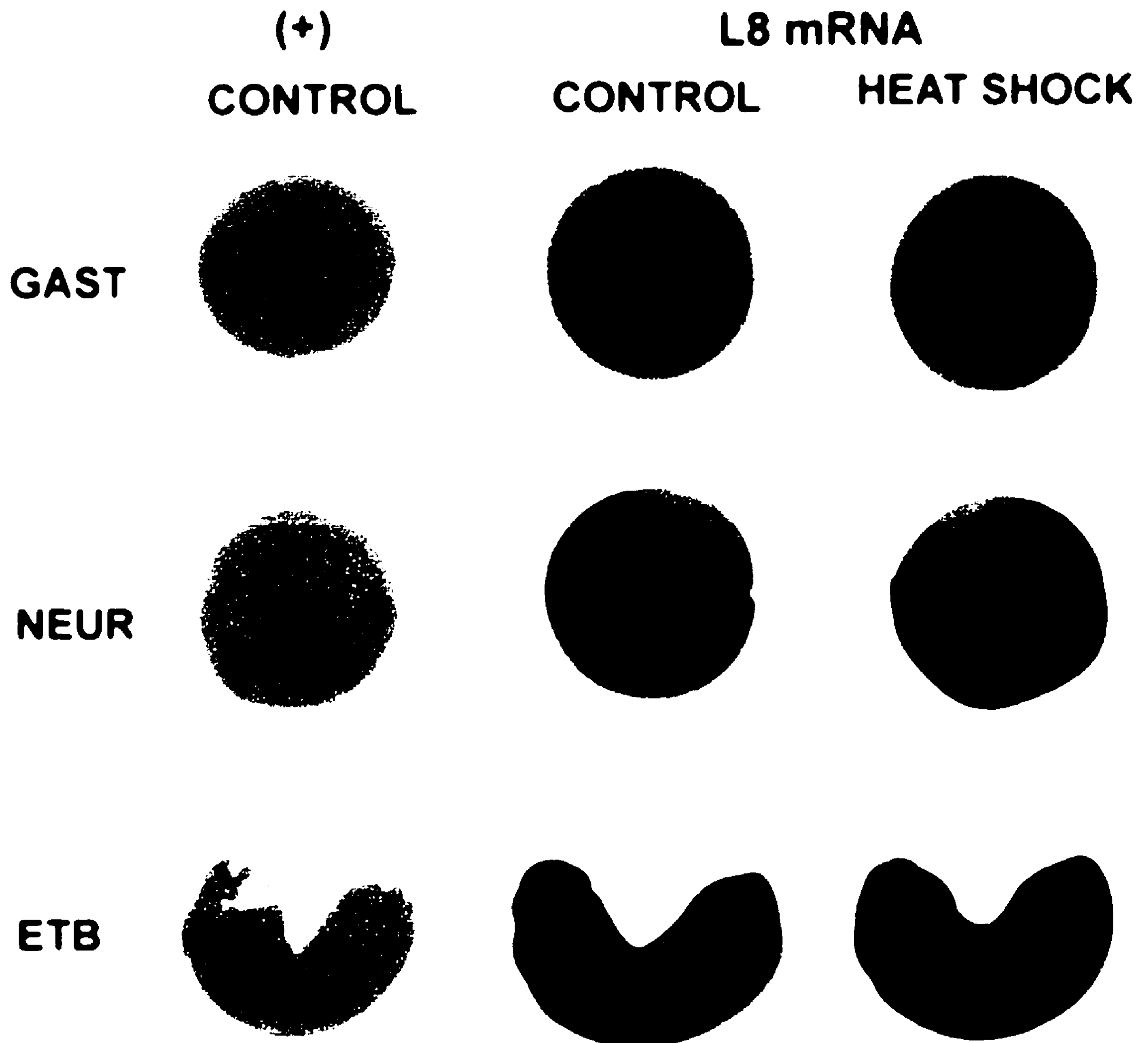
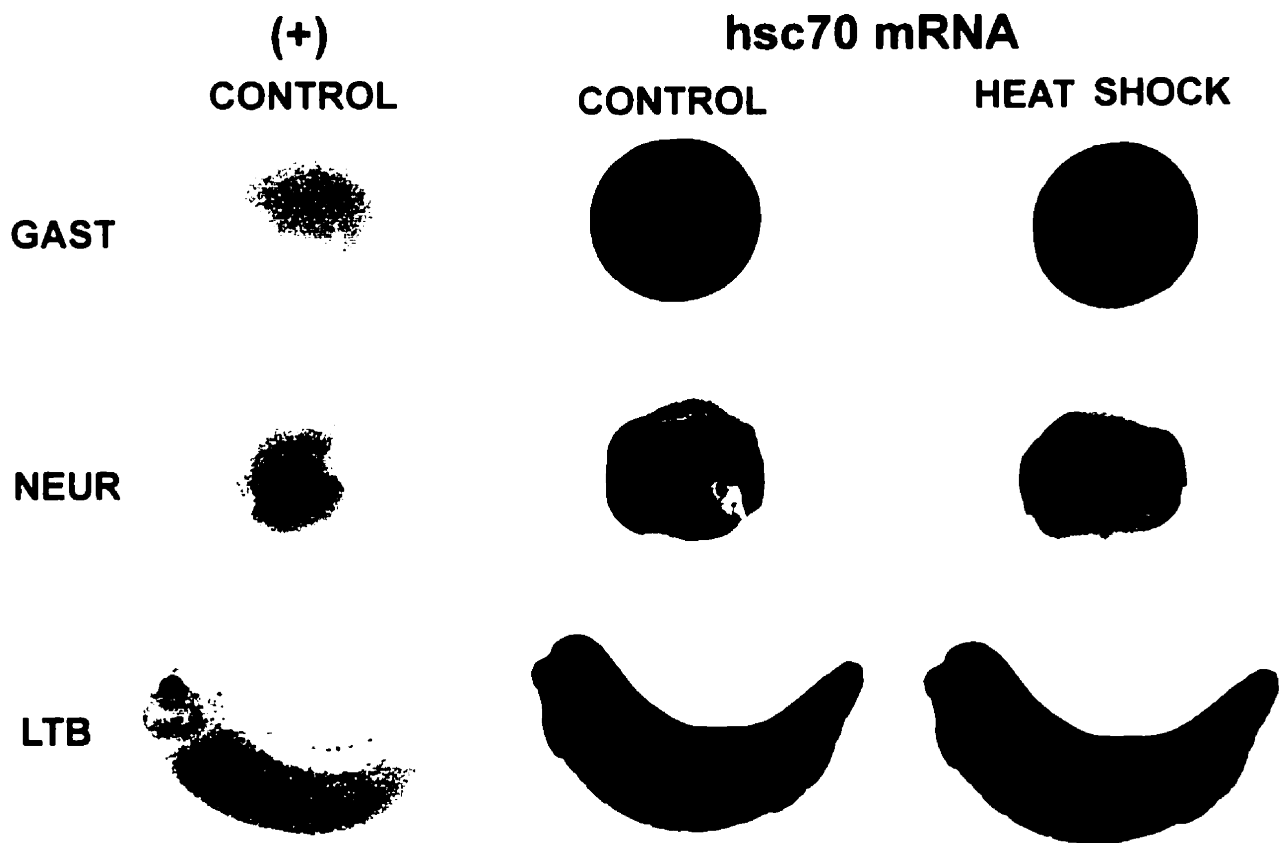

(+)
L8 mRNA
CONTROL
CONTROL
HEAT SHOCK
GAST
NEUR
ETB

Figure 30. Effect of heat shock on the spatial pattern of hsc70 mRNA accumulation in *Xenopus* embryos. Whole mount *in situ* hybridization with DIG-labeled hsc70 antisense and sense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at gastrula (GAST), neurula (NEUR) and late tailbud (LTB) stages.


(+)
CONTROL
hsc70 mRNA
CONTROL
HEAT SHOCK
GAST
NEUR
LTB

temperature to 33°C for 1 h did not appear to increase the relative level of hsc70 mRNA or produce a difference in its spatial distribution.

3.4.2. Effect of heat shock on the spatial pattern of hsp70 mRNA accumulation in *Xenopus* embryos

In previous studies it has been reported that heat shock induced accumulation of hsp70 mRNA first occurs after the midblastula stage of *Xenopus* development (Heikkila et al, 1985). *In situ* hybridization analysis of *Xenopus* embryos with DIG-labeled hsp70 antisense RNA probe confirmed that hsp70 mRNA was not constitutively present but was heat shock-inducible at blastula, gastrula (Fig. 31), neurula and tailbud stages (Fig. 32). Interestingly, heat shock-induced accumulation of hsp70 mRNA in early tailbud embryos was enriched in the head and tailbud regions as described in more detail below. Given the strong induction of hsp70 gene expression at 33°C we decided to examine the effect of a range of temperatures from 22°C to 35°C on hsp70 mRNA accumulation in early tailbud stage embryos (Fig. 33a). While exposure of embryos to 26°C for 1 h did not induce the accumulation of hsp70 mRNA, placement of embryos at 30°C resulted in the accumulation of hsp70 mRNA in the olfactory placode, lens vesicle, optic cup, cranial nerves, otic vesicle, spinal cord and in the pearl-like somatic region along the spinal cord (Fig. 33b). A similar but more intense pattern was observed in early tailbud embryos exposed to 33°C for 1 h (Fig. 33a). Additionally, hsp70 mRNA was

Figure 31. Spatial pattern of hsp70 mRNA accumulation in blastula and gastrula embryos after heat shock. Whole mount *in situ* hybridization with DIG-labeled hsp70 antisense and sense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at late blastula (BLAST) and gastrula (GAST) stages.

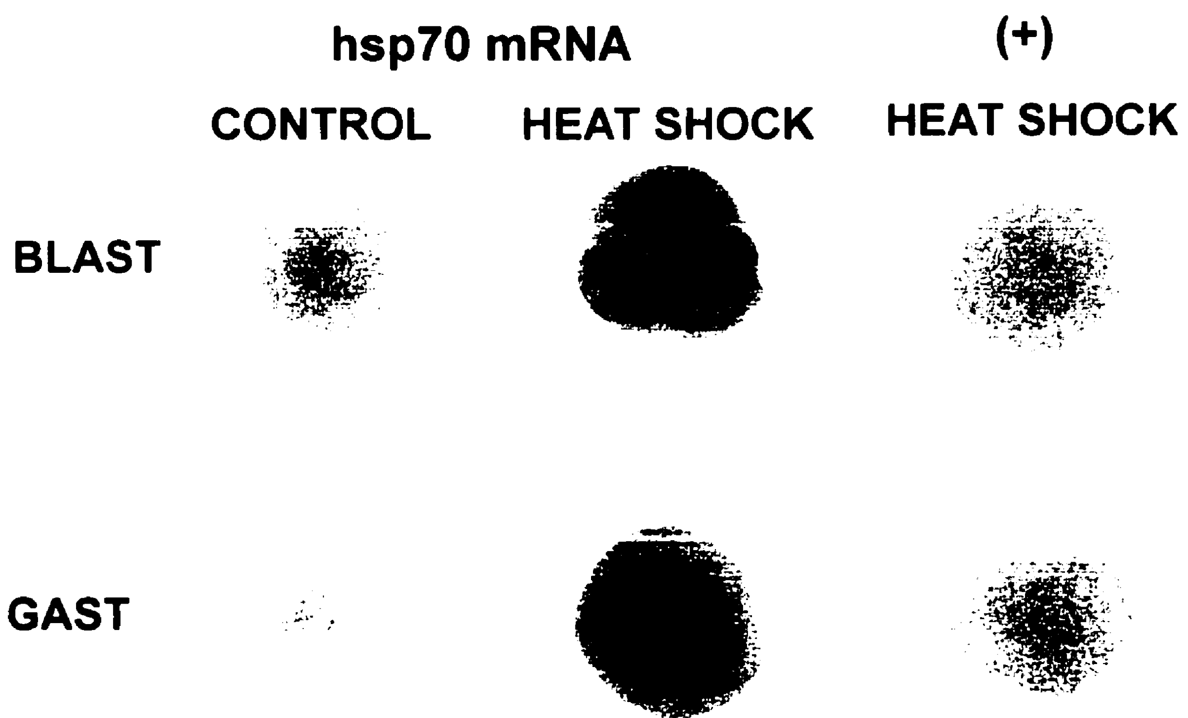

hsp70 mRNA
(+)
CONTROL
HEAT SHOCK
HEAT SHOCK
BLAST
GAST

Figure 32. Spatial pattern of hsp70 mRNA accumulation in neurula, early and late tailbud embryos after heat shock. Whole mount *in situ* hybridization with DIG-labeled hsp70 antisense and sense RNA probe was carried out with control and heat-shocked (1 h at 33°C) *Xenopus* embryos at neurula (NEUR; transverse view from the anterior side of the embryo), early (ETB) and late tailbud (LTB) stages.

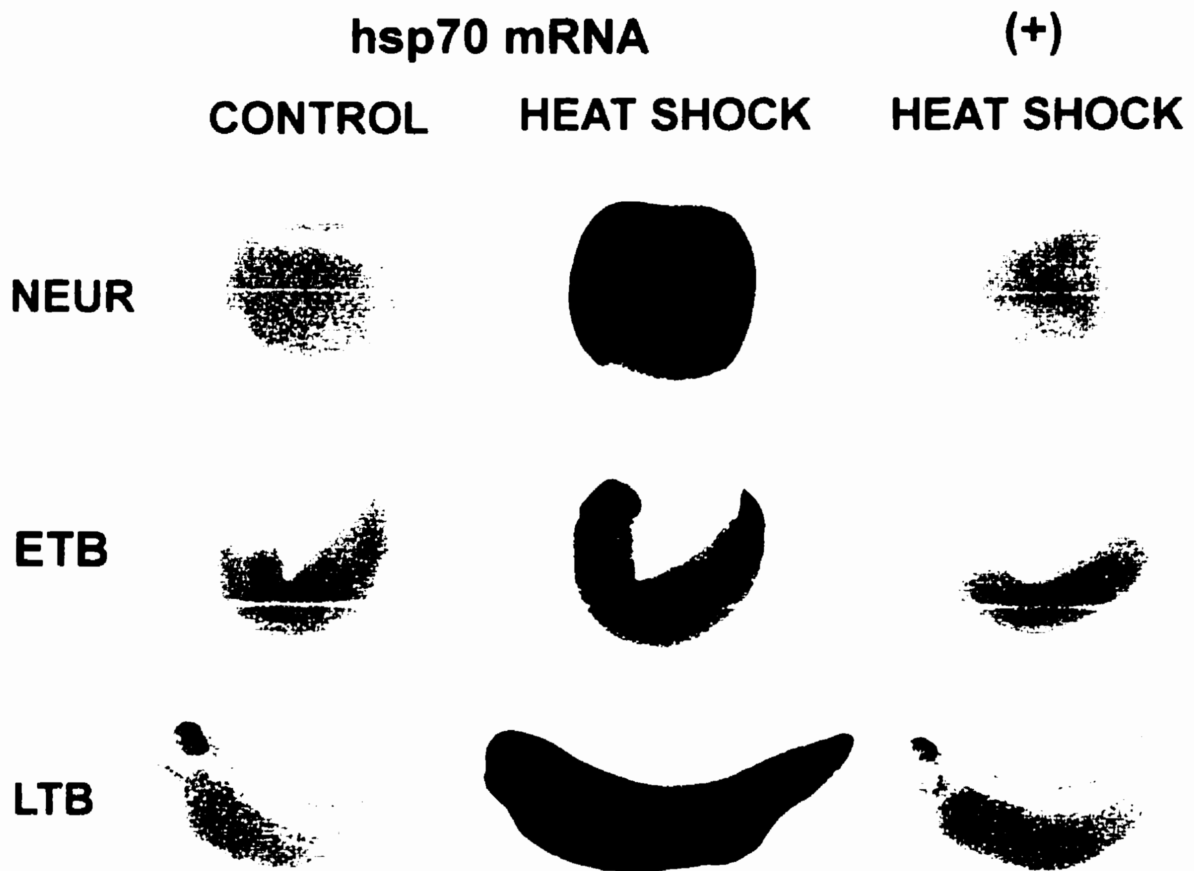

hsp70 mRNA
(+)
CONTROL
HEAT SHOCK
HEAT SHOCK
NEUR
ETB
LTB

Figure 33a. Spatial pattern of hsp70 mRNA accumulation in early tailbud stage *Xenopus* embryos exposed to different temperatures. Whole mount *in situ* hybridization with DIG-labeled hsp70 antisense RNA probe was carried out with *Xenopus* early tailbud (stage 22-23) embryos exposed to different heat-shock temperatures, ranging from 22°C to 35°C, for 1 h.

22°C

26°C

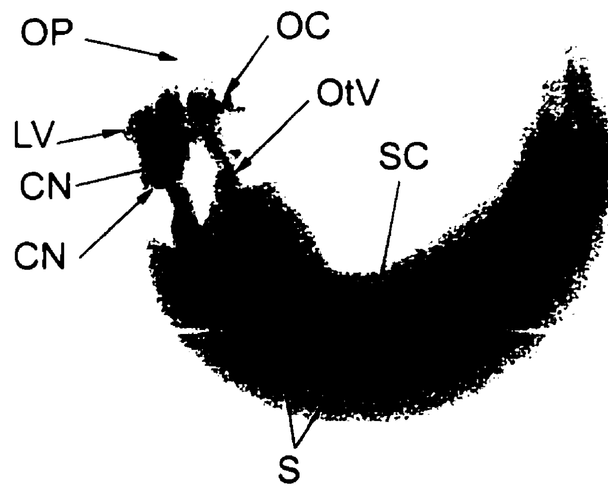
30°C

33°C

35°C

Figure 33b. Spatial pattern of hsp70 mRNA accumulation in early tailbud stage *Xenopus* embryo exposed to 30°C for 1 h. OP, olfactory placode; OC, optic cup; LV, lens vesicle; OtV, otic vesicle; CN, cranial nerve; SC, spinal cord; S, somites; L, liver.

ETB, 30°C



abundant in the head, somites and growing tail. At 35°C the pattern of hsp70 mRNA distribution was global and more intense than found at 33°C.

Figure 34a displays sagittal views of late tailbud stage embryos which were exposed to different heat shock temperatures for 1 h and then hybridized with DIG labeled hsp70 antisense RNA. Hsp70 mRNA accumulation was first induced at 26°C in the somitic region of the growing tail. At 30°C hsp70 mRNA was more pronounced in the somitic region and was also present in the forebrain, cranial nerves, otic vesicle, heart and liver (Fig. 34b), but absent from the cement gland. Hsp70 mRNA accumulation in the late tailbud stage embryos exposed to 33 and 35°C was strongly induced and displayed a global pattern of distribution. Once again it should be mentioned that eye tissue and melanocytes undergo a normal increase in pigmentation at this stage of *Xenopus* development.

For comparison we have also carried out *In situ* hybridization analysis of *Xenopus* early and late tailbud stage embryos exposed to 21°C, 30°C, and 35°C with a DIG-labeled cytoskeletal actin antisense RNA probe. As shown in Figure 35, actin mRNA was constitutively present in a global fashion throughout the embryo except in the cement gland of the late tailbud embryos. Furthermore actin mRNA levels were not enhanced by heat shock.

Figure 34a. Spatial pattern of hsp70 mRNA accumulation in late tailbud stage *Xenopus* embryos exposed to different temperatures. Whole mount *in situ* hybridization with DIG-labeled hsp70 antisense RNA probe was carried out with *Xenopus* late tailbud (stage 32-33) embryos exposed to different heat-shock temperatures, ranging from 22°C to 35°C, for 1 h.

22°C

26 °C

30°C

33°C

35°C

Figure 34b. Spatial pattern of hsp70 mRNA accumulation in late tailbud stage *Xenopus* embryo exposed to 30°C for 1 h. FB, forebrain; CG, cement gland; CN, cranial nerve; OtV, otic vesicle; H, heart; S, somites; MC, melanocytes.

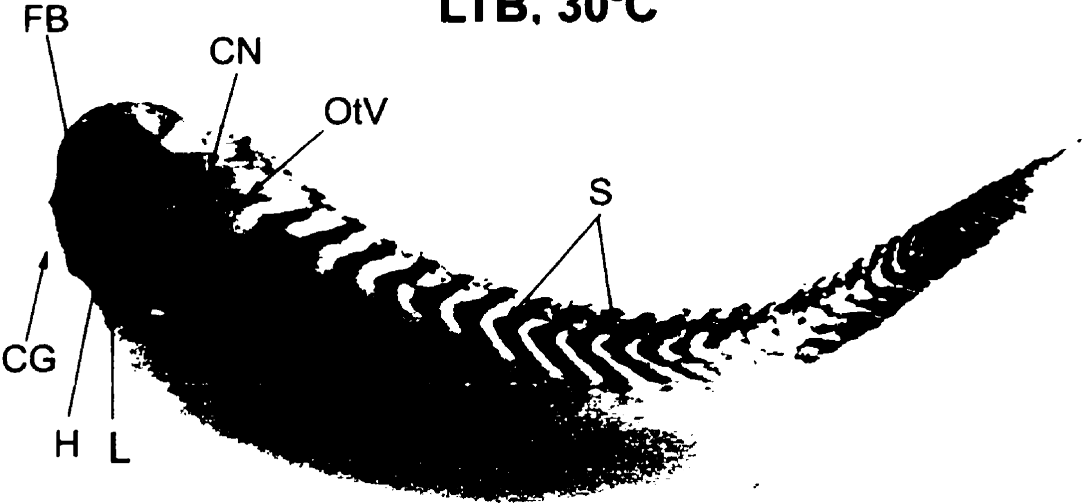
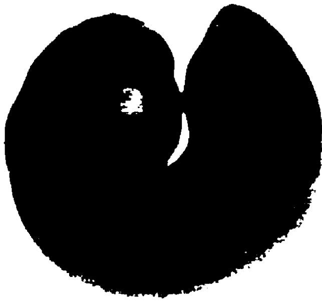
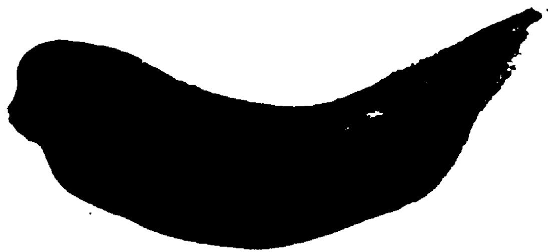

LTB, 30°C
FB
CN
OtV
S
CG
H
L

Figure 35. Spatial pattern of actin mRNA accumulation in early and late tailbud stage *Xenopus* embryos exposed to different temperatures. Whole mount *in situ* hybridization with either DIG-labeled actin sense or antisense RNA probe was carried out with early tailbud (ETB; stage 22-23) and late tailbud (LTB; stage 32-33) *Xenopus* embryos exposed to different temperatures, ranging from 22°C to 35°C, for 1 h.

Actin mRNA

	ETB	LTB
22°C		
30°C		
35°C		

(+)

33°C		

4. DISCUSSION

In the present study we have sequenced and characterized a full length cDNA for the *Xenopus laevis* immunoglobulin binding protein, BiP. Examination of the *Xenopus* BiP cDNA coding region revealed that there were two possible translational initiation sites separated by 9 nucleotides. According to Kozak (1991a; 1991b), the optimal consensus sequence for initiation of translation is GCCA/GCCAUGG. After analysis of the two AUG sequences we concluded that the second AUG from the 5' end represented the main translation initiation site because of its greater identity with the Kozak consensus sequence. The *Xenopus* cDNA nucleotide sequence contained an open reading frame that encoded a 70,192 Da protein which has an identity of approximately 90% with chicken, rat and human BiP. The conservation of the amino acid sequence was maintained along the length of the protein except for the amino and carboxyl terminal regions. Recently, another cDNA for *Xenopus* BiP has been isolated (Beggah *et al.*, 1996). The open reading frame of this cDNA encoded a *Xenopus* BiP protein having 97.6% amino acid sequence identity with the predicted amino acid sequence of the BiP protein coded by the cDNA which we have sequenced. Most of the differences with respect to amino acid sequence (10/16) occur between amino acids 572 and 605. This finding suggests the presence of at least 2 BiP genes in *Xenopus.* Previous results from our group employing 2D-PAGE determined that BiP exists as 3 isoforms

(Winning *et al.*, 1989; Winning *et al.*, 1991). One polypeptide was detected in embryos and in adult cells (A6 and white blood cells), the second polypeptide was detected only in embryos while the third was found only in the adult cells. These results suggested a developmental switch from an embryonic to an adult pattern of BiP gene expression. One explanation for these findings could be the presence of the second *Xenopus* BiP gene. Developmental gene switching has been reported previously in *Xenopus* for the keratin (Miyatani *et al.*, 1986), globin (Hentschel, 1979) and myosin heavy chain (Radice and Malicinski 1989) gene families.

Interestingly, in the present study, we have found that the identity between *Xenopus* BiP and *Xenopus* hsp70 and hsc70.I was only 57% and 55.2%, respectively. Thus, *Xenopus* BiP had greater identity with BiP from other vertebrates than with the cytosolic members of the *Xenopus* hsp70 family. Other studies have also shown that BiP from different species have a higher identity than other members of the hsp70 family from the same organism (Haas, 1994). For example, hamster and rat BiP share 98% sequence identity while hamster BiP and hsp70 have only 62% sequence identity. It has been suggested that BiP genes share a common ancestor with other hsp70 genes, and that they diverged from each other at the time of the first appearance of eukaryotes (Nicholson *et al.*, 1990).

The N-terminal regions of *Xenopus* and chicken BiP are quite different having only 43% identity in the first 30 amino acids. However, the N-terminal amino acid sequence of *Xenopus* BiP is still typical for a secretory leader

sequence having a positively charged second amino acid (lysine) and hydrophobic amino acids thereafter, as well as nonpolar amino acids at positions 12 and 14 (Stoeckle *et al.*, 1988; Munro and Pelham, 1986). In comparison with chicken and hamster BiP, it is likely that cleavage of the signal peptide of *Xenopus* BiP occurs after the valine in position 14 (Stoeckle *et al.*, 1988). All members of the hsp70 family have ATPase activity and bind ATP in the N-terminal portion of the protein, following the leader sequence. The amino acid sequence identities between the ATP-binding domains of *Xenopus* BiP, hsc70 and hsp70 as well as with mammalian and avian BiP are quite high suggesting that *Xenopus* BiP has ATPase activity. Gaut and Hendershot (1993) have shown that 3 specific amino acids in the ATP-binding domain of hamster BiP were involved in the ATP-release of proteins. An examination of our amino acid sequence data revealed the corresponding residues namely, Thr-37, Glu-199 and Thr-227 in *Xenopus* BiP. *Xenopus*, chicken, rat, and human BiP share the carboxyl terminal sequence, KDEL, which has been reported in other soluble resident ER proteins such as glucose-regulated protein 94 and protein disulfide isomerase (Munro and Pelham, 1986; Haas, 1994). It has been suggested that ER proteins containing this signal are retrieved from post-ER compartments via interaction with a specific receptor (Haas, 1994).

As mentioned above the carboxyl portion of *Xenopus* BiP is significantly different from chicken BiP. For example, in a comparison of *Xenopus* and chicken BiP, the identity in the carboxyl end (amino acid 603-655) was 73%.

The difference between *Xenopus* BiP and hsp70 and hsc70.I was even greater in this region. The C-terminal portion of the hsp70 family of proteins appears to be involved in peptide binding activity (Gething, 1994; Haas, 1994). Thus, the differences between BiP and hsp70 are expected, because the two *Xenopus* hsp70 family members are localized in different subcellular compartments and probably interact with different sets of unfolded or malfolded proteins.

In the 3' UTR of BiP mRNA, we have detected the polyadenylation consensus sequence, AAUAAA, and the adenylation control element (ACE), UUUUUAU. All mRNAs in *Xenopus* immature oocytes require the presence of a poly(A) tail to be translated (Varnum *et al.,* 1992; Simon and Richter, 1994). After oocyte maturation, the germinal vesicle breaks down and releases deadenylation factors which deadenylate all mRNAs without the ACE sequence, while those mRNAs possessing the ACE sequence are polyadenylated with a cytoplasmic poly(A) polymerase (Varnum *et al.,* 1992; Simon and Richter, 1994). Thus, it is likely that *Xenopus* BiP mRNA is efficiently translated during oogenesis as well as after oocyte maturation. Finally, a mRNA instability element, UAUUUA, was also found in the 3' UTR of BiP mRNA. This sequence element has been shown to be involved in mRNA instability in interferon, c-fos and c-myc mRNAs (Brawerman, 1987; Shaw and Kamen, 1986; Vriz and Mechali, 1989). This sequence is also present in *Xenopus* hsp30 mRNAs which are unstable at control temperatures (Krone and Heikkila, 1988; Krone *et al*, 1989, Ohan and Heikkila, 1995).

An examination of BiP mRNA levels in *Xenopus* A6 kidney epithelial cells revealed that this message was present constitutively and that its levels were enhanced by a variety of agents or treatments that have been shown to induce BiP gene expression in mammalian cells (Gething *et al.*, 1994). For example, exposure of A6 cells to galactose-free media, tunicamycin, 2-deoxyglucose, 2-deoxygalactose, glucosamine, dithiothreitol and the calcium ionophore A23187 for 24 h resulted in an increase in the relative level of BiP mRNA. The treatments employed in the present study, which enhanced the relative level of BiP mRNA, may ultimately lead to an increase in the level of unfolded or denatured protein (Haas, 1994). It has been suggested that prolonged association of unfolded protein with BiP results in a drop in the level of active BiP which then initiates a feed-back regulatory signal ultimately activating BiP gene transcription (Haas, 1994). The pathway leading from the ER's unfolded protein response to BiP gene expression is still unclear in vertebrates. In yeast accumulation of unfolded proteins in the ER is sensed in the lumen and transferred across the ER membrane to the nucleus (Mori *et al.*, 1992; Cox and Walter, 1996; Kawahara *et al.*, 1997; Mori *et al.*, 1998). There it induces the synthesis of the basic leucine-zipper transcription factor, hac1p, which binds specifically to the UPRE in the promoter of BiP and possibly other genes leading to their increased expression. This is a long process involving *de novo* protein synthesis (Kim *et al.*, 1987) and may explain why only sustained treatment with various inducers for 4-24 h increased the relative level of BiP mRNA in A6 cells. However, exposure of A6 cells to 33°C for only 1 h significantly

enhanced BiP mRNA accumulation. A possible explanations for this result in response to heat shock will be discuss later in the section dealing with the heat shock induction of BiP mRNA accumulation during *Xenopus* development.

Also we have examined the effect of homocysteine on BiP mRNA accumulation. Under conditions explained in Material and Methods we were not able to detect any change in BiP mRNA accumulation in *Xenopus* kidney epithelial cells after treatment with 5 mM homocysteine for 24 h. This is in contrast to the findings of Kokame *et al.* (1996) who reported up-regulation of BiP gene expression in human vascular endothelial cells after treatment with 3-10 mM homocysteine for 4 h. The lack of enhanced BiP mRNA accumulation following homocysteine treatment suggests tissue- or species- specific differences with respect to its action. This contention is supported by Outinen *et al.* (1998) who reported an increase in the accumulation of BiP mRNA in human umbilical vein endothelial cells after treatment with 0.1 - 5 mM homocysteine for 18h but the same treatment did not change the amount of BiP mRNA in human megakaryocytic Dami cell line (Sood *et al.*, 1996).

In the present study we have examined the relative levels of BiP mRNA in selected tissues of adult *Xenopus*. While constitutive levels of BiP mRNA were detected in all tissues examined, the relative levels differed dramatically between certain tissues. For example, liver displayed a very high level of BiP mRNA while muscle tissue had relatively low levels. These findings suggest that the expression level of the BiP gene is adapted to the cellular requirements of a particular tissue. It is possible that liver cells contain a relatively high level

of protein destined for the ER which requires proper folding and assembly by BiP. This idea is feasible given the number of proteins continuously secreted by the liver such as albumin, transferrin, and lipoproteins (Lodish *et al.*, 1995).

Although there is a lot of information available about the activation of BiP gene expression during different stress conditions in cultured cells, little is known about BiP gene expression during development. In our study we have examined BiP mRNA accumulation and its spatial distribution during *Xenopus* development. Northern blot analysis revealed that BiP mRNA was present at constant levels from unfertilized egg to gastrula. *In situ* hybridization studies determined that BiP mRNA was enriched in the animal pole region of the unfertilized egg, a pattern which was retained after fertilization in cleavage and blastula stage embryos. At the gastrula stage, BiP mRNA was present throughout the embryo with relatively less accumulation in the yolk plug region. The finding of constitutive levels of BiP mRNA in unfertilized eggs, cleavage and early blastula stages embryos indicated that these messages were maternal in origin since the zygotic genome transcription is not activated until after the midblastula stage of development (Kimelman *et al.*, 1987). This result was not unexpected given our finding of an ACE sequence in the 3' UTR region of BiP mRNA. Previous studies have shown that *Xenopus* mRNAs which contain the ACE sequence are polyadenylated and maintained after oocyte maturation in contrast to mRNAs without the ACE sequence which are deadenylated and degraded (Varnum *et al.*, 1992). The presence of BiP mRNA in pre-MBT embryos probably reflects the need of the early embryos to produce BiP protein

which interacts with newly synthesized membrane and secretory proteins in the ER.

Our results revealed that BiP mRNA accumulation was enhanced further in neurula and tailbud embryos compared to earlier stage embryos. At the neurula stage, BiP mRNA accumulation was preferentially enriched in the neural plate, around the blastopore and along the neural folds. This pattern was maintained in early and late tailbud stage embryos where BiP mRNA was distributed primarily along the dorsal part of the embryo in the somitic region, spinal cord, cranial nerves, otic vesicle and in the forebrain. It was also present in the heart, gills, liver diverticulum, pronephros, pronephric duct and around the anus. A possible explanation for the increase in BiP mRNA in neurula and tailbud stage embryos compared to earlier stages and the preferential localization of BiP mRNA may involve the shift towards the organogenesis at neurula and tailbud stages. During organogenesis the precise arrangement of tissues is achieved by secondary induction in which certain groups of cells affect the behavior of the neighbouring set of cells, causing changes in their developmental pathway. This influence is conveyed through the secretion of various secretory and membrane proteins (Gilbert, 1994). Developmental interactions are also mediated by hormones, which travel long distances from their sites of production to the target tissues. In either case, the newly synthesized inducer proteins and/or peptide hormones must pass through the ER and become transiently bound with BiP for proper folding. This possibility is supported by the work of Dorner *et al.*, (1989), who reported an increase in yeast BiP gene expression after increased accumulation of some

secretory proteins in the ER. Recently, Chen *et al.* (1998) offered a more direct demonstration of the role of BiP during secondary induction. They reported that the mitogen-activated protein kinase ($p38^{MAPK}$) pathway is involved in the rapid enhancement of BiP gene expression in 9L rat brain tumor cells, suggesting a role of BiP gene expression during cell division. Since MAP kinases are able to phosphorylate and activate different transcription factors that enter the nucleus it is likely that these activation pathways may be one of the mechanisms implicated in a transduction of a signal from the cell surface to the nucleus during secondary induction.

In an attempt to further characterize the pattern of BiP gene expression during *Xenopus* development, we examined the effect of various BiP gene inducers on the accumulation of BiP mRNA. For example, treatment of *Xenopus* embryos with the antibiotic tunicamycin, an inhibitor of protein glycosylation, induced BiP mRNA accumulation in neurula embryos. It should be mentioned that the requirement of a 12 h treatment period precluded the analysis of cleavage and blastula stages. Previously we found that BiP mRNA was first tunicamycin inducible at the tailbud stage (Winning *et al.,* 1991). However, this latter result probably reflected the lack of sensitivity because of the heterologous rat BiP cDNA probe used in the analysis. In the present study we have also examined the effect of the Ca^{2+} ionophore A23187 on BiP mRNA accumulation in *Xenopus* embryos. Since the required treatment was only 3 h we were able to examine BiP gene inducibility beginning at the blastula stage. Our Northern blot analysis indicated that enhanced BiP mRNA accumulation

was first inducible by A23187 at the neurula stage. This result was confirmed by *in situ* hybridization. At the neurula stage, A23187 enhanced BiP mRNA accumulation throughout the embryo. However, at the tailbud stage A23187 induced BiP mRNA accumulation occurred primarily in the head region, along the spinal cord, in the somites, heart, liver, pronephros and pronephric duct. While the exact mechanism for enhanced BiP gene expression by A23187 is not known, it is thought that Ca^{2+} depletion leads to the disruption of protein folding and inhibition of protein synthesis due to the phosphorylation of the translation initiation factor eIF-2α (Morris *et al.*, 1997). As mentioned previously, the accumulation of unfolded and misfolded proteins occurs under Ca^{2+} deprivation and results in the increased expression of the BiP gene. It has been shown that BiP is responsible for maintaining ER membrane permeability as well as the preservation of the Ca^{2+} concentration gradient (Plemper *et al.*, 1997; Pilon *et al.*, 1997). Also, BiP appears to have a direct role as a Ca^{2+} storage protein (Lievremont *et al.*, 1997). Given these functions of BiP in the maintenance of the Ca^{2+} concentration gradient as well as its chaperone role during protein synthesis, it is possible that enriched BiP mRNA accumulation in these regions represents one of the first steps in the cell's protective mechanism against ER stress. In support of a protective role in the cell for BiP, it has been reported that prior overexpression of BiP in CHO cells can prevent A23187-induced general inhibition of mRNA translation (Morris *et al.*, 1997). Furthermore, Liu *et al.*, (1997) showed that pretreatment of porcine renal epithelial cells with tunicamycin or thapsigargin, which increased BiP

gene expression, induced tolerance to subsequent treatment with the alkylating toxicant, iodoacetamide. Given our finding that BiP mRNA was enriched in the pronephros and pronephric duct after treatment with A23187, it is interesting to mention that kidney proximal tubular epithelium represents an important target for chemical toxicants (Liu *et al.*, 1997).

In the present study we have also found that heat shock induced BiP mRNA accumulation begins at the gastrula stage. Also, *in situ* hybridization analysis revealed that heat shock enhanced BiP mRNA levels in the neural plate, along the neural folds, around the blastopore and in portions of the epidermis. This pattern was preserved in early tailbud stage embryos. It is interesting to note that at the late tailbud stage heat induced accumulation of BiP mRNA increased dramatically in a global manner throughout the embryo. Given these results it is possible that heat shock-induced BiP gene expression contributes along with the accumulation of other heat shock proteins in the protection of the embryo from thermal stress. The mechanism responsible for heat-shock induction of BiP mRNA accumulation is not known. This phenomenon may be regulated at the level of mRNA stability given the presence of a mRNA instability sequence at the 3' UTR. This element has been shown to be involved in mRNA instability in interferon (Brawerman, 1987), c-fos (Shaw *et al.*, 1986) and c-myc mRNAs (Vriz and Mechali, 1989) as well as in rat insulin-like growth factor binding protein (Ooi *et al.*, 1993), *Xenopus* Eg2 (Duval *et al.*, 1990) and hsp30 mRNAs (Ohan and Heikkila, 1995). In this context, heat shock-induced reduction of general protein

synthesis reduces the level of factor(s) responsible for BiP mRNA degradation which, under normal conditions are continually synthesized and bound to the instability element.

Alternatively, it is possible that heat shock treatment may cause a rapid increase in the level of unfolded proteins in the ER which could lead to the activation of BiP gene expression via the unfolded protein response. Interestingly, in contrast to heat shock treatment, exposure of gastrula stage embryos to A23187 did not enhance BiP mRNA accumulation. It is possible that the mechanism leading to an unfolded protein response by means of A23187 was not functional at this stage due to the absence of the transcription factors which are essential for this induction. Previous studies have shown that different cell types exhibit different sensitivity to A23187. Freedman *et al.,* (1981) reported that porcine lymphocytes were more sensitive to A23187 toxicity than murine lymphocytes. Furthermore, low concentrations of A23187 induced mitogenic activation of normal T lymphocytes, but it did not have any effects on tumor T- and B- cell lines. They suggested that different sensitivity to A23187 resulted from variance in membrane proteins between cell lines.

On the other hand, since the *Xenopus* BiP gene has not been isolated, we cannot rule out the possibility that the BiP promoter may contain a functional heat shock element. Previous studies have shown that heat shock can induce BiP gene expression in a variety of fungi (van Gameren *et al.,* 1997; Techel *et al.,* 1998). For some of these fungi the promoter region of the BiP gene was sequenced and a functional HSE was detected (Mori *et al.,* 1992; Stedman and

Buck., 1996). Furthermore, it was reported in *S. cerevisiae* that BiP gene expression was induced by a depletion of hsp70 through a HSE-mediated pathway (Oka *et al.*, 1997). The situation in plants appears to be more complex. BiP gene expression was not induced by heat shock in soybean and spinach (Koizumi, 1996). However, BiP gene expression was induced by heat shock in *Arabidopsis* seedlings but it is not known as yet whether a functional HSE exists in the BiP gene promoter (Koizumi, 1996). Given the presence of HSF throughout *Xenopus* development (Ovsenek and Heikkila, 1990), the possible existence of a HSE in the *Xenopus* BiP promoter might explain heat induction of BiP gene expression immediately after MBT once zygotic genome is activated. However, this possibility requires the isolation and sequence analysis of the *Xenopus* BiP gene promoter.

It should be mentioned at this point that the accumulation and spatial distribution of mRNA for the large ribosomal subunit protein, L8, was used as a control in northern blot and *in situ* hybridization analysis of BiP mRNA accumulation after different treatments. Our experiments revealed that L8 mRNA was ubiquitously present in *Xenopus* embryos at all stages of development, and that its expression was unaffected by heat shock. Previous studies in *Xenopus* embryos have also shown that L8 was constitutively present during *Xenopus* development (Shi and Liang, 1994; Stolow *et al.*, 1996). This latter study reported that maternal L8 mRNA was present in the oocytes and that embryonic L8 mRNA was activated after MBT. Furthermore, the expression of L8 mRNA did not show any tissue specificity. Our results

confirmed these latter findings in contrast to the selective spatial distribution of BiP mRNA.

Since the BiP gene is the ER member of the hsp70 family, we were interested in comparing its spatial pattern of expression with cytosolic hsc70 and hsp70 in *Xenopus* embryos. *In situ* hybridization analysis revealed that hsc70 mRNA was present constitutively at all stages of *Xenopus* development. In gastrula stage embryos, hsc70 mRNA was ubiquitously distributed but at the neurula stage it was enriched in the regions which will develop into the central neural system and somites. These findings are in good agreement with results obtained with zebrafish embryos (Santacruz *et al.*, 1997). These researchers hypothesized that the hsc70 gene might be involved in differentiation events that participate in neurogenesis and somitogenesis. Similar results were obtained by Herberts *et al.* (1993). In this latter study, constitutive hsp70-related proteins were localized in both animal and vegetal blastomeres of blastula stage embryos. At the gastrula stage, accumulation of hsp70-related proteins showed a global pattern, including endodermal yolk mass cells, blastoporal collar, cells of the involuting dorsal mesoderm and the sensorial layer of the neuroectoderm. Given the role of hsc70 as a molecular chaperone in protein assembly, it is possible that it interacts with other proteins involved in these differentiation events. In the present study we have shown that pattern of hsc70 mRNA accumulation was unaffected by heat shock. These results agree with earlier Northern blot hybridization experiments in our laboratory in which placement of gastrula and neurula embryos at 33°C for 1 h

did not enhance hsc70 mRNA accumulation (Ali *et al.*, 1996). Similar results with respect to the ineffectiveness of heat shock in increasing the relative levels of hsc70 mRNA has been reported in early stages of development of the amphibian, *Pleurodeles waltl* (Delelis-Fanien *et al.*, 1997) as well as in a chinook salmon embryo cell line, CHSE (Zafarullah *et al.*, 1992). However, O'Malley *et al.* (1985) has reported that heat shock produced an increase in relative level of hsc70 mRNA in rat PC12 and human HeLa cells. Although hsc70 mRNA accumulation was not induced by heat shock, the enhanced accumulation of hcs70 after MBT may be associated with the increase in thermoresistance of *Xenopus* embryos (Heikkila *et al.*, 1985, Ali *et al.*, 1996). In support of this possibility it has been reported that heat-induced apoptosis was blocked in cells lines in which the expression of hsp70 was either constitutively elevated or transiently induced (Mosser *et al.*, 1997). It is possible that after exposure of embryos to heat shock hsc70 interacts with denatured and/or misfolded proteins until effective levels of hsp70 are synthesized to further protect the embryo from the thermal stress.

Previous results from our laboratory have shown that heat shock induced accumulation of hsp70 mRNA occurs immediately after MBT, at the late blastula stage (Heikkila et al, 1985, 1987; Ovsenek and Heikkila, 1988; Krone and Heikkila, 1989). Our *in situ* hybridization experiments confirmed these results. A heat shock treatment of 33°C for 1 h induced hsp70 mRNA accumulation at both animal and vegetal pole in the late blastula embryo. It was present at the animal pole as well as in the yolk plug at gastrula stage. A

frontal transverse view of the neurula stage embryo showed a more dorsal distribution of hsp70 mRNA in the anterior part of the embryo after heat shock. The same pattern was preserved in the early tailbud stage embryo. The distribution of hsp70 mRNA after heat shock at the late tailbud stage resembled the distribution of BiP mRNA after heat shock. Hsp70 mRNA was distributed in a global manner throughout the embryo including the epidermis. Similar expression was observed in 2-day-old zebrafish embryos in which the epidermal epithelial cells exhibited very high levels of hsp70 mRNA throughout the embryo after 2 h exposure to 37°C (Lele *et al.*, 1997). It is likely that the cells on the surface of the embryo would be the first to sense an increase in temperature and respond to it by accumulating hsp70 mRNA.

Given the strong induction of hsp70 gene expression at 33°C we decided to examine the effect of a range of temperatures from 22°C to 35°C on hsp70 mRNA accumulation in the early and late tailbud stage embryos. While placement of early tailbud embryos at 26°C did not result in the accumulation of hsp70 mRNA, treatment of embryos at 30°C resulted in the accumulation of hsp70 mRNA in the olfactory placode, lens vesicle, optic cup, otic vesicle, cranial nerves, spinal cord and in the somitic region along the spinal cord. In late tailbud embryos hsp70 mRNA accumulation was first detected at 26°C in the posterior somitic region and in the area of hind limb muscle precursors. At 30°C hsp70 mRNA was present in central nervous system and somites as well as in the heart and liver. In contrast to the above results, placement of either early or late tailbud embryos at 33°C or 35°C yielded a global response with

respect to hsp70 mRNA accumulation. In these studies actin mRNA was detected constitutively in a global fashion throughout the embryo at both tailbud stages and was not heat-shock inducible. Therefore this study has shown a preferential induction of hsp70 gene expression in selected tissues of tailbud embryos at lower heat shock temperatures. It is possible that the more heat sensitive tissues such as the central nervous system, somitic region and heart may have a lowered temperature set point with respect to hsp70 gene induction. In support of this possibility Ali *et al.* (1997) have shown that placement of adult frogs at 26°C resulted in the preferential accumulation of hsp70 mRNA and activation of HSF in heart tissue. Different HSF activation temperature set points have been described in mouse tissue with pachytene spermatocytes displaying a 7°C lower HSF1 activation temperature then somatic testis cell types or other tissues (Sarge, 1995; Sarge *et al.*, 1995). Taken together all of these results suggest that the HSF activation temperature may not necessarily have a fixed value in a particular organism and that it can vary in a tissue-dependent manner. A number of studies have suggested that HSF activation and hsp gene transcription are triggered by an increase in the level of unfolded protein (reviewed by Morimoto *et al.*, 1994). Interestingly, Ali *et al.* (1997) was able to correlate the preferential activation of HSF and hsp70 gene expression in adult frog heart with a lowered temperature for protein denaturation relative to other tissues studied. A similar situation may exist in *Xenopus* tailbud embryos. It is likely that the heat-induced preferential expression of hsp70 in selected embryonic tissues may have a protective

function through its role as a cellular chaperone. The role of hsp70 as a cellular chaperone during heat shock in *Xenopus* embryos is probably supported by other members of its family including hsc70 and BiP.

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