Molecular Analysis of Encystment and Diapause in *Artemia* : Gene Expression and Stress Response

by

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•••dedicated to my parents, Wangxi Qiu and Zhengen Liao

Table of Contents

List of Figures

List of Tables

Abstract

During oviparous development, *Artemia* embryos encyst and enter diapause, a condition characterized by low metabolic activity and high tolerance to environmental stress. Entry into diapause is thought to involve differential gene expression leading to synthesis of regulatory and structural proteins that modulate organismal behavior, and during diapause stress proteins are may protect encysted embryos. Subtractive hybridization was utilized to recover several up-regulated genes, including those that encode the small heat shock proteins (sHSPs) ArHsp21 and ArHsp22, and p8, a co-transcription factor. Full length cDNAs of ArHsp21 and ArHsp22 encode 181 and 190 amino acid residues, respectively, with a conserved α-crystallin domain. ArHsp21 and ArHsp22 form oligomers and exhibit chaperone activity, functional characteristics of most sHSPs. These sHSPs are detected during oviparous but not ovoviviparous development and upon exposure of adult *Artemia* to thermal stress, synthesis of ArHsp22 was observed. In contrast, instar II larvae synthesized neither ArHsp21 nor ArHsp22 in response to heat shock. ArHsp21 and ArHsp22 have the potential to protect *Artemia* embryos during development, whereas only ArHsp22 is stress inducible. *Artemia* p8 contains 66 amino acid residues and its sequence is similar to p8 from other organisms. Probing of Southern blots indicated a single p8 gene in *Artemia*. p8 transcripts are present in low amounts in oocytes prior to fertilization and increase rapidly, peaking at one day post-fertilization during oviparous but not ovoviviparous development. p8 protein is present at two days post-fertilization and is found in encysted embryos released from females, where it was detected in nuclei by immunofluorescent staining. The properties of p8 are consistent with a role in regulation of *Artemia* entry into encystment. As a representative of *Artemia* sHSPs, p26 gene structure was analyzed, revealing four exons and three introns, with one intron in the 5'-untranslated region. Cis-acting elements such as putative estrogen and Ap-1 binding sites, and heat shock elements, were found in the first intron and the 5'-flanking upstream region of the p26 gene, and they may contribute to developmental regulation of this gene.

Abbreviations and Symbols

3'-RACE	rapid amplification of 3' cDNA ends
5'-RACE	rapid amplification of 5' cDNA ends
ATP	adenosine 5'-triphosphate
DAPI	4'6-diamidino-2-phenylindole, dihydrochloride
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FITC	fluorescein isothiocyanate
hr	hour
HSE	heat shock element
HSP	heat shock protein
HSF	heat shock factor
HSP	heat shock protein
HST	high salt Tween
HPC	Hexylene glycol, pipes and $CaCl_2$ buffer
IgG	immunoglobulin G
IPTG	isopropylthio-β-galactosidase
kDa	kilodalton
min	minute
PBS	phospate buffered saline
Pipes	1,4-peperazinediethanesulfonic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
TAE	Tris, acetate, EDTA buffer
TBS	Tris buffered saline
TE	Tris, EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-(hydroymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
X-Gal	5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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I. Introduction

1.1 Diapause and Development

1.1.1 Diapause

For many organisms environmental conditions vary between favorable and unfavorable with season and location. In order to survive adverse environments, the ability to suppress metabolic activities, ranging from 20-30% reduction to virtually ametabolic states, has evolved and this includes hibernation, estivation and diapause [1-3]. Hibernation is a metabolic suppression phenomenon whereby many organisms such as small mammals and amphibians survive cold winter weather, whereas estivation is a means by which organisms such as lungfish deal with arid conditions accompanied by heat and lack of food [2]. Diapause in insects and their arthropod relatives allows escape from insults such as temperature extremes, desiccation and lack of food [3, 4]. Once diapause terminates [5, 6] and upon exposure of organisms to favorable conditions, growth and development resume.

In the transition to diapause, enzyme activities are often modulated through phosphorylation or dephosphorylation, and thus they contribute to new homeostasis [1-3]. During diapause, metabolism decreases, cell division is reduced or eliminated, morphological development slows or arrests, and resistance to stress increases due to elevated synthesis of molecular chaperones. For example, the pea aphid *Acyrthosiphon pisum* exhibits slow but continuous morphological development throughout diapause [7], whereas during brine shrimp diapause, development stops completely [8]. Diapause occurs at different stages in various organisms including embryos of silkmoth, larvae of the European corn borer, pupae of flesh flies and adults of ladybird beetles, but within

each species it usually is restricted to a single developmental stage [3]. The length of diapause (dormancy) ranges from a few hours in some birds to many years in a selection of plants and animals [1, 3].

Diapause regulation involves factors such as proteins and hormones [9], and it is subject to genetic control [4]. Based on induction signals, diapause is divided into two categories. In one case as in fly *Sarcophaga bullata*, diapause is induced in response to environmental conditions such as day length and temperature [10]. In the other, as in *Artemia*, diapause is obligatory, occurring independent of environmental conditions [4]. After signals are received and transducted within cells, organisms up-regulate the expression of some genes, down-regulate others and adjust enzyme activities, leading to suppression of synthesis and degradation of macromolecules involved in cell cycles, including DNA, mRNA, proteins, membrane phospholipids and ATP, and to elevated synthesis of stress proteins playing a protective role for other molecules [9, 11-16]. During diapause in the flesh fly, *Sarcophaga crassipalpis*, the genes for protease inhibitor Scys-B, two small heat shock proteins 23 kDa and pScD14, hsp 70, and pScD 86 which encodes a DNA-repair protein are up-regulated [17-21]. The genes for pScD56 and proliferating cell nuclear antigen (PCNA) promoting cell cycle regulation, are down regulated [17]. In the Colorado potato beetle, *Leptinotarsa decemlineata*, three diapause-associated proteins known as DAT-1, DAT-2 and DAT-3 are detected and they may be desiccation proteins [22]. In the pea aphid *A. grandis*, the diapause-specific protein Ag-SP1, possibly functioning as an amino acid reservoir, is observed [23]. In *C. elegans*, the insulin/IGF pathway and TGFβ pathway, including 16 proteins, among which is DAF-16,

a Forkhead type O (FOXO) transcription factor inhibiting growth and promoting diapause [24, 25], are involved and subject to extensive investigation.

1.1.2 *Artemia* diapause

The brine shrimp, *Artemia*, is distributed around the world, and it inhabits locations with hypersalinity, high ultraviolet radiation, low oxygen tensions and temperature extremes [26-30]. To survive these adverse environmental factors, *Artemia* exploits two developmental pathways to complete its life cycle, namely the ovoviviparous and oviparous pathway. In the former, zygotes undergo a five day growth period in the ovasac and are released from females as nauplii. In the latter, zygotes develop into cysts containing approximately 4,000 nuclei and they leave females as developmentally arrested gastrulae (cysts) which enter diapause characterized by extremely low metabolic activity [31]. Cysts tolerate stress such as anoxia, desiccation, temperature extremes and ultraviolet irradiation [28]. For example, continuous anoxia for 18 months resulted in only modest decreases in viability and at least 60% of cysts give rise to viable nauplii after continuous anoxia for 4 years at temperature of 20-23 °C when fully hydrated [29, 30, 32]. In contrast to many organisms, in *Artemia* during diapause metabolism is reduced to a point where its detection is difficult, indicating that protein synthesis, energy metabolism and macromolecular degradation are greatly reduced [29, 30, 33].

The stability of diapause cysts raises questions as to what confers protection on embryos. Large amounts of trehalose and glycerol occur in diapause embryos, these being compatible solutes which may stabilize proteins and other cellular constituents against denaturation [34, 35]. Trehalose may protect membrane structure and counteract the effects of desiccation [35-37]. Additinally, two proteins, p26 and artemin, are found

in cysts but not in nauplii. p26 is a well-characterized, abundant small heat shock protein in cysts and its synthesis occurs in cyst-destined embryos from day 2 to day 5 post-fertilization [32, 33]. This small heat shock protein is thought to play an important role in protecting other proteins in diapause embryos [38, 39], exemplified by the finding that p26 binds tubulin *in vitro* and prevents its denaturation [40]. Artemin, making up 10-15% of the total non-yolk protein in cysts, is exceptionally thermostable and under certain conditions is associated with RNA [8, 41, 42]. Artemin may protect RNA during diapause and after diapause termination RNA is available for rapid translation into proteins, ensuring resumption of growth and development [41].

Artemia diapause is obligatory and not induced by environmental signals whereas termination of diapause requires dehydration. However, except for p26 and artemin, other genes involved in *Artemia* diapause remain to be characterized. Additionally, regulation of p26 gene expression and diapause induction deserve exploration.

1.2 Heat Shock Proteins (HSPs)

1.2.1 HSP synthesis

Since the heat shock response was first observed in *Drosophila* [43], HSPs have been found in all organisms [44-46]. On the basis of size, structure and function, HSPs are divided into five categories, namely Hsp100, Hsp90, Hsp70, Hsp60 and the small HSPs (sHSPs) [47]. The larger HSPs are ATP-dependent, whereas the sHSPs are ATP-independent. Most species possess more than one type of HSP and these localize to different compartments, such as nuclei, mitochondria, chloroplast and endoplasmic reticulum (ER), and to different organs [48, 49]. For instance, Hsp22 localizes to the

mitochondrial matrix in *Drosophila* [50], Hsp47 is an endoplasmic reticulum resident [51] and the αA-crystallin is expressed almost exclusively in the mammalian lens [52].

The synthesis of some HSPs is either developmentally regulated or stress-induced. Developmentally regulated HSPs are synthesized in the absence of stress at specific developmental stages, whereas stress-induced HSPs are expressed in response to environmental insults. *Drosophila* Hsp68 and Hsc70 genes are expressed at normal growth temperature during the blastoderm stage of growth and they are slightly stress inducible thus they belong to the developmental group of HSPs. In *Caenorhabditis elegans*, SEC-1, a small developmentally regulated chaperone protein of 159 amino acids, is present in early embryogenesis and is thought to inhibit nascent polypeptide aggregation, promote correct folding of newly synthesized proteins and facilitate the normal progression of embryogenesis because at this stage other HSPs such as Hsp70 are absent [53]. p26, a sHSP found in *Artemia*, is developmentally regulated. During oviparous development, p26 is abundantly produced, undoubtedly protecting cysts, but heat shock does not induce p26 gene expression [31]. C. *elegans* genes encoding the HSPs, Hsp16-1, Hsp16-2, Hsp16-41 and Hsp16-48 are arranged on chromosome V, with Hsp16-1 paired with Hsp16-48 and Hsp16-2 with Hsp16-41 [54, 55]. In contrast to developmentally regulated HSPs in *C. elegans*, these genes are virtually inactive in unstressed animals, but upon exposure to heat shock, they are transcribed at all developmental stages. mRNA levels for these genes are greatest in embryos and the first larval stage.

In contrast, other HSPs are both synthesized in response to stress and at certain developmental stages in the absence of stress. For instance, the *Drosophila* genes for

Hsp22, 23, 26 and 27, located in the 67B region of chromosome 3, increase expression rapidly upon exposure to heat shock and other stress, but they also show developmental regulation. Hsp23 is expressed during embryogenesis in specific glial cells [56], but the mRNA of Hsp22 is not detected in early development. Glaser et al. [57] identified Hsp26 expression in *Drosophila* larvae, pupae and adults. Throughout the larval stages, Hsp27 expression is limited to the CNS and gonads [45]. In the testes, Hsp22 is strongly induced by heat shock, but Hsp23 and Hsp27 are not. *Xenopus* Hsp30 mRNA and protein accumulate preferentially in cement gland, somitoc negi, lens and proctodeum upon heat shock, but they are constitutively expressed in early and midtailbud embryo [58].

1.2.2 HSP functions

HSPs play critical roles in metabolism, differentiation and development, especially when adverse environmental conditions are encountered. Under normal conditions, proteins may misfold or aggregate during synthesis, thus resulting in permanent loss of function, but constitutive HSPs interact with newly synthesized proteins and stabilize their structure, providing another opportunity to fold appropriately [49, 59, 60]. HSPs also influence protein import into organelles and this has been reviewed in detail [44]. Proteins also unfold, misfold, aggregate and degrade during stress. HSPs can prevent undesirable protein interactions and restore unfolding proteins to functional conformations, as exemplified by *in vitro* protection of citrate synthase with HSPs [61] and complex formation of murine Hsp25 and Hsp26 with nonnative proteins [49]. As well, HSPs assist with the degradation of denatured proteins whose structure cannot be recovered. A subunit of the proteasome associates with sHSPs, and interaction occurs between *Drosophila* Hsp23, Hsp27 and DmUbc 9, the ubiquitin conjugating enzyme, that

participates in the degradation of S and M phase cyclins [62]. Moreover, HSPs are implicated in other processes necessary for normal growth and development. For example, some sHSPs such as human hsp27 and *Artemia* p26, which are phosphorylated on serine residues, regulate microfilament dynamics, modifying actin response to physiological agonists and stabilizing the actin cytoskeleton [63, 64]. Mammalian sHSPs appear to participate in signal transduction related to microfilaments, this mediated by changes in chaperone phosphorylation. Heat shock cognate gene HSC70 is associated with intermediate filaments and *Artemia* p26 with tubulins [40], the building blocks of microtubules. Finally, HSPs are associated with many diseases, with Hsp90, Hsp70 and Hsp27 constitutively overexpressed in the tumor cells of breast, uterine, renal, osteosarcoma and various leukemias, where they increase resistance to oncogenesis-induced apoptosis through a protective antitumor immune response [65, 66].

It is worthwhile, in the content of function, to mention the relationship between HSPs and diapause. Many HSP genes are up-regulated during diapause, suggesting HSPs protect proteins including structural proteins and enzymes when metabolism is low. For example, in diapause pupae of the flesh fly *S. crassipalpis*, pScD14 showing 85% identity to the lens protein α-crystallin was observed [17]. As well, Hsp23 and Hsp70 are up-regulated, but Hsp90 down-regulated. HSPs may facilitate the shut down of cell activities, contributing to cell cycle arrest that characterizes diapause [18, 67, 68], but further effort is required to confirm this.

1.2.3 HSP structure and substrate interaction

HSP100, 90, 70 and 60 are composed of N-variable, C-variable, ATPase and peptide binding domains, with the ATP-binding domains exhibiting high similarity

among different species [69]. In contrast, sHSPs, which exhibit ATP-independent function, are made up of three domains, namely amino- and carboxy-terminals which are highly variable in size and sequence and a conserved α-crystallin domain of approximately 90 residues [47, 70]. sHSPs usually form high molecular weight oligomeric complexes [71, 72]. The N-terminal domain may be buried within oligomers and assists in subunit assembly whereas the C-terminal domain is exposed at the surface and contributes to the solubility of substrate-chaperone complexes [73-75]. However, there are exceptions to these general characteristics with N-terminal truncated Oshsp16.9 capable of forming complexes similar to those formed by the wild type protein [76]. sHSP quaterntary structure is variable, ranging from toroids to roughly globular particles [77-79]. For example, human recombinant αB-crystallin forms a hollow, globular shell with a molecular mass of 650 kDa and an asymmetric appearance under the electron microscopy [80], whereas frog retinal and lens α-crystallin reveals a torus-like shape [81]. Wheat HSP16.9 is a dodecamer consisting of two disks, each comprising six α-crystallin domains organized in a trimer of dimers [82].

sHSP structure influences its chaperone activity. There is a relationship between oligomerization and chaperone activity. Hsp12.6 from *C. elegans* contains short amino acid residues at its N-terminus and C- terminus, therefore exists as monomers that cannot prevent the thermally-induced aggregation of citrate synthase [83, 84]. Deletion of an N-terminal region of *C. elegans* Hsp 16-2 leads to loss of oligomerization and chaperone properties [84]. However, other experiments suggest that the native oligomeric state of α-crystallin is not essential for suppression of non-specific protein aggregation and oligomerization provides structural stability, so that molecular chaperone function

persists because protein turnover is limited [85]. For example, 70% of the human αB-crystallin was below 100 kDa after 2 h of trypsin digestion, and it retained 65% of its chaperone activity [86]. Additionally, despite being similar to wild-type αB crystallin in aggregate molecular mass, the chimeric αANBC consisting of the N-terminal domain of αA crystallin and the C-terminal region of αB crystallin possessed no chaperone-like activity [87]. Thus, it seems there is no direct relationship between oligomeric size and chaperone activity. Furthermore, the substrate–binding domain has been reported to be located within the α-crystallin domain. Therefore, important to chaperone function is the α-crystallin domain because binding is a prerequisite for the interaction between sHSPs and unfolded proteins [86, 88]. Other experiments reveal that truncation of the C terminus of α-crystallin decreases chaperone-like activity and this is believed to be one of the post-translational modifications that compromises sHSP chaperone function *in vivo*.

1.3 Regulatory Regions of HSP Genes and Transcription Factors

The synthesis of HSPs results from the interaction of gene specific transcription factors such as heat shock factors (HSFs) with general transcription machinery proteins including TFII A, B, D, E, F, H, J and RNA polymerase II, or from the interaction of gene-specific transcription factors with the nucleosome [89-91]. Activated transcription factors associate with gene regulatory regions, facilitating binding of the general transcription machinery or the movement of polymerase II along genes. Some HSP genes are turned on upon exposure to stress such as heat shock, toxic chemicals, ATP depletion, endotoxins, and hyperosmolarity, whereas others do not react to stress. The different

responses depend on variations in upstream elements and transcription factors available in the cell.

1.3.1 Upstream sequences of HSP genes

The region upstream of the transcription start site contains the core promoter, regulatory sequences and enhancers which bind transcription factors, resulting in gene transcription. Transcription regulatory sequences are divided into the basal regulatory elements such as GC box, which are necessary for basal transcription, and specific sequences like the HSEs, these required for inducible transcription. The former contribute to limited expression of HSP genes under normal growth conditions and the latter are responsible for elevated expression of developmental and stress-induced HSP genes upon exposure to appropriate signals.

Developmentally regulated and stress-induced HSPs share some basal expression elements, but some basal elements required by HSP genes in different organisms differ. In rodent cells, for example, the elements required for basal Hsp70 expression are the proximal CAT box, GC box (Sp1 site), and the TATA box, whereas in human cells these and additional elements including CAAT and AP2 sites are required for basal transcription. Basal transcription of the Hsp27 gene in human breast cancer cells, on the other hand, is controlled by sequences in the proximal 200 bp region of the promoter including the TATA box, GC box and AP2 site [92].

Stress-induced HSP genes are mainly dependent on stress elements for their transcription, including HSEs and stress regulatory elements (STREs). Located in the regulatory region, the HSEs, composed of inverted repeats of nGAAn such as nGAAnnTTCn, drive stress-induced HSP genes and a few developmental HSP genes by

interaction with HSFs as described in the following section. In the consensus sequence nGAAn "G" at position 2 is absolutely conserved, with base substitutions abolishing heat induced expression, whereas "A" at positions 3 and 4 is less conserved and "n" represents any nucleotide. HSE structure varies for different organisms and heat shock factors [93, 94]. For example, two inverted repeats are sufficient for *Drosophila* HSF1 binding, but optimal binding is obtained with three repeats [95]. By contrast, S. *cerevisiae* HSF1 binds to six contiguous inverted repeats of nGAAn. Embryonic HSF2 purified from F9 embryonal carcinoma cell tumors requires at least three 5'-nGAAn-3' motifs and its optimal binding sequence entails a palindromic 8-mer core 5'-TTCTAGAA-3' representing two inverted repeats of AGAA.

Other arrangements of the repeat nGAAn pertain. For example, there are two HSEs in the rat αB-crystallin gene promoter: a dimeric HSE arranged in a head:head fashion at position -391 (GGAAGATTCC) and a trimeric element arranged in tail:tail/head:head order at position -54 (GTTCCAGAAGCTTCA)[96, 97]. Although there are many HSEs in the upstream region of some HSP genes, not every HSE mediates stress-dependent induction. This is exemplified by *Drosophila* Hsp26 which possesses seven different HSEs, with only HSE1 and HSE2, located at -62 and -72, respectively, and HSE6 located at -350, necessary for full heat shock induction. In addition to upstream regulatory sequences, a HSE found in the first intron of the rat hsp27 gene, designated i-HSE [98], reduces transcription of hsp27 in contrast to up-regulation, as is the case for most HSEs.

In the promoter of human Hsp27 the stress-related element known as STRE, whose consensus sequence is A/TGGGGA/T, binds lens epithelium derived growth factor (LEDGF), a regulatory factor required for activation during stress [99]. Moreover, in C.

elegans, a novel HSE (GGGTGTC), different from previously described HSEs, was identified, suggesting that it interacts with a novel transcription factor under heat shock conditions [100]. These observations improve our understanding of stress-induced HSP activation and suggest sequences that are involved in stress response in *Artemia*.

In contrast to HSEs and STREs, an ecdysterone regulatory element (EcRE) [101, 102] resides in the upstream region of the *D. melanogaster* Hsp27 gene, stimulating expression upon interaction with the ecdysterone receptor. A number of estrogen response element (ERE) half sites ($GGGCGGG(N)_{10}GGTCA$) mediate expression of the human Hsp27 gene in MCF-7 human breast cancer cells. In the upstream sequence of the murine αB-crystallin gene, the elements αBE-1 (AATGTCCCTG), αBE-2 (CCAAGATAGTTGCT GGCTCAATTCCCCTGGCAT) and αBE-3 (GGAGGAGGAGGG) are crucial for transcription [103, 104]. These elements are enhancers which promote αB-crystallin gene transcription. αBE-1 and αBE-2 are utilized for expression of the αB-crystallin gene in the lens, skeletal muscle and lung, where αBE-3 enhances synthesis of αB-crystallin in the lens and skeletal muscle. Additionally, the MRF sequence essential for αB-crystallin expression in skeletal muscle is ACCAGCAGCTGCTTGGGA [103, 105, 106]. In the murine αB-crystallin gene there are lens-specific regulatory regions (LSR), namely LSR1 (GTGAGTACCGGGTATGTGTCACCCTGCCAA) and LSR2 (GGATAATAAAACCC CTGACCTCACCATTCCAGA) [107, 108]. Pax-6 interacts with both LSR1 and LSR2 for αB-crystallin gene expression in the lens. By contrast, the αB-crystallin gene in duck lacks all those αBE elements, but contains block 1 which corresponds to αBE-2 and block 2 different in sequence from αB elements [109].

1.3.2 HSP transcription factors

Many transcription factors bind to the regulatory elements just described and regulate either developmental or stress induced HSP gene expression. These include the HSFs, HSF1, HSF2, HSF3 and HSF4 [110-112], GAGA factor, ecdysterone receptor [102, 113], Pax-6, and estrogen receptor. Their structure and function are partially characterized at molecular levels, but how they control HSP gene expression is not completely understood.

Four HSFs are found in different organisms and HSF isoforms are produced by alterative splicing, thus increasing their number. HSF1, HSF3 and HSF4 are directly related to stress, while HSF2 is not [114]. The main functional HSF domains are those that bind DNA, participate in oligomerization and activate transcription. The DNA binding region, located in the amino-terminus, is a helix-turn-helix domain. The carboxyl terminal activation domain is comprised of activation-induced and suppressive trimerization subdomains, the former activating HSP gene expression and the latter controlling HSF trimerization.

HSF1, an inactive monomer in most cell types, is phosphorylated at the regulatory domain, decreasing its activity [115]. However, upon exposure to stress, HSF1 is converted to a hyperphosphorylated trimer localized to the nucleus [116], where it binds DNA at highly conserved HSEs. In contrast to monomeric HSF1, HSF2 exists as an inert dimer [117], with two isoforms, HSF2-α and HSF2-β, detected. The latter is more abundantly expressed than the former throughout spermatogenesis and embryogenesis, acting as a negative regulator of transcription. HSF2, not activated in response to heat shock or other cellular stresses, is responsible for the high spontaneous expression of HSP genes observed in the absence of stress in embryonal carcinoma cells (EC) during mouse embryogenesis and spermatogenesis. HSF2 is activated by high extracellular

potassium [118] or during hemin-induced differentiation of erythroleukemic cells during mouse spermatogenesis. Upon activation it converts to a DNA-binding trimer that is capable of inducing HSP gene transcription [119]. HSF3 is specific to avian cells and is an inactive dimer during ordinary conditions. Upon activation, dimeric HSF3 is converted into trimers capable of binding HSEs, but the temperature needed for association of HSF3 subunits is higher than for HSF1, suggesting that the former has a role during severe and persistent stress in avian cells. HSF4 is novel, occurring as two isoforms due to splicing. HSF4a functions as a repressor of HSP gene expression and HSF4b has the potential to transactivate HSP genes [120]. Both isoforms lack the hydrophobic heptad repeat sequence (HR-C) necessary for suppression of HSF trimer formation, leading to constitutive trimerization and DNA binding activity *in vitro* [110]. HR-C, a peptide of 33 amino acid residues, is well conserved among the vertebrate HSFs including HSF1 HSF2 and HSF3. HSF-4a contains a DNA-binding domain and N-terminal hydrophobic heptad repeats (HR-A/B), but lacks a transcription activation domain, acting as a repressor of other HSFs through binding directly to HSEs or by oligomerizing with other members of the family [121]. Other evidence indicates that HSF-4a represses basal transcription by interaction with TFIIF through the RNA polymerase II-associating protein [120].

HSFs do not substitute functionally for each other, but interactions between different HSFs are coordinated in order to mediate HSP gene transcription. For instance, HSF3 may regulate HSF1 activity because deletion of HSF3 in chicken cells eliminates HSF1 binding ability after heat shock [122]. Additionally, human HSF2 is associated with

HSF1 before and after heat shock, mediating the response of HSF2 to increased temperature [123].

By comparison with HSFs which mediate stress-induced activation of genes, the understanding of transcription factors involved in the developmental regulation of HSP gene expression is modest. As an EcRE binding protein responsible for initiating developmental expression of HSP genes, the 20-hydroxyecdysone receptor has been described in *Drosophila* [101]. 20-hydroxyecdysone, a steroid hormone, is synthesized at a specific developmental stage in *Drosophila* and activates its receptor directly or through a regulator, thus mediating HSP gene expression. For example, promoters in *Drosophila* hsp27, hsp22, and hsp26 are directly regulated by the ecdysterone receptor bound to ecdysterone, but the hsp23 promoter depends on a poorly defined putative regulator which mediates binding between ecdysterone and its receptor.

Pax-6 is a member of the paired-domain family of transcription factors contributing to developmental regulation of HSPs and it is essential for eye development [124]. Pax-6 is expressed in presumptive lens cells of the ectoderm overlying the outgrowing optic vesicle, prior to initiation of mouse lens formation and the expression of crystallins. Pax-6 interacts with LSR1 and LSR2 and activates the αB-crystallin promoter in the lens [124].

In addition to regulating several housekeeping and developmentally expressed genes other than those for HSPs, the GAGA factor has a critical role in promoting attachment of the TFII D complex to target sequences such as the hsp26 gene in *Drosophila* [89]. The GAGA factor interacts with two $(CT)_n \cdot (GA)_n$ elements and may modify chromatin structure, helping to maintain the promoter in an open conformation. In this context,

analysis of hsp70 gene promoter sequences indicates that the GAGA factor, in conjunction with NUFR, an ATP-dependent remodeling complex, disrupts chromatin. Also, the GAGA factor interacts with dSAP18, a member of the dSin3 complex that displays histone deacetylase activity and contributes to remodeling of nucleosome, leading to access of transcription factors to the promoter [125]. Other factors affect transcription of HSP genes and as an example, BRCA1, involved in breast cancer, may modulate mammalian heat shock response pathways [126].

1.4 p8, a Co-transcription Factor

The co-transcription factor p8 was discovered when studying the molecular response of the injured rat pancreas, and its expression, structure and function have been investigated extensively [127, 128]. p8 is up-regulated during pancreatitis; however p8 mRNA expression is constitutively high in salvary glands, moderate in stomach, colon, liver and kidneys, slight in lungs, heart, duodenum, jejunum and ileum, and absent in brain, spleen, testes, thymus and skeletal muscles in healthy control animals. The p8 gene has been detected in several other species including human, mouse, frog and fruit flies [129].

p8 plays an important role in diseases such as pancreatitis and cancer [130]. Acute pancreatitis is the most frequent disease of the pancreas, during which hydrolytic enzymes are activated, leading to digestion of pancreatic and peripancreatic tissues [130]. In response to this stress, total cellular RNA decreases by 40% and transcripts encoding trypsinogen I, chymotrypsinogen B, procarboxypeptidase A, proelastase I and amylase, decrease by greater than 50%. However, p8 is over-expressed and the mRNA encoding

p8-dependent pancreatitis associated protein (PAP), an anti-inflammatory factor, increases more than 200 times [129]. The absence of functional p8 alters the course of acute pancreatitis. For example, p8-knock-out animals develop a more severe disease phenotype with serum levels of amylase and lipase higher than those observed in wild type animals [129]. p8 is also involved in cancer progression but research results vary. Some experiments demonstrate that p8 is conducive to development of pancreatic cancer and that suppression of p8 in cancer cells impedes tumour progression [130, 131], whereas in other experiments, p8 inhibits the growth of human pancreatic cancer cells [132, 133]. A better understanding of p8 effects on cancer cells is required in order to determine its role in cancer progression and metastasis.

p8 cDNA has been cloned from mouse, rat and human. In humans, p8 is 82 amino acids long and shows an overall similarity of 74% with rat p8 which contains 80 amino acid residues [134]. The human p8 gene consists of three exons and two introns and it maps to chromosome 16. The sizes of exons I, II and III are 214, 150 and 329 nucleotides, respectively. Neither proximal authentic CAAT, TATA nor the initiator sequence which directs the site of initiation and basal level of transcription in TATA-less promoters was present in the 5'-flanking region. The mouse p8 polypeptide is 80 amino acids long and shows 91% and 75% identity with rat and human counterparts, respectively. Exons I, II and III are 148, 144 and 334 nucleotides, respectively. The mouse p8 promoter contains putative regulatory elements, including a TATA box in position +43 as well as C/EBP, Sp1 Oct-1, Ap-1, NFkB, IL-RE, CREB and Myc binding sits. The site-directed mutagenesis of CAAT, the C/EBP binding site, decreased promoter activity to 5% [134].

Different elements upstream of p8 gene between human and mouse predict the difference in regulatory mechanisms of the gene transcription.

Biochemical and biophysical analysis demonstrate that p8 is similar to the HMG-I/Y protein although the identity between p8 and HMG-I/Y protein is only 35% and the AT hook motif is not conserved. p8 binds DNA because it possesses a helix-loop-helix motif, characteristic of some families of DNA-interacting proteins [135]. Furthermore, the deduced human p8 amino acid sequence contains a canonical, bipartite, nuclear targeting signal at position 63 (KLVTKLQNSERKKRGA) and the protein is detected almost exclusively within the nucleus of COS-7 cells transfected with p8 expression plasmids [134]. Like some transcription factors, p8 is a phosphoprotein, and its DNA binding affinity is enhanced when phosphorylated by protein kinese. p8 is thought to promote gene activation through relieving histone H1-mediated repression of transcription and by facilitating the formation of enhanceosomes as a consequence of both protein/DNA and protein/protein interactions because proteins like p8 have the capacity to bend, straighten, unwind and induce loops or supercoil formation in linear DNA molecular *in vitro* [136]. p8 is also acetylated by p300 and interacts with this protein, enhancing the effect of p300 on Pax2A and Pax2B transcription of glucagon gene promoters, whereas p300 in isolation increased Pax2 trans-activation activity weakly and p8 alone had no significant effect [131]. Taken together, p8 is a co-transcription factor which modulates the expression of other genes.

Clearly, diapause is not just the simple cessation of cell activity and induction of diapause requires coordination of many enzymes and proteins. This led to the central

hypothesis, namely that entry into diapause is due to the differential expression of genes, including those encoding regulatory and structural proteins, and maintenance of cellular integrity during diapause requires protection provided by molecular chaperones such as the small HSPs. *Artemia* was used in this study as a model animal to explore, by subtractive hybridization, gene expression during encystment. This work leads to the discovery of several up-regulated genes, including those for the sHSPs ArHsp21 and ArHsp22, and p8, a co-transcription factor. In addition, gene structure and regulation were examined for p26, a well characterized, developmentally regulated sHSP thought to have a role in *Artemia* diapause. The results indicate that ArHsp21 and ArHsp22 provide protection to other molecules during *Artemia* diapause, and p8 modulates partial gene expression involved in *Artemia* diapause. Additionally, p26 gene contains one intron in the 5'-UTR and some transcription factor binding sites, which may regulate its developmental expression during *Artemia* diapause transition.

II. Materials and Methods

2.1 *Artemia* Strains and Culture

The strains used were *A. franciscana* (Great Salt Lake [GSL]), *A. franciscana* (San Francisco Bay [SFB]), *A. urmiana, A. sinaca, A. tibetiana, A. parthenogenetic* and *A. persimilis. A. franciscana* (GSL) was purchased from Brine Shrimp Direct, Ogden, Utah, USA, while other strains were from Dr. Peter Bossier, Agricultural Research Center-Ghent/Department of Sea Fisheries (CLO-DVZ), Ankerstraat 1, 8400 Oostende, Belgium. Cysts were hydrated in cold distilled water overnight at 4 °C and then rinsed three times with cold distilled water. To obtain nauplii, the hydrated cysts were incubated at 27 °C with shaking for 20 h at 200 RPM in Hatch Medium containing 422 mM NaCl, 9.4 mM KCl, 25.4 mM $MgSO_4 \cdot 7H_2O$, 22.7 mM $MgCl_2 \cdot 6H_2O$, 1.4 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $NaHCO_3$ and 0.1% disodium tetraborate. Nauplii were transferred to glass tanks containing sea water and fed with ISO algae and Roti-Rich Liquid Invertebrate Food (Florida Aqua Farms Inc. USA) two times per day. Waste was removed from tanks every day and the water was changed once per week. As required for developmental studies, *Artemia* females with embryo-filled lateral pouches coupled with a male were placed in 6-well culture plates and examined with a dissecting microscope as development progressed. At one day intervals after fertilization, lateral pouches were removed in Hatch Medium and embryos developing into either cysts or nauplii were collected and frozen in liquid nitrogen.

2.2 Preparation of Genomic DNA from *A. Franciscana* (GSL)

Five grams of nauplii were homogenized on ice in 20 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) using a Dounce homogenizer and filtered through a single layer of Miracloth (Calbiochem, La Jolla, CA). The homogenate was centrifuged for 5 min at 2,000 x g, and the pelleted nuclei were resuspended in 20 ml of SSC. SDS was added to 1% and the solution was extracted twice with one volume of phenol (Invitrogen, Carlsbad, CA, USA) /chloroform (Fisher Scientific, New Jersey, USA) /isoamyl alcohol (Fisher Scientific, New Jersey, USA) (25:24:1) The aqueous phase was dialyzed against 2 changes of SSC at 4 °C for 36 hr, incubated with alpha-amylase (Sigma, ST Louis, MO, USA) and RNase (Invitrogen) at 37 °C for one hr followed by treatment with proteinase K (Invitrogen) for one hr at 37 °C. The DNA solution was dialyzed against 2 changes of 10 mM Tris, 1 mM EDTA, pH 7.4 for 36 hr. DNA quality was determined by measuring the 260/280 ratio and by electrophoresis in 0.4% agarose gels.

2.3 Identification of Genes Up-regulated Early in Oviparous Development of *Artemia* Embryos

2.3.1 cDNA preparation for subtractive hybridization

Ovoviviparous and oviparous embryos two days post-fertilization were harvested and their total RNA was isolated with Trizol (Invitrogen). One μg of total RNA was incubated at 72 °C for 2 min, cooled on ice for 2 min and then reverse transcribed to first-strand cDNA at 42 °C for 1 hr in a 10 μl reaction mixture containing 1 μl of 5'BD SMART CDS primer II A (5'-AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$VN-3), 1 μl of BD SMART II A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAAGTACG CGGG-3'), 2 μl of 5 X first-strand, 1μl of 20 mM DTT, 1 μl of 10 mM dNTP and 1μl of

BD PowerScript Reverse Transcriptase (BD Bioscience, Mississauga, ON, Canada). Forty µl of TE buffer was added to each tube, which was then heated at 72 °C for 7 min. PCR was performed in 100 µl reactions containing 1 µl of first-strand cDNA, 10 µl of 10 x BD Advantage 2 PCR Buffer, 2 µl of 50 x dNTP (10 mM of each dNTP), 2 µl of 5'PCR Primer II A (5'-AAGCAGTGGTATCAACGCAGAGT-3') and 2 µl of 50 x Advantage 2 Polymerase Mix (BD Bioscience). The reaction conditions were 95 °C for 1 min and then 18 cycles of 95 °C for 15 sec, 65 °C for 30 sec and 68 °C for 6 min. The pooled PCR products were purified with phenol:choloroform:isoamyl alcohol (25:24:1) and concentrated with butanol extraction until the volume was 40-70 µl. The concentrated PCR products were applied to a BD CHROMA SPIN 1000 Column (BD Bioscience) to remove small PCR fragments and two washes were performed with sequentially 25 µl and 150 µl of TNE buffer (BD Bioscience). The cDNA was eluted with 350 µl of TNE buffer.

2.3.2 Subtractive hybridization

In order to obtain cDNA representatives of genes up-regulated during encystment, cDNA from oviparously developing embryos 2 days post-fertilization was used as tester and cDNA from the ovoviviparous pathway 2 days post-fertilization as driver. Both cDNAs were digested at 37 °C for 3 hr with Rsa I (MBI, Fermentas, Hanover, MD, USA) and the reaction was terminated by addition of 8 µl of 0.5 M EDTA. A mixture containing 170 µl of Rsa I-digested cDNA, 680 µl of NT2 buffer and 17 µl of Nucleo Trap Suspension (BD Bioscience) was incubated at room temperature with gentle agitation every 2-3 min. After centrifugation at 10,000 x g for 1 min, the pellet was washed twice with NT3 buffer and the cDNA was eluted with TE buffer. The DNA was

precipitated with 2 volumes of ethanol containing ammonium acetate, dried and dissolved in 6.7 μl of TNE buffer.

Tester cDNA was diluted five-fold with sterile H_2O and divided into two parts for ligation with Adaptor 1 (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCC GGGATGGT-3') and Adaptor 2R (5'-CTAATACGACTCCACTATAGGGCAGCG TGGTCGCGGCCGAGGT-3') in a 10 μl mixture containing 2 μl of diluted tester cDNA, 2 μl of adaptor, 2 μl of 5 x ligation buffer and 1 μl of T4 DNA ligase (400 units/μl) (BD Bioscience). The mixture was incubated at 16 °C overnight, then heated at 72 °C for 5 min to inactivate the ligase. The first hybridization was performed in a 4 μl mixture containing 1.5 μl of Rsa I–digested driver cDNA, 1.5 μl of Adaptor-ligated Tester and 1 μl of Hybridization buffer at 98 °C for 1.5 min and 68 °C for 8 hr. One μl of denatured driver cDNA was mixed with the products of the first hybridization reactions and incubated at 68 °C overnight for the second hybridization. Two hundred μl of dilution buffer was added to the second hybridization mixture followed by heating at 68 °C for 7 min.

2.3.3 Construction of a subtractive hybridization library

One μl of hybridization product was used as template for PCR in a mixture of 25 μl containing 2.5 μl of 10 x PCR reaction buffer, 0.5 μl of dNTP (10 mM), 1 μl of PCR primer 1 (10 μM) (5'-CTAATACGACTCACTATAGGGC-3') and 0.5 μl of 50 x Advantage cDNA Polymerase Mix. The mixture was incubated at 75 °C for 5 min followed by 27 cycles of 94 °C for 30 sec, 66 °C for 30 sec and 72 °C for 1.5 min. These PCR products were diluted 10-fold and 1 μl was used in a second PCR, for which the

forward primer was nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and the reverse primer was nested PCR primer 2R (5'-AGCGTGGTCGCGGCCGAG GT-3'). The reaction conditions were 12 cycles of 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 1.5 min. The PCR products were pooled, purified with the NucleoTrap PCR Purification Kit (BD Bioscience), and inserted into a T/A cloning vector (Invitrogen), followed by transformation of TOPO10 competent *E. coli* (Invitrogen).

2.3.4 Screening the subtractive library

Transformed *E. coli* were plated and white colonies picked for culture in a 96 well block. To select clones containing inserts more than 700 bp in length, PCR was performed using the M13 Forward Primer (5'-GTAAAACGACGGCCA-3') and the M13 Reverse Primer (5'-CAGGAAACAGCTATGAC-3'). Reaction conditions were 95 °C for 5 min followed by 25 cycles of 95 °C for 1 min, 45 °C for 1 min, 72 °C for 3 min. Sequencing was done at Bio S&T Inc, Lachine, Quebec.

2.3.5 Verification of differential gene expression during embryo development

Total RNA was prepared with Trizol (Invitrogen) from embryos 2 days post-fertilization undergoing ovoviviparous and oviparous development. Contaminating DNA was digested with the TURBO DNA-free kit (Ambion, Austin, TX, USA), and first-strand cDNA was synthesized at 37 °C for 1 hr in a mixture of 33 µl containing 11 µl of First-strand Reaction Mix (Amersham Bioscience, Baie d'Urfe, Quebec, Canada), 1 µl of primer (Not I-d$(T)_{18}$), 1 µl of DTT and 20 µl of RNA. To determine the expression of candidate genes, PCR was performed with the Advantage-2 PCR Enzyme System (BD Bioscience) in a total volume of 25 µl containing tubulin control gene primers and gene specific primers (Table1). The reaction conditions were 1 min at 95 °C, followed by 25

Table 1. Primers for detection of differentially expressed candidate genes

No.	Protein name	Primer sequences
Conti-04	neuralized-related protein	5'-CCTCCAGTGCTCCATCAAGTAGT-3' 5'-AAGTTGTCGAGAAATGGAGATGC-3'
Conti-11	sacbrood virus polyprotein	5'-CCACGGCTCATTGTTTCTCTTAC-3' 5'-CTACCGGATTTTTCAGCCAGTTC-3'
Conti-16	steroid dehydrogenase	5'-ACGAAAGTTTGTGGAGTTGGTAT-3' 5'-GCACTACTACCTACCCCTCTCAT-3'
Conti-20	FLILRR associated protein	5'-ATCAGCTAGAATCAGTTGTGGAA-3' 5'-AGTGTTGAAGATTTTTGGAGATG-3'
Conti-27	Erv1-like growth factor	5'-GACATAAGCTGAGAAGGTGGTA-3' 5'-GTTTTCCTACTTTTTCGTTGAC-3'
Conti-29	chitinase like precursor	5'-TGTAAAGGAAGTTGACGGAAGA-3' 5'-AGAGGCATTGACGGTAACTTTC-3'
1A-06	superoxide dismutase	5'-TTCCAGGTGGCAGATTGA-3' 5'-ACGCGGGGTTATGACAGT-3'
1B-03	N33	5'-AACAAAAAGCACACAAATACCAT-3' 5'-GAATATATGCCGTTGTTGGTCTA-3'
1B-10	PTPA	5'-GACCACTTCCATAATCAATCCT-3' 5'-TGAAGCAGTGAAGAATAAACCA-3'
1B-11	sidekick	5'-TTGAACAGACCTTGATTG-3' 5'-TGATCAAAACAGAAAGGA-3'
1C-08	cathepsin	5'-ATTCATACTTGGGGATTTTT-3' 5'-AGGTAGTAGGATCAGGTGTGACGA-3'
1C-09	p8	5'-TCGGAAGTCCGGGACCTATAGAAT-3' 5'-ACATGATGTTTGAGGTTTTG-3'
1C-10	uncoupling protein	5'-AAAAAGCAAATAAGAATAGCA-3' 5'-AAACAGCTGTGAAAGAAGGAC-3'
1D-01	chloride channel protein	5'-GAAAACAACACCAACAAAACAGG-3' 5'-AGAAGAAAGACAAAGGGCAAAAG-3'
1D-09	fatty-acyl reductase	5'-CAGGACAAGATGCTAAAAAT-3' 5'-AATGAAAGACAATGGAGACA-3'
1E-10	CDK5 activating protein	5'-CAGCAACGCCTACATTACAAG-3' 5'-ACTTGGACGTTCTCTTGGTTC-3'
2A-01	DDH	5'-CAGTTTTGCCTATGGGTCAGAGC-3' 5'-ATGGCGGTAGAGGAAAATCGTAT-3'
2B-03	BAP1	5'-GACCCTGGTTTATTCACACTGCT-3' 5'-AATAATTCCGCTTCATCCACTTG-3'
2C-09	P26-like (ArHsp22)	5'-CCAAGCGACATTACAGTGAACA-3' 5'-CTGAATCCTTCTTTGCCTTGGCTC-3'
2E-04	PMCBP	5'-CACCAATCCCATAGAGCAT-3' 5'-GAATGCCCGAATAGACAAC-3'
2F-03	QM	5'-TAGGCAGAAAGAAGGCATCA-3' 5'-AGCTGGTGGTTCTCCTTCAT-3'

Table 1, continued

2F-06	chromatin-remodeling factor	5'-TTTGGGATTATTGGGAGTTG-3' 5'-ACGAGTGGATGAACGAAGAA-3'
2G-03	cytochrome P450	5'-ACGTTCTTAGGTTGGTTCTTCC-3' 5'-TCGGTGACTTTGTGGGTTATTA-3'
2G-09	GPI	5'-AACCAATGGTCAACACGCTTTC-3' 5'-CACAGCCGTCTTACCCTTCATC-3'
3B-01	peritrophin-like protein	5'-GTACTACCCCTCGCCTGAA-3' 5'-TCCTCCCGAGTTTATGCTA-3'
3C-11	CoA desalturase	5'-TTGCTGGTGCTGGAAATAATGG-3' 5'-CAGTAAGCGGCAGCACAGAAAT-3'
3C-12	hsp16 (ArHsp21)	5'-AGACCACAATCCCGTCACCTGTTT-3' 5'-TCAATCTTGACGTCCTCGGGCTTA-3'
3F-03	RNA-binding protein	5'-GTTTCTCTCCTTTTTCCAATCA-3' 5'-ATACAGAGGAAGAAGCAGAACG-3'
3F-04	ALHD	5'-TCTTGGGAGAATGATACTGCTT-3' 5'-TCCATATGGCTGCTCTAAGTAA-3'
3F-07	hVPS16	5'-CTTTCGCCAACTGATTTCT-3' 5'-GGTTACTTTGGACATCTTT-3'
3F-09	takeout	5'-GGAAGATGTGTTGGATGAGGTG-3' 5'-GGCAATAGAAAGCACCGAATAA-3'
3G-07	F-box and WD-40 domain protein	5'-GTCATGCCAGGATAAAGC-3' 5'-TCCAAGGTATGTAGACGA-3'
3H-06	LTA4	5'-CATATGGGATTGAGGAGAAAGC-3' 5'-TAGCCCATGAAATAGCACACAG-3'
Control	Tubulin	5'-CTGCATGCTGTACAGAGGAGATGT-3' 5'-CTCCTTCAAGAGAGTCCATGCCAA-3'

cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 30 sec. PCR products were electrophoresed in 2% agarose gels and stained with Gel Star (Gambrex Bio Science, Rockland, Inc. USA).

2.4 Characterization of *Artemia* Small Heat Shock Proteins

2.4.1 5'-RACE for ArHsp21 and ArHsp22

Total RNA was prepared from oviparous embryos 2 days post-fertilization as described above. Ten μg of total RNA was incubated with calf intestine alkaline phosphatase (CIP) (Ambion) at 37 °C for 1 hr and then purified with acid phenol:chloroform. The purified RNA was treated with tobacco acid pyrophosphatase (TAP) (Ambion) at 37 °C for 1 hr and ligated with the 5'-RACE Adaptor (5'-GCUGAUGGCGAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3') using T4 RNA ligase. Ligated RNA was reverse transcribed to cDNA at 42 °C for 1 hr in a mixture containing random decamers and M-MLV reverse transcriptase (Ambion) and dNTP. Nested PCR was performed using 5'-RACE outer primer (5'-GCTGATGGCGATGAATGAACACTG-3'), 5'-RACE inner primer (5'-CGCGGATCC GAACACTGCGTTTGCTGGCTTTGATG-3'), gene-specific outer primer (ArHsp22 outer primer: 5'-CGGCCATTCTTCTTCATTCTCACC-3'; ArHsp21 outer primer: 5'-GTCTAAGAATATCAGTCCTCTCGTTTGCT-3'), and gene-specific inner primer (ArHsp22 inner primer: 5'-CTGAATCCTTCTTTGCCTTGGCTC-3'; ArHsp21 inner primer: 5'-CGATTGATAGGGATTTCTTTAGGCATGG-3') based on sequences from subtractive hybridization. Outer and inner 5'RLM-RACE PCR conditions were 94 °C for 3 min and then 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min, followed

by 72 °C for 7 min. The PCR products were inserted into a T/A cloning vector (Invitrogen), followed by transformation into TOPO10 Competent *E. coli* (Invitrogen) for sequencing. Gene Runner was used for protein translation.

2.4.2 3'-RACE for ArHsp21 and ArHsp22

For reverse transcription, 1 μg of total RNA was incubated at 42 °C for 1 hr with 3 μl of 3' RACE Adaptor (5'-GCGAGCACAGAATTAATACGACTCACTATAGGT$_{12}$ VN-3'), 4 μl of dNTP mix, 2 μl of 10 x RT buffer, 1 μl of RNase inhibitor and 1 μl of M-MLV Reverse Transcriptase. Two nested PCRs for 3' RLM-RACE were performed with denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min, and an extension of 72 °C for 7 min. The PCR primers were: 3'-RACE outer primer (5'-GCGAGCACAGAATTAATACGACT-3'), 3'-RACE inner primer (5'-CGCGGATCCGAATTAATACGACTCACTCACTATAGG-3'), gene-specific outer primer (ArHsp22 outer primer: 5'-CCAAGCGACATTACAGTGAAC AGC-3') and gene-specific inner primer (ArHsp22 inner primer: 5'-AACGATGAA TTCTGGCGAGTTAAGC-3') based on the sequences from subtractive hybridization. 3'-RACE was not necessary for ArHsp21 because its complete 3'-end cDNA was obtained by subtractive hybridization. The products were cloned and sequenced as described for 5'-RACE .

2.4.3 Detection of ArHsp21 and ArHsp22 genes on Southern blots

DNA was isolated from nauplii with phenol/chloroform/isoamyl alcohol (25:24:1). ArHsp21 and ArHsp22 cDNAs were labeled with digoxigenin-11-dUTP by using PCR Dig Labeling Mix (Roche, Mississauga, Ontario, Canada). PCR was performed with ArHsp21 forward primer: 5'-GCCTCGAGATGTCAGGTATGAGACTT-3'; ArHsp21

reverse primer: 5'-GCAAGCTTGAATATCAGTCCTCTCGT-3'; ArHsp22 forward primer: 5'-GCGTCGACATGACTACCCTAGTACCTTGGA-3'; ArHsp22 reverse primer: 5'-CGCTGCAGATTACGGCCATTCTTCTTCATT-3'. The conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 30 sec, 72 °C for 45 sec. The PCR products were gel purified using the GFX PCR and Gel Band Purification Kit (Amersham Bioscience) according to manufacturer's instructions. Fifteen μg of genomic DNA was digested with restriction enzymes, including Bam HI, Hind III, Pst I, Xho I, Sal I and Eco RI for which no recognition sites existed in ArHsp21 and ArHsp22 cDNAs, precipitated with ethanol and dissolved in 20 μl of TE buffer. Digested DNA was electrophoresed in 0.7% agarose gels which were submerged twice for 15 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl) at room temperature, followed by immersion twice in neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 min, and once in 20 x SSC for 10 min. DNA was transferred to nylon membranes (Roche) using the Rapid Downward Transfer System/Buffer Tray (Schleicher & Schuell, New Hampshire, U.S.A.). The membranes were washed in 2 x SSC, baked at 80 °C for 2 h, prehybridized in Dig Easy Hyb (Roche) at 42 °C for 6 hr and then hybridized overnight with purified probes in Dig Easy Hyb at 46 °C. The membranes were washed twice at room temperature in 2 x SSC containing 0.1% SDS and twice at 68 °C in 0.5 x SSC containing 0.1% SDS. The membranes were reacted with CDP-Star (Roche) following the manufacturer's instruction and exposed to X-ray film (FUJI photo film Co., Ltd, Tokyo, Japan).

2.4.4 Expression of ArHsp21 and ArHsp22 in *E. coli BL21* (DE3) pLysS

Total RNA was isolated from oviparous embryos 2 days post-fertilization and genomic DNA contamination eliminated with the TURBO DNA-free kit (Ambion). RT-PCR was performed with Pfu DNA polymerase (MBI) and primers based on sequences from 5' and 3'-RACE. The ArHsp21 forward primer was 5'-GCCTCGAGATGTCAG GTATGAGACTT-3' including an Xho I restriction site, and the reverse primer was 5'-GCAAGCTTGAATATCAGTCCTCTCGT-3' including a Hind III site. The ArHsp22 forward primer was 5'-GCGGATCCATGACTACCCTAGTACCTTGGA-3' including Bam HI, site and the reverse primer was 5'-CGCTGCAGATTACGGCCATTCTTCTTC ATT-3 including a Pst I site. After addition of adenine with A-Addition kit (Qiagen) all RT-PCR products were cloned into a T/A vector (Invitrogen), followed by transformation of TOPO10 competent *E. coli* (Invitrogen). Plasmid DNA was purified, digested with the enzymes mentioned above and resoved in agarose gels. Insert bands were removed and purified. The corresponding enzymes were used to digest the His-tagged expression vector pRSET A (Invitrogen) which was gel purified. Inserts were ligated into the linearized pRSET A vector followed by transformation into TOP 10 F' *E. coli*. The plasmid was purified, the insert was sequenced, and the plasmid was transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen) for expression. Cells were grown to an OD_{600} of 0.6 and induced by incubation in 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) for 6 hr.

2.4.5 Purification of ArHsp21 and ArHsp22

Bacteria were harvested by centrifugation at 2,000 x g for 15 min at 4 °C, washed with PBS and resuspended in Equilibration/Wash buffer (BD Bioscience) followed by addition of protease inhibitor solution A (40 µl/ml) and inhibitor solution B (20 µl/ml)

and lysozyme (0.75 mg/ml in sterilized water) (Sigma). Mixtures were incubated at room temperature for 20 min, frozen in liquid nitrogen and thawed in a water bath at 42 °C three times, and then sonicated three times for 10 sec, with cooling on ice for 30 sec between each sonication. The cell lysate was centrifuged at 12,000 x g for 10 min and the soluble extract incubated with the BD TALON metal affinity resin equilibrated in W/E buffer (BD Bioscience) for 20 min. The resin was washed twice for 10 min with 15 bed volumes of buffer consisting of 10 mM imidazole, 50 mM sodium phosphate and 500 mM NaCl, pH 7.5, placed in a column, washed once with 10 bed volumes of the same buffer and eluted with 5 bed volumes of Elution Buffer (BD Bioscience). The eluate was collected in 500 µl fractions which were electropheresed in 12.5% SDS/polyacrylamide gels. Fractions containing polyhistidine-tagged proteins were pooled, dialyzed in 10 mM phosphate buffer, pH 7.2 for 4 hr at room temperature with a change of buffer, and overnight at 4 °C using Slide-A-Lyzer 7 K Dialysis Cassettes (Pierce, Woburn, MA, USA), followed by concentration in Microcon YM-10 centrifugal filter devices (Millipore Corporation, Bedford, MA, USA).

2.4.6 Preparation of antibodies to ArHsp21 and ArHsp22

ArHsp21 and ArHsp22 purified from bacteria were emulsified with Titermax Gold Adjuvant (Sigma), injected subcutaneously into rabbits three times at 15-day intervals and the serum was harvested 45 days after the first injection. Rabbits were obtained from Charles River Canada (St. Constant, Quebec, Canada) and care for in accordance with guidelines in "Guide to Care and Use of Experimental Animal" available from Canadian Council on Animal Care. To test antibody activity, bacteria extracts containing ArHsp21 and ArHsp22 were mixed with 4 x treatment buffer, placed in a boiling water bath for 5

min and electrophoresed in 12.5% SDS polyacrylamide gels, followed by blotting to nitrocellulose membranes. The blots were probed with antibodies to ArHsp21 and ArHsp22 followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson Immunologicals, West Grove, PA, USA) diluted 1:10000 in HST. Enhanced chemiluminescence reagent (PerkinElmer Life Science, Boston, MA, USA) was applied to the membrane for 1 min and then the blot exposed to X-ray film (FUJI photo film., Ltd, Tokyo, Japan).

2.4.7 Oligomerization of ArHsp21 and ArHsp22

Ten ml 10-50% continuous sucrose gradients in 0.1 M Tris-glycine (pH 7.4) were formed with a sucrose gradient maker (MRA, Boston, MA, U.S.A.) Four hundred µl samples of protein extract from bacteria containing p26, ArHsp21 and ArHsp22 were loaded individually on gradients and centrifuged at 200,000 x g for 16 hr at 4 °C in a Beckman SW41 T1 rotor. The tubes were bottom punctured with a 21-gauge needle and 800 µl fractions were collected. Thirty µl from each fraction was mixed with 4 x treatment buffer and 8 µl of the mixture was electrophoresed in 12.5% SDS ployacrylamide gels for immunodetection with Omni-probe (Santa Cruz Biotech, Santa Cruz, CA, USA), an antibody recognizing the $(His)_6$ tag. The molecular mass markers of 669 kDa (thyroglobulin), 443 kDa (apoferritin), 200 kDa (α-amylase), 66 kDa (bovine serum albulin) and 29 kDa (carbonic anhydrase) (Sigma) were centrifuged individually as described and the A_{280} of each fraction was measured using a SPECTRAmax PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine their location in the gradient.

2.4.8 Chaperone activity of ArHsp21 and ArHsp22

Citrate synthase (Sigma) at 150 nM was mixed with purified ArHsp21 and ArHsp22 at final concentrations of 37.5 nM, 75 nM, 150 nM, 300 nM and 600 nM, in 40 mM HEPES/KOH buffer, pH 7.5. One ml reaction mixtures were placed in a cuvette and heated at 43 °C in a water bath with the aggregation of citrate synthase monitored by measuring light scattering at 360 nm using a SPECTRAmax PLUS spectrophotometer (Molecular Devices) at 2 min intervals for 1 hr.

Ten mg of insulin (Sigma) was dissolved in 1 ml 0.1 N NaOH and adjusted to pH 7.2 with 0.5 M sodium phosphate buffer, pH 6.8, yielding a stock solution which was diluted with 10 mM phosphate buffer containing 100 mM NaCl to produce a working solution. Protection against reduction induced protein denaturation was performed in a mixtue containing insulin at 0.023 mg/ml (4 μM), DTT at 20 mM and purified ArHsp21 and ArHsp22 at 0.05 μM, 0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM or1.6 μM. Reaction mixtures were 300 μl and protein aggregation was measured at A_{400} in a 96-well plate at 25 °C with a SPECTRAmax PLUS spectrophotometer (Molecular Devices) at 2 min intervals for 30 min. Bovine serum albumin (Sigma) and bovine immunoglobulin (IgG) (Sigma) were used to control for non-specific protection of citrate synthase and insulin.

2.4.9 ArHsp21 and ArHsp22 synthesis during *Artemia* development

Encysted *Artemia* embryos, emerged nauplli (EIII), instar I nauplii, instar II larvae and adult males were harvested, washed once with cold Hatch Medium, transferred to mini Dounce homogenizers and homogenized on ice for 10 min in PIPES buffer (100 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.5) containing protease inhibitor solutions A and B. The homogenate was centrifuged at 12000 x g for 10 min at 4 °C, after which the supernatant was transferred to a fresh tube, and centrifuged at the same speed for 5

min. Samples of supernatant were mixed with 4 x treatment buffer and placed in a boiling water bath for 5 min. Twenty µl of each sample was electrophoresed in 12.5% SDS plolyacrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose and probed with antibodies to p26, ArHsp21 and ArHsp22.

Embryos undergoing either oviparous or ovoviviparous development were collected at daily intervals from 8 females at days 0 through 5 post-fertilization. The embryos were rinsed with Hatch Medium and homogenized in 0.4 ml of Trizol (Invitrogen) with a mini-homogenizer (Radnoti, Monrovia, CA, USA). The homogenate was incubated for 5 min at room temperature followed by addition of 80 µl of chloroform. The mixture was centrifuged at 12,000 x g for 15 min at 4 °C and the upper protein-containing phase was mixed with 120 µl of ethanol followed by incubation at room temperature for 3 min. After centrifugation at 2,000 x g for 5 min at 4 °C, the supernatant was incubated with 600 µl of isopropyl alcohol for 10 min at room temperature and then centrifuged at 12,000 x g for 10 min at 4 °C. The pellet was washed three times in 95% ethanol containing 0.3 M guanidine hydrochloride and once in ethanol, and then dissolved in 30 µl of 1% SDS. The resulting solution was mixed with 4 x treatment buffer, placed in a boiling water bath for 5 min, electrophoresed in 12.5% SDS polyacrylamide gels, and either stained with Coomassie blue or blotted to nitrocellulose membranes, which were probed with antibodies to ArHsp21 or ArHsp22.

2.4.10 Quantification of ArHsp21 and ArHsp22 mRNA during *Artemia* embryo development

Total RNA was prepared with Trizol (Invitrogen) at daily intervals as described above from embryos of 4 females at day 0 to day 5 post-fertilization during oviparous

and ovoviviparous development. Genomic DNA was eliminated with the TURBO DNA-free kit (Ambion) and the RNA was reverse transcribed with the First-strand cDNA synthesis kit (Amersham Biosciences) following the manufacturer's instructions. PCR was performed in 96-well PCR plates (Bio-Rad, Mississauga, Ontario, Canada) using the iCycler (Bio-Rad) in a mixture of 25 µl which contained 0.5 µl of cDNA, 12.5 µl of Platinum SYBR Green qPCR supermix-UDG (Invitrogen), 0.5 µl of Rox reference dye (Invitrogen), 50 ng of forward primer and 50 ng of reverse primer. The reaction conditions were 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 15 sec, 55 °C for 30 sec, 72 °C for 30 sec, followed by an extension of 72 °C for 5 min. Gene specific primers were: ArHsp21 forward primer: 5'-AGACCACAATCCCGTCACCTGTTT-3', reverse primer: 5'-ATCAATCTTGACGTCCTCGGGCTT-3'; ArHsp22 forward primer: 5'-AGTGAACAGCACGGACAAGGAGAT-3, reverse primer: 5'-AGCCGGCATGATC TGAATCCTTCT-3'. Control gene primers were α-tubulin forward primer: 5'-CTGCAT GCTGTACAGAGGAGATGT-3' and reverse primer: 5'-CTCCTTCAAGAGAGTCCAT GCCAA-3'. Linear plasmids containing cDNA inserts were used as templates for PCR standards.

2.4.11 ArHsp21 and ArHsp22 synthesis during heat shock of *Artemia* larvae and adult males

Instar II larvae and adult males were put into 100 ml of well aerated Hatch Medium in 250 ml flasks, incubated at 39 °C for 1 hr in a water bath, then transferred to 27 °C for 2, 4 and 6 hr with shaking. The animals were homogenized on ice in PIPES buffer containing protease inhibitor solutions A and B. The homogenate was centrifuged at 12,000 x g for 10 min at 4 °C, the supernatant was recovered and centrifuged for another

5 min. Similar amounts of protein from each sample was electrophoresed in 12.5% SDS polyacrylamide gels and either stained with Commassie blue or blotted to nitrocellulose for immunodetection with antibodies to p26, ArHsp21 or ArHsp22.

2.5 Characterization of the Co-transcription Factor, p8

2.5.1 Cloning of full-length p8 cDNA

Based on the p8 partial sequence obtained from subtractive hybridization, 5'- and 3'-RACE were performed to obtain the full-length cDNA sequence. For 5'-RACE, total RNA was processed and transcribed to cDNA as described above. Nested PCR was performed with p8 outer primer (5'-CACCAAGAGCCCTACATGTTGCTA-3'), p8 inner primer (5'-TCGGAAGTCCGGGACCTATAGAAT-3'), adaptor outer primer (5'-GCTGATGGCGATGAATGAACACTG-3') and adaptor inner primer (5'-CGCGGATC CGAACACTGCGTTTGCTGGCTTTGATG-3'). For 3'-RACE, p8 outer primer (5'-AGGTAGTAGGATCAGGTGTGACGA-3') and inner primer (5'-ACCCTAGTGGACA CTCAAAGAAAGC-3'), adaptor outer primer (5'-GCGAGCACAGAATTAATACGAC T-3') and inner primer (5'-CGCGGATCCGAATTAATACGACTCACTCACTATAGG-3') were used. The reaction conditions were the same as those used for 5'- and 3'-RACE described above. All PCR products were cloned for sequencing as described above.

2.5.2 Detection of the p8 gene on Southern blots

p8 cDNAs were labeled with PCR Dig Labeling Mix (Roche) using forward primer 5'-GCGGATCCATGTCAGAAGATCATTTTGATA-3'and reverse primer 5'-CGCTGC AGAGTCATTTTTTGTCAGCACG-3'. Fifteen μg of genomic DNA was digested with restriction enzymes, including Bam HI, Hind III, Pst I, Xho I, Sal I and Eco RI,

precipitated with ethanol and then dissolved in 20 µl of TE buffer. Digested DNA was electrophoresed overnight in 0.7% agarose gels which were then submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) twice for 15 min at room temperature, followed by neutralization solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) twice for 15 min and then in 20 x SSC for 10 min. DNA was transferred to nylon membranes (Roche) with the Rapid Downward Transfer System/Buffer Tray (Schleicher & Schuell). Membranes were washed in 2 x SSC, baked at 80 °C for 2 h., prehybridized in Dig Easy Hyb (Roche) at 42 °C for 6 hr and hybridized in Dig Easy Hyb containing purified probes at 43 °C overnight. The remaining steps were as described for ArHsp21 and ArHsp22 genes.

2.5.3 Expression of p8 in *E. coli* BL21 (DE3) pLysS

Total RNA from oviparously developing embryos at day 2 post-fertilization was used as template to perform PT-PCR with the forward primer (5'-GCGGATCCATGTCA GAAGATCATTTTGATA-3'), including a Bam HI site and the reverse primer (5'-CGC TGCAGAGTCATTTTTTGTCAGCACG-3'), including a Pst I site. After addition of adenosine (Qiagen), the RT-PCR products were cloned into a T/A cloning vector (Invitrogen), followed by digestion with Bam HI and Pst I. The purified insert was ligated into the His-tagged pRSET A vector linearized with Bam HI and Pst I and transformed into *E. coli* TOP 10 F' (Invitrogen). Plasmid DNA recovered from these cells was transformed into *E. coli* BL 21 (DE3) pLysS (Invitrogen) for protein expression.

2.5.4 Production of antibody to p8

Bacteria containing p8 were harvested, centrifuged at 2,000 x g for 15 min at 4 °C and washed with PBS buffer. The pellets were resuspended in Equilibration/Wash buffer

(BD Bioscience) followed by addition of protease inhibitor solutions A (40 µl/ml) and B (20 µl/ml) and lysozyme (0.75 mg/ml) (Sigma). The mixture was incubated at room temperature for 20 min, frozen and thawed three times and then sonicated three times as described previously. The cell lysate was centrifuged at 12,000 x g for 10 min and the supernatant incubated with equilibrated BD TALON metal affinity resin for 20 min. The resin was washed twice for 10 min with 15 bed volumes of buffer (pH 7.5) containing 10 mM Imidazole, 50 mM sodium phosphate and 500 mM NaCl, loaded into a column, then washed with 10 bed volumes of the same buffer before elution of the protein with 5 bed volumes of Elution Buffer (BD Bioscience). The eluate was collected in 500 µl fractions, electrophoresed in SDS polyacrylamide gels and fractions containing polyhistidine-tagged proteins were pooled. Purified p8 was emulsified with Titermax Gold Adjuvant (Sigma) and injection into rabbits three times at 15-day intervals, with the serum harvested 45 days after the first injection

2.5.5 Immunodetection of p8 during embryo development

Embryos were collected from 12 females at daily intervals from day 0 through day 5 post-fertilization during both oviparous and ovoviviparous development. Protein samples were prepared as described previously and the resulting solution was mixed with 4 x treatment buffer, boiled for 5 min and applied to 12.5% SDS polyacrylamide gels which were either stained with Coomassie blue or blotted to nitrocellulose membrane. The blots were probed with antibodies to p8 as described previously.

2.5.6 Quantification of p8 mRNA during embryo development

Total RNA was reverse transcribed as described above. PCR was performed in 96-well PCR plates (Bio-Rad) using the iCycler (Bio-Rad) with 25 µl mixtures containing

0.5 µl of cDNA, 12.5 µl of Platinum SYBR Green qPCR supermix-UDG (Invitrogen), 0.5 µl of Rox reference dye (invitrogen), 50 ng of forward primer and 50 ng of reverse primer. The reaction conditions were 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 15 sec, 55 °C for 30 sec, 72 °C for 30 sec, followed by an extension at 72 °C for 5 min. The p8 forward primer was 5'-AGGTAGTAGGATCAGGTGTGACGA-3' and the reverse primer was 5'-TCGGAAGTCCGGGACCTATAGAAT-3'. The α-tubulin forward primer was 5'-CTGCATGCTGTACAGAGGAGATGT-3' and the reverse primer was 5'-CTCCTTCAAGAGAGTCCATGCCAA-3'.

2.5.7 p8 localization in *Artemia* nuclei

Ten g of hydrated cysts and instar II larvae were homogenized separately on ice for 5 min by hand in chilled mortars in 35 ml of HPC (0.5 M hexylene glycol, 0.05 M Pipes-free acid, 1 mM $CaCl_2$, pH 7.6) and the homogenates were filtered through one layer of Miracloth, followed by centrifugation at 2,000 x g for 10 min. The pellets were suspended in 40 ml of HPC buffer and then centrifuged twice at 2,000 x g for 10 min. The resulting pellets were suspended in 17.5 ml of HPC buffer and suspensions were layered on 25 ml of 75% Percoll (Sigma). Centrifugation was carried out in a Beckman JS-13 swinging bucket rotor at 16,000 x g for 30 min, the nuclear band was transferred to a fresh tube and diluted to a final volume of 15 ml with HPC buffer. The nuclei were placed on a fresh 25 ml cushion of 75% Percoll and centrifuged. The nuclear band was transferred to a fresh tube, mixed with an equal volume of HPC buffer and centrifuged. The nuclear pellet was suspended in 1 ml of HPC buffer.

Nuclei from cysts and instar II larvae were placed on slides coated with poly-L-lysine, fixed in 4% (w/v) paraformaldehyde at room temperature for 20 min, hydrated in

PBS for 5 min and then incubated with p8 antibody for 30 min. The samples were washed 3 min with PBSAT three times, followed by incubation with FITC-conjugated goat-anti-rabbit antibody (Jackson Immunologicals, West Grove, PA, USA) for 30 min. The samples were washed three times with PBS for 3 min, incubated with 0.001 μg/ml DAPI for 5 min, washed with water for 5 min and mounted in mounting buffer (0.5 M 1,4-diazabicyclo[2.2.2] octane [DABCO] in 80% glycerol). Slides were examined with epifluorescence microscopy.

2.5.8 Identification of putative p8 transcription regulatory sequences

Genomic DNA was digested overnight at 37 °C with Sau 3AI, Sal I, EcoR I, Hind III, Pst I and Xba I, and the fragments were ligated into corresponding cassettes (TaKaRa, Shiga, Japan) at 16 °C for 30 min. Nested PCR was performed with primers C1 (5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3'), S1 (5'-CAGCCTTCCAC TTTGCCCGGAGAAGAGATG-3'), C2 (5'-CGTTAGAACGCGTAATACGACTCAC TATAGGGAGA-3') and S2 (5'-TTGTCCATATCAAAGTTAAAGTGTTCAAATCTA TC-3'). C1 and C2 were based on cassette sequence, whereas S1 and S2 were designed on the basis of p8 cDNA sequence. The reaction conditions were 35 cycles of 94 °C for 30 sec, 60 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 5 min. The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) for sequencing and analysis with the Genomax program in website for transcription factor binding sites.

2.6 Characterization of the p26 Gene

2.6.1 Sequencing of p26 cDNA from several *Artemia* species

Total RNAs were prepared from cysts of *A. franciscana* (GSL), *A. franciscana* (SFB), *A. urmiana*, *A. sinaca*, *A. tibetiana*, *A. parthenogenetic* and *A. persimilis* and reverse transcribed as described previously. PCR was performed at 94 °C for 2 min, 40 cycles of 94 °C for 30 sec, 55 °C for 45 sec, 72 °C for 1.5 min, and extension at 72 °C for 5 min. The forward primer was 5'-GCGCGGATCCACCATGGCACTTAACCCATG-3' and the reverse primer was 5'-CGCGCCTCGAGTTAAGCTGCACCTCCTGTCT-3'. PCR products were cloned into pGEM-T Easy vector (Promega) for sequencing at the DNA Sequencing Facility, Hospital for Sick Children (Toronto, ON).

2.6.2 Construction of an *Artemia* genomic library

Genomic DNA was partially digested at 37 °C for 30 min with Sau 3A I (Promega), at 0.125 units/μg DNA and the extent of digestion was determined by electrophoresis in 0.4% agarose gels. DNA fragments of 9-23 kb were extracted twice with ethanol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1) and then precipitated with ammonium acetate and ethanol. To avoid cloning two or more small DNA fragments into the same vector, fragments less than 14 kb were removed by centrifugation in 10-40% discontinuous gradients containing 9 ml each of 10%, 20%, 30% and 40% sucrose in buffer composed of 1M NaCl, 20 mM Tris-HCl and 5 mM EDTA (pH 8.0). The gradients were stored for 3 hr at room temperature, 200 μg of DNA was applied to each gradient and centrifugation was at 83,000 x g (25,000 rpm) for 22 hr at 20 °C in a Beckman SW 28 rotor. Fractions of 400 μl were collected and DNA size was analyzed by electrophoresis in 0.6% agarose gels. Fractions containing DNA fragments of 14-23 kb were pooled, the DNA was purified and then incubated with Lamda Vector BamH I Arms (Promega) for 3 hr at room temperature and overnight at 4

°C. Recombinant phage were packaged with the Packagene Extract (Promega), the titer was determined using *E. coli* LE392 and 10 plaques were picked randomly, amplified, purified, digested with restriction enzymes and electrophoresed in 0.7% agarose gels to check insert size. Phage were stored in buffer containing 20 mM Tris-HCH, 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin and 7% DMSO, pH 7.4.

2.6.3 Screening the *Artemia* genomic library

Full-length p26 cDNA was labeled with the PCR Dig Labeling Mix (Roche) containing digoxigenin-11-dUTP with a forward primer of 5'-GCGCGGATCCACCAT GGCACTTAACCCATG-3' and a reverse primer of 5'-CGCGCCTCGAGTTAAGCT GCACCTCCTGATCT-3'. DNA from plaques was transferred to duplicate sets of nylon membranes (Roche), denatured in 5 ml of denaturation solution (0.5 M NaOH 1.5 M NaCl) for 5 min, neutralized in 5 ml of neutralization solution (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4) for 15 min and then incubated in 5 ml 2 x SSC for 10 min. The membrane was baked at 80 °C for 2 hr prior to prehybridization in Dig Easy Hyb (Roche) for 6 hr at 42 °C. The labeled probe was denatured for 5 min at 96 °C, cooled on ice immediately, placed in fresh Dig Easy Hyb (Roche) and incubated with membrane overnight at 46 °C. The membrane was washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min, followed by two washes in 0.5 x SSC, 0.1% SDS at 68 °C for 15 min. Plaques that hybridized to the probe were detected with a Chemiluminescent Assay (Roche) as described by the manufacturer, using X-ray film (Labscientific, Inc, Livinston, NJ, USA). Each positive clone was purified by four rounds of screening.

2.6.4 Sequencing p26 genomic clones

Phage DNA was amplified and incubated with pancreatic DNase and RNase at 1 μg/ml for 30 min. Samples were centrifuged at 11,000 x g and phage were collected by centrifugation at 83,000 x g for 3 hr at 4 °C in a Beckman SW27 rotor. Approximately 2 ml of SM buffer (0.01% gelatin, 50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$, pH 7.5) was added to each tube which was then incubated overnight at 4 °C on a shaking platform, followed by incubation with proteinase K at 50 μg/ml for 1 hr at 50-55 °C. SDS was added to 0.5%, followed by extraction with phenol and phenol/chloroform prior to DNA precipitation with ethanol. The presence of p26 DNA in clones was determined by probing Southern blots, then each clone was digested with one or two different restriction enzymes, including Bam HI, Eco RI, Hind III, Xba I and Sac I prior to electrophoresis in 0.4% agarose gels. DNA fragments containing p26 DNA were selected by Southern hybridization, cloned into the pBluescript II SK (+) vector (Stratagene, Cedar Creek, TX, USA), and sequenced at the DNA Sequencing Facility, Hospital for Sick Children (Toronto, ON, Canada).

2.6.5 Sequencing p26 upstream DNA

Ten μg of Genomic DNA , isolated as described above, was digested to completion overnight at 37 °C with Sau 3AI, Sal I, EcoR I, Hind III, Pst I and Xba I, and the fragments were ligated into cassettes (TaKaRa) at 16 °C for 30 min. After incubation at 94 °C for 2 min, the first PCR was performed for 35 cycles at 94 °C for 30 sec, 62 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 5 min. The primers were C1 (5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3') and S1 (5'-CCACCTCC GAAGCCACCAAATC-3'), based respectively on the cassette and nucleotides 86-107 of the p26 cDNA. One μl of the first PCR reaction product was denatured 1min prior to 25

cycles of 94 °C for 30 sec, 61 °C for 1 min, 72 °C for 2 min and a final 5 min extension. The primers were C2 (5'-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3') and S2 (5'-AGTCATACCACCAAATCCTCCGT-3'), based respectively on cassette and nucleotides 42-63 of the p26 cDNA. The PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham) and cloned into the pGEM-T Easy vector (Promega).

2.6.6 Identification of the p26 gene transcription start site

One g of hydrated cysts was homogenized in Trizol (Invitrogen) using a mini Dounce homogenizer. The homogenate was shaken vigorously in chloroform and centrifuged at 12,000 x g for 15 min. The upper aqueous phase containing RNA was incubated with isopropyl and centrifuged to collect the RNA. The pellet was washed with 75% ethanol, centrifuged and dissolved in DEPC-treated water. mRNA was recovered according to manufacturer's instructions on oligo(dT)-cellulose using an mRNA Purification Kit (Amersham). First-strand cDNA was synthesized using 1 μg of poly $(A)^+$ RNA in 10 μl of reaction mixture containing 1μl of Smart II A Oligonucleotide (5'-AAG CAGTGGTATCAACGCAGATACGCGGG-3'), 1μl of 5'-CDS primer (5'-(T) 25 N-1N-3'), 2 μl of 5 x First-strand buffer, 1 μl of DTT, 1 μl of dNTP mix and 1 μl of PowerScript Reverse Transcriptase. The 5'-RACE reaction was performed using the SMART RACE cDNA Amplification kit (BD Biosciences). Briefly, amplification was performed with the adapter primer Universal Primer A Mix containing long, 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'and short 5'-CTAATACGACTCACTATAGGGC-3') primers and a p26 cDNA primer (5'-GTAGTTCCCCCTTCAATCCTTCCAA-3') at 25 cycles of 94 °C for 30 sec, 68 °C for

30 sec, 72 °C 2 min. The PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced.

2.6.7 Detection of p26 mRNA on northen blots

Total RNA was isolated from embryos obtained from females two days post-fertilization using Trizol (Invitrogen) as described above. Five μg of total RNA was electrophoresed in 1.5% agarose gels containing formaldehyde and blotted to nylon membranes (Roche) in Transfer Buffer (Ambion) with the Rapid Downward Transfer System/Buffer Tray (Schleicher & Schuell). Blots were prehybridized in Dig Easy Hyb (Roche) for 6 hr at 50 °C using roller bottles and then hybridized at 50 °C overnight with full-length p26 cDNA and a 520 bp fragment of 18S cDNA, both labeled by PCR with digoxigenin-11-dUTP using PCR Dig Labeling Mix (Roche). Hybridization was detected as described for screening of genomic DNA library.

2.6.8 Quantification of p26 mRNA

Total RNA was prepared from embryos of two animals at day 0 to day 5 post-fertilization with the RNeasy Protect Mini Kit (Qiagen) and Qiashredder (Qiagen) followed by purification of mRNA with Oligotex (Qiagen). RNA was reverse transcribed to cDNA using Supermix (Sigma) with primers designed to prevent amplification of genomic DNA. p26 primers were forward 5'-GGACACGTCCAAAGAGAATTTCGAC G-3' and reverse 5'-CTGCACCTCCTGATCTTGTTGTTCT-3', and the probe was 5'-/6-FAM/AGCGCCAGCTGTTGGAAGGATTGAA/3BHQ_2/-3'. Elongation factor primers used for normalization of data were forward: 5'-GGCTCTCAAGATTTCTTTGCTCAG GTT-3', reverse: 5'-CGGACAGCAAATCGACCAAGAG-3' and the probe was 5'-/6-FAM/TGCGGCCATGATCACTTTGGTACCTT/3BHQ_2/-3'. PCR was performed in

25 µl containing 12.5 µl of IQ Supermix (Bio-Rad), 0.5 µl of cDNA, 150 nM of probe, and primers. Conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min with the iCycler (Bio-Rad).

III. Results

3.1 Differential Gene Expression during *Artemia* Encystment and Diapause

3.1.1 cDNA preparation for subtractive hybridization

cDNA was synthesized by PCR using mRNA from embryos at day 2 post-fertilization (Fig.1). The cDNA size range was from 0.25 kb to more than 10 kb, but most products were 0.8 kb to 1.5 kb in length. To improve subtractive hybridization efficiency, cDNA smaller than 0.3 kb was removed.

3.1.2 Identification of encystment and diapause specific *Artemia* genes

To decrease the chance of cloning repeat sequences and to obtain longer inserts for sequencing, PCR was used to screen 1152 clones from the subtractive hybridization library from which 288 clones were sequenced. Of the sequences identified from the library, 56.2% matched sequences in GenBank, including known and hypothetical genes, among which there were 30 contigs containing more than one copy, 40.3% did not correspond to any sequences in GenBank and the remaining sequences were not usable (Table 2). Fifty-five genes had similarity with known genes from other organisms (Table 3). The average sequence length was 500 bp due to digestion of cDNA with Rsa I during subtractive hybridization library construction. All the sequences were deposited in GenBank. In order to determine if any of the identified genes were differentially expressed in oviparous and ovoviviparous development embryos, semi-quantitative RT-PCR was utilized to quantify their transcript abundance (Fig 2, Table 4, and 5).

3.2 Small Heat Shock Proteins, ArHsp21 and ArHsp22

3.2. 1 ArHsp21 and ArHsp22 cDNAs

Figure 1. Synthesis of cDNA from embryos at day 2 post-fertilization

Total RNA was prepared from embryos 2 days post-fertilization during either oviparous or ovoviviparous development and reverse transcribed to cDNA which was amplified using PCR as described in "Materials and Methods". Lane 1, cDNA from oviparous development; lane 2, cDNA from ovoviviparous development; M, DNA size markers; bp, base pair.

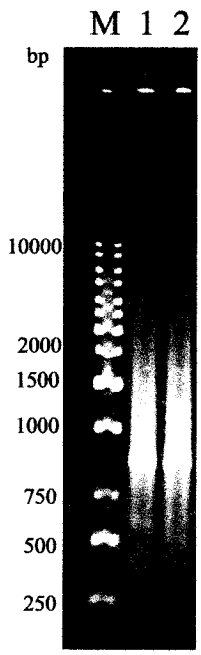


Figure 1

Table 2. Artemia cDNA clones sequenced from the subtractive hybridization library

Clone category	Number of clones	Total clones (%)
Unreadable sequences	10	3.5
Sequence similar to know proteins	92	31.9
Hypothetical protein	70	24.3
Unidentified	116	40.3
Total	288	100

Table 3. Subtractive hybridization clone sequences matched to protein encoding sequences in GenBank*

Accession number	Best sequence match in GenBank	Length (bp)	Copy number	Representative species	E Value
	Metabolism				
DW678203	steroid dehydrogenase	494	3	*H. sapiens*	3e-22
DW678204	cytochrome c oxidase subunit III	393	2	*A. frnaciscana*	1e-38
DW678171	fatty-acyl reductase	209	1	*B. mori*	1e-08
SW678169	extensin-like protein	402	1	*L. esculentum*	4e-07
DW678177	Alg2	334	1	*R. pusillus*	2e-16
DW678175	dimeric dihydrodiol dehydrogenase	483	1	*H. sapiens*	4e-20
DW678184	calcium-binding protein	338	1	*C. elegans*	4.5
DW678185	cytochrome P450	485	1	*R. norvegicus*	4e-15
DW678194	aldehyde dehydrogenase	355	1	*C. elegans*	1e-38
DW678186	glucose-6-phosphate isomerase	443	1	*D. yakuba*	1e-63
DW678188	mucin-like peritrophin	284	1	*C. elegans*	2e-07
DW678190	stearoyl-CoA desaturase	418	1	*A. americanum*	3e-04
DW678199	leukotriene A4 hydrolase	586	1	*H. sapiens*	5e-74
DW678180	peritrophic membrane chitin binding protein2	493	1	*T. ni*	0.005
DW678181	peritrophic membrane chitin binding protein1	579	1	*T. ni*	0.004
DW678201	peritrophic matrix insect intestinal mucin	268	7	*P. xylostella*	0.014
DW678209	chitinase like precursor	477	2	*C. elegans*	0.011
DW678167	uncoupling protein	261	1	*S. scrofa*	1e-10
DW678168	chloride channel protein	533	1	*O. cuniculus*	2e-33
	Cellular Processes				
DW678200	Neuralized-like protein	850	12	*M. musculus*	7e-04
DW678202	Cytokinesis protein	655	5	*C. elegans*	0.054
DW678206	nonmuscle myosin-II	628	2	*D. melanogaster*	4e-41
SW678159	phosphotyrosyl phosphatase activator PTPA	501	1	*H. sapiens*	2e-41
DW678165	Cathepsin	411	1	*P. chilotes*	4e-12
DW678172	CDK5 regulatory subunit	508	1	*H. sapiens*	0.42
DW678176	BRCA1 associated protein	362	1	*H. sapiens*	4e-30
DW678178	PDZ domain	194	1	*H. sapiens*	0.012
DW678179	ArHsp22	582	1	*A. franciscana*	2e-18
DW678183	chromatin remodeling complex	327	1	*H. sapiens*	0.010
DW678189	p26	438	1	*A. franciscana*	4e-70
DW678191	ArHsp21	343	1	*A. franciscana*	0.018
DW678193	RNA binding protein	335	1	*A. thaliana*	0.40
	Genetic Information Processing				
DW678208	ribosomal protein L18	611	2	*B. lanceolatum*	1e-70

Table 3, continued

Accession number	Best sequence match in GenBank	Length (bp)	Copy number	Representative species	E Value
DW678210	ribosomal protein L35A	234	2	*S. frugiperda*	0.011
DW678161	ELF2 alpha	563	1	*S. frugiperda*	3e-32
DW678173	elongation factor 1-alpha	361	2	*Artemia. sp.*	5e-65
DW678174	60s ribosomal protein L5	307	1	*S. clava*	7e-38
DW678162	40S ribosomal protein S25	371	1	*R. norvegicus*	1e-25
DW678163	60S ribosomal protein L11	272	1	*D. melanogaster*	1e-31
DW678207	erv1-like growth factor	476	1	*S. cerevisiae*	1e-30
DW678164	40S ribosomal protein S17	284	1	*H. sapiens*	4e-24
DW678170	40S ribosomal protein S23	393	1	*D. variabilis*	6e-62
SW678182	QM protein	528	1	*H. sapiens*	1e-71
DW678187	60S ribosomal protein L23a	506	1	*M. musculus*	3e-54
DW678192	60S ribosomal protein L5	204	1	*H. sapiens*	5e-11
DW678197	60s acidic ribosomal protein p2 (el12) Environmental Information Processing	480	1	*Brine shrimp*	5e-34
DW678205	FLI-LRR associated protein-1	664	2	*M. musculus*	0.12
DW678157	N33 protein	377	1	*R. norvegicus*	1e-07
DW678156	superoxide dismutase	198	1	*B. suppressaria*	8e-04
DW678158	alpha1G T-type calcium channel	99	1	*B. taurus*	1.2
DW678160	Sidekick	178	1	*D.melanogaster*	1e-09
DW678166	p8	553	1	*H. sapiens*	2e-06
DW678195	vacuolar protein sorting 16	149	1	*H. sapiens*	9e-06
DW678196	Takeout	385	1	*D. melanogaster*	2e-06
SW678198	F-box and WD-40 domain protein	246	1	*H. sapiens*	0.011

"*": Categories are based on the KEGG PATHWAY at www.genome.ad.jp/keg/pathway .html.

Figure 2. Gene regulation during *Artemia* oviparous development

Total RNA was prepared from embryos 2 days post-fertilization during either oviparous or ovoviviparous development, and RT-PCR was performed with primers based on putative diapause-specific genes selected by subtractive hybridization. The products were electrophoresed in 1.5% agarose gels and stained with Gel Star. Odd numbers, ovoviviparous development, even numbers, oviparous development. A, 1, 2, superoxide dismutase; 3, 4, PTPA; 5, 6, sidekick; 7, 8, p8; 9, 10, cathepsin; B, 1, 2, chloride channel protein; 3, 4, erv1-like growth factor; 5, 6, fatty-acyl reductase; 7, 8, peritrophin-like protein; 9, 10, CoA desaturase; 11, 12, RNA-binding protein; 13, 14, F-box and WD-40 domain protein; C, lanes 1, 2, takeout protein; 3, 4, lung-inducible neuralized-related C3H4 RING domain protein; 5, 6, glucose-6-phosphate isomerase; 7, 8, cytochrome P450; 9, 10, peritrophic membrane chitin binding protein; 11, 12, QM protein; 13, 14, Cdk5 activator binding protein; 15, 16, dimeric dihydrodiol dehydrogenase. D, 1, 2, sacbrood virus polyprotein; 3, 4, similar to N33 protein; 5, 6, uncoupling protein 3 isoform; 7, 8, leucine rich repeat (in FLII) interacting protein; 9, 10, chitinase like precursor; 11, 12, aldehyde dehydrogenase; 13, 14, cytokinesis protein cej-1 precursor; 15, 16, steroid dehydrogenase; E, 1,2, hVPS 16; 3, 4, LTA4; F, 1, 2, chromatin-remodeling factor; 3, 4, Bap-1; G, 1, 2, ArHsp21; 3, 4, ArHsp22. Tub, tubulin.

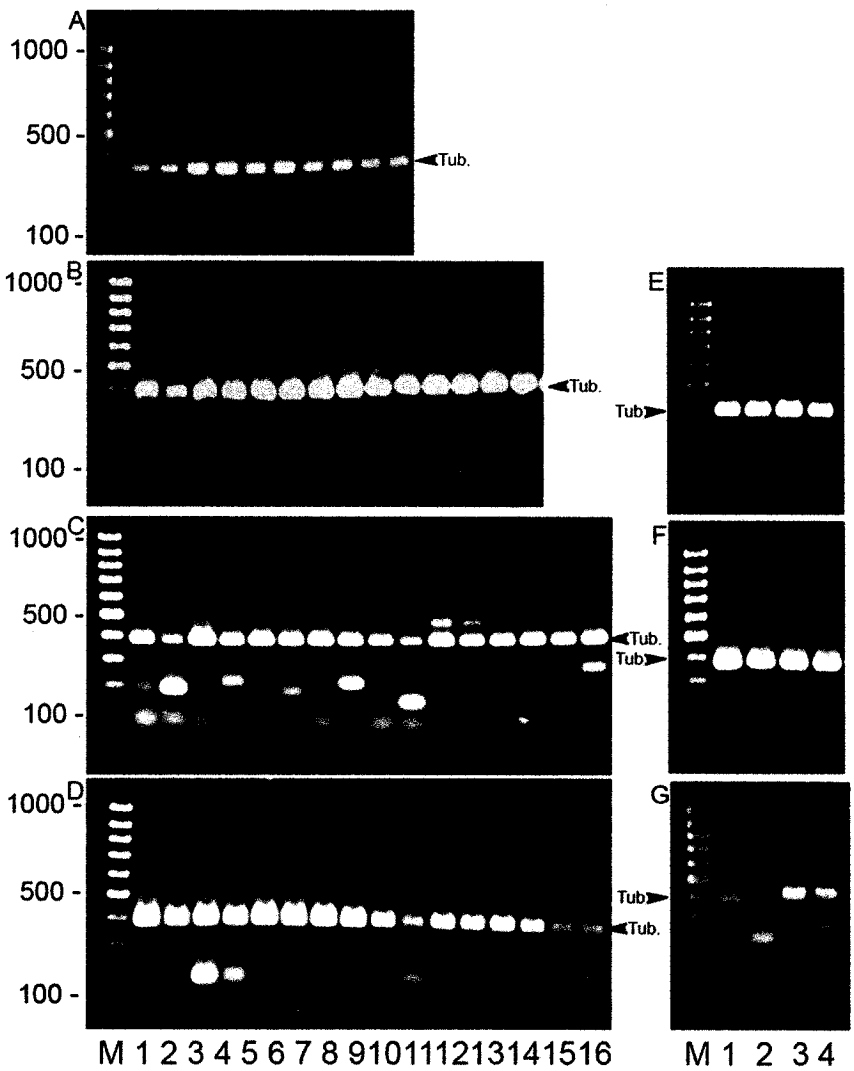

A
1000 -
500 -
100 -
Tub.
B
1000 -
500 -
100 -
Tub.
C
1000 -
500 -
100 -
Tub.
Tub.
D
1000 -
500 -
100 -
Tub.
Tub.
E
Tub.
F
G
Tub.
Tub.
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
M 1 2 3 4

Figure 2

Table 4. Genes up-regulated during early oviparous development

Gene	Functional category	Specific function
Takeout	extracellular transport	circadian rhythm
Neuralized-related protein	development	notch signaling protein
GPI	carbohydrate metabolism	glycolysis pathway
Cytochrome p450	lipid metabolism	oxidative metabolism
Cdk5 activator binding protein	cell growth and death	suppressor
DDH	lipid metabolism	catalyze oxidation
Uncoupling protein	energy metabolism	thermogenesis
Aldehyde dehydrogenase	carbohydrate metabolism	catalyze oxidation
Chitinase like protein	carbohydrate metabolism	chitin synthesis
Cytokinesis protein cej-1	carbohydrate metabolism	chitin synthesis
Steroid dehydrogenase	lipid metabolism	steroid hormone
p8	signal translation	gene regulation
p26	cell growth and death	chaperone
ArHsp21	cell growth and death	chaperone
ArHsp22	cell growth and death	chaperone
BAP1	cell growth and death	growth suppressor
PMCBP	secretory pathway	chitin synthesis
Ervl-like growth factor	protein turnover	growth control

Table 5. Genes down-regulated during early oviparous development

Gene	Functional category	Specific function
QM	translation	suppressor
N33	signal transduction	gene regulation
FLAP	signal transduction	receptor ligand binding

On the basis of the partial cDNA sequences obtained by subtractive hybridization, RACE was performed to yield 375 bp products for ArHsp22 3'-RACE, and 650 bp and 640 bp products for 5'-RACE of ArHsp21 and ArHsp22, respectively (Fig. 3), which were cloned into T/A cloning vectors for sequencing. The ArHsp21 full length cDNA sequence was 774 nucleotides, including an open reading frame (ORF) of 546 bp, a 78 bp 5' untranslated (UTR) region, a 120 bp 3' UTR and a poly (A) tail (Fig. 4). The "A" at the 5'end, 78 bp upstream of the translation initiation site is the transcription start site. The ArHsp22 full length cDNA is 744 nucleotides, consisting of an ORF of 573 bp, a 48 bp 5' UTR, a 113 bp 3' UTR and a poly (A) tail (Fig. 4). The "A" at the 5'end 47 bp upstream of the translation initiation site is the transcription start site. Similarity between the nucleotide sequences of p26, ArHsp21 and ArHsp22 was limited when determined by NCBI blast 2 sequence program.

3.2.2 Deduced amino acid sequences of ArHsp21 and ArHsp22

Amino acid sequences of ArHsp21 and ArHsp22 were deduced from cDNA sequences, yielding polypeptides of 181 and 190 residues, respectively, relative to 192 residues in p26 (Fig. 5). The molecular masses of p26, ArHsp21 and ArHsp22 are 20.8 kDa, 21.1 kDa and 22.4 kDa, respectively, as calculated with Gene Runner. ArHsp21 and ArHsp22 each possessed an α-cystallin domain and the Blast search showed they are small heat shock proteins. p26 shared 38% identity and 50% similarity with ArHsp 22, and 29% identity and 51% similarity with ArHsp21. The amino acid sequences of ArHsp21 and ArHsp22 were 27% identical and 52% similar. All three *Artemia* sH SPs share a highly conserved arginine residue in the α-cystallin domain and a V/IXI/V motif in the C-terminal extension (Figs. 5 and 6).

Figure 3. Cloning ArHsp21 and ArHsp22 cDNAs

Total RNA was prepared from embryos two days post-fertilization during *Artemia* oviparous development and 5'- and 3'-RACE were performed based on the partial sequences of ArHsp21 and ArHsp22 cDNAs. The products were electrophoresed in 1% agarose gels. Lane 1, negative control for 3'-RACE; lane 2, ArHsp22, 3'-RACE; lane 3, negative control for 5'-RACE; lane 4, ArHsp21, 5'-RACE; lane 5, ArHsp22, 5'-RACE.

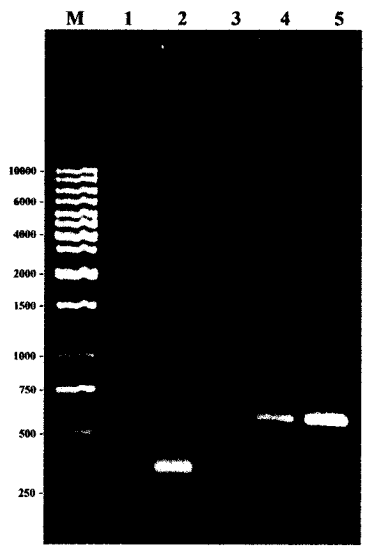


Figure 3

Figure 4. Nucleotide sequences of *Artemia* sHSP cDNAs

cDNA sequences of p26 (Accession number AF031367), ArHsp21 and ArHsp22 were aligned by Clustal W. "*", identical nucleotides; blank, nonidentical nucleotides; boxed ATG, translation start site; boxed TAA and TGA, stop codons; boxed AATAA, AATTA and ATATAA, poly-adenylation signals.

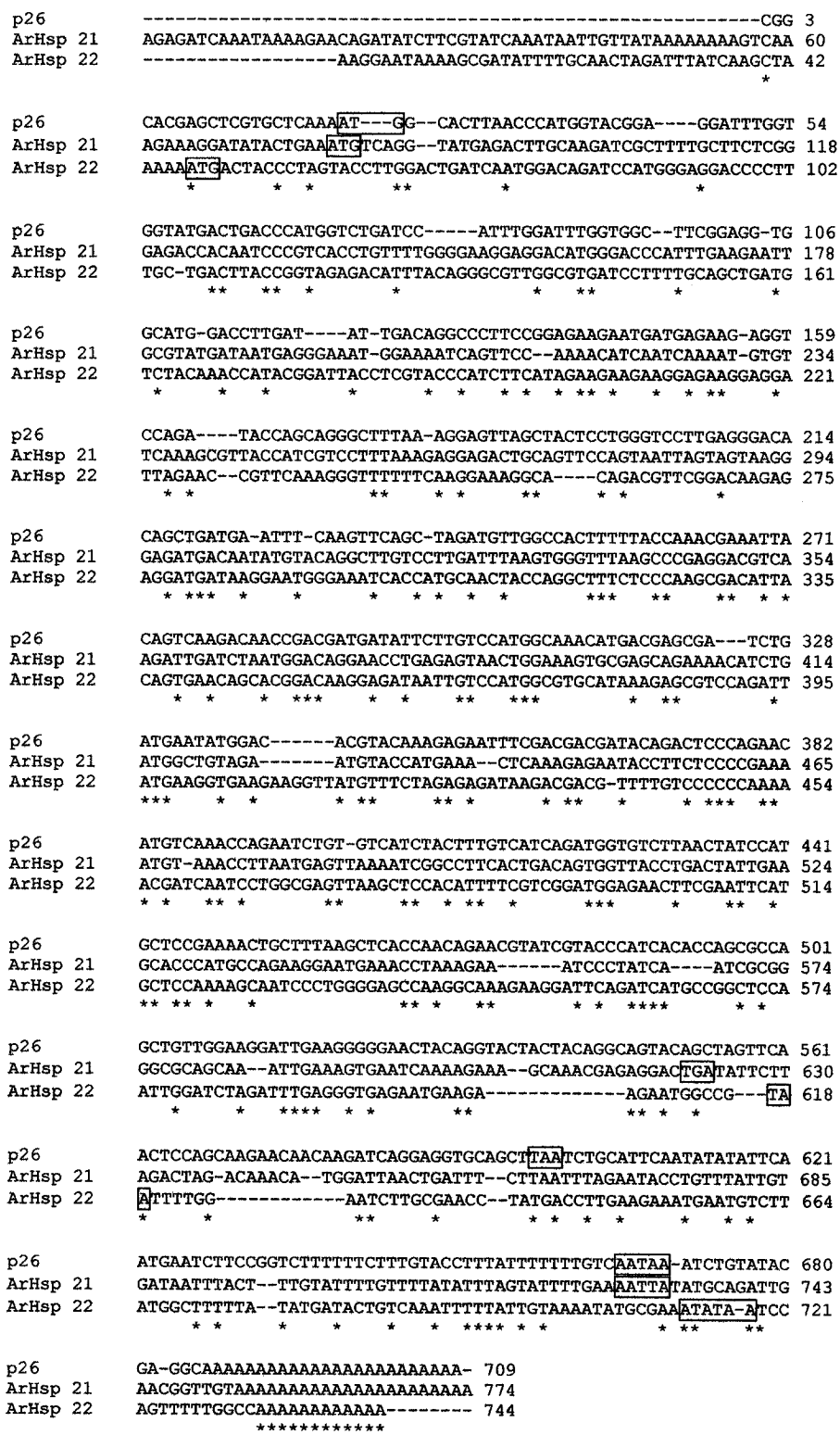

p26 ----------CGG 3
ArHsp 21 AGAGATCAAATAAAAGAACAGATATCTTCGTATCAAATAATTGTTATAAAAAAAAGTCAA 60
ArHsp 22 ----------AAGGAATAAAAGCGATATTTTGCAACTAGATTTATCAAGCTA 42
p26 CACGAGCTCGTGCTCAAAAT---GG--CACTTAACCCATGGTACGGA----GGATTTGGT 54
ArHsp 21 AGAAAGGATATACTGAAATGTCAGG--TATGAGACTTGCAAGATCGCTTTTGCTTCTCGG 118
ArHsp 22 AAAAATGACTACCCTAGTACCTTGGACTGATCAATGGACAGATCCATGGGAGGACCCCTT 102
p26 GGTATGACTGACCCATGGTCTGATCC-----ATTTGGATTTGGTGGC--TTCGGAGG-TG 106
ArHsp 21 GAGACCACAATCCCGTCACCTGTTTTGGGGAAGGAGGACATGGGACCCATTTGAAGAATT 178
ArHsp 22 TGC-TGACTTACCGGTAGAGACATTTACAGGGCGTTGGCGTGATCCTTTTGCAGCTGATG 161
p26 GCATG-GACCTTGAT----AT-TGACAGGCCCTTCCGGAGAAGAATGATGAGAAG-AGGT 159
ArHsp 21 GCGTATGATAATGAGGGAAAT-GGAAAATCAGTTCC--AAAACATCAATCAAAAT-GTGT 234
ArHsp 22 TCTACAAACCATACGGATTACCTCGTACCCATCTTCATAGAAGAAGAAGGAGAAGGAGGA 221
p26 CCAGA----TACCAGCAGGGCTTTAA-AGGAGTTAGCTACTCCTGGGTCCTTGAGGGACA 214
ArHsp 21 TCAAAGCGTTACCATCGTCCTTTAAAGAGGAGACTGCAGTTCCAGTAATTAGTAGTAAGG 294
ArHsp 22 TTAGAAC--CGTTCAAAGGGTTTTTTCAAGGAAAGGCA----CAGACGTTCGGACAAGAG 275
p26 CAGCTGATGA-ATTT-CAAGTTCAGC-TAGATGTTGGCCACTTTTTACCAAACGAAATTA 271
ArHsp 21 GAGATGACAATATGTACAGGCTTGTCCTTGATTTAAGTGGGTTTAAGCCCGAGGACGTCA 354
ArHsp 22 AGGATGATAAGGAATGGGAAATCACCATGCAACTACCAGGCTTTCTCCCAAGCGACATTA 335
p26 CAGTCAAGACAACCGACGATGATATTCTTGTCCATGGCAAACATGACGAGCGA---TCTG 328
ArHsp 21 AGATTGATCTAATGGACAGGAACCTGAGAGTAACTGGAAAGTGCGAGCAGAAAACATCTG 414
ArHsp 22 CAGTGAACAGCACGGACAAGGAGATAATTGTCCATGGCGTGCATAAAGAGCGTCCAGATT 395
p26 ATGAATATGGAC------ACGTACAAAGAGAATTTCGACGACGATACAGACTCCCAGAAC 382
ArHsp 21 ATGGCTGTAGA-------ATGTACCATGAAA--CTCAAAGAGAATACCTTCTCCCCGAAA 465
ArHsp 22 ATGAAGGTGAAGAAGGTTATGTTTCTAGAGAGATAAGACGACG-TTTTGTCCCCCCAAAA 454
p26 ATGTCAAACCAGAATCTGT-GTCATCTACTTTGTCATCAGATGGTGTCTTAACTATCCAT 441
ArHsp 21 ATGT-AAACCTTAATGAGTTAAAATCGGCCTTCACTGACAGTGGTTACCTGACTATTGAA 524
ArHsp 22 ACGATCAATCCTGGCGAGTTAAGCTCCACATTTTCGTCGGATGGAGAACTTCGAATTCAT 514
p26 GCTCCGAAAACTGCTTTAAGCTCACCAACAGAACGTATCGTACCCATCACACCAGCGCCA 501
ArHsp 21 GCACCCATGCCAGAAGGAATGAAACCTAAAGAA------ATCCCTATCA----ATCGCGG 574
ArHsp 22 GCTCCAAAAGCAATCCCTGGGGAGCCAAGGCAAAGAAGGATTCAGATCATGCCGGCTCCA 574
p26 GCTGTTGGAAGGATTGAAGGGGGAACTACAGGTACTACTACAGGCAGTACAGCTAGTTCA 561
ArHsp 21 GGCGCAGCAA--ATTGAAAGTGAATCAAAAGAAA--GCAAACGAGAGGACTGATATTCTT 630
ArHsp 22 ATTGGATCTAGATTTGAGGGTGAGAATGAAGA----------AGAATGGCCG---TA 618
p26 ACTCCAGCAAGAACAACAAGATCAGGAGGTGCAGCTTAATCTGCATTCAATATATATTCA 621
ArHsp 21 AGACTAG-ACAAACA--TGGATTAACTGATTT--CTTAATTTAGAATACCTGTTTATTGT 685
ArHsp 22 ATTTTGG----------AATCTTGCGAACC--TATGACCTTGAAGAAATGAATGTCTT 664
p26 ATGAATCTTCCGGTCTTTTTTCTTTGTACCTTTATTTTTTTGTCAATAA-ATCTGTATAC 680
ArHsp 21 GATAATTTACT--TTGTATTTTGTTTTATATTTAGTATTTTGAAAATTATATGCAGATTG 743
ArHsp 22 ATGGCTTTTTA--TATGATACTGTCAAATTTTTATTGTAAAATATGCGAAATATA-ATCC 721
p26 GA-GGCAAAAAAAAAAAAAAAAAAAAAAA- 709
ArHsp 21 AACGGTTGTAAAAAAAAAAAAAAAAAAAAA 774
ArHsp 22 AGTTTTTGGCCAAAAAAAAAAA-------- 744

Figure 4

Figure 5. Amino acid sequence alignment for *Artemia* sHSPs

The deduced amino acid sequences of p26, ArHsp21 and ArHsp22 were aligned by Clustal W. Red, small and hydrophobic residues; blue, acidic residues; magenta, basic residues; green, residues containing hydroxyl or amine groups; "*", identical residues; ":", conserved substitution; ".", semi-conserved substitution. The α-crystallin domains of p26, ArHsp21 and ArHsp22 consist of residues 61-152, 69-151 and 73-159, respectively. A highly conserved arginine in the α-crystallin domain and the carboxy-terminal extension motif, V/IXI/V, are boxed.

```
p26             -MALNPWYGGFGGMTDPWSDPFG------FGG-FGGGMDLDIDRPF--------RRRMMR 44
ArHsp21         -MSGMRLARSLLLLGRPQSRHLF------WGRRTWDPFEELRMIMREMENQFQNINQNVF 53
ArHsp22         MTTLVPWTDQW---TDPWEDPFADLPVETFTGRWRDPFAADVYKPYGLPRTHLHRRRRRR 57
                  :             * .  : . . .::     . :                 .:

p26             RGPDTSRALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDERSD- 103
ArHsp21         KALPSSFKEETAVPVISSKGDDNMYRLVLDLSGFKPEDVKIDLMDRNLRVTGKCEQKTS- 112
ArHsp22         RIRTVQRVFSRKGTDVRTREDDKEWEITMQLPGFLPSDITVNSTDKEIIVHGVHKERPDY 117
                :    .   .   .    :   . :.: :::  * *.::.:.  * :: * *  .::..

p26             --EYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPTERIVPITPAPA 161
ArHsp21         --DGCRMYHETQREYLLPENVNLNELKSAFTDSGYLTIEAPMPEGMKPKE--IPINRGAQ 168
ArHsp22         EGEEGYVSREIRRRFVPPKTINPGELSSTFSSDGELRIHAPKAIPGEPRQRRIQIMPAPI 177
                ..:   : :* :*.:  *: ::  .:.*:::..* * *.** .   .* :   : * : :

p26             VGRIEGGTTGTTTGSTASSTPARTTRSGGAA 192
ArHsp21         QIESESKE--------SKRED---------- 181
ArHsp22         GSRFEG--------ENEEEWP--------- 190
                   *
```

Figure 5

Figure 6. Alignments of sHSPs from Artemia and other organisms

The deduced amino acid sequences of *Artemia* sHSPs were aligned with human αA-crystallin (h-A) (P02489), human αB-crystallin (h-B) (P02511), wheat HSP16.9 (WHSP16.9) (S21600) and *Drosophila* hsp26 (Drohsp26) (AAA28636) by Clustal W. "*", identical residues; ":", conserved substitution; "." Semi-conserved substitution; boxed "R", highly conserved arginine residue in the α-crystallin domain; boxed I/VXV/I, conserved motif in the carboxy-terminal extension.

```
h-A             ---MDVTIQHPWFKRT-------LGPFY--PSRLFDQFFGEGLFEYDLLPF----LSSTI 44
h-B             ---MDIAIHHPWIRRP-------FFPFHS-PSRLFDQFFGEHLLESDLFP-----TSTSL 44
ArHsp22         -----MTTLVPWTDQW---TDPWEDPFADLPVETFTGRWRDPFAADVYKPYGLPRTHLHR 52
p26             ------MALNPWYGGFGGMTDPWSDPFG------FGG-FGGGMDLDIDRPF--------R 39
WHSP16.9        ---------MSIVRRS--------NVFDP-----FADLWADPFDT--------------F 24
Drohsp26        ---MSLSTLLSLVDELQ---EPRSPIYELGLGLHPHSRYVLPLGTQQRRSINGCPCASPI 54
ArHsp21         MSGMRLARSLLLLGRPQ-----SRHLFWG--RRTWDPFEELRMIMREMEN--------QF 45
                                        :               :

h-A             SPYYR--QSLFRT--VLDSGISEVRSDR----DKFVIFLDVKHFSPEDLTVKVQDDFVEI 96
h-B             SPFYLRPPSFLRAPSWFDTGLSEMRLEK----DRFSVNLDVKHFSPEELKVKVLGDVIEV 100
ArHsp22         RRRRRRIRTVQRVFSRKGTD---VRTREDD--KEWEITMQLPGFLPSDITVNSTDKEIIV 107
p26             RRMMRRGPDTSRALKELATPG-SLRDTA----DEFQVQLDVGHFLPNEITVKTTDDDILV 94
WHSP16.9        RSIVPAISGGSSETAAFANARVDWKETP----EAHVFKVDLPGVKKEEVKVEVEDGNVLV 80
Drohsp26        CPSSPAGQVLALRREMANRNDIHWPATAHVGKDGFQVCMDVAQFKPSELNVKVVDDSILV 114
ArHsp21         QNINQNVFKALPSSFKEETAVPVISSKGD--DNMYRLVLDLSGFKPEDVKIDLMDRNLRV 103
                                    :         .   . :::  .  .::.:.  .  : :

h-A             HG--KHNERQD---DHGYISR---EFHRRYRLPSNVDQSALSCSLSADGMLTFCGPKIQT 148
h-B             HG--KHEERQD---EHGFISR---EFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQ- 151
ArHsp22         HG--VHKERPDYEGEEGYVSR---EIRRRFVPPKTINPGELSSTFSSDGELRIHAPKAIP 162
p26             HG--KHDERSD---EYGHVQR---EFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTAL 146
WHSP16.9        VSGERSREKEDKNDKWHRVERSSGKFVRRFRLPEDAKVEEVKAGLEN-GVLTVTVPKAEV 139
Drohsp26        EG--KHEERQD---DHGHIMR---HFVRRYKVPDGYKAEQVVSQLSSDGVLTVSIPKPQA 166
ArHsp21         TG--KCEQKTS---DGCRMYH---ETQREYLLPENVNLNELKSAFTDSGYLTIEAPMPEG 155
                  .     :: .   .   : :   .  *.:  *   .   : . :  .* * .  *

h-A             GLDATHAERAIPVSR-EEKP-------------TSAPSS-------- 173
h-B             ---VSGPERTIPITR-EEKPAV-----------TAAPKK-------- 175
ArHsp22         GEPRQRR---IQIMPAPI-GSRFEG--------ENEEEWP---------- 190
p26             SSPTERI---VPITPAPA-VGRIEGGTTGTTTGSTASSTPARTTRSGGAA 192
WHSP16.9        KKPEVKA---IEISG---------------------------------- 151
Drohsp26        VEDKSKERI-IQIQQVGPAHLNVKANESEVKGKENGAPNGKDK----- 208
ArHsp21         MKPKE-----IPINRGAQQIESESKESKRED-------------------181
                         : :
```

Figure 6

3.2.3 ArHsp21 and ArHsp22 genes

To identify sHSP genes, *Artemia* DNA was digested with Bam HI, Sac I and Xho I for ArHsp21 and Sal I and Hind III for ArHsp22, then probed with representative cDNAs. There were no restriction sites for these enzymes in the ArHsp21 and ArHsp22 cDNA. For ArHsp21, Southern blots, using *Artemia* DNA digested with Bam H I, Sac I and Xho I, contained single bands of approximately 9.1 kb, 8.8 kb and 4.0 kb (Fig. 7). For ArHsp22 two bands of approximately 8.8 kb and 8.7 kb for Sal I digestion and five bands of 7.0 kb, 6.5 kb, 5.5 kb, 5.0 kb and 4.0 kb were obtained upon Hind III digestion (Fig. 8).

3.2.4 Synthesis and purification of p26, ArHsp21 and ArHsp22

In order to analyze ArHsp21 and ArHsp22 functions *in vitro*, these proteins and p26 were synthesized in transformed bacteria and purified (Fig. 9). ArHsp21 yield was high, with a very strong band in Coomassie blue stained gels, but the yield of ArHsp22 was lower, possibly due to arginine enrichment in the amino-terminus. The p26 yield was between ArHsp21 and ArHsp22, with no corresponding band visible in Coomassie blue stained gels. Although the calculated molecular mass of p26 was the smallest of the three *Artemia* sHSPs, when purified from bacteria it ran parallel to ArHsp22 and behind ArHsp21 in SDS polyacrylamide gels. The sizes of purified p26, ArHsp21 and ArHsp22 were estimated by SDS polyacrylamide gel electrophoresis to be 32, 28 and 32 kDa.

3.2.5 sHSP oligomer formation

To determine the oligomer size of p26, ArHsp21 and ArHsp22, cell free extracts from bacteria containing these proteins were centrifuged in sucrose gradients, revealing

Figure 7. The ArHsp21 gene

Genomic DNA (10 μg) prepared from *Artemia* was digested to completion, electrophoresed in 0.7% agarose gels and probed with ArHsp21 cDNA labeled with PCR Dig Labeling Mix. The lanes contained DNA digested with, 1, Bam HI; 2, Sac I; 3, Xho I; 4, linearized plasmid containing the ArHsp21 cDNA insert. DNA fragment length is indicated on the right.

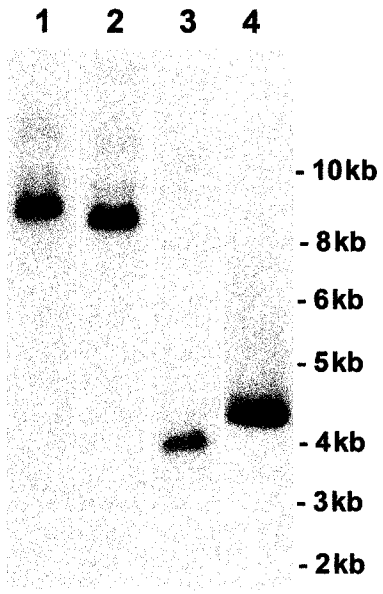


Figure 7

Figure 8. The ArHsp22 gene

Artemia DNA (10 μg) was digested to completion, electrophoresed in 0.7% agarose gels, blotted to nylon membranes and probed with ArHsp22 cDNA labeled with Dig labeling mix. The lanes contained DNA digested with, 1, Sal I; 2: Hind III; Lane 3, linearized plasmid containing ArHsp22 cDNA insert. DNA fragment length is indicated on the right.

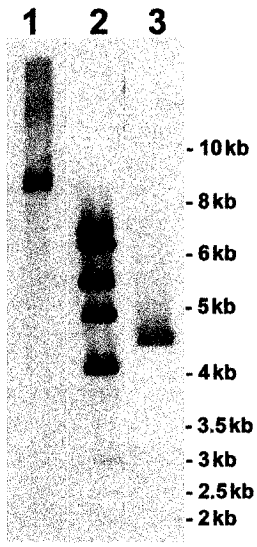


Figure 8

Figure 9. Synthesis and purification of *Artemia* sHSPs

p26, ArHsp21 and ArHsp22 were synthesized in *E. coli* and purified to apparent homogeneity on TALON affinity columns. Samples were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (A, C) or blotted to nitrocellulose membranes and detected with Omniprobe, a monoclonal anti-$(His)_6$ antibody (B, D). Panels A and B, bacterial extract; C and D, purified proteins. Lane 1, ArHsp22; 2, p26; 3, ArHsp21; 4, extract from bacteria transformed with vector only; M, molecular mass markers of 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa. A, each lane contained 30 μg of protein; B, lanes 1, 2 and 4, 10 μg of protein, lane 3, 1 μg of protein; C, lanes 1 and 2, 2 μg of protein, lane 3, 4μg of protein; D, lanes, 1 and 2, 0. 4 μg of protein, lane 3, 0.6 μg of protein.

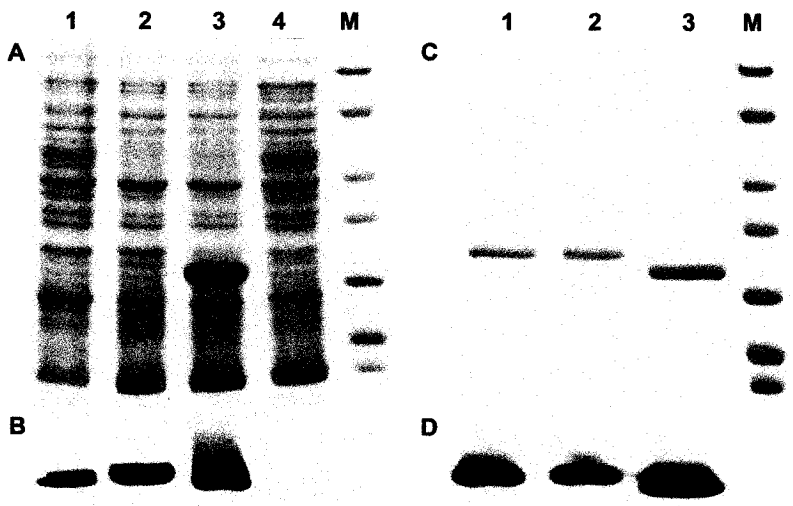


Figure 9

oligomers of almost the same size and ranging from approximately 29 kDa to 320 kDa in molecular mass (Fig. 10). Maximal monomer number per oligomer was 13.

3.2.6 Production and immunological cross reactivity of antibodies raised against *Artemia* sHSPs

Rabbit antiboies generated against p26, ArHsp21 and ArHsp22 were tested against each sHSP. The antibodies to each of p26, ArHsp21 and ArHsp22 reacted with the corresponding protein but not with the other sHSPs, demonstrating lack of cross-reactivity (Fig. 11).

3.2.7 Chaperone activity of *Artemia* sHSPs

3.2.7.1 *Artemia* sHSPs inhibit heat induced denaturation of citrate synthase

Aggregation of citrate synthase assessed by light scattering was used to analyze the protective effects of p26, ArHsp21 and ArHsp22. In contrast to bovine serum albumin and bovine immunoglobulin (controls), ArHsp21 and ArHsp22 greatly inhibited citrate synthase aggregation, especially at 600 nM, and as sHSP concentration decreased, citrate synthase aggregation increased (Fig. 12). At the same concentration of each sHSP, there was little difference in light scattering, indicating p26, ArHsp21 and ArHsp22 were equally effective in protecting citrate synthase from heat induced denaturation. The experiments were performed in duplicate using independently prepared samples with similar results.

3.2.7.2 *Artemia* sHSPs inhibit reduction induced denaturation of insulin

ArHsp21 and ArHsp22 reduced the aggregation of insulin caused by DTT at 25 °C and their protective effect improved with an increase in concentrations from 0.1 μM to 1.6 μM. ArHsp22 was somewhat better at preventing insulin denaturation than was

Figure 10. Oligomerization of *Artemia* sHSPs

Artemia sHSPs synthesized in bacteria were centrifuged through 10-50% continuous sucrose gradients prior to fractionation and detection of proteins on western blots with Ominoprobe. A, p26; B, ArHsp21; C, ArHsp22. The positions of molecular mass markers 29, 66, 200, 443 and 669 kDa are indicated by numbered arrows.

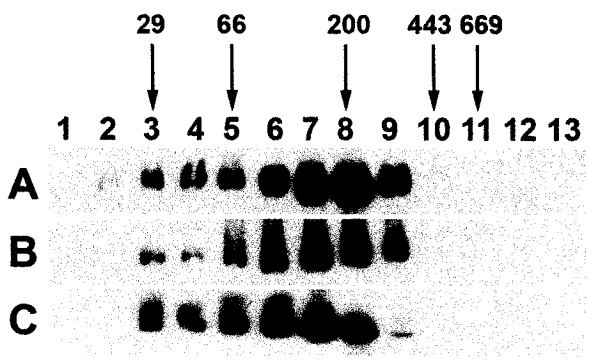


Figure 10

Figure 11. Production of antibodies to *Artemia* sHSPs

Bacterial extracts containing *Artemia* sHSPs were electrophoresed in 12.5% SDS polyacrylamide gels and either stained with Coomassie blue (A) or blotted to nitrocellulose and immunostained with antibodies to ArHsp21 (B), p26 (C) and ArHsp22 (D). Lane 1, bacteria extract containing ArHsp22; 2, p26; 3, ArHsp21; 4, vector only; M, molecular mass markers x 10^{-3} kDa.

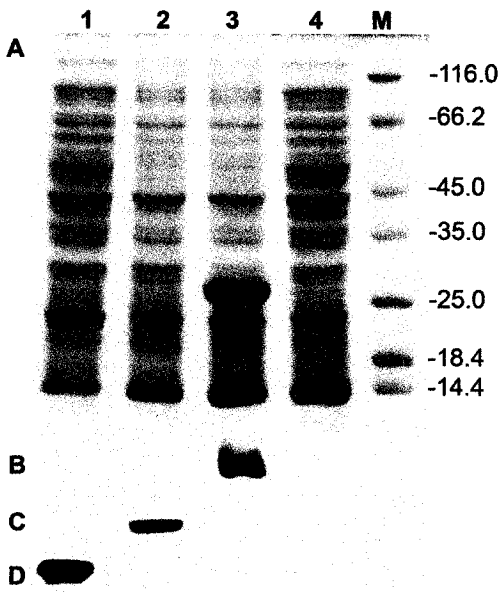


Figure 11

Figure 12. Citrate synthase thermal aggregation is suppressed by *Artemia* sHSPs

Purified sHSPs were incubated with citrate synthase (150 nM) at 43 °C and turbidity increases were measured at A_{360}. In graphs labeled ArHsp22 and ArHsp21 the curves represent: 1, no sHSP added; 2, 37.5 nM sHSP; 3, 75 nM sHSP; 4, 150 nM sHSP; 5, 300 nM sHSP, 6, 600 nM sHSP. In the graph labeled control, the lanes represent: 1 no sHSP added; 2, 600 nM bovine immunoglobulin; 3, 600 nM bovine serum albumin; 4, 300 nM p26; 5, 600 nM p26.

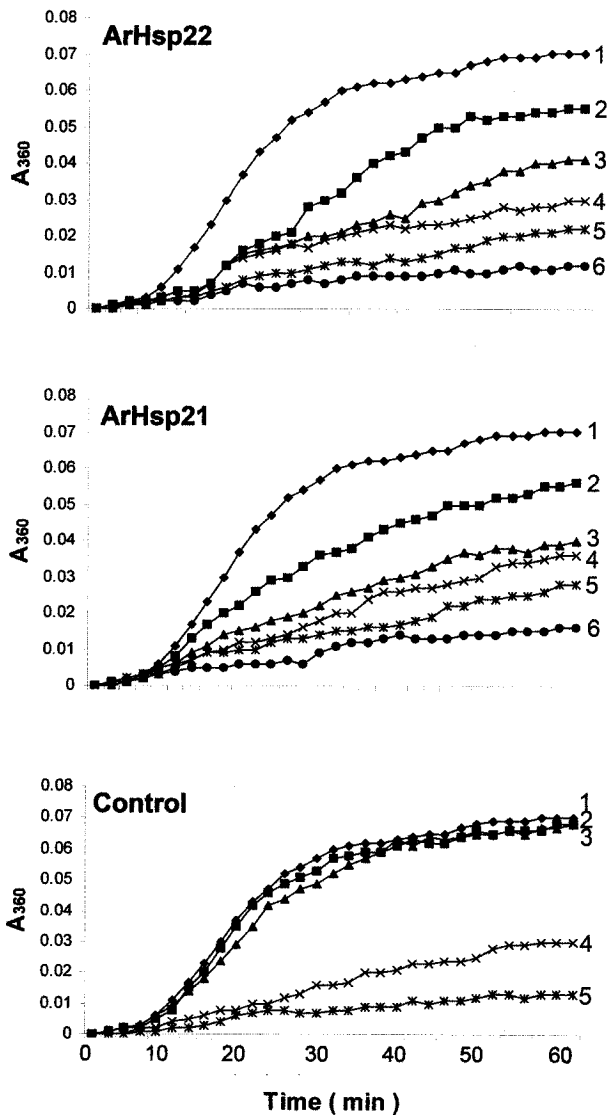

ArHsp22
ArHsp21
Control
A360
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
2
3
4
5
6
0
10
20
30
40
50
60
Time (min)

Figure 12

ArHsp21 (Fig. 13). Bovine serum albumin and bovine immunoglobulin had very little impact on reduction-induced aggregation of insulin. The experiments were performed in duplicate using independenltly prepared samples with similar results.

3.2.8 Developmental expression of ArHsp21 and ArHsp22 in *Artemia*

3.2.8.1 Quantification of ArHsp21 and ArHsp22 mRNA during embryo development

During oviparous development, ArHsp21 mRNA increased from day 0 to day 5 post-fertilization, reaching a peak of expression at day 5, whereas during ovoviviparous development, ArHsp21 mRNA increased from day 0 to day 3 and then decreased (Fig. 14, ArHsp21). There were obvious differences in ArHsp21 mRNA levels during oviparous and ovoviviparous development. ArHsp22 mRNA increased in embryos from day 0 to day 5 during oviparous development, but during ovoviviparous development, only a tiny amount of ArHsp22 mRNA was detected at days 2 and 3 (Fig 14, ArHsp22). At Day 5 during oviparous development, ArHsp22 mRNA was 35% more abundant than ArHsp21 mRNA.

3.2.8.2 Synthesis of ArHsp21 and ArHsp22 during *Artemia* embryo development

ArHsp21 was detected in embryos at day 3 post-fertilization during oviparous development by immunoprobing of western blots. The protein band representing ArHsp21 at day 4 was almost the same in intensity as at day 3, but stronger at day 5 (Fig. 15 B). No ArHsp21 was observed during ovoviviparous development (Fig. 15 E). In contrast, ArHsp22 was just visible at day 3 post-fertilization during oviparous development, but the bands were equally strong at days 4 and 5 (Fig. 15 C). ArHsp22 was not observed in embryos undergoing ovoviviparous development (Fig. 15 F).

Figure 13. Suppression of reduction induced insulin aggregation by *Artemia* sHSPs

Purified *Artemia* sHSPs were incubated with 4 μM insulin at 25 °C and light scattering was measured at A_{400} upon addition of DTT. In the graphs labeled ArHsp22 and ArHsp21, the curves represent: 1, no sHSPs added; 2, 0.1 μM sHSP; 3, 0.2 μM sHSP; 4, 0.4 μM sHSP; 5, 0.8 μM sHSP; 6, 1.6 μM sHSP. In the graph labeled control, the curves represent: 1 no sHSPs added; 2, 1.6 μM bovine immunoglobulin; 3, 1.6 μM bovine serum albumin ; 4, 1.6 μM p26.

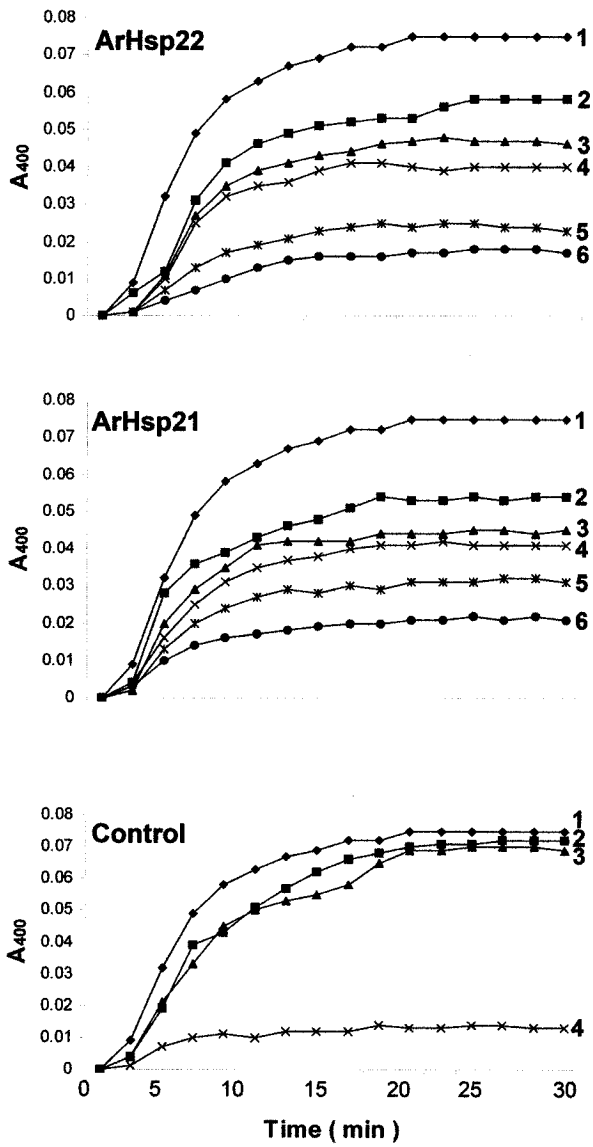

ArHsp22
ArHsp21
Control
A400
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
2
3
4
5
6
0
5
10
15
20
25
30
Time (min)

Figure 13

Figure 14. sHSP mRNAs during *Artemia* embryo development.

Total RNA was prepared at daily intervals following fertilization from *Artemia* embryos developing either oviparously or ovoviviparously. The RNA was reverse transcribed and ArHsp21 and ArHsp22 mRNAs were quantified by Real-Time PCR. Light shaded bars, oviparous development; dark shaded bars, ovoviviparous development.

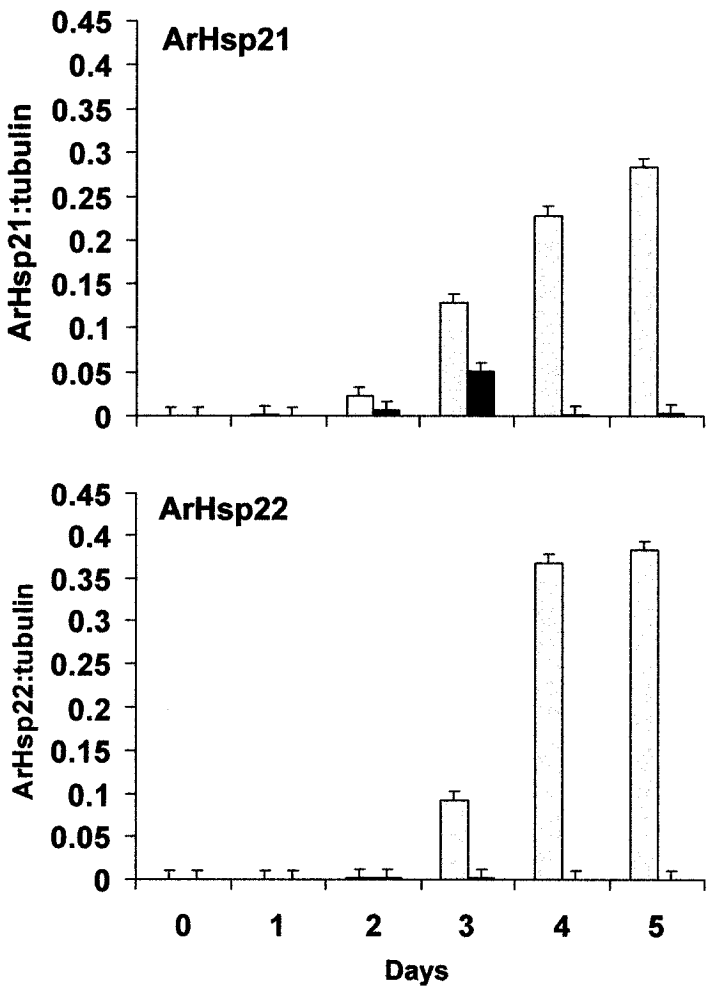

ArHsp21
ArHsp21:tubulin
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ArHsp22
ArHsp22:tubulin
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
Days

Figure 14

Figure 15. sHSPs during *Artemia* embryo development

Protein extracts prepared from *Artemia* embryos at daily intervals post-fertilization were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (A, D) or blotted to nitrocellulose and stained with antibodies to ArHsp21 (B, E) and ArHsp22 (C, F). A-C, oviparous development; D-F, ovoviviparous development. Lane 1, day 0; 2, day 1; 3, day 2; 4, day 3; 5, day 4; 6, day 5. Thirty μg of protein was loaded in each lane.

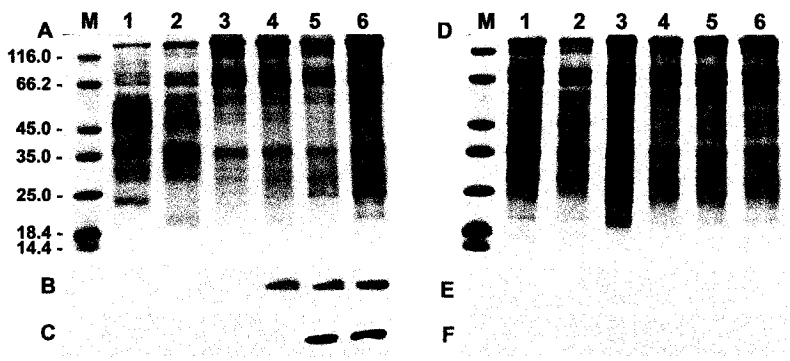


Figure 15

3.2.8.3 Expression of ArHsp21 and ArHsp22 in *Artemia* cysts, larvae and adults

Although ArHsp21 and ArHsp22 were discovered in embryos, it was of interest to examine their expression at other developing stages in order to better understand their functions *in vivo*. ArHsp21 and ArHsp22 appeared in cysts, emerged larvae (EIII) and instar I larvae, but not in Instar II larvae and adults (Fig. 16). p26 also occurred in cysts, EIII larvae and Instar I larvae, but was less abundant in instar II larvae than were the other two sHSPs.

3.2.9 Heat induction of ArHsp21 and ArHsp22

After heating instar II larvae and adults at 39 °C for one hr and allowing recovery for 2, 4 and 6 hr, neither p26 nor ArHsp21 was found (Fig. 17 B, C, F, G). However, ArHsp22 was detected in adults, with the highest amount occurring after 6 hr of recovery (Fig. 17 D). ArHsp22 expression was not induced in instar II larvae (Fig. 17 H) after heating.

3.3 p8, a Co-transcription Factor

3.3.1 Full length cDNA sequence of p8

Based on the partial p8 cDNA sequence obtained by subtractive hybridization, 3'- and 5'RACE were performed to obtain the full length p8 cDNA sequence (Fig. 18). The 3'-RACE reaction gave one DNA fragment of 480 bp, whereas 5'-RACE yielded products of 240 bp and 273 bp, suggesting two transcription start sites. The longer p8 cDNA sequence was 577 bp and the shorter was 544 bp. p8 full length cDNA consisted of an ORF of 201 bp, 5'- UTR of either 43 or 10 bp, and a 322 bp 3'-UTR with poly(A) tail (Fig. 19). The deduced p8 amino acid sequence was 66 residues, beginning with

Figure 16. sHSP degradation during post-diapause *Artemia* development

Protein extracts were prepared from post-diapause developing *Artemia* in the presence of protease inhibitors and electrophoresed in SDS polyacrylamide gels. The gels were either stained with Coomassie blue (A) or transferred to nitrocellulose membranes and probed with antibodies to p26 (B), ArHsp21 (C) and ArHsp22 (D). Lane 1, undeveloped cysts; 2, emerged larvae; 3, instar I larvae; 4, instar II larvae; 5, adult males; M, molecular mass markers x 10^{-3}. Fifty μg of extract was loaded in each lane.

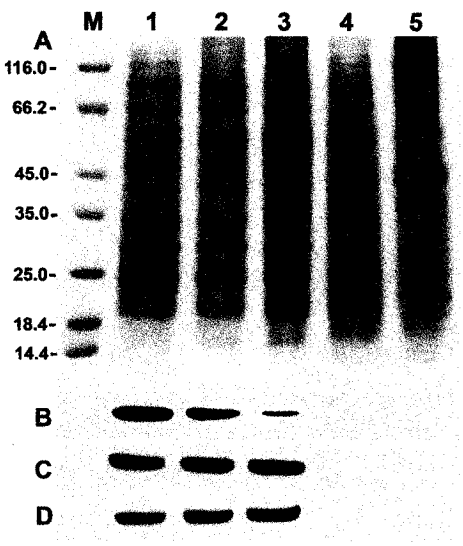


Figure 16

Figure 17. Heat induced synthesis of *Artemia* sHSPs

Artemia adult males (A-D) and instar II larvae (E-H) were heated at 39 °C for 1 hr, transferred to 27 °C and allowed to recover. Samples were homogenized, electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (A, E) or blotted to nitrocellulose and stained with antibodies to p26 (B, F), ArHsp21 (C, G) and ArHsp22 (D, H). Lanes 1, 3 and 5 are unheated controls, whereas lanes 2, 4 and 6 are from heat shocked organisms allowed to recover for 2, 4 and 6 hrs respectively; lane 7 is protein extract from cysts; M, molecular mass markers x 10^{-3} kDa. Forty-five μg of protein extract was loaded in each lane.

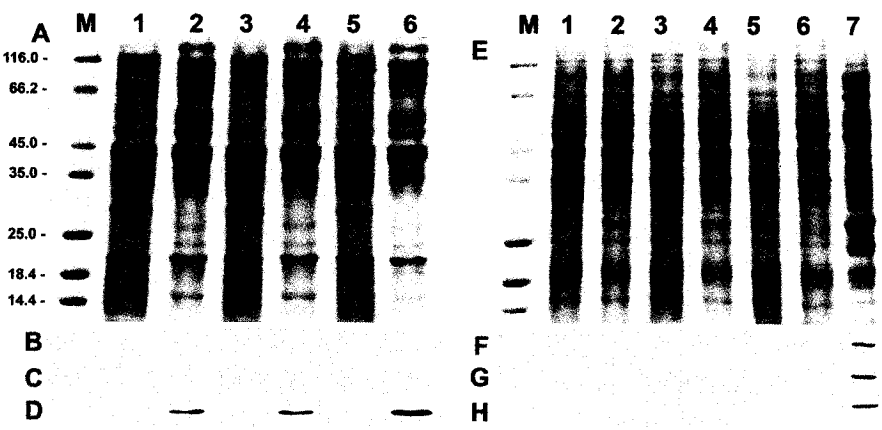


Figure 17

Figure 18. Cloning p8 cDNA

Total RNA was isolated from oviparously developing embryos two days post-fertilization and 3'- and 5'-RACE were performed using primers designed on the partial p8 sequence obtained by subtractive hybridization. RACE products were electrophoresed in agarose gels and stained with Gel Star. Lane1, 3'-RACE reaction lacking cDNA; 2, 3'-RACE; 3, 5'-RACE reaction lacking cDNA; 4, 5'-RACE, M, size markers in bp.

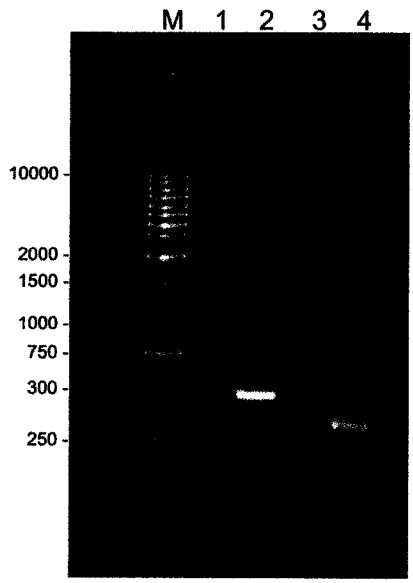


Figure 18

Figure 19. p8 cDNA and deduced amino acid sequences

The complete p8 cDNA sequence and its deduced amino acid sequence are shown. Boxed C and boxed A, transcription start sites; boxed ATG translation start site; boxed TGA stop codon; boxed AATAAA, polyadenylation signal.

```
                                                    M  S  E  D
   CATTTGAAAGGAAGAGTTTCAGGTAGTAGGATCAGGTGTGACGATGTCAGAAGATC 56
H  F  D  R  F  E  H  F  N  F  D  M  D  K  H  L  F  S  G  Q
ATTTTGATAGATTTGAACACTTTAACTTTGATATGGACAAGCATCTCTTCTCCGGGCAA 115
 S  G  R  L  R  T  K  Q  E  A  A  L  H  T  N  R  F  D  P  S
AGTGGAAGGCTGCGGACGAAACAGGAAGCTGCTCTTCATACAAACCGATTTGACCCTAG 174
  G  H  S  R  K  L  V  T  K  M  K  N  T  E  I  K  K  R  A
TGGACACTCAAGAAAGCTAGTAACGAAAATGAAAAACACGGAAATTAAAAAACGTGCTG 233
D  K  K
ACAAAAAATGACTTCTCCAGGGTTGTGAATTCTATAGGTCCCGGACTTCCGATCTCGTT 292
TTATGGCCTTCGTAATTGCAGTGGGACTGATTTTTTTTCAATTAATTGTATTAATTGTG 351
GACAATGGTGCTGGTAATTTAGCAACATGTAGGGCTCTTGGTGACAAAAGTGATAAAAA 410
TAGTTCCAAAAATGTGATATTTTGGGTTTTATATTTACTCCTTTCTTTTGCTGGCAACA 469
CTGTCTGAACATGTTTTTTTTTAATATTGTACTCGGTTGTGGTTTTCTTGCGTATACTG 528
TCTTTTGTATTCATTTTATCTGATAATAAACCTTTTGTAAAAAAAAAAA           577
```

Figure 19

methionine and ending with lysine (Fig. 19). The p8 molecular mass calculated with Gene Runner was 7.9 kDa.

3.3.2 p8 sequence comparison

The amino acid sequence of *Artemia* p8 was compared to p8 from other organisms (Fig. 20). p8 from *Artemia* was the shortest of the compared sequences and it shared high amino acid identity and similarity with p8 from other organisms (Table 6). The comparison of nucleotide sequences indicated limited similarity (Table 6).

3.3.3 Detection of the *Artemia* p8 gene

Southern blots containing DNA from *Artemia* digested with Xho I, Pst I, Sal I, Bam HI and Hind III, enzymes which do not cut *Artemia* p8 cDNA gave a single band for each digestion when hybrided with p8 probe(Fig. 21). The results indicate a single p8 gene in *Artemia*.

3.3.4 Production of antibody to p8

Extracts from *E. coli* BL21 transformed with the expression vector containing p8 cDNA were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue or transferred to nitrocellulose and probed with OmniProbe. The size of p8 as determined by SDS pplyacrylamide gel electrophoresis was larger than that determined by Gene Runner (Fig. 22). Purified p8 was injected into rabbits and polyclonal antibody specifically recognizing the protein was obtained.

3.3.5 p8 expression during embryo development

3.3.5.1 Quantitative analysis of p8 mRNA

p8 transcripts increased remarkably in embryos from day 0 to day 1 post-fertilization during oviparous development and then decreased until transcripts were no

Figure 20. Sequence comparison between p8 from *Artemia* and other organisms

The *Artemia* p8 amino acid sequence deduced from cDNA was aligned with p8 from *Drosophila* (NP_609539), human (AAC19384), mouse (NP_062712), rat (AAB94673) and *Xenopus* (BAB33387) by ClustalW. Red, small hydrophobic residue; blue, acidic residue; magenta, basic residue; green, residue with hydroxyl or amine group. "*", identical residues; ":", conserved substitutions; ".", semi-conserved substitution.

```
mouse          MATLPPTANPSQQPLNLEDEDGILDEYDQYSLA-HPC-VVGGGRKGRTKREAAANTNRPS 58
rat            MATLPPTAHTSQQPVNIEDEDGILDEYDQYSLA-QSY-VVGGGRKGRTKREAAANTNRPS 58
human          MATFPPATSAPQQPPGPEDEDSSLDESDLYSLA-HSY-LGGGGRKGRTKREAAANTNRPS 58
Xenopus        MKTSYIEAN-KVQPTDFEVQ--YFDEYEYYNLT-DRYSLPTAARKGRTKKEAEANTNRES 56
Drosophila     ------------------MSEAHFDEYEHYNFDHDKHIFSGHSGKQRNKREANEHTNHFD 42
Artemia        ------------------MSEDHFDRFEHFNFDMDKHLFSGQSGRLRTKQEAALHTNRFD 42
                                  .   :*. : :.:  .   .   . : *.*:**  :**: .

mouse          PGGHERKLLTKFQNSE-RKK--AWR-- 80
rat            PGGHERKLLTKFQNSE-RKK--AWR-- 80
human          PGGHERKLVTKLQNSE-RKKRGARR-- 82
Xenopus        PCGHERKISSKLQRSECKKKLKVAKV- 82
Drosophila     PSGHSRKILTKLMNTNNNNKKAAACKN 69
Artemia        PSGHSRKLVTKMKNT--EIKKRADKK- 66
               * **.**: :*: .:  . *  .
```

Figure 20

Table 6. Comparison of *Artemia* p8 with p8 from other organisms

Organism	Nucleotide (encoding region) Identity %	Amino acid Similarity %	Identity %
Mouse	52.5	63	43
Rat	50.5	63	43
Human	51.0	71	55
Xenopus	53.5	64	37
Drosophia	59.5	82	68

Figure 21. *Artemia* contains a single p8 gene

Artemia genomic (10 μg) DNA was digested with restriction endonucleases, electrophoresed in 0.6% agarose gels, transferred to nylon membranes and probed with labeled p8 cDNA. Lanes 1, 2, 3, 4, and 5 were loaded with DNA digested respectively with Xho I, Pst I, Sal I, Bam H I and Hind III. Lane 6, plasmid containing p8 cDNA. Fragment size is indicated on the left in kb.

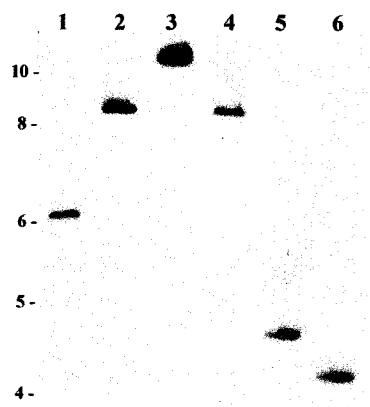


Figure 21

Figure 22. Synthesis and purification of p8

p8 was synthesized in transformed *E. coli* and purified using TALON affinity resin. Samples was electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (A and C) or transferred to nitrocellulose and probed with Omniprobe (B and D). Panels A and B, lane 1, bacterial extract containing p8; 2, extract from bacterial transformed with vector only. Panels C and D, lane 1, purified p8. M, molecular mass markers $x10^{-3}$. A, each lane received 30 μg of protein; B, 10 μg of protein; C, 3 μg of protein; D, 0.4 μg of protein.

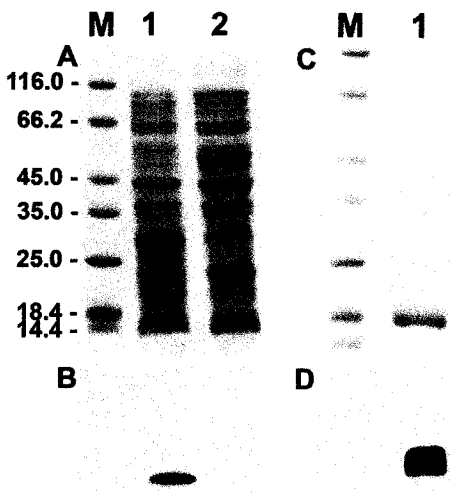


Figure 22

longer detectable at day 4 (Fig. 23). In contrast, there was a minor amount of p8 mRNA at day 1 during ovoviviparous development, which decreased throughout embryo development. (Fig. 23). There was a 111-fold difference in p8 mRNA at day 1 when oviparously and ovoviviparously developing embryos were compared.

3.3.5.2 Immunodetection of p8 during embryo development

p8 protein was first detected in embryos at day 2 post-fertilization and persisted until day 5 (Fig. 24), although after day 4 p8 mRNA was, as determined by RT-PCR, at very low levels (Fig. 23). In contrast, p8 protein was not detected in embryos at any stage of ovoviviparous development, as reflected by the low mRNA measurements.

3.3.6 Putative p8 gene regulatory elements

To explore the regulation of p8 gene transcription, 828 bp of sequence upstream of the p8 gene transcription start site was obtained by DNA walking (Fig. 25), and analyzed by computer to yield putative regulatory cis-acting elements (Fig. 25). The TATA box is located 57 bp upstream of the transcription start site, and elements binding Sp2, AP-1, CEBPB were observed. Two heat shock elements were found.

3.3.7 Localization of p8 to nuclei

p8 from other organisms is a co-transcription factor, suggesting it should reside in nuclei. Probing of western bots with antibody raised to p8 revealed the protein in *Artemia* cysts but not instar II larvae (Fig. 26 I). Immunofluorescent staining demonstrated p8 protein distributed in a speckled pattern within nuclei purified from cysts (Fig. 26 II A).

3.4 Diversity, Structure and Expression of the p26 Gene

3.4.1 p26 cDNA and protein sequence comparisons across *Artemia* species

Figure 23. p8 mRNA expression is developmentally regulated

Total RNA was prepared from *Artemia* embryos at daily intervals post-fertilization, reverse transcribed and quantified by Real-Time PCR using α-tubulin mRNA for comparison. Red, p8 mRNA in oviparous embryos; blue, p8 mRNA in ovoviviparous embryos.

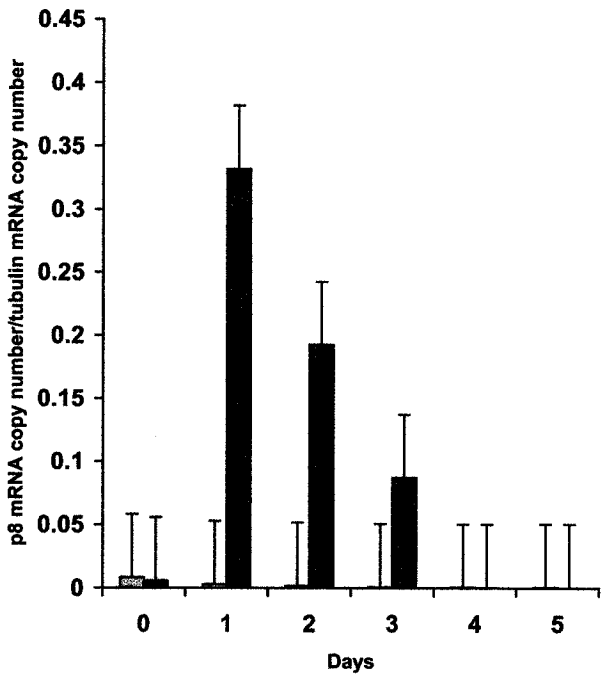

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
p8 mRNA copy number/tubulin mRNA copy number
0
1
2
3
4
5
Days

Figure 23

Figure 24. p8 synthesis during embryo development

Protein extracts were prepared from oviparously (A and B) and ovoviviparously (C and D) developing *Artemia* embryos at daily intervals post-fertilization, electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (A and C) or transferred to nitrocellulose and probed with antibody to p8 (B and D). Lane 1, day 0 (fertilization); lane 2, day 1 post-fertilization ; lane 3, day 2; lane 4, day 3; lane 5, day 4; lane 6, day 5. M, molecular weight markers x 10^{-3}. Thirty μg of protein extract was loaded in each lane.

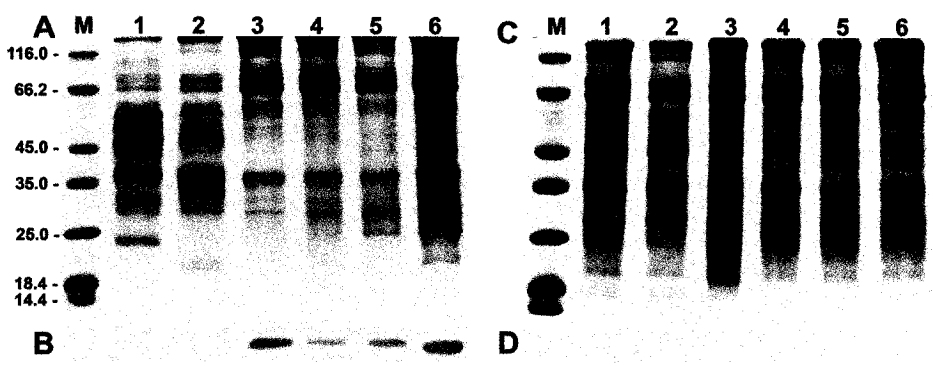


Figure 24

Figure 25. The p8 gene 5'-regulatory region

The upstream sequence of the p8 gene was obtained using LA PCR *in vitro* cloning. Boxed TATAA, TATA box; bold C with arrow, transcription start site; putative cis-acting regulatory sequences identified by computer assisted search are underlined and labeled in the figure.


CTTGTGTTTATTATATAACCGATAAGCACACAAAAAAGTG-792
CTATCTTAGCATTATTACAATCAATTTTTTGACTATAGCA-752
GATA2
TCGAAAATCGCTGTGCAGCACGCAATTATAGCACTTTAGG-712
AACTGACCGTGTCGGCAACCCTAATTCTTCTCCATTGAAC-672
ATAGTCTAGAGCCACCACGCGTACGTCTTCAGGCCTTATT-632
CATGCCTTCTAATCTTTTCATGACCATCATCTTCAGATGG-592
HSE
AGGGTCCGGCCCTACCGATCCTAGGCCCTTGAATTTGTAG-552
Sp2
TCCGCTATGGTCGGCTCAACCCACCCTAATAGGATCGAGG-512
CAGCACCCTGAAGAGGCAGCAGTCCATAACTGGTATACCC-472
TGCCGCAGTTGTGCTGCTATAGAGGATCCTCATACGAGGT-432
TATTCTAATAAAAAAAGGTAAAGATCAGGTTGCATTTGAT-392
AAATTTTAAGTGGTGTGGGTCTGTGCTTCATTGTTGCTTT-352
TCCTGTGAATTTCTTACCGCTTACCTTCTTAGTCACGAGG-312
Ap1
ATCCCTCCCTTTATTCTATGGAGCCCGCTTAATAGCCTCC-272
CTGGAAGCATCCTTGACAGTTCACATGTACACAACATATC-232
HSE
AGCTTTATCTAAAAAATGATGACGAGGTAATTGCATCATG-192
CEBPB
TTTAAAGCATTTCGTAAAATGTTTTGAGATTATTTGTGAA-152
CEBPB
AAAACTGCGCAGTTAAGGTTTTTTTGGCACAGTTGCTAGA-112
TTTTTTTTAACAAAAATGATAAGAGGCTATAACAGCGCCA -72
TCAGAATCCATATAAGTAACAAAGGTTTTGGATCAGTATC -32
AAAACTCGTTTTAGTTTTCTGTTAATGAAGTCATTT

Figure 25

Figure 26. p8 localizes to nuclei of encysted *Artemia* embryos

I. Western blots. Protein extracts from *Artemia* cysts (1) and instar II larvae (2) were electrophorsed in SDS-polyacrylamide gels, transferred to membranes and probed with antibodies to p8. II. Localization. Nuclei prepared from *Artemia* cysts (A, B, E, F) and instar II larvae (C and D) were fixed with paraformaldehyde and double stained with antibody to p8 and DAPI. Nuclei visualized in panels E and F received only secondary antibody followed by DAPI. Panels A, C and E, anti-p8 staining; panels B, D and F, DAPI staining.

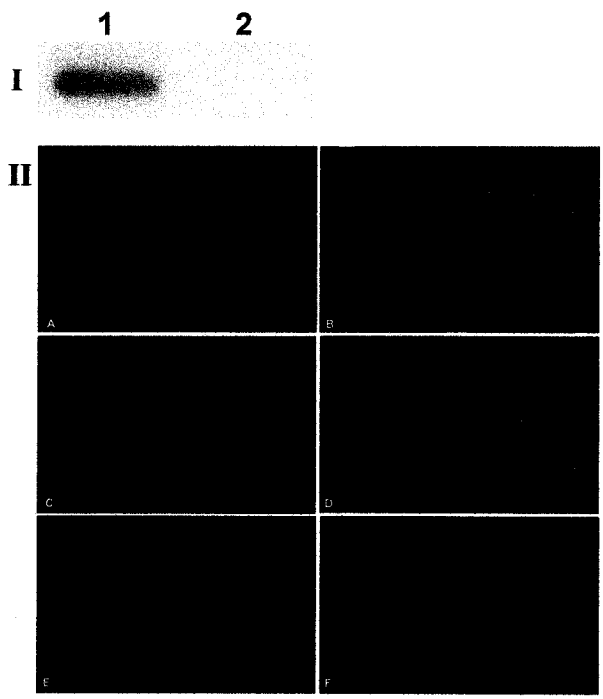


Figure 26

In order to further expand the study, p26 cDNAs were obtained from additional *Artemia* species and the *A. franciscana* p26 gene was cloned and sequenced. With stop codons included ORFs for p26 cDNAs from two *A. franciscana* strains and *A. sinica* were 579 nucleotides, *A. urmiana* p26 cDNA consisted of 576 nucleotides, *A. parthenogenetica* contained 585 and *A. persimilis* p26 cDNA was 588 nucleotides (Fig. 27). Comparison of nucleotide sequences demonstrated varying degrees of similarity between p26 cDNAs from disparate *Artemia* species with *A. persimilis* exhibiting the most variation (Fig. 27, Table 7), but generally the p26 cDNA nucleotide sequences were closely related.

Alignment of amino acid sequences deduced from p26 cDNAs indicated that some nucleotide substitutions led to residue changes (Fig. 28, Table 8). The carboxy-terminal extension contained the most modifications with 25.0% (10/40) of residues differing across species boundaries, followed by the amino-terminal region with 16.9 % (10/59) and the α-crystallin domain with 6.5% (6/93). Y18 in the p26 sequence 17-WYDPF-21 of *A. franciscana* from the GSL (ARC1520), representing a widely conserved sHSP motif, was S18 in all other *Artemia* species. This substitution introduces a large bulky amino acid for a smaller residue, although both are potential phosphorylation sites. Other modifications include the loss or gain of several potential phosphorylation sites, the loss of a negatively charged residue at position 35 in *A. parthenogenetica* (ARC1407) p26, and the short insertions, 184-STI-186 and 185-STR-187 in the carboxy-terminal extensions of *A. parthenogenetica* (ARC1407) and *A. persimilis* (ARC1321), respectively. R114 in the α-crystallin domain of p26 from the *Artemia* species examined is equivalent to a highly conserved arginine found in sHSPs from other organisms, and the conserved

Figure 27. p26 cDNAs from different *Artemia* species.

Nucleotide sequences of p26 cDNAs from *A. franciscana* (GSL) (1520) (Accession Number AF031367), *A. franciscana* (SFB) (1258) (Accession Number DQ310577), *A. sinica* (1218) (Accession Number DQ310576), *A. urmiana* (1511) (Accession Number DQ310580), *A. parthenogenetica* (1407) (Accession Number DQ310579) and *A. persimilis* (1321) (Accession Number DQ310578) were aligned by ClustalW. Nucleotide numbers are on the right. *, identical nucleotides; space, non-identical nucleotides. GSL, Great Salt Lake; SFB, San Francisco Bay.

```
1520    ATGGCACTTAACCCATGGTACGGAGGATTTGGTGGTATGACTGACCCATG 50
1258    ATGGCACTTAACCCATGGTGCGGAGGATTTGGTGGTATGACTGACCCATG 50
1218    ATGGCACTTAACCCATGGTACGGAGGATTTGGTGGTATGGGCGACCCATG 50
1511    ATGGCACTTAACCCATGGTACGGAGGATTTGGTGGTATGAGCGACCCATG 50
1407    ATGGCACTTAACCCATGGTACGGAGGATTTGGTGGTATGAGCGACCCATG 50
1321    ATGGCACTTAACCCATGGTACGGAGGATATGGCGGTATGACTGATCCGTG 50
        ********************* ******** *** ******   ** ** **

1520    GTATGATCCATTTGGATTTGGTGGCTTCGGAGGTGGCATGGACCTTGATA 100
1258    GTCTGATCCATTTGGATTTGGTGGCTTCGGAGGTGGCATGGACCTTGATA 100
1218    GTCTGATCCGTTTGGATTTGGTGGCATCGGAGGTGGCATGGACCTCGACA 100
1511    GTCTGATCCATTTGGATTTGGTGGCGTCGGAGGTGGCATGGACCTCGACA 100
1407    GTCTGATCCATTTGGATTTGGTGGCGTCGGAGGTGGCATGGACCTCGACA 100
1321    GTCAGATCCATTTGGATTTGGTGGCTTCGGAGGTGGTATGGACCTTGACA 100
        **  ***** ********************** ********** ******** ** *

1520    TTGACAGGCCCTTCCGGAGAAGAATGATGAGAAGAGGTCCAGATACCAGC 150
1258    TTGACAGGCCCTTCCGGAGAAGAATGATGAGAAGAGGTCCAGATACCAGC 150
1218    TTGACAGGCCCTTCCGGAGAAGAATGATGAGAAGAGCTCCAGATACCAGT 150
1511    TTGACAGGCCCTTCCGGAGAAGAATGATGAGAAGAGCTCCGGACACCAGT 150
1407    TTTACAGGCCCTTCCGTAGAAGAATGATTAGAAGAGCTCCGGACACCAGT 150
1321    TTGATAGACCCTTCAGGAGAAGAATGATGAGAAGAACTCCAGATACTAGT 150
        ** * ** ****** * ********** ******  *** ** ** **

1520    AGGGCTTTAAAGGAGTTAGCTACTCCTGGGTCCTTGAGGGACACAGCTGA 200
1258    AGGGCTTTAAAGGAGTTAGCTACTCCTGGGTCCTTGAGGGACACAGCTGA 200
1218    AGGGCTTTAAAAGAGCTAGCTACTCCTGGGTCTCTGAGGGACACTGCTGA 200
1511    AGGGCTATAAAAGAGCTAGCTACTCCTGGGTCCTTGAGGGACACTGATGA 200
1407    AGGGCTTTAAAAGAGCTAGCTACTCCTGGGTCCTTGAGGGACACTGCTGA 200
1321    CGGGCTCTAAAGGAGTTATCTACTCCTTGGACCTTAAGGGACACTGCTGA 200
         ***** **** *** ** ******** ** *  * ******** * *** 200

1520    TGAATTTCAAGTTCAGCTAGATGTTGGCCACTTTTTACCAAACGAAATTA 250
1258    TGAATTTCAAGTTCAGCTAGATGTTGGCCACTTTTTACCAAACGAAATTA 250
1218    TGAATTTCAAGTTCAGCTAGATGTTGGTCACTTTCTACCAAATGAAATTA 250
1511    TGAATTTCAAGTTCAGCTAGATGTTGGTCACTTTCTACCAAATGAAATTA 250
1407    TGAATTTCAAGTTCAGCTAGATGTTGGTCACTTTCTACCAAATGAAATTA 250
1321    TGAATTTCAAGTTCAACTAGATGTTGGCCACTTTCTACCAAATGAGATTA 250
        *************** *********** ****** ******* ** ****

1520    CAGTCAAGACAACCGACGATGATATTCTTGTCCATGGCAAACATGACGAG 300
1258    CAGTCAAGACAACCGACGATGATATTCTTGTCCATGGCAAACATGACGAG 300
1218    CAGTCAAGACCACCGACGATGATATTCTTGTCCATGGCAAACACGACGAG 300
1511    CAGTCAAGACCACTGACGATGATATTCTTGTCCATGGCAAACATGACGAG 300
1407    CAGTCAAGACCACTGACTATGATATTCTTGTCCATGGCAAACATGACGAG 300
1321    CAGTCAAGACCACTGATGACGATATTCTTGTTCATTGCAAACATGACGAG 300
        ********** ** **  * *********** *** ******* ******

1520    CGATCTGATGAATATGGACACGTCCAAAGAGAATTTCGACGACGATACAG 350
1258    CGATCTGATGAATATGGACACGTCCAAAGAGAATTTCGACGACGATACAG 350
1218    CGGTCCGATGAATATGGACACGTCCAAAGAGAATTTCGACGACGATACAG 350
1511    CGGTCCGATGAATATGGACACGTCCAAAGAGAATTTCGACGACGATACAG 350
1407    CGGTCCGATGAATATGTACACGTCCAAAGAGAATTTCGACGACGATACAG 350
1321    CGGTCCGATGAATATGGACACGTCCAAAGAGAATTTCGACGACGATACAG 350
        ** ** ********** ************************************

1520    ACTCCCAGAACATGTCAAACCAGAATCTGTGTCATCTACTTTGTCATCAG 400
1258    ACTCCCAGAACATGTCAAACCAGAATCTGTGTCATCTACTTTGTCATCAG 400
1218    ACTCCCAGAACATGTCAAGCCAGAATCTGTGTCATCTACTTTGTCATCAG 400
1511    ACTCCCAGAACATGTCAAACCGGAATCTGTGTCATCTACTTTGTCATCAG 400
1407    ACTCCCAGAACATGTCAAACCGGAATCTGTGTCATCTACTTTGTCATCAG 400
1321    ACTACCAGAGCATGTCAAACCCGAATCTGTGTCATCTACTTTGTCATCAG 400
        *** ***** ******** ** ****************************

1520    ATGGTGTCTTAACTATCCATGCTCCGAAAACTGCTTTAAGCTCACCAACA 450
1258    ATGGTGTCTTAACTATCCATGCTCCGAAAACTGCTTTAAGCTCACCAACA 450
1218    ATGGTGTCTTAACTATCCATGCTCCTAAAACTGCTTTGAGCTCACCAACG 450
1511    ATGGTGTCTTAACTATCCATGCTCCAAAAACTGCTTTGAGCTCACCAAAA 450
1407    ATGGCGTCTTAACTATCCATGCTCCAAAAACTGCTTTGAGCTCACCAACA 450
1321    ATGGTGTTTTAACTATACATGCTCCAAAAACTGCTTTGAGCTCACCAACG 450
        **** ** ******** ******** *********** **********

1520    GAACGTATCGTACCCATCACACCAGCGCCAGCTGTTGGAAGGATTGAAGG 500
1258    GAACGTATCGTACCCATAACACCAGCGCCAGCTGTTGGAAGGATTGAAGG 500
1218    GAACGTATCGTACCCATCACACCAGCGCCAGCTGTTGGAAGGATTGAAGG 500
1511    GAACGTATCGTACCCATCACACCAGCGCCAGCCGTTGGAAGGATTGAAGG 500
1407    GAACGTATCGTACCCATCACACCAGCGCCAGCCGTTGGAAGGATTGAAGG 500
1321    GAACGTATCGTACCCATCACGCCAGCGCCAGCTGTTGGAAGGATTGAAGG 500
        ***************** ** *********** *****************

1520    GGGAACTACAGGTACTACTACAGGCAGTACAGCTAGTTCAACTACAGCAA 550
1258    GGGAACTACAGGTACTACTACAGGCAGTACAGCTAGTTCAACTCCAGCAA 550
1218    GGGCACTGCCGGTACTACTACAGGCAGTACAGCTAGTTCAACTCCAGCAA 550
1511    GGGCACTACCGGTACTAC---AGGCAGTACAGCTAGTTCAACTCCAGCAA 547
1407    GGGCACTACCGGTACTAC---AGGCAGTACAGCTAGTTCAACTCCAGCAA 547
1321    TGGAACGACGGGTTCTACTGCAGGTAGTACAGCTAGTTCAACTCCAGCAA 550
         ** **  * *** ****   *** ****************** ******

1520    GAACAACAA---------GATCAGGAGGTGCAGCTTAA 579
1258    GAACAACAA---------GATCAGGAGGTGCAGCTTAA 579
1218    GATCAACAA---------GATCAGGAGGTGCAGCTTAA 579
1511    AATCAAAAA---------GATCAGGAGGTGCAGCTTAA 576
1407    GATCAACAATATCAACAAGATCAGGAGGTGCAGCTTAA 585
1321    GATCAACAAGATCAACAAGATCAGGAGGTGCAGCTTAA 588
         * *** **          ********************
```

Figure 27

Table 7. Comparison of p26 cDNA sequences. p26 cDNA clones obtained from *Artemia* species by RT-PCR were sequenced and compared as described in Materials and Methods.

	A. fran (GSL)	*A. fran* (SFB)	*A. sinica*	*A. urmiana*	*A. parthen*	*A. persimilis*
A. franciscana (GSL) (ARC1520)	100%					
A. franciscana (SFB) (ARC1258)	99%	100%				
A. sinica (ARC1218)	94%	94%	100%			
A. urmiana (ARC1511)	93%	93%	96%	100%		
A. parthenogenetica (ARC1407)	93%	93%	95%	97%	100%	
A. persimilis (ARC1321)	90%	90%	90%	90%	90%	100%

Figure 28. p26 amino acid sequences

Amino acid sequences of p26 from *A. franciscana* (GSL) (1520), *A. franciscana* (SFB) (1258), *A. sinica* (1218), *A. urmiana* (1511), *A. parthenogenetica* (1407) and *A. persimilis* (1321) were aligned by ClustalW. Amino acid residue numbers are on the right. (*), identical residues, (:), conserved substitution; (.), semi-conserved substitution; space, residue missing or nonconserved substitution.

```
1520  MALNPWYGGFGGMTDPWYDPFGFGGFGGGMDLDIDRPFRRRMMRRGPDTS 50
1258  MALNPWCGGFGGMTDPWSDPFGFGGFGGGMDLDIDRPFRRRMMRRGPDTS 50
1218  MALNPWYGGFGGMGDPWSDPFGFGGIGGGMDLDIDRPFRRRMMRRAPDTS 50
1511  MALNPWYGGFGGMSDPWSDPFGFGGVGGGMDLDIDRPFRRRMMRRAPDTS 50
1407  MALNPWYGGFGGMSDPWSDPFGFGGVGGGMDLDIYRPFRRRMIRRAPDTS 50
1321  MALNPWYGGYGGMTDPWSDPFGFGGFGGGMDLDIDRPFRRRMMRRTPDTS 50
      ****** **:*** *** *******.******** *******:** ****

1520  RALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDE 100
1258  RALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDE 100
1218  RALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDE 100
1511  RAIKELATPGSLRDTDDEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDE 100
1407  RALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDYDILVHGKHDE 100
1321  RALKELSTPWTLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHCKHDE 100
      **:***:** :**** ********************** ***** ****

1520  RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT 150
1258  RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT 150
1218  RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT 150
1511  RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPK 150
1407  RSDEYVHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT 150
1321  RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT 150
      ***** ******************************************.

1520  ERIVPITPAPAVGRIEGGTTGTTTGSTASSTTAR---TTRSGGAA 192
1258  ERIVPITPAPAVGRIEGGTTGTTTGSTASSTPAR---TTRSGGAA 192
1218  ERIVPITPAPAVGRIEGGTAGTTTGSTASSTPAR---STRSGGAA 192
1511  ERIVPITPAPAVGRIEGGTTG-TTGSTASSTPAK---SKRSGGAA 191
1407  ERIVPITPAPAVGRIEGGTTG-TTGSTASSTPARSTISTRSGGAA 194
1321  ERIVPITPAPAVGRIEGGTTGSTAGSTASSTPARSTRSTRSGGAA 195
      ******************:* *:*******.*:   :.******
```

Figure 28

Table 8. Comparison of p26 proteins. Amino acid sequences of p26 from *Artemia* species were deduced from their corresponding cDNAs and compared as described in Materials and Methods. Values not in brackets represent amino acid identity and those in brackets indicate similarity.

	A. fran (GSL)	*A. fran* (SFB)	*A. sinica*	*A. urmiana*	*A. parthen*	*A. persimilis*
A. franciscana (GSL) (ARC1520)	100%					
A. franciscana (SFB) (ARC1258)	98% (98%)	100%				
A. sinica (ARC1218)	96% (96%)	96% (96%)	100%			
A. urmiana (ARC1511)	93% (95%)	94% (96%)	95% (96%)	100%		
A. parthenogenetica (ARC1407)	92% (93%)	93% (94%)	94% (95%)	93% (94%)	100%	
A. persimilis (ARC1321)	92% (94%)	93% (95%)	92% (94%)	92% (95%)	92% (95%)	100%

carboxy-terminal extension V/IPI/V motif as 154-VPI-156 occurs in these *Artemia* species.

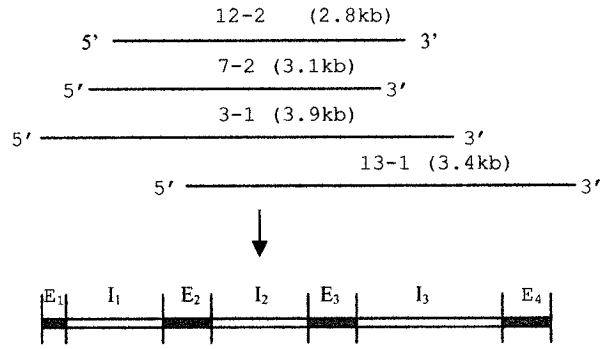
3.4.2. The *A. franciscana* p26 gene

The titer of the genomic library prepared from *A. franciscana* GSL (ARC1520) was 1.2 x 10^7 plaque forming units (PFU)/ml and ten clones picked randomly from the library contained inserts of 15 to 23 kb. Screening approximately 4.4 x 10^6 plaques with labeled full-length p26 cDNA yielded four reactive clones termed 12-2, 7-2, 3-1, and 13-1 with inserts of 17, 17.8, 19 and 15 kb respectively (Fig. 29 A). Overlapping DNA fragments from the clones yielded the p26 gene sequence including structural regions, and 5'- and 3'-UTRs (Fig. 29) deposited in the NCBI database under Accession Number DQ310575. Comparison of *A. franciscana* p26 cDNA (Accession Number AF031367) with the genomic sequence revealed three introns, with intron 1 in the 5'-UTR and the insertion sites of introns 2 and 3 disrupting codons (Fig. 29B). Agarose gel electrophoresis of 5'-RACE products gave one DNA fragment indicating a single transcription start site (Fig. 30), this away from the ATG translation initiation codon in p26 cDNA and genomic sequences by 53 bp and 1348 bp, respectively (Fig. 29B, 30B). Computer-assisted analysis indicated intron 1 contains many putative cis acting elements of potential interest for p26 gene expression during development, including heat shock factors (HSFs) and Ap1 binding sites (Fig. 29B). The introns of 1295, 1261 and 1283 bps begin with GT and terminate in AG, consistent with the intron boundary rule. The exons, composed of 47, 113, 225 and 333 bps do not correspond to p26 domain structure (Fig. 31), nor are they consistent in position and number to intron/exon locations in other sHSP genes deposited in the NCBI data base. Approximately 5 kb of non-coding sequence upstream of the p26

Figure 29. Cloning and sequencing of the p26 gene from *A. franciscana* (ARC1520) GSL

A, the p26 genomic clones, 12-2, 7-2, 3-1 and 13-1 were restriction digested, electrophoresed in agarose gels and blotted to membranes. DNA fragments revealed on Southern blots to hybridize with labeled p26 cDNA probes, for which sizes are indicated in brackets, were sequenced and aligned to yield the p26 gene (Accession Number DQ310575). E_1-E_4, exons; I_1-I_3, introns. B, p26 gene sequence. Boxed A, transcription start site; boxed ATG, translation start site; boxed TAA, stop codon; boxed AATAAA, polyadenylation signal; exons, upper case letters in bold; introns, lower case letters; HSEs, lower case letters in bold; underline, Ap1 binding site; nucleotides 391-1390 are identical to those obtained by LA PCR *in vitro* cloning; nucleotide numbers are on the right.

A



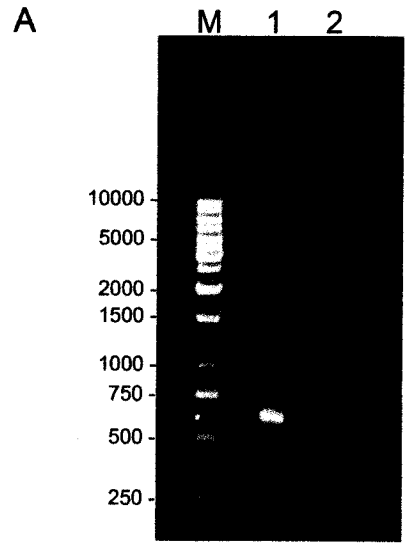
B

5'-ACCATAAGTTTGAACTCGATTGACAGGGACAGACTCATATTAACATTgtaagttcttgtttttttctgtttgggattcatttgttttataat 92
aagtcagtaattccaaattatgatctatgtttaccggtaggagttgagtcatacgaagtaaatttttgtcgtcctggagaaccaaaaataaattaaatg 192
aatttctgtaatatttggaaagtcagatttggcaaatacccttctgaaccccttgtgaagtattgttgaaaacttcagctatgcagcttttcactacctc 292
aaatttttttaaaacaaagataatgaagcttctagaaatgagtaaattagggtcagggttgtttgtgagatcccacttttctttgcctgctcgtagatc 392
aaatactctgaatttttctacacagctacaaaaatgggtaagattctagttaattgacttcttgctacctcggaaagggtttaggttaggaaaatgaaa 492
cagggatgggtttacaggctaaactatgtcccgggaaggtatttaaagtacccacctccactctttctccctctagagggccctgaaatttgcctaca 592
acaggtctacctactgaaattttgacaaaacattttaccttaatttttagttactacttgctttttctctgccttttagttctgaaaatgcaattcctg 692
atttgagtataatttttgagcccaatcaatgttttttcaaaatttaggaaatgtgttcgcatatctttaaaaccttacaaaatggaattgagcaatgttg 792
gatgaaaacaattttgttgtactttaattaagcagaagatgtattttgcaaggtttcacttttataacacatttttaaaggtcatcaaagctcagggc 892
tctagagggagaaggagtggaggtgggtacttcaaaatactttcccaggacaaacttcagcttgtagacccgtccctgaaactcattttcctaaccta 992
ccctttttgaaatagcaagaagtcaactagaattttaccagacgcaaaataaaaactaacgactagaaattattcagtatataagaggggctgcccttt 1092
ctcatccctcactctagactaagtttgacttttttccatttttatttttttttttttaagaactcttgaaacaagagggtcattaaatttaaatggg 1192
atatttttaatgaacttgaagactagcaagagtgaaggttaaggaagggggatccccctcataagggataattatttatcgttttaatttttcggatt 1292
ttcaattattgagaacagggctatccaatgtattgatatttctttttagATCAAAATGGCACTTAACCCATGGTACGGAGGATTTGGTGGTATGACTGA 1392
CATGGTCTGATCCATTTGGATTTGGTGGCTTCGGAGGTGGCATGGACCTTGATATTGACAGgtaagtatcatttctaagttctaatccaataaggtat 1492
gagttggcacggaaatgcttgtcttaaatgcagaggggctatttcttatttagtctgtagccagagatcgcccactttaccgttcgaataaatttttc 1592
agggtaactccccttgatgcgttgatgtgggagactaaggttttagcgatttttaaacacgattatattatctatagtctttcgagacatttttctgtt 1692
acattatattttcaaaaatattttgtgaagtgtttctgtttggaattttgaactggtcctcatacgagcatgttttccagtaaagttatagacccata 1792
accattacatcatatttttttcacccaggccctgtttatagaaggagttatcgcagaagcttaagaagtagctaatataattgaaaataaacttgtctt 1892
ttaaaagtaaaaatgattagagggttatcagcccctcccccgcccattatttcctcaacatgttgaatcgaaatttgacatgggcaatttttttttag 1992
tagttgaaaggtcatttttttcagagggcactgtcatttctaggacagggaattttgaaattaacatagaaaaactagtaatttaaaaacaaataagc 2092
catcgtgaagataggcctccaccgtgtgatcatgccatgtgctgtgcaaccccgttatttgagaaaggataactcgattatttctcagaaagaattgc 2192
aattggggtgcttcagatggaaattaaagttttttttaactgaaagtaaggagcgacattaaaacttaaaacgaacagaaattacttcgtatatggaa 2292
ggctgcttcctcatcaacgccccgctctttacgctaaagtttgactctcttcttttttaaaacttcttcttttttaaaattcttcttttttgaaacattct 2392
tacttttaggggtgtttccccctatttttctaaaaaacgcaaatttttctcagactcggtaacttttgatgggtaagacctaaacttgatgaaacttat 2492
atttaaaatcagcattaaattccatttttttagagttatggtttctattgagccgggtcgctccttactacagttctttaccacgaactgtttgaaaaa 2592
ctaagaacaaggggaacacatttcgactacagttgttttatgttcgttggttcttccagtgtatgaaaaggcttgttgtttaaccgccaaaacagaa 2692
ttagaaatggttatatttgcagGCCCTTCCGGAGAAGAATGATGAGAAGAGGTCCAGATACCAGCAGGGCTTTAAAGGAGTTAGCTACTCCTGGGTCC 2792
GAGGGACACAGCTGATGAATTTCAAGTTCAGCTAGATGTTGGCCACTTTTTACCAAACGAAATTACAGTCAAGACAACCGACGATGATATTCTTGTCC 2892
GGCAAACATGACGAGCGATCTGATGAATATGGACACGTCCAAAGAGAgtgaggcttatcttttttctccttttctttttttttctttaatacttagct 2992
aggatcggggatctagaggggggaatcgggatgaagcggagtggaggaatcgggatgaagcggagtggaggaatc 3092
gatgaagcttggtggatggaatatataaatattcattatgtccattaaaatgtccatgacagcgaattttctgtctccttactcctgggatattctcc 3192
tgcaattatgactacttttgcagtctccatttttggacggtaagcacatgaatgtaatcttttaatgctaaaaaaagttaaagaacttgtctcccagc 3292
cacccatattaggcttagtgcaaatgaagctaaaataagacattaatttcaactcatctgacacgttatgggattccctgctccttccacttttatca 3392
tagttcgtggtaaggaactagtaaggagcggccaggcccaaaagtaaccgacactaaaaaacaaaattttgatacccatagttacatcaaaagataaa 3492
ttttaattctgattttgactcttgactccacttttttaaaatagtaaaaaatttagcgtaaagagcggggtgttgaggagggaacaaccccttttcgtat 3592
ggagtaatttctgttcgttttaagctttaatgtcgctccttacttttggttgaaaaaactaaggtcagttaaaaaactttaatttaacaaagtaagg 3692
ccgaatgcaacctttatatcatagaatatcaacatccatattgcgctcttcttgaaaacaactcctccaaataacgggaggttagggtaaatctgt 3792
tgctatgtgggaccggctgactatgtccatattgatatgtaggacaggctgactaatttggcccatattgtgaggcttccggtcattcacggaatgat 3892
gcgtcctccgacactgaaatatatcattcttaagataggaaaactgatttggatataaaaatatcgaatttccaacatcttgttaagcaaagcagaga 4092
ctctatgaaacacttaggaagattaagcttaaacttgttaaatttaccccctcacaccatttaaagaaaaataagtaataagaattaaaatagtgtca 4192
atatgtttggcttgtggattatttggcttgtagccaacgcgaatggatcatggtgatgacttatgctaaaaaattcatgactgttggtccaagccctа 4292
tttttacatttattatacattattttgcagATTTCGACGACGATACAGACTCCCAGAACATGTCAAACCAGAATCTGTGTCATCTACTTTGTCATCAG 4392
GGTGTCTTAACTATCCATGCTCCGAAAACTGCTTTAAGCTCACCAACAGAACGTATCGTACCCATCACACCAGCGCCAGCTGTTGGAAGGATTGAAGG 4492
GAACTACAGGTACTACTACAGGCAGTACAGCTAGTTCAACTCCAGCAAGAACAACAAGATCAGGAGGTGCAGCTTAATCTGCATTCAATATATATTCA 4592
GAATCTTCCGGTCTTTTTTCTTTGTACCTTTATTTTTTTGTCAATAAATCTGTATACGAGGCAAttgaagattgttccttcgct-3' 4679

Figure 29

Figure 30. Identification of the p26 gene transcription start site

A, the products of 5'-RACE reactions were electrophoresed in agarose gels and visualized by staining with Gel Star. Lane M, size markers, bp; 1, 5'-RACE products; 2, 5'RACE products in the absence of template DNA. B, sequence of DNA fragment generated by 5'RACE. Boxed A, transcription start site; boxed ATG, translation start site. Nucleotide numbers are on the right.



B

```
5'-ACCATAAGTTTGAACTCGATTGACAGGGACAGACTCATATT   41
AACATTATCAAAATGGCACTTAACCCATGGTACGGAGGATTTGG   85
TGGTATGACTGACCCATGGTCTGATCCATTTGGATTTGGTGGCT  129
TCGGAGGTGGCATGGACCTTGATATTGACAGGCCCTTCCGGAGA  173
AGAATGATGAGAAGAGGTCCAGATACCAGCAGGGCTTTAAAGGA  217
GTTAGCTACTCCTGGGTCCTTGAGGGACACAGCTGATGAATTTC  261
AAGTTCAGCTAGATGTTGGCCACTTTTTACCAAACGAAATTACA  305
GTCAAGACAACCGACGATGATATTCTTGTCCATGGCAAACATGA  349
CGAGCGATCTGATGAATATGGACACGTCCAAAGAGAATTTCGAC  393
GACGATACAGACTCCCAGAACATGTCAAACCAGAATCTGTGTCA  437
TCTACTTTGTCATCAGATGGTGTCTTAACTATCCATGCTCCGAA  481
AACTGCTTTAAGCTCACCAACAGAACGTATCGTACCCATCACAC  525
CAGCGCCAGCTGTTGGAAGGATTGAAGGGGGAACTAC-3'      562
```

Figure 30

Figure 31. Comparison of p26 domain and exon positions

Schematic representations of p26 protein and cDNA were drawn to scale and aligned. N, amino-terminal region; α, α-crystallin domain; C, carboxy-terminal extension; E_1-E_4, exons. Numbers above each schematic indicate p26 amino acid residues within protein domains (upper half of figure) and encoded by exons (lower half of figure).

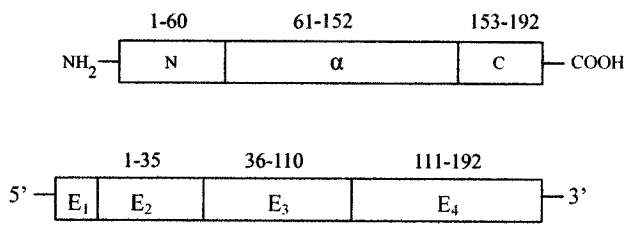


Figure 31

transcription start site was obtained, and of this approximately 1.0 kb is shown revealing TATA and CCAAT boxes 22 and 392 bp upstream, respectively, from the transcription start site (Fig. 32). Representative transcription factor binding sites include those for HSFs and the estrogen receptor.

3.4.3 p26 gene expression in *Artemia* embryos

As determined by Real-Time PCR, p26 mRNA first appeared in oviparously developing *Artemia* embryos two days post-fertilization and increased until cyst release from females, whereas p26 mRNA was observed in ovoviviparous embryos at 2 and 3 days post-fertilization only (Fig. 33). Oviparous embryos possessed approximately 10 times more p26 mRNA than ovoviviparous embryos at three days post-fertilization. A strong p26 band was observed on northern blots containing mRNA from oviparous embryos two days post-fertilization while similar amounts of mRNA from ovoviviparous embryos yielded a weak band (Fig. 33, insert). p26 was detected on western blots containing protein extracts from oviparous but not ovoviviparous embryos (not shown).

Figure 32. The noncoding sequence 5' to the p26 gene transcription start site

The noncoding region 5' to the p26 transcription start site was obtained by sequencing genomic clones. Boxed A, transcription start site; boxed tata, TATA box; boxed ccaat, CCAAT box ; lower case letters in bold, heat shock elements; underline, estrogen receptor binding site; nucleotide numbers are on the right.


cctactgaaaaaaaaaacgggagatatatcagtgctag-1027
cgatgcaaaagggattttccttttttgttcaaggagggg -987
aaccataatttccttgtaagaggttttcgaccgcactg -947
attccaatggtagactttattttgacctggcgccattt -907
tcgaagggatgttcaggcttttctttcagataaatttt -867
ctttcgcaataaacttttaagacgaaccgaaataatag -827
ttaccattcctgagttagtgtcagatggcaatttttttt -787
cactaggcatttttttcaaaatgcgtttttttaactttt -747
ggttactatgggctgtggtttgctctttactaggttgc -707
atctaccactgcaaccacgatcagcattttatttactt -667
atatgacaattatatcatctcccccattacagattcat -627
gtgcatatttaaggtttcgtgatgtttacgtaaatatc -587
ttgtgttataaatcaaaatttgaattcatccccgcttt -547
taacagaactcatcgcaccagattacagttaattataa -507
taaattattgaataaacctaaaagcttcagtttattaa -467
atgtgaattgggcatcaaaatgaatttcttaacaaaat -427
gtcttgtaaaccaatggaatctttgagagtaaccaaat -387
ggcttatactaataaataaatattcaaagaatttgtat -347
ctttatgatagttcttaataagtgcttttgaacccaat -307
tgaactattaaaactgactatataacgccacaaatagc -267
aaaaattatttgtgataagcttattgaaagcagggcat -227
atcaaaccttgtttttggtttgtaacacaaagaaaaat -187
aagcagctgaattttcagatatccgggattccaattct -147
ataacaacattttgatctagccctatttcacagaaacc -107
gtagcttttgttaaggcctgttttagccaggtttaaac -67
tgaaaatatggtctcgccactgcacgttctctgcaaat -27
atataaccaagtaaaaacgttgatcaACCATAA 7

Figure 32

Figure 33. Quantification of p26 mRNA during *Artemia* embryo development

p26 mRNA was quantified in *Artemia* embryos by Real-Time PCR as described in Materials and Methods using tubulin mRNA as an internal standard. Insert, p26 mRNA and 18S rRNA obtained two days post-fertilization were electrophoresed in agarose, blotted to membranes and hybridized to labeled probes. C, oviparously developing embryos; N, ovoviviparously developing embryos. Each lane of the northern blot received 4.8 μg of total RNA.

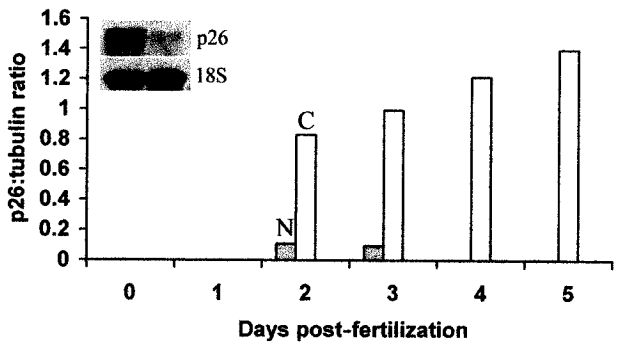

p26
18S
C
N
p26:tubulin ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Days post-fertilization

Figure 33

IV. Discussion

4.1 Gene Expression in Diapause-destined *Artemia* Embryos

Diapause, characterized by the arrest of development and metabolism, is utilized by insects and their arthropod relatives to exploit seasonal resources and escape harsh conditions such as winter and dry seasons [14]. Some organisms such as the flesh fly *Sarcophaga bullata* enter diapause after receiving an environmental change cue like a period of short daylength plus low temperature [3]. The *C. elegans* third instar larvae enter diapause (dauer) when they encounter unfavourable circumstances [4]. In contrast, *Artemia* diapause is obligatory, and occurs normally as part of the life cycle. As a simple example of this, when *Artemia* are cultured in our laboratory under identical conditions, some females release swimming nauplli and others release cysts, entering diapause. What induces *Artemia* embryos to enter diapause is not known.

In order to examine the differential gene expression during *Artemia* encysment, subtractive hybridization was employed to obtain genes that were up-regulated in oviparous but not ovoviviparous embryos 2 days post-fertilization. The proteins encoded by these genes may contribute to diapause induction or maintenance through participating in different metabolic activities. A gene similar to *Drosophila* takeout was highly expressed and is presumably associated with signal transduction during *Artemia* diapause. The takeout gene belongs to the group of circadian clock genes and is induced by starvation. Under starvation, the average death time is 30 hr for wild type flies and 20 hr for takeout gene mutant flies [137, 138]. Furthermore, in *Manduca* larval epidermis, a gene in the takeout gene family is regulated by hormones and the protein encoded by this gene can stabilize juvenile hormone (JH) [139]. In diapause induction, hormones play an

important role in signal transfer. As a case in point, diapause hormone (DH), a neuropetide appears to promote diapause in commercial silkmoth *Bombyx mori*, [3]. Therefore, the up-regulated takeout in *Artemia* might influence the hormones that are presumably involved in signal transduction during diapause.

p8 was overexpressed in oviparous as compared to ovoviviparous development in *Artemia*. In human and mouse, p8 is a co-transcription factor and is involved in stress such as pancreatitis, minor pancreatic injuries and cancer where it suppress growth [133, 135]. Therefore, in *Artemia*, p8 may trigger the transcription of genes encoding proteins implicated in controlling shutdown of metabolism. Detailed discussion on p8 will be presented in later sections, and is especially interesting because other up-regulated transcription factors were not detected as *Artemia* embryos underwent oviparous development.

Three small HSPs p26, ArHsp21 and ArHsp22 are highly expressed during encystment, and they may enhance protection of other proteins during diapause. Likewise, in the flesh fly *Sarcophaga crassipalpis*, transcripts of hsp23 and hsp70 are absent in unstressed, nondiapause pupae but highly expressed in diapausing pupae without thermal stress, which suggests these molecular chaperones modulate the rapid increase in stress tolerance that occurs during early diapause [17, 21]. In addition, hsp23 and hsp70 were suggested to have a function in cell cycle regulation within the diapause program [18]. In *Artemia*, although p26 enters cyst nuclei, it does not appear to operate as a specific transcription regulator [39, 140-142], and thus is unlikely to promote *Artemia* diapause.

Genes involved in cell growth suppression were up-regulated in encysting *Artemia* embryos. These included neuralized-related C3HC4 RING protein, cyclin-dependent protein kinase 5 (Cdk5) activator binding protein and BRCA1 associated protein-1 (Bap1). Neuralized-related C3HC4 RING protein is induced in the human lung during endotoxemia and has RING domain-depend auto-ubiquitination or E3 ligase activity [143], thus participating in the turnover of protein substrates [144-146]. In *Drosophila* and *Xenopus*, this protein is implicated in the Notch signaling pathway and may regulate the fate of cells early in differentiation and development [143]. Therefore, neuralized-related C3HC4 RING protein in diapause-destined *Artemia* embryos may suppress cell division and molecular synthesis. Cdks play critical roles in cell division regulation, with Cdk5 known to modulate cell differentiation [147, 148]. cDNAs for three Cdk5 activator binding proteins, designated C42, C48, and C53, were cloned in rat, with C42 shown to inhibit Cdk5 activation [149, 150]. Therefore, high expression of Cdk5 activator binding protein in *Artemia* may lead to inhibition of cell differentiation in embryos. As an ubiquitin hydrolase, BAP1 is a tumor suppressor protein that interacts with BRCA1, a breast cancer susceptibility gene product [151, 152]. BAP1 and BRCA1 co-localize in nuclei as dot-like structures, and BAP1 binds to the BRCA1 RING finger motif. Full-length BRCA1 binds to BAP1 *in vitro* which enhances the growth suppression properties of BRCA1 in colony formation assays [153]. In the *Artemia* subtractive library, BAP1 mRNA was observed but BRCA1 mRNA was not identified, suggesting either that not enough clones were sequenced to obtain BRCA1 mRNA or that BAP1 suppresses cell division and growth through interaction with a protein not closely related to BRCA1 in sequence. cDNA for QM, a putative tumor suppressor interacting with c-Yes was

recovered from the *Artemia* library, but it was present in two days post-fertilization during both cyst and nauplii development. c-Yes contributes to cell stability, division, proliferation, migration and differentiation, whereas QM reduces c-Yes kinase activity by 70%, leading to suppression of cell activity [154, 155]. QM expression at the protein and mRNA levels in embryos from day 3 to day 5 post-fertilization must be determined in order to better analyze its effects on diapause.

A new metabolic homeostasis is established during diapause, and up-regulated enzymes were identified, including glucose-6-phosphate isomerase, cytochrome p450, dimeric dihydrodiol dehydrogenase, aldehyde dehydrogenase and steroid dehydrogenase. During *Artemia* diapause transition, oxygen provision might decrease due to cyst shell construction and the highly expressed glucose-6-phosphate isomerase catalyzes D-glucose-6-phospate to D-fructose-6-phosphate in the glycolysis pathway, supplying ATP for maintenance of cell activity [156-158]. Likewise, during diapause in the mosquito *Culex pipiens*, blood-digesting enzymes are down-regulated and the enzymes associated with sugar feeding are up-regulated [159]. In organisms such as human, rat, pig and in plants, dimeric dihydrodiol dehydrogenase, cytochrome p450 and aldehyde dehydrogenase remove excess metabolic intermediates and potentially toxic substances. [160-167], whereas steroid dehydrogenase may be involved in hormone metabolism [168]. During *Artemia* diapause transition, metabolic activity decreases, potentially leading to accumulation of intermediates which are toxic to cells, but up-regulated dimeric dihydrodiol dehydrogenase, cytochrome p450 and aldehyde dehydrogenase could attenuate their effects, helping to re-establish homeostasis.

Interestingly, peritrophic membrane chitin binding protein and chitinase-like precursor cDNAs were recovered during oviparous development and they are implicated in chitin synthesis [169-171]. The shell contains chitin and it is involved in UV resistance because the death rate greatly increases if *Artemia* embryos without shells are exposed to UV irradiation [26]. Uncoupling protein 3 isoform, a membrane protein, was up-regulated in encysting embryos and it modulates substance consumption to release heat energy and maintain body temperature in other organisms [172-174], however, its function in *Artemia* remains to be determined.

On the basis of the gene expression profiles described above, a hypothetical model of *Artemia* diapause development modulated by up-regulated gene expression is proposed (Fig. 34). In *Artemia*, unknown signals involved in diapause induction are transferred to and activate transcription factors, thus leading to up-regulation of other transcription factors and their effectors such as p8. As a consequence, enzymes (G-6P, p450), suppressor proteins (C3HC4, Bap1) and stress proteins (ArHsp21, ArHsp22 and p26) are synthesized and embryos enter diapause. Results obtained by subtractive hybridization therefore provide insight into the mechanism by which diapause initiation is regulated. However, only a limited number of clones were sequenced in this study, and many other genes involved in diapause undoubtedly remain to be detected. Additionally, false positive clones such as housekeeping gene clones were observed in the library demonstrating the importance of verifying that genes are actually up-regulated. In future research, up-regulated genes identified in this study will be characterized in detail. Screening of subtractive libraries will be extended, likely revealing additional up-regulated and down-regulated genes implicated in *Artemia* diapause.

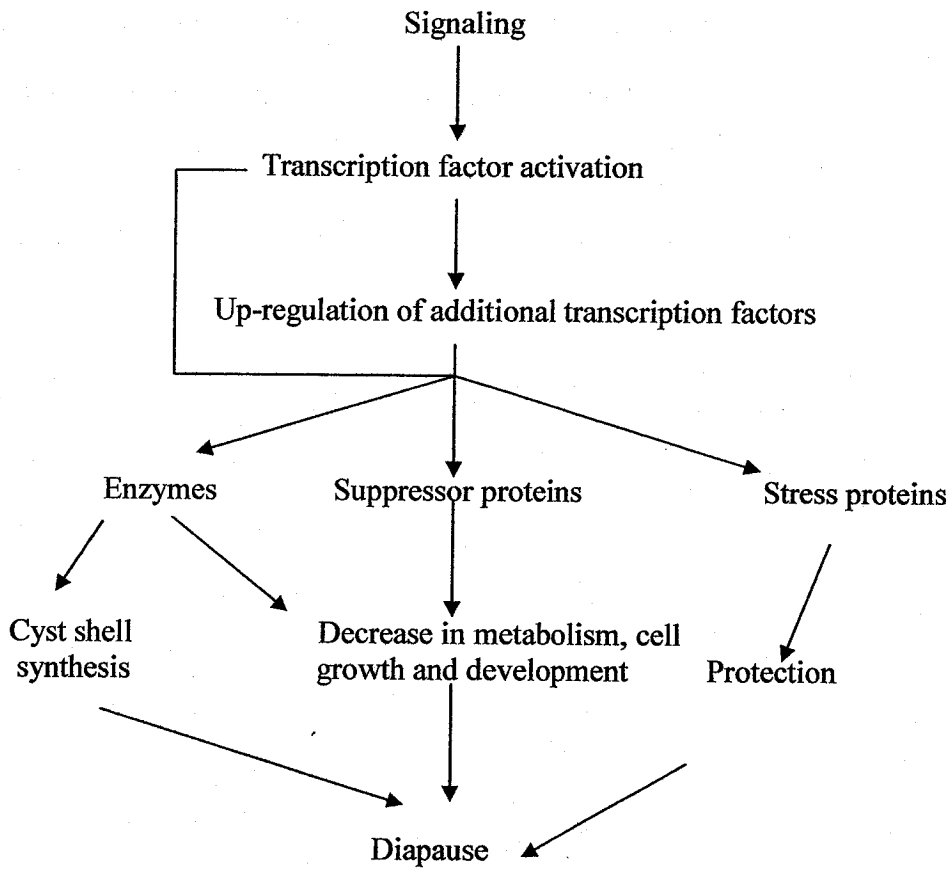


Fig 34. Regulation of diapause by up-regulated genes

4.2 Identification and Characterization of ArHsp21 and ArHsp22, Small Heat Shock Proteins Upregulated in Encysting *Artemia*

Partial cDNA sequences of ArHsp21 and ArHsp22 were obtained by screening the subtractive hybridization library, complete cDNAs were generated through 5'- and 3'-RACE, and their corresponding genes were detected on Southern blots, demonstrating the cDNAs are not likely to be RT-PCR artifacts. For ArHsp21, Southern blots of Bam HI, Sac I and Xho I digested genomic DNA yielded one band, implying that ArHsp21 is encoded by a single gene in *Artemia*. In contrast, for ArHsp22, the blots of Sal I and Hind III digested genomic DNA displayed two and five strong bands, respectively, suggesting either that there are multiple genes or there are introns in the *Artemia* ArHsp22 gene because the cDNA does not contain these restriction sites. However, the number of ArHsp22 gene copies in *Artemia* remains uncertain and characterization of ArHsp21 and ArHsp22 genomic clones is necessary. By comparison, in other organisms, the number of sHSP gene copies and the introns therein vary. For example, in the *C. elegans* genome, the sec-1 gene, encoding a sHSP, occurs as a single copy [43], whereas in plants, five sHSP gene families have been detected, each with multiple members [175]. *Drosophila* sHSP genes including hsp22, hsp23, hsp26 and hsp27 do not contain introns [176], but *C. elegans* Hsp16 genes possess an intron [177].

Alignment of the three known sHSP cDNAs from *Artemia* indicated low similarity although their lengths were similar. The stop codons of p26, ArHsp21 and ArHsp22 were TAA, TGA and TAA, respectively, and comparison of the deduced amino acid sequences of ArHsp21 and ArHsp22 against the NCBI database demonstrated they are similar to one another and to other sHSPs. Comparison with other sHSPs clearly displays the

presence of three domains within ArHsp21 and ArHsp22, namely the C-terminus, N-terminus, and α-crystallin domains. Additionally, ArHsp21 possesses an arginine at position 123 and ArHsp22 has an arginine at position 130, both corresponding to the most highly conserved residue in sHSP α-crystallin domain. The conserved carboxy-extension motif, I/VPV/I exists in ArHsp21 as 161IPI163 and in ArHsp22 as 170IQI172 [82]. Although p26, ArHsp21 and ArHsp22 exhibit a conserved α-crystallin domain and other similarities, antibodies to these proteins did not cross-react.

4.3 Oligomerization of ArHsp21 and ArHsp22

There are different opinions about the relationship between oligomerization and chaperone activity in sHSPs, but most researchers believe oligomerization is involved in chaperone activities [73, 76, 178, 179]. For example, Hsp12.6 from *C. elegans* is monomeric due to very short N- and C-terminal regions and does not exhibit molecular chaperone activity *in vitro* [84]. The present work showed that like p26, ArHsp21 and ArHsp22 oligomerize. The protein complexes yielded by p26 in the present experiment were consistent in mass with those examined in previous experiments and smaller than those in extracts from *Artemia* [180]. Purified p26 from *Artemia* assembles into oligomers as large as 670 kDa, whereas oligomers ranging from 29 to 320 kDa were obtained with p26 synthesized in bacteria. Therefore, it can be inferred that ArHsp21 and ArHsp22 in *Artemia* embryos may be bigger than determined in this study by synthesis in bacteria. By way of comparison, α-crystallin from the lens yields multimers with molecular masses ranging from approximately 300 kDa to greater than 1 MDa [181]. Pore exclusion limit electrophoresis demonstrated that Hsp27 forms oligomers of 200-

800 kDa [182]. Additionally, Hsp20 exists in a concentration dependent equilibrium between a dimer of 43 kDa and a large oligomer of 470 kDa [183].

4.4 Chaperone Activity of ArHsp21 and ArHsp22

Although, sHSP functions are not completely resolved, stabilization of partially folded intermediates during polypeptide folding, assembly and disasembly is important as is preventing aggregation of denatured and partially unfolded proteins [184-187]. Like other sHSPs such as p26, ArHsp21 and ArHsp22 inhibit the aggregation of citrate synthase (CS) under elevated temperature and prevent DTT-induced aggregation of insulin at 25 °C. There was no significant difference between the chaperone activities of ArHsp21 and ArHsp22, and they were concentration-dependent, with elevation of sHSP concentration increasing chaperone activity in *in vitro* assay. In many previous experiments, CS and insulin were used to analyze the chaperone activity of HSPs. For example, Hsp22 and Hsp23 from *Drosophila* prevented heat-induced aggregation of CS, although with different efficiencies [186], and in the presence of either murine Hsp25 or yeast Hsp26, the thermal aggregation of citrate synthase was effectively reduced [39]. Additionally, bovine lens α-crystallin, and human αA- and αB-crystallins are effective in preventing the aggregation of destabilized insulin by DTT [79, 188]. Thus, ArHsp21 and ArHsp22 can be thought to be small HSPs.

4.5 The Synthesis of ArHsp21 and ArHsp22 Is Developmentally Regulated

p26 is expressed during oviparous but not ovoviviparous development, potentially playing an important role in *Artemia* diapause [31]. Like p26, ArHsp21 and ArHsp22

were synthesized in oviparous but not ovoviviparous embryos, and thus like sHSPs in several other organisms, they are developmentally regulated. For example, in *C. elegans*, small embryonic chaperone (SEC-1) is regulated by intrinsic development factors rather than extrinsic stress [53]. In amphibians, the genes for sHSPs such as hsp30 are developmentally regulated under both normal and stress conditions [189]. The expression of three sHSPs p26, ArHsp21 and ArHsp22 during *Artemia* diapause development indicates that more than one stress protein is required to stabilize and protect other proteins. Likewise, in the flesh fly *S. crassipalpis*, transcripts of hsp23 and hsp70 are highly expressed in diapausing pupae, and in *C. elegans* dauer larvae, Hsp-20, Hsp-70 and daf-21, a sHSP, are up-regulated [25].

Immunodetection on western blots revealed that at day 3 post-fertilization during oviparous development, the ArHsp21 band was stronger than the ArHsp22 band. The expression of these sHSPs and p26 increased from three days to five days post-fertilization in oviparous embryos. mRNA of all three sHSPs exhibited similar profiles, with no transcripts at day 0 and reaching their highest amount at day 5, although the final expression levels varied. The amount of ArHsp22 mRNA was higher than for ArHsp21 and less than for p26. By the end of instar I, the three sHSPs were completely degraded, although at slightly different rates. Upon heat shocking of adults, ArHsp22 expression was detected but p26 and ArHsp21 were not, and no sHSPs were observed in heat shocked larvae. These findings suggest that p26, ArHsp21 and ArHsp22 play different roles in diapause although their functions may overlap, especially in protection of other proteins. p26 is present in cyst nuclei, presumably conferring protection to nuclear proteins [39, 141], but the localization of ArHsp21 and ArHsp22 was not determined. By

comparison, *Drosophila* hsp22, hsp23, hsp26, hsp27 and *l(2)efl* occur in different cells, organelles and development stages although they are all sHSPs, but their activities in each location are unknown.

In other organisms, expression of sHSP genes is regulated by transcription factors, including the HSFs (HSF1, HSF2, HSF3 and HSF4)[110-112], GAGA factor, ecdysterone receptor [102, 113], Pax-6, Phr and estrogen receptor. Analysis of the upstream region of these three *Artemia* sHSP genes is needed to further understand differences in their expression.

4.6 p8, a Developmentally Regulated Co-transcription Factor in *Artemia* Embryos

As a co-transcription factor, p8 is expressed rapidly in response to acute pancreatitis in human, mouse and rat and it is not restricted to these organisms [128, 129]. p8 functions in cell cycle regulation, initiation of luteinizing hormone beta (LHβ) gene expression, and control of tumor progression [128, 129]. In the present experiments, a partial p8 cDNA was recovered from *Artemia* embryos during oviparous development. Results, obtained by 5'- and 3'-RACE, revealed two transcription start sites in the p8 gene, but only one ORF and a typical polyadenylation signal (AATAAA). This suggests that two sets of transcription factors turn on p8 gene transcription at different binding sites, but details are lacking. Southern blots of *Artemia* genomic DNA digested with Xho I, Pst I, Sal I, Bam HI and Hind III and probed with a labeled p8 cDNA yielded one band, suggesting one p8 gene in *Artemia*. In comparison, only one p8 gene resides in humans and mice, with the human p8 gene mapped to chromosome 16 [134, 190].

The deduced amino acid sequence of p8 was very similar to p8 from other organisms, sharing 66% and 55% identity with *Drosophila* and human p8 respectively, with the protein from *Artemia* and *Drosophila* shorter than for other organisms. Sequence alignment with p8 from other organisms demonstrates three conserved peptides near the C-terminus, helix I (QEAALHT), loop (NRFDPSG) and helix II (HSRKLVTKM) [128], suggesting p8 is a helix-turn-helix transcription factor. The remainder of the amino-terminal sequence is more variable than the helix-turn-helix region. Interestingly, although sequence homology with HMG-I/Y proteins is low, p8 can be considered as a HMG-I-Y-like protein, because it is unstructured in solution and binds to DNA in a sequence-independent manner [135]. Moreover, p8 is phosphorylated by various kinases such as protein kinase C and protein kinase A (PKA), and phosphorylated p8 (PKAp8) binds DNA strongly [128]. p8 proteins lack signal peptides and typical transmembrane regions, but its carboxy-terminal region contains a nuclear localization signal and may modulate nuclear entry, as shown to occur in transiently transfected cells [190].

4.7 Developmental Regulation of p8

p8 protein occurs at very low levels in healthy human pancreas but is highly expressed during the acute phase of pancreatitis or upon encountering minor pancreatic injuries. The mRNA is rapidly and stably induced by endothelin-1 (ET-1) and requires recruitment of the phosphatidylinositol 3-kinase (PI3K) pathway and its effector NFAT4 [191]. p8 plays a functional role in the initiation of luteinizing hormone (LH) gene expression in the gonadotrope [136]. Interestingly, in mouse, p8 was found to greatly induce the expression of pancreatitis-associted protein (PAP), an anti-inflammatory factor

involved in defending the pancreas against various insults [127, 129, 192]. The available results indicate that p8 is an inducible gene and its expression may stimulate transcription of other genes although p8 function differs from cell to cell [132, 133]. [121]. In human and mouse, p8 interacts with the general co-activator p300 [131], further supporting the possibility that it functions as a co-transcription factor.

In *Artemia*, p8 was developmentally regulated. Real-Time PCR revealed that p8 mRNA peaks at one day post-fertilization in oviparous embryos, but was at a much lower level during ovoviviparous development. p8 protein appeared at day 2 post-fertilization and remained till day 5 in oviparous embryos, although mRNA was essentially absent after day 4. No p8 was present during ovoviviparous development. The expression pattern of p8 suggests that it plays a role in regulating *Artemia* diapause. p8 mRNA and protein appear earlier than other proteins such as p26, ArHsp21 and ArHsp22, indicating potential activity as a co-transcription factor as mentioned above in other organisms. That is, it is possible that p8 mRNA is rapidly transcribed at one day post-fertilization and translated into protein, which turns on genes suppressing metabolism and development, thus leading to diapause. However, if this actually occurs in *Artemia* and the genes under p8 control are not known and remain to be studied.

The transcriptional regulation of genes is a complex process which usually involves interaction between multiple cis-acting elements and their binding partners. To investigate p8 gene transcription, its upstream region was analyzed, revealing putative regulatory elements, including a TATA box, and Sp2, Ap1, GATA2, C/EBP β and other transcription factor binding sites. By comparison, in mouse p8 gene 5'-flanking region, C/EBP, Sp1, Oct-1, Ap-1, NFkB, IL6-RE, CREB and Myc binding sites were found,

whereas the human p8 5'-flanking region lacks a initiator sequence of PyPyANT/APyPy, CAAT and TATA boxes [134, 190]. Functional analysis demonstrated that C/EBPα and C/EBPβ interact with the ATTGCATCAG site of the upstream region in the mouse p8 gene to activate promoter activity with co-operation of other unknown trans-acting factors on the promoter. Alternatively, the C/EBP trans-acting factors could bind directly to a less conserved C/EBP cis-acting DNA sequence because in mouse p8 promoter, the region between nucleotides -100 and +36, without the classic C/EBP cis-acting sequence, still responds to the C/EBP trans-acting factors [134]. p8 gene expression was regulated by GATA2 on the basis of the relationship between the levels of p8 and GATA2 in gonadotropes, and of several potential GATA-binding sites located in the 5'-flanking region of the murine p8 gene[136]. These results suggest experimental approaches for the analysis of *Artemia* p8 gene promoter regulation.

4.8 Localization of p8 in *Artemia*

As a co-transcription factor, p8 is expected to localize to the nucleus at least transiently, if not at all times, and in this context, *Artemia* p8 was found in cyst nuclei, the expected location. By comparison, when human p8 cDNA was expressed in COS-7 cells, it was present almost exclusively in the nucleus as determined by immunostaining [190]. In follicular tumors, the nuclear localization of p8 was predominant, but p8 was dominantly in the cytoplasm in more aggressive tumor phenotypes such as those which are large or poorly differentiated. The significance of this observation is unknown [193].

4.9 Sequence Variation in p26 from Different *Artemia* Species

Different *Artemia* species contain comparable amounts of p26 [194] and, as demonstrated here, p26 amino acid sequences are similar from species to species with few suggestions of significant changes in protein structure and hence in function. The α-crystallin domain is modified at only six of ninety-three residues in all *Artemia* species examined and position 114 of p26 is occupied by arginine, a highly conserved sHSP residue which when mutated in human sHSPs leads to reduced chaperone activity and disease [66]. p26 from all *Artemia* species investigated contained the conserved carboxy-terminal motif 154-VPI-156, thought to promote oligomer stability by interacting with a hydrophobic fold between β-strands 4 and 8 in the α-crystallin domain of neighboring monomers [82]. The largest modifications in primary sequence were three residue insertions in the carboxy-terminal extensions of p26 from the geographically separated species *A. parthenogenetica* and *A. persimilis*. The insertions increased the hydrophilic character of the carboxy-terminal extensions, thus promoting sHSP solubility, and they may stabilize quaternary structure by increasing the number of residues available to interact with neighboring monomers [66, 70, 82]. A three residue insertion, Asp-Gly-Lys, occurs in the carboxy-terminal of Hsp26 from various *Drosophila* populations but its effect on structure and function are unknown [195].

Amino acid substitutions in p26 from different *Artemia* species are positioned randomly and some appear to have limited influence on the protein. On the other hand, the A66D and T150K substitutions in *A. urmiana* p26 might produce a salt bridge should the carboxy-terminal extension fold back on the α-crystallin domain. Structure would be stabilized and the introduction of K150 into the carboxy-terminal extension also increases protein solubility. The G96C substitution in β-strand 5 of *A. persimilis* p26 could lead to

disulphide bridge formation and stabilization of higher order structure. Testing these possibilities caused by the naturally occurring amino acid substitutions observed in this study awaits characterization of p26 either from cysts of different *Artemia* species or as bacterial expression products.

4.10 Structure and Expression of the p26 Gene from *A. Franciscana*

Sequencing revealed three introns in the *A. franciscana* (GSL; ARC 1520) p26 gene, in agreement with previous results [32]. In comparison, plant, yeast and many invertebrate sHSP genes lack introns. The *Drosophila* sHSP genes, with exception of a single intron in the *l(2)efl*, are intronless [48]; however, the four *C. elegans* Hsp16 genes each contain one intron coinciding in position with mammalian α-crystallin gene intron 1. The *C. elegans* SEC-1 gene has a single intron of 56 bp that disrupts codon 93, and Hsp12.3 has two introns, with the second approximating mammalian α-crystallin gene intron 2 in location [48, 53, 177]. The p26 gene introns, two of which interrupt codons, are not positioned at protein domain interfaces, and intron 1 is in the 5'-UTR, an arrangement reported for a sHSP gene from the honey bee *A. mellifera*, but no others.

sHSP genes from plants contain clusters of heat shock elements (HSEs) formed of frequently imperfect, palindromic, modular repeats of (aGAAn) and (nTTCt), often as 5'-nGAAnnTTCnnGAAn-3'[196], an arrangement observed for other organisms as described below. Modules are considered defective if the invariant G or C and/or the 2 highly conserved A and T (upper case letters) are missing [197, 198]. Until now, putative cis-acting regulatory sites in the *Artemia* p26 gene, expressed mainly in oviparous embryos but indifferent to stress [31,199] were unidentified. In this work, HSEs

composed maximally of two perfect palindromic repeats were found upstream of the p26 transcription start site, but their role in transcriptional regulation, as for the HSEs in intron 1, has yet to be tested. Although mammalian HSEs are generally not involved in developmental control of sHSP genes, a HSE in the first intron of *Hsp27* may repress transcription [98], and this could potentially be true for the HSEs in the first intron of p26. Additionally, the sunflower *Ha hsp17.6 G1* gene, which is expressed in seeds but is not heat responsive, contains a degenerative HSE distal to the TATA box involved in developmentally regulated sHSP synthesis [197, 200].

Drosophila hsp22 contains three copies of the consensus heat shock element CTnGAAnTTCnAG required for stress induction, and developmental regulation is partially dependent on these sequences [201]. The *Drosophila* genes *hsp22*, *hsp23*, *hsp26* and *hsp27* respond to20-hydroxyecdysone, an arthropod, receptor-binding, steroid hormone that interacts with HERE, a 20-hydroxyecdysone response element [113, 202, 203]. A distal estrogen receptor binding site was recognized in the p26 gene upstream sequence, this suggestive of regulation by a steroid hormone as occurs in *Drosophila* for sHSP genes. Other transcriptionally important sites in the *Drosophila hsp16* gene family are (CT)n segments, recognized by the chromatin remodeling GAGA factor (GAF) [204], but these were not observed in the p26 gene. Heat induces cell-specific synthesis of *Drosophila* sHSPs [45] demonstrating the complexity of gene regulation for these chaperones. The *C. elegans hsp16* HSEs consist of three nGAAn motifs of alternating polarity with the *hsp16-1* and *hsp16-2* sequences as CtcGAAtgTTCtaGAAa and *hsp16-41* and *hsp16-48* as CtaGGAccTTCtaGAAcaTTCt [177]. These genes are differentially induced by heat during development and in assorted tissues. Conversely, the *C. elegans*

gene encoding sHSP SIP-1 lacks HSEs and is not induced by stress, whereas *hsp26* and *hsp43* are expressed constitutively and insensitive to stress [53, 177].

As a final comparison to p26, mammalian *hsp25* and α-crystallin genes contain stress-responsive HSEs [205, 206] and their expression is developmentally regulated in diverse embryonic cells and tissues [203]. Several cis-acting elements influence mammalian sHSP gene expression during development and stress, including the upstream transcription factor (USF), a member of the basic helix loop helix zipper transcription factor family, AP-1, Pax-6, MyoD and CREB/ATF family members, MEF2 and SRF as members of the MADS-box family, glucocorticoid, estrogen and retinoic acid receptors, GAGA factors and HET (HSP27-ERE-TATA-binding protein) [52, 124, 203, 207]. A putative Ap-1 binding site occurs in intron 1 of p26 and an estrogen binding site in the 5' region of the p26 gene. These may contribute to developmental transcription of p26 gene in *Artemia*.

p26 mRNA was detected previously only in oviparous embryos [31], however, in this study low levels of p26 gene transcripts were identified in ovoviviparous embryos. The detection of p26 mRNA in ovoviviparous embryos reflects the greater sensitivity of PCR as compared to probing of northern blots, and substantially more RNA was used for northern blots than in past work. p26 protein was not observed in ovoviviparous embryos indicating either a very small amount of the protein in these embryos or inhibition of p26 mRNA translation.

4.11 Conclusions

Artemia embryos undergo either ovoviviparous or oviparous development to initiate their life cycle. In the latter, encysted embryos are released from females, and they enter diapause. As integrated developmental programs, encystment and diapause involve the coordinated synthesis and activity of many molecules, including signal transductors, transcription factors, suppressor proteins, stress proteins, enzymes and other molecules. Along with p26 characterized previously, ArHsp21 and ArHsp22, which form oligomers and possess chaperone activity, are thought to protect other molecules during diapause, and ArHsp22 also protects *Artemia* adults from stress. As a co-transcription factor, p8, potentially influences entry into encystment and diapause, and its synthesis precedes that of stress proteins such as p26, ArHsp21 and ArHsp22. The p26 gene contained one intron in the 5'-UTR and an assortment of transcription factor binding sites, which may regulate expression of this gene during *Artemia* diapause. The results support the hypothesis as proposed earlier, but clearly, much remains to be determined within the context of *Artemia* diapause with the results presented here indicating several interesting areas such as p8, *in vivo* functional analysis, p26 putative transcription factor binding site functions, three small HSP localization and relationships of other identified genes with diapause for future research.

Appendix I. Solutions and Recipes

Solutions for *Artemia* culture

Hatch Medium (1L)

24.70 g	NaCl
0.70g	KCl
6.26g	$MgSO_4 \cdot 7H_2O$
4.41g	$MgCl_2 \cdot 6H_2O$
0.21g	$CaCl_2 \cdot 2H_2O$
0.04g	$NaHCO_3$
1.00g	$Na_2B_4O_7 \cdot 10H_2O$

Pipes Buffer (1L)

100 mM	Pipes (30.24 g)
1mM	$MgCl_2$ (0.31 g)
1mM	EGTA (0.38 g)
pH 6.5	

Solutions for SDS-polyacrylamide gel electrophoresis

1B:	18.3 g	Tris
	2.5 ml	20% SDS stock solution
	pH 8.8	
	Add dH_2O to 100 ml	
1C:	0.1ml	TEMED
	Add dH_2O to 50 ml	
1D:	50 mg	Ammonium persulfate
	Add dH_2O to 10 ml	
2 B:	6.0 g	Tris
	2.5 ml	20% SDS stock solution
	pH 6.8	
	Add dH_2O to 100 ml	
2 C:	0.2 ml	TEMED
	Add dH_2O to 10 ml	

10% Running gels:

1A 4.0 ml, 1B 4.0 ml, 1C 2.0 ml, dH_2O 4 ml, 1D 2 ml.

12.5% Running gels:

1A 5.0 ml, 1B 4.0 ml, 1C 2.0 ml, dH_2O 3 ml, 1D 2 ml.

Stacking gels:

1A 1.0 ml, 2B 2.5 ml, 2C 1.25 ml, dH_2O 4 ml, 1D 1.25 ml.

4 × Treatment Buffer (20 ml)

0.6 g	Tris
1.6 g	SDS
8.0 ml	Glycerol
4.0 ml	2-mercaptoethanol
0.2%	Bromophenol blue
pH 6.8	

Running Buffer (4 L)

12.0 g	12.0 g
57.6 g	Glycine
8.0 ml	20% SDS stock solution

Coomassie Blue Staining Solution (2 L)

10 g	Coomassie brilliant blue
140 ml	Acetic acid
800 ml	Methanol
Stir 1 hr and filter through miracloth	

Destaining Solution (2 L)

400 ml	Methanol
140 ml	Acetic acid
100 ml	Glycerol

Solutions for Western blotting

Blot electrode buffer (4 L)

12.0 g	Tris
56.7 g	Glycine
800.0 ml	Methanol

TBS buffer (1 L)

1.21 g	Tris
8.18 g	NaCl
Adjust pH to 7.4 with HCl	

TBS-Tween buffer (1 L)

1.0 ml	Tween 20
Add TBS buffer to 1 L	

HST buffer (1 L)

1.21 g	Tris
58.40 g	NaCl
5.0 ml	Tween 20

Adjust pH to 7.4 with HCl

2% Ponceau S solution

2.0 g	Ponceau S
30.0 g	Trichloroacetic acid (TCA)

Add dH_2O to 100 ml. Dilute 1:9 in dH_2O for staining blots

ECL stripping buffer

100 mM	2-mercaptoethanol
2%	SDS
62.5 mM	Tris-HCl, pH 6.7

Immunofluorescence staining

8.0 g	NaCl
0.2 g	KCl
0.15 g	Na_2HPO_4
0.2 g	KH_2PO_4

pH 7.4 as mixed

PBSAT buffer

0.25 g	BSA (bovine serum albumin)
0.376 g	Triton X-100

Add PBS buffer to 50 ml

10×HPC buffer (500 ml)

0.735 g	$CaCl_2 \cdot 2H_2O$
0.075 g	PIPES

Adjust pH to 6.5, autoclave

1×HPC solution

50 ml	10×HPC buffer
32 ml	Hexylene glycol
418 ml	dH_2O

10×NaCl/$MgCl_2$/Tris solution 100 ml)

8.7 g	NaCl
2.8 g	$MgCl_2 \cdot 6H_2O$
1.9 g	Tris

Adjust pH to 6.5 with HCl, autoclave

Percoll solution

188 ml	Percoll
25 ml	10×NaCl/$MgCl_2$/Tris solution
37 ml	Autoclaved dH_2O

Solutions for DNA, including Southern blotting, constructing and screening library

TE

10 mM	Tris-HCl pH 8.0
1 mM	EDTA

6×DNA loading buffer

0.25%	Bromophenol blue
0.25%	Xylwne cynol
15%	Ficoll (type 400)

50×TBE

242.0 g	Tris
57.1 ml	Glacial acetic acid
100.0 ml	0.5 M EDTA (pH 8.0)
Add dH_2O to 1 L	

Denaturation solution

0.5 M	NaOH
1.5 M	NaCl

Neutralization solution (for Southern transfer)

0.5 M	Tris-HCl, pH 7.5
1.5 M	NaCl

Neutralization solution (for plaque hybridization)

1.0 M	Tris-HCl, pH 7.4
1.5 M	NaCl

20×SSC (1 L)

88.2 g	Trisodium citrate
176.3 g	NaCl
Adjust pH to 7.0 with HCl and autoclave	

Phage buffer

20 mM	Tris-HCl, pH 7.4
100 mM	NaCl

10 mM	$MgSO_4$

SM buffer

0.01%	Gelatin
50 mM	Tris-HCl, pH 7.5
100 mM	NaCl
8 mM	$MgSO_4$

Top agarose

1.0 g	Bacto-trypton
0.8 g	NaCl
0.6 g	Agarose

Solutions for Northern blot

10×MOPs

0.2 M	MOPs(3-N-morpholino propanessulfonic acid
50 mM	Na AoC
10mM	EDTA

Ajust pH to 7.2 with NaOH, with DEPC treated dH2O for dilution

Other solutions

Protease inhibitors

Solution A

10 mg	Leupeptin
10 mg	Soybean trypsin inhibitor

Dissolved in 10 ml Pipes buffer

Solution B

10 mg	Pepstatin
20 mg	PMSF

Dissolved in 10 ml ethanol

SOC medium (100 ml)

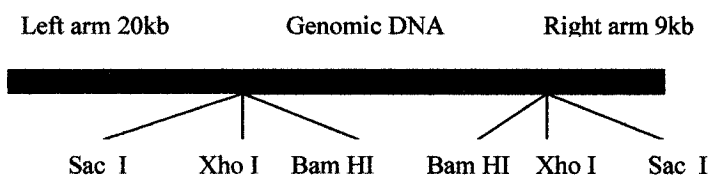
2.0 g	Bacto-tryptone
0.5 g	Bacto-yeast extract
1.0 ml	1 M NaCl
0.25 ml	1 M KCl

Dissolved in dH_2O and autoclave

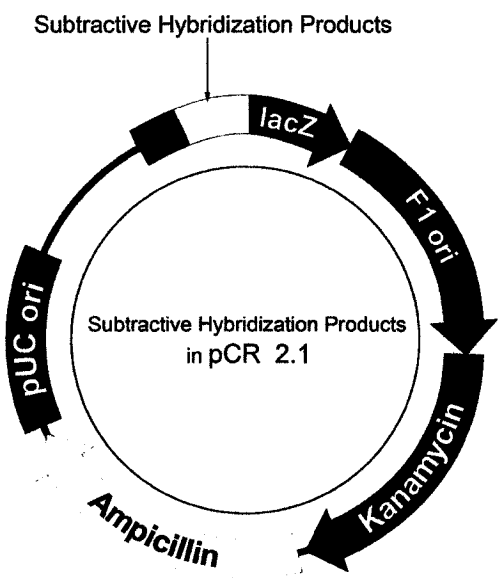
1.0 ml	2 M Mg^+
1.0 ml	2 M Glucose

Filter sterilize

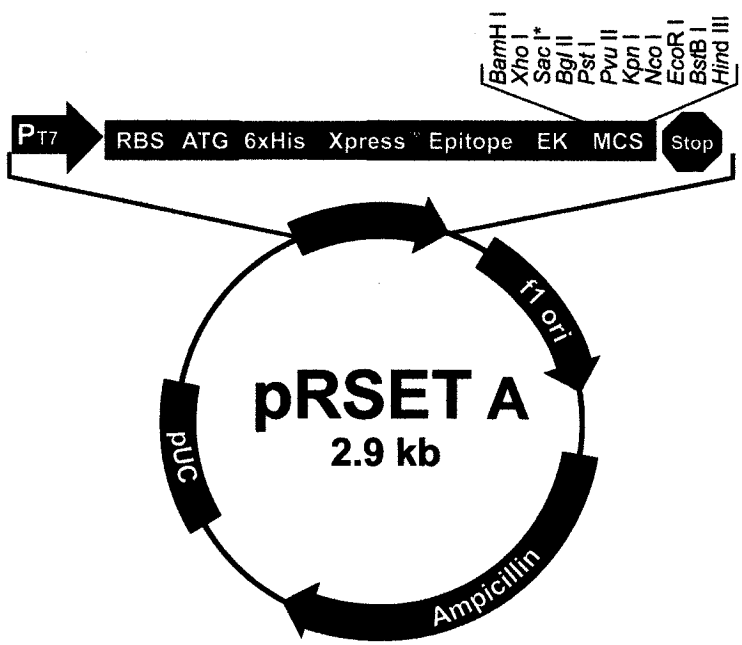
Appendix II. Schematic Representation of *Artemia* Genomic Library



Appendix III. Schematic Representation of *Artemia* Subtractive Hybridization Library



Appendix IV. Expression Vector Applied in ArHsp21, ArHsp22 and p8 cDNA Cloning



Appendix V. Accession Numbers of Sequences Submitted to GenBank

Gene (mRNA or clone) name	Accession number	Gene (mRNA or clone) name	Accession number
ArHsp21	DQ361273	clone 2G03	DW678185
ArHsp22	DQ361274	clone 2G09	DW678186
p8	DQ361275	clone 3A11	DW678187
p8 gene upstream	DQ361276	clone 3B01	DW678188
p26 from *A.franciscana*(SFB)	DQ310577	clone 3B07	DW678189
p26 from *A. sinica*	DQ310576	clone 3C11	DW678190
p26 from *A.urmiana*	DQ310580	clone 3C12	DW678191
p26 from *A. parthenogenetica*	DQ310579	clone 3E12	DW678192
p26 from *A. persimilis*	DQ310578	clone 3F03	DW678193
p26 gene	DQ310575	clone 3F04	DW678194
clone 1A06	DW678156	clone 3F07	DW678195
clone 1B03	DW678157	clone 3F09	DW678196
clone 1B09	DW678158	clone 3F10	DW678197
clone 1B10	DW678159	clone 3G07	DW678198
clone 1B11	DW678160	clone 3H06	DW678199
clone 1C01	DW678161	clone contig 04	DW678200
clone 1C03	DW678162	clone contig 05	DW678201
clone 1C06	DW678163	clone contig 11	DW678202
clone 1C07	DW678164	clone contig 16	DW678203
clone 1C08	DW678165	clone contig 18	DW678204
clone 1C09	DW678166	clone contig 20	DW678205
clone 1C10	DW678167	clone contig 25	DW678206
clone 1D01	DW678168	clone contig 27	DW678207
clone 1D03	DW678169	clone contig 28	DW678208
clone 1D04	DW678170	clone contig 29	DW678209
clone 1D09	DW678171	clone contig 30	DW678210
clone 1E10	DW678172		
clone 1F03	DW678173		
clone 1F10b	DW678174		
clone 2A01	DW678175		
clone 2B03	DW678176		
clone 2B04	DW678177		
clone 2C01	DW678178		
clone 2C09	DW678179		
clone 2E04	DW678180		
clone 2E12	DW678181		
clone 2F03	DW678182		
clone 2F06	DW678183		
clone 2G01	DW678184		

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