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Regulation and Function of Small Heat Shock Protein Genes During Amphibian Development

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Abstract Small heat shock proteins (shsps) are molecular chaperones that are inducible by environmental stress such as elevated temperature or exposure to heavy metals or arsenate. Recent interest in shsps has been propelled by the finding that shsp synthesis or mutations are associated with various human diseases. While much is known about shsps in cultured cells, less is known about their expression and function during early animal development. In amphibian model systems, shsp genes are developmentally regulated under both normal and environmental stress conditions. For example, in *Xenopus*, the shsp gene family, hsp30, is repressed and not heat-inducible until the late neurula/early tailbud stage whereas other hsps are inducible at the onset of zygotic genome activation at the midblastula stage. Furthermore, these shsp genes are preferentially induced in selected tissues. Recent studies suggest that the developmental regulation of these shsp genes is controlled, in part, at the level of chromatin structure. Some shsps including *Xenopus* and *Rana* hsp30 are synthesized constitutively in selected tissues where they may function in the prevention of apoptosis. During environmental stress, amphibian multimeric shsps bind to denatured target protein, inhibit their aggregation and maintain them in a folding-competent state until reactivated by other cellular chaperones. Phosphorylation of shsps appears to play a major role in the regulation of their function. J. Cell. Biochem. 93: 672–680, 2004.

Key words: heat shock; development; *Xenopus*; mRNA; transcription; chaperone; chromatin; histone; acetylation; cement gland

Environmental stressors such as elevated temperature, heavy metals, and arsenate can induce the synthesis of families of heat shock proteins (hsps) including hsp90, hsp70, and small hsps (shsps) [Feige et al., 1996]. Members of the hsp families are expressed normally within the cell and are involved in protein folding, assembly, and transport. It is likely that during cellular stress these molecular chaperones bind to and prevent irreversible aggregation or misfolding of damaged or denatured protein. Therefore these molecular chaperones are essential under normal growth conditions as well as serving to protect the cell from the adverse effects of stress. Transcription of hsp genes is mediated by the heat shock element (HSE) found in the 5′ upstream regions of these genes and interacts with a transcription activating protein known as heat shock factor (HSF). HSF preexists within the cell as an inactive monomer that is converted into an active trimer upon heat shock that is capable of binding to the HSE and facilitating transcription of hsp genes. One of the triggers for HSF activation is the accumulation of unfolded protein. While the function and expression of the hsp70 and hsp90 gene families have been intensively studied relatively less is known about shsps. Recent interest in shsps has been spurred, at least in part, by the finding that shsp synthesis or mutations are associated with diseases such as multiple sclerosis, various neuropathologies, and muscle myopathy [MacRae, 2000; Van Montfort et al., 2002; Irobi et al., 2004].

Shsps range in size from 12 to 43 kDa and include α-crystallin since this lens protein is stress-inducible and possesses many of the same physical and functional properties found with other shsps [MacRae, 2000; Van Montfort et al., 2002]. In contrast to hsp70 and hsp90, shsps exhibit a low degree of conservation with

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the exception of an 80–100 amino acid domain that is also found in α-crystallins. Shsps are capable of forming large, highly polymeric structures within the cell and may be necessary for proper functioning. The various in vivo functions suggested for shsps include actin capping/decapping activity, cellular differentiation, chaperone activity, and modulation of redox parameters. Cells in which shsps are overexpressed can acquire thermotolerance. In these situations it is likely that shsps protect cellular proteins by preventing their aggregation or misfolding and maintaining their solubility.

Developmental regulation of heat shock-induced hsp gene expression has been documented in a number of plant and animal systems. Our laboratory and others have been involved in an examination of the regulation and function of shsps during early amphibian development [Bienz, 1984; Helbing et al., 1996; Heikkila et al., 1997]. Since amphibian embryogenesis takes place in an aquatic environment, these organisms are particularly susceptible to environmental stressors. The predominant amphibian model embryonic system is the South African Clawed frog, *Xenopus laevis*. These frogs produce large quantities of eggs that can be fertilized in vitro and are amenable to microinjection studies [Heikkila et al., 1997]. Also a vast amount of information has accumulated regarding *Xenopus* early embryonic development at both the cellular and molecular level. Early frog embryogenesis is characterized by a relatively rapid series of cleavages during which the zygotic genome is essentially transcriptionally quiescent. During this phase of development, new protein synthesis utilizes pre-exisiting maternal mRNA. At the midblastula stage or transition (MBT) the zygotic genome is activated with the transcription of selected genes. In response to a series of inductive events the embryos then develop through gastrula, neurula, tailbud, and tadpole stages.

The following review will examine the developmental regulation of shsp gene expression in amphibians as well as outline evidence for their regulation at the level of chromatin structure. Also the function of these genes as molecular chaperones in response to environmental stress will be examined. Finally, it is hypothesized that constitutive expression of some of these shsps may function normally during amphibian development in certain tissues to prevent apoptosis.

AMPHIBIAN SHSP FAMILIES

Mammalian cells synthesize six distinct small hsps including hsp27, hsp20, αA-crystallin, αB-crystallin, hspB2, and hspB3 [MacRae, 2000]. In amphibians three families of shsps have been described so far as shown in Table I. The most intensively studied amphibian shsps are members of the hsp30 family, which has been found in *Xenopus laevis* and *Rana catesbeiana*. A total of five *Xenopus* hsp30 genes have been isolated (hsp30A to E). Hsp30A contains an insertional mutation with a stop codon in the coding region while hsp30B is a pseudogene [Bienz, 1984]. Only partial sequence information is available for hsp30E [Krone et al., 1992]. The hsp30C and hsp30D genes are intronless, share a high degree of similarity, and their 24 kDa proteins contain a conserved α-crystallin domain in the C-terminal region found in other eukaryotic shsps [Krone et al., 1992]. The 5′ regulatory region of the hsp30C and D gene contains HSEs that are responsible for their stress inducibility. The only other amphibian hsp30 gene isolated was from the bullfrog, *Rana catesbeiana* [Helbing et al., 1996]. *Rana* hsp30 shared 76% amino acid identity with *Xenopus laevis* when conservative amino acid changes were taken into account. The *Xenopus* and *Rana*

TABLE I. Selected Amphibian Small Heat Shock Proteins

Amphibian	Shsp	Reference
Cynops pyrrhogaster	αA-crystallin	Mizuno et al., 2002
Rana berlandieri	αA- and αB-crystallin	Deretic et al., 1994
Rana catesbeiana	hsp30	Helbing et al., 1996
	αA- and αB-crystallin	Lu et al., 1995
Rana temporaria	αA- and αB-crystallin	Brahma et al., 1987
Xenopus laevis	αA-crystallin	Brunekreef et al., 1997
	hsp30A	Bienz, 1984
	hsp30C, hsp30D	Krone et al., 1992
	Bshsps	Ohan et al., 1998

hsp30 genes were thought to be unique until comparable shsp genes were detected in desert topminnow and more recently in quail [Norris et al., 1997; Katoh et al., 2004]. The second family of *Xenopus* shsps is the basic small heat shock proteins (Bshsps) [Ohan et al., 1998]. Unfortunately, little is known about these proteins with the exception that their stress-inducible expression is regulated at the transcriptional level and that they are distinct from the more acidic hsp30 family based on charge and immunoreactivity. It is tempting to speculate that these shsps may be the counterpart of the mammalian hsp27 family given the studies with desert topminnow. Amphibian α-crystallin gene expression and function has been documented in embryonic lens tissue [Table I]. Their expression appears to involve the transcription factor L-Maf [Reza and Yasuda, 2004]. However, mammalian αA- and αB-crystallins are stress-inducible and can function as molecular chaperones in non-lenticular tissue [MacRae, 2000; Van Montfort et al., 2002]. Unfortunately similar studies have not been carried out in amphibian systems.

HSP30 GENE EXPRESSION IN EMBRYOS

Heat shock induced expression of hsp70 and hsp90 genes during *Xenopus* development coincides with the activation of the zygotic genome at MBT [Heikkila et al., 1997]. Interestingly, hsp30 genes are not stress inducible until later in development. For example, hsp30A and hsp30C genes were first heat-inducible at the late neurula/early tailbud stage while hsp30D was not expressed until the midtailbud stage, 1 day later in development. Additionally hsp30 mRNA and protein preferentially accumulated in selected tissues of heat shocked midtailbud embryos including the cement gland (CG), lens placode (LP), somites, and proctodeum (Fig. 1A–D) [Lang et al., 1999]. In contrast, actin mRNA displayed a more generalized pattern of accumulation in embryos that was not altered with heat shock. These experiments

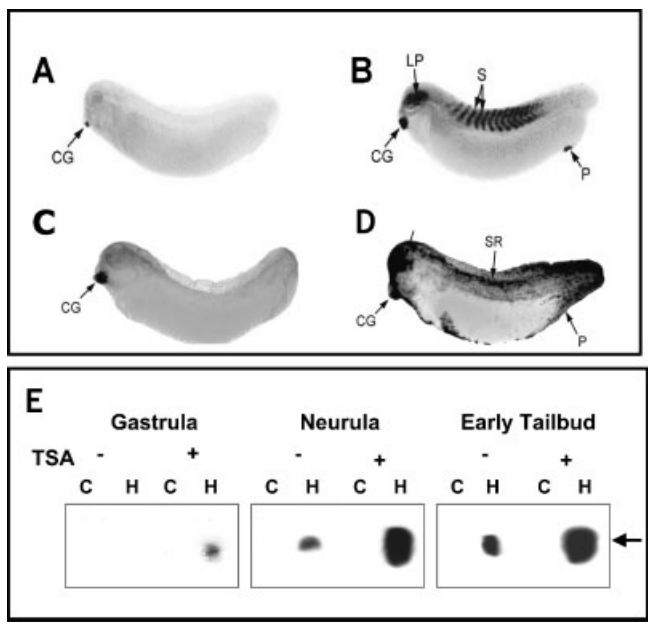

Fig. 1. Hsp30 gene expression in control and heat shocked *Xenopus* embryos. Whole mount in situ hybridization with dioxygenin-labeled hsp30 antisense riboprobe was carried out with control (**A**; 22°C) and heat shocked (**B**; 1 h at 33°C) midtailbud stage embryos. Constitutive hsp30 mRNA accumulation was observed in cement gland (CG). Preferential heat-induced accumulation of hsp30 mRNA was detected in lens placode (LP), somites (S), proctodeum (P), and CG. Immunohistochemical analysis using an anti-hsp30 antibody revealed hsp30 accumulation in CG of control tailbud embryos (panel **C**). In heat shocked embryos hsp30 was detected in CG, LP, somitic region (SR), and proctodeum (panel **D**). In panel **E**, treatment of *Xenopus* embryos with 30 nM trichostatin A (TSA) resulted in precocious heat-induced accumulation of hsp30 mRNA at the gastrula stage rather than at the late neurula-early tailbud stages as found in non-HDI treated embryos. Additionally, HDI treatment enhanced heat-induced hsp30 mRNA accumulation in neurula and tailbud embryos. These experiments suggest that hsp30 gene expression during development is regulated at the level of chromatin structure. In this study total RNA was isolated from control (C; 22°C) and heat shocked (**H**; 1 h at 33°C) embryos and subjected to Northern blot analysis. Arrows indicate the positions of hsp30 mRNA.

suggested that certain embryonic tissues in the midtailbud embryos were more sensitive in the activation of hsp30 gene expression than others. Preferential heat shock-induced shsp gene expression in selected tissues has been documented in other developmental systems including *Drosophila* spermatogenesis [Michaud et al., 1997].

The mechanism(s) responsible for the repression of *Xenopus* hsp30 gene expression prior to late neurula/early tailbud is not known. Various studies including Hair et al. [1998] have suggested that the generalized repression of transcription that occurs during the cleavage stages is the result of a large pool of histone binding to DNA that blocks the binding of transcription factors. At MBT this pool of histone is depleted and increases the accessibility of transcription factors to *cis*-acting sites associated with various genes. It is likely that the regulatory features that activate the expression of zygotic genes at MBT are also responsible for the heat-inducible expression of hsp70 and hsp90 genes. The mechanism associated with the continued repression of hsp30 gene expression after MBT until the late neurula/early tailbud stage of development may also reside at the level of chromatin structure. This possibility was suggested previously since microinjection experiments failed to define specific *cis*-acting sequences responsible for hsp30 gene developmental regulation [Ali et al., 1993].

Changes in chromatin organization have been reported in many biological systems that exhibit developmental or tissue-specific controls in gene expression. For example, relief from chromatin-mediated repression has been associated with the tissue-specific activation of the α-fetoprotein gene in endothelium-derived cells [Crowe et al., 1999]. The lysine residues of core histone molecules can undergo a wide range of post-translational modifications, including acetylation, phosphorylation, and methylation [Spencer and Davie, 1999]. The histone code theory proposes that the post-translational modifications of histone amino-termini are an epigenetic chromatin marking system that can regulate "on–off" states of transcription [Jenuwein and Allis, 2001]. Furthermore recent studies found that transcriptional activation is associated with the recruitment of histone acetyl transferases (HATs) while repression involves histone deacetylases (HDACs) [Ahringer, 2000].

A number of transcriptional coactivators possess HAT activity including cyclic adenosine monophosphate response element-binding protein (CBP) and p300 [Ahringer, 2000]. The transcriptional coactivator, p300/CBP stimulates the transcription of specific genes by interacting, either directly, or through cofactors, with transcription factors, such as c-Fos, c-Jun, and c-Myb and nuclear hormone receptors. Acetylation is thought to be an integral characteristic of these processes. Deacetylation of core histone molecules stabilizes higher-order chromatin structure, and in doing so, excludes the basal transcriptional machinery from DNA. The Sin3/Rpd3 complex, which is a component of the NuRD deacetylase complex, can interact with a variety of corepressors including *Drosophila* hunchback and Groucho [Ahringer, 2000]. In yeast, Sin3/Rpd3 is recruited to various promoter regions by specific DNA binding proteins and associated repressors [Alland et al., 1997]. The result is highly localized deacetylation of histone molecules and repression of transcription.

Recently the role of histone hyperacetylation on the expression of hsp30 genes during early *Xenopus laevis* development was investigated [Ovakim and Heikkila, 2003]. Histone hyperacetylation was achieved by incubation of *Xenopus* embryos in the presence histone deacetylase inhibitors (HDIs) such as trichostatin A. HDIs loosen chromatin structure by enhancing the acetylation of lysine residues of histone tails by reducing their positive charge, and weakening electrostatic interactions with DNA and other nucleosomes [Hong et al., 1993]. Incubation of embryos with HDI resulted in precocious heat-induced accumulation of hsp30 mRNA at the gastrula stage rather than the late neurula/early tailbud as found with non-HDI treated embryos (Fig. 1E). HDI-induced premature or enhanced expression has been found with a variety of developmentally regulated genes including γ-globin and histone H1 [Almouzni et al., 1994; McCaffrey et al., 1997]. The mechanism responsible for the HDI associated precocious expression and enhancement of heat shock-induced hsp30 mRNA accumulation in *Xenopus* embryos is not known. It is possible that HDIs may alter the conformational state of hsp30 chromatin such that it is more accessible to transcription factors (Fig. 2). Future studies will be required to determine whether the putative repressed state of hsp30

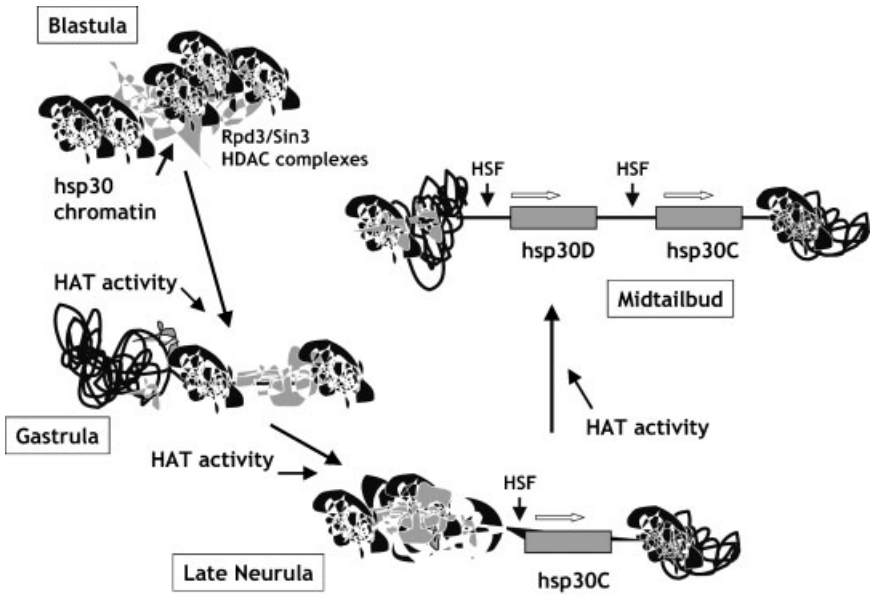

Fig. 2. Involvement of chromatin structure in the regulation of heat-induced hsp30 gene expression during *Xenopus* development. At the blastula and gastrula stage hsp30 chromatin is present in a repressed condition possibly due to Rpd3/Sin3 histone deacetylase complexes. After the gastrula stage, post-translational modification of histones such as acetylation by histone acetyl transferases (HAT) induce a loosening of hsp30 chromatin structure such that at late neurula the hsp30C gene is accessible to transcription factors including heat shock factor (HSF). This process continues with development such that other hsp30 genes such as hsp30D can be transcribed at midbtailbud stage. The open arrows indicate heat shock-induced transcription of hsp30 genes.

chromatin in pre-neurula embryos is associated with histone deacetylase and co-repressor complexes such as Rpd3 and Sin3, which have been detected during early *Xenopus* development [Sachs et al., 2001]. While it is possible that chromatin acetylation may be involved in the transcriptional activation of *Xenopus* hsp30 genes, one cannot rule out other types of modification such as phosphorylation.

Interestingly, HDI treatment did not alter the spatial pattern of heat-induced *hsp30* mRNA accumulation in neurula and tailbud embryos. It is likely that this aspect of *hsp30* gene expression is not governed by chromatin conformation. A number of mechanisms may account for this phenomenon including the possibility that specific hsp30C and D regulatory elements and/or transcription factors are involved in HSF-mediated expression at tailbud once hsp30 chromatin is derepressed. Alternatively, it is possible that certain tissues of the midtailbud stage embryo may have a lower temperature set point for HSF activation than in other tissues. In support of this possibility it was found that adult heart tissue had a lower HSF activation temperature than other tissues examined such as liver [Ali et al., 1997].

HSP30 FUNCTION DURING ENVIRONMENTAL STRESS

The in vivo function of hsp30 in heat-shocked frog embryos is not known. However, a number of studies carried out with recombinant hsp30C and D protein described below, have demonstrated their ability to act as molecular chaperones (Fig. 3). These proteins, which form multimeric complexes both in vivo and in vitro, inhibited heat- or chemical-induced aggregation of citrate synthase (CS) and luciferase (LUC) by maintaining them in a soluble form [Fernando and Heikkila, 2000; Abdulle et al., 2002]. Furthermore, using an in vivo *Xenopus* oocyte microinjection refolding assay, it was determined that hsp30 maintained its target protein in a folding competent state such that it could be reactivated in the presence of other molecular chaperones [Abdulle et al., 2002]. For example, LUC heat denatured alone did not regain significant enzyme activity following heat treatment and microinjection into *Xenopus* oocytes. However, heat treatment of LUC in the presence of hsp30 resulted in 100% enzyme reactivation following injection. Mutagenesis studies of hsp30C revealed that deletion of the

last 25 amino acids from the C-terminal end or reduction of the net negative charge of the C-terminal tail by converting two aspartic acid residues to glycines inhibited its chaperone activity and altered its secondary structure [Fernando and Heikkila, 2000; Abdulle et al., 2002; Fernando et al., 2002]. The inhibition of chaperone activity in these C-terminal mutants is likely due to their reduced ability to maintain target protein in a soluble state (Fig. 3).

In a recent study, it was found that heat shock-induced hsp30 was phosphorylated by mitogen-activated kinase-activated protein kinase-2 (MAPKAPK-2) in *Xenopus* A6 kidney epithelial cells primarily during recovery from stress [Fernando et al., 2003]. Phosphorylation induced the formation of smaller multimeric hsp30 complexes as well as a loss of hsp30 α-helical and β-sheet structure. Furthermore phosphorylated hsp30 had a reduced ability to inhibit heat-induced aggregation of CS and LUC in vitro compared to unphosphorylated hsp30C. It is possible that phosphorylation during recovery from stress may accelerate the release of tightly bound *Xenopus* hsp30 particles from its target proteins by inducing a change in the oligomeric assembly of hsp30 (Fig. 3). The released target proteins then undergo refolding and reactivation in an ATP-dependent manner by other molecular chaperones including hsp/hsc70. Various studies have shown that shsps play an essential role in maintaining the integrity of actin and intermediate filaments [Mounier and Arrigo, 2002]. It was suggested that phosphorylated shsps present in small oligomers interact directly or indirectly to protect microfilaments against further disruption by inhibiting the action of actin-severing proteins activated by the stress response and then promoting their recovery.

CONSTITUTIVE HSP30 GENE EXPRESSION AND FUNCTION

Whole mount in situ hybridization analysis of *Xenopus* embryos revealed constitutive hsp30

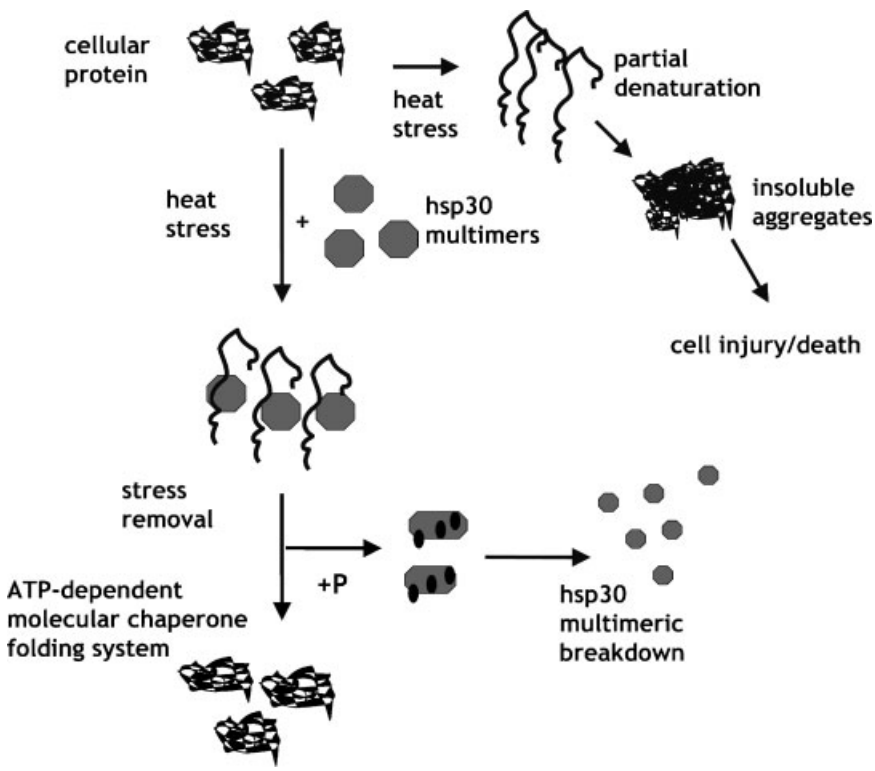

Fig. 3. Model of hsp30 function in *Xenopus* cells. Exposure of cells to elevated temperatures can result in protein denaturation and the formation of potentially lethal insoluble aggregates. However heat shock-induced hsp30 multimeric complexes can bind to partially denatured protein and maintain them in a soluble and folding competent state. Once the stress is removed, other molecular chaperones in the presence of ATP can refold the proteins back to their native state. Meanwhile the hsp30 multimeric complexes are phosphorylated which results in a change in secondary structure as well as a loss of chaperone activity and their ability to bind target protein. The black oval shapes represent phosphorylation sites. This is followed by the breakdown of the hsp30 multimeric complexes into smaller oligomers.

mRNA accumulation only in the CG of early and midtailbud embryos (Fig. 1A) [Lang et al., 1999]. This mucus-secreting structure, found at the anterior end of the embryo, permits attachment of the newly hatched larva to a solid support. The CG is derived from the epithelial layer of frog ectoderm and is the first ectodermal organ to differentiate [Sive and Bradley, 1996]. Hsp30 mRNA accumulation in the CG was transient since it was not evident in late tailbud or later stage embryos. These CG hsp30 transcripts appear to be an as yet unidentified member(s) of the hsp30 family. Whole mount immunohistochemical analysis revealed hsp30 protein in the CG of late tailbud and early tadpole stage embryos. Thus, while hsp30 message accumulated in the early and midtailbud stages before decaying, hsp30 protein was present until at least early tadpole.

The regulatory mechanism associated with constitutive expression of hsp30 gene(s) in the CG of *Xenopus* tailbud embryos is not known. It is unlikely that the CG undergoes a localized stress-like response since hsp70 mRNA was not detected in this region under control conditions. It is possible that hsp30 genes in the *Xenopus* CG are induced by an, as yet, unidentified hormone or other inductive agent. In support of this possibility, hsp30 gene expression has been observed in the livers of thyroid hormone-treated metamorphosing tadpoles of *Rana catesbeiana* [Helbing et al., 1996].

Hsp30 may function as a molecular chaperone and interact with cytosolic proteins in the mucus-secreting cells of the CG. It is also possible that hsp30 may be involved in the prevention of apoptosis of the CG, which is a transient organ and eventually lost by means of apoptosis at the tadpole stage [Poitras et al., 2003]. In mammalian cells, shsps are transiently expressed during the cell division to differentiation transition and appear to be essential for preventing differentiating cells from undergoing apoptosis [Arrigo, 1998]. Since *Xenopus* hsp30 mRNA is lost by the late tailbud stage whereas the protein is still detectable, it is conceivable that hsp30 could function in the prevention of apoptosis until it is degraded. As mentioned above bullfrog hsp30 is constitutively expressed in tadpole. While we have found recently that *Rana* hsp30 can act as a molecular chaperone [Kaldis and Heikkila, unpublished results], its function in metamorphosing tadpole liver remains to be determined. However, it is possible that *Rana* hsp30 may function to block apoptosis in liver cells.

FUTURE DIRECTIONS

Clearly there are a number of unresolved questions regarding the regulation of gene expression and function of shsps during amphibian development. The use of chromatin immunoprecipitation (ChIP) assays with antibodies directed at various proteins including acetylhistone H3 and H4, histone deacetylase 1, and Sin3A will assist in the understanding of chromatin associated with hsp30 promoters during frog development. Since the analysis of hsp30 molecular chaperone function during environmental stress was carried out with in vitro or cultured cell systems it will be interesting to examine the chaperone activity of shsp multimeric complexes formed in vivo in embryos. This is of particular interest since different hsp30 isoforms are synthesized at different stages of embryogenesis [Heikkila et al., 1997]. The possible involvement of hsp30 in inhibiting CG apoptosis could be addressed using antisense morpholino oligonucleotide technology. Theoretically these oligos should block translation of hsp30 mRNAs and enhance CG apoptosis. Finally, the analysis of Bshsp and α-crystallin gene expression and function in amphibians is an area that requires additional study.

Studies with *Xenopus laevis* and other amphibians have made major contributions to our understanding of vertebrate development and the regulation and function of shsps. However, the fact that many amphibian species such as *Xenopus laevis* normally take 1–2 years to reach sexual maturity does not make them ideal for carrying out key genetic experiments. Also the generation of knockout mutants with *Xenopus laevis* is hampered by the fact that this species is tetraploid. Future research using *Xenopus tropicalis* may circumvent these problems since this diploid species has a generation time of less than 5 months [Hirsch et al., 2002]. Finally transgenic technology has been developed for *Xenopus tropicalis* as well as being the subject of a major effort with respect to genomics and proteomics.

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