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Molecular Characteristics, Developmental Regulation And Cellular Localization Of A Small Heat Shock/α-Crystallin Protein From *Artemia franciscana*

by

Ping Liang

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
November, 1997

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Molecular Characteristics, Developmental Regulation and Cellular Localization of a Small Heat Shock/α-Crystallin Protein from Artemia franciscana"

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To My Parents

梁庄发 (Liang, Zhuangfa)
向秀娥 (Xiang, Xiue)
梁庄寿 (Liang, Zhuangshou)
黄爱华 (Huang, Aihua)

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Abstract

During diapause and quiescence, encysted *Artemia* gastrulae bring their metabolism to a reversible standstill and demonstrate a remarkable resistance to environmental stress. Previous results suggested that p26, an abundant, low molecular weight protein in *Artemia* cysts, is a small heat shock/α-crystallin protein and that it has a protective function in these embryos. In this study, p26 was purified to apparently homogeneity from cell-free extracts of *Artemia* cysts. p26 existed as multimers up to 700 kD in mass and multiple isoforms of the protein were detected on 2D-gels. A complete cDNA for p26 was cloned and sequenced, revealing an encoded polypeptide of 192 amino acids with a conserved "α-crystallin domain". Unusual characteristics of p26 were the presence of glycine- and arginine-rich regions in the N-terminal domain, the longest C-terminal extension yet observed in a small heat shock/α-crystallin protein, a lack of predicted α-helix structure in α-crystallin domain and the absence of the "Arg-X-X-Ser" phosphorylation motif. *Artemia* had multiple p26 genes and they were exclusively expressed in embryos undergoing encystment. During encystment, p26 mRNA first appeared in two day embryos, while p26 protein appeared in three day embryos. Both p26 mRNA and protein reached their highest amounts before the cysts were released and they remained at this level in activated cysts. p26 mRNA decreased rapidly during post-gastrula development and disappeared completely after emergence. The protein remained at a high level until emergence, after which it decreased quickly and had disappeared from the western blots of instar II larvae cell-free extracts. p26 occurred in the cytoplasm as well as in nuclei, wherein it was sometime found in discrete compartments. p26 had chaperone activity *in vivo*, as demonstrated by the enhanced thermoresistance of bacteria expressing p26. In conclusion, p26 represents the only known small heat shock/α-crystallin protein from a crustacean and it is the only protein in this family thought to function in diapause. p26 may bind to proteins and other macromolecules in encysted *Artemia* embryos under stress and prevent their irreversible aggregation. This allows spontaneous and/or assisted folding of proteins, permitting rapid resumption of cyst metabolism under favourable growth conditions.

Abbreviations and symbols

ATP	adenosine 5'-triphosphate
CS	citrate synthase
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
dH_2O	distilled water
DTT	dithiothreitol
ECL	enhanced chemiluminescence
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
HSF	heat shock factor
HSP	heat shock protein
HST	high salt Tween
IEF	isoelectric focusing
IgG	immunoglobulin G
IPTG	isopropylthio-β-galactosidase
kDa	kilodaltons
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase-chain reaction
Pipes	1,4-peperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
rpm	rotation per minute
RT	room temperature
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TCP-1	t-complex polypeptide α-chain
TEMED	N,N,N',N'-tetramethylethylenediamine
TEMN	Tris-EDTA-$MgCl_2$-NaCl buffer
Tris	Tris-(hydrosymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate

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I. Introduction

1.1 Heat shock proteins (HSPs)

Tissières *et al.* (1974) demonstrated a rapid and selective increase in the synthesis of a group of proteins following exposure of *Drosophila* larvae to temperatures above those optimal for normal growth and development. These proteins were referred to as heat shock proteins (HSPs) due to the method of induction, but subsequent studies revealed that several other stress factors, such as metals, amino acid analogs and anoxia induce a similar response. HSPs exist in all organisms from bacteria to humans. They are divided into five major families, HSP70, HSP60, HSP90, HSP100, and small HSPs, according to their size, structure and function (Table 1)(Craig *et al.* 1994; Morimoto *et al.* 1991, 1994).

1.2 Many HSPs are molecular chaperone

The major role of HSPs in cells is as molecular chaperones (Hendrick & Hartl 1993, 1995; Ellis & van der Vies 1991; Georgopoulos & Welch 1993). The term, molecular chaperone, was first used by Laskey *et al.* (1978) to describe the role of nucleoplasmin in the assembly of nucleosomes *in vitro*, wherein it binds to purified histones and promotes nucleosome assembly by donating histone to assembling chromatin. This term was subsequently applied to members of several structurally unrelated protein families that interact with non-native proteins (Ellis 1987; Ellis & van der Vies 1991). Molecular chaperones either assist in the

Table 1. Major classes of HSPs and molecular chaperones

Family	Specific protein	Location	Mass (kDa)	Cofactors	References
HSP70					
Prokaryotes	DnaK	Cyt[1]	70	DnaJ, GrpE	Hartl 1996; Rassow *et al.* 1997
Yeast	Ssa, Ssb	Cyt	70	YDJ1	Deshaies *et al.* 1988
	Ssc, Ssh	Mit[2]	70		Murakami *et al.* 1988
	Kar2, Ssi1p	ER[3]	70	Sec63	Brodsky 1996; Rassow *et al.* 1997
Higher	HSP70, 73	Cyt /nucleus	70	HSP40	Hartl 1996;
eukaryotes	Hsc70	Cyt/nucleus	70	HSP40, Hip/Hop	Frydman & Höhfeld 1997; Höhfeld 1995
	Grp75	Mit	75	Tim44	Stuart *et al.*1994; Rassow & Pfanner 1996
	HSP70	Chl[4]	70		Hähfeld & Hartl 1994
	Grp78(BiP)	ER	78	Sec63	Brodsky 1996; Blond-Elguidi *et al.* 1993
HSP60 (Chaperonin)					
Prokaryotes	GroEL	Cyt	60	GroES	Hartl 1996; Fenton *et al.* 1996
Archaebac[5]	TF55		55		Trent *et al.* 1991
Yeast	HSP60	Mit	60	HSP10	Martin *et al.* 1992
	Tcp-1α/β	Cyt	60		Stoldt *et al.* 1996; Chen *et al.* 1994
Plants	Cpn60 (Rubisco BP)	Chl	60	Cpn10	Hähfeld & Hartl 1994; Boston *et al.* 1996
Animals	Tcp-1 (TRiC, CCT)	Cyt/nucleus	55		Sternlicht *et al.* 1993; Melki & Cowan 1994; Joly *et al.* 1994
HSP100 (Clp)					
Prokaryotes	ClpA/B/C	Cyt	100		Squires & Squires 1992; Horwich 1995
Yeast	HSP104	Cyt/ Mit	104		Chernoff *et al.* 1995
Chicken	HSP100	Cyt	100		Koyasu *et al.* 1989
Plants	HSP100(ClpB)	Cyt	100		Horwich 1995; Boston *et al.* 1996
HSP90					
Prokaryotes	HtpG	Cyt	68		Spence & Georgopoulos 1989
Yeast	HSP90	Cyt	90	Sti1	Parsell & Lindquist 1993
Mammals	HSP90/83	Cyt	90	Hop, p23	Jakob & Buchner 1994; Frydman & Höhfeld 1997
Eukaryotes	Grp94(Erp99)	ER	94		Melnick *et al.* 1994; Jakob & Buchner 1994

Continued

Family	Specific protein	Location	Mass (kDa)	Cofactors	References
Small HSPs					
Prokaryotes	IbpA/B, Clpp		14/16.4		Verbon *et al.* 1992; Allen *et al.* 1992
	BPSBP[6]	Periplasm			Richarme & Caldas 1997
N. crassa	HSP30	Mit	30		Plesofsky-Vig & Brambl 1990; 1995
Plants	Hs11, 22	Cyt/Mit/Chl/ER	11-28		Boston *et al.* 1996; Water *et al.* 1996
Yeast	Hs12, 22, 26		12-26		Susek & Lindquist 1989; Rossi &
	HSP42		42.8		Lindquest 1989; Wotton *et al.* 1996
S. mansoni	p40		40		Nene *et al.* 1986; de Jong *et al.* 1988
C. elegans	HSP12.6, Sec-1		12.6 – 25		Leroux *et al.* 1997a,b; Linder *et al.*
	HSP16-1/2/41/48				1996; Jones *et al.* 1986
Drosophila	HSP22/23/26	Cyt/Mit/Chl	22-27		Arrigo & Landry 1994; Michaud *et al.*
	HSP27				1997; Pauli & Tonka 1987
Xenopus	HSP30A/C/D/E		30		Helbing *et al.* 1996; Heikkila *et al.*
					1997; Tam & Heikila 1995
Chicken	IAP (HSP25)		25		Miron *et al.* 1988, 1991
Mammal	HSP27(25/28)	Cyt/Nucleus	27		Landry *et al.* 1982; 1989; Huot *et al.*
					1991,1996; Arrigo & Welch 1987
	p20		20		Kato *et al.* 1994b
Vertebrates	α-Crystallin A/B	Cyt/Nucleus	20		Groenen *et al.* 1994; Klemenz *et al.*
					1991,1993; Dasgupta *et al.* 1992

Cyt[1], cytosol; Mit[2], mitochondria; ER[3], endoplasmic reticulum; Chl[4], chloroplast; Archaebac[5], Archaebacteria; BPSBP[6], bacterial periplasmic substrate-binding protein.

correct folding, or prevent the misfolding and aggregation of other proteins in cells under normal growth conditions as well as under stress (Hendrick & Hartl 1993; Hartl 1996). Molecular chaperones mediate several cellular processes including the folding and oligomerization of nascent polypeptides, intracellular transport of proteins, restoration of denatured proteins and prevention of their aggregation (Hartl 1996), organization of the cytoskeleton (Arrigo & Landry 1994; Huot *et al.* 1996; Lavoie *et al.* 1993a,b, 1995; Liang & MacRae 1997; Jakob & Buchner 1994), and regulation of signal transduction pathways (Frydman & Höfeld 1997; Bohen *et al.* 1995) and apoptosis (Mehlen *et al.*

1996a, b).

Among the known HSP families, HSP70 and HSP60 are well established as molecular chaperones (Craig *et al.* 1994; Morimoto *et al.* 1991, 1994; Georgopoulos & Welch 1993; Ellis & van der Vies 1991; Hartl & Martin 1992; Hendrick & Hartl 1993, 1995; Martin *et al.* 1992; Hartl 1996). HSP70 is a family of proteins approximately 70 kDa in molecular mass, found in diverse organisms and cellular compartments. It includes stress-inducible and constitutive members, the latter usually termed HSC70 (Hightower *et al.* 1994). The major functions of HSP70 include binding to nascent polypeptide chains on ribosomes, maintaining the membrane translocation-competent state of precursor proteins, importing proteins into mitochondria, chloroplasts and the endoplasmic reticulum, and protection of proteins in cells under stress. A universal theme behind these actions is the ATP-dependent binding of HSP70 to short hydrophobic regions of 7-9 amino acids on target proteins in extended or unfolded conformations (Flaherty *et al.* 1990; Flynn *et al.* 1991; Blon-Elguindi *et al.* 1993; Hartl 1996; Rassow *et al.* 1997; Beckmann *et al.* 1990). The HSP60 or chaperonin family is a group of proteins with a distinct ring-shaped or toroid (double donut) quaternary structure. Included are GroEL from bacteria, rubisco-binding protein from chloroplasts, HSP60 from mitochondria, and the t-complex polypeptide 1 (TCP-1, also called CCT or TRiC) from eukaryotic cytosol (Gupta 1995; Hartl 1996; Willison & Kubota 1994). Proteins from this family preferentially bind folding intermediates, the so called "molten globules", that are

characterized by a significant degree of secondary structure but little overall compactness (Hayer-Hartl *et al.* 1994; Creighton 1997). In cooperation with HSP70 and probably other chaperones, chaperonins actively mediate the folding of proteins into the native state in an ATP-dependent manner (Frydman & Hartl 1996; Frydman *et al.* 1994; Hendrick & Hartl 1995; Martin *et al.* 1992; Martin & Hartl 1994; Morimoto *et al.* 1994). During this process, HSP70 and its cofactor HSP40 interact with nascent polypeptides and prevent their premature (mis)folding, at least until a domain capable of forming a stable structure is synthesized. For many proteins, completion of folding requires subsequent interaction with a chaperonin, wherein fully folded proteins are produced in the central cavity of the toroid complex by ATP-dependent cycles of release and rebinding. This model also applies to refolding of denatured proteins (Frydman & Hartl 1996; Martin *et al.* 1992), although differences between these two situations have been noticed recently. For example, release into the cytosol as nonnative intermediates may not be necessary for the folding of nascent polypeptides (Frydman & Hartl 1996).

HSP90 is an abundant, essential cytosolic protein under nonstress conditions, and it increases markedly during stress (Georgopoulos & Welch 1993; Morimoto *et al.* 1991, 1994). Studies *in vitro* suggest that HSP90 suppresses aggregation or denaturation of citrate synthase and casein kinase II, while *in vivo* studies indicate it acts only on specific protein targets in a maturational and regulatory capacity (Redmond *et al.* 1989; Craig *et al.* 1994;

Jacob *et al.* 1995). As a well known regulatory example, the binding of HSP90, in cooperation with Hsc70 and the cofactors Hip and Hop, is required for steroid hormone receptors to be in a high-affinity hormone binding state (Redmond *et al.* 1989; Boston *et al.* 1996; Frydman & Höhfeld 1997). HSP90 has also been implicated in regulation of signal transduction probably, by modulating tyrosine kinase activity (Boston *et al.* 1996; Jakob & Buchner 1994).

HSP100, and its close bacterial homologue ClpB, promote survival of organisms exposed to extreme stress (Craig *et al.* 1994; Parsell & Lindquist 1994; Lindquist & Kim 1996). In the absence of HSP104, the survival of yeast under heat shock conditions is reduced by several orders of magnitude. In addition, moderate amounts of HSP104 are required for the maintenance and propagation of the non-Mendelian factor, [*psi+*], while high levels of HSP104 cure cells of [*psi+*] (Patino *et al.* 1996). Interestingly, rather than preventing the aggregation of target proteins, HSP100 appears to mediate protein disaggregation in an ATP-dependent manner, leading either to the renaturation of individual proteins or to their proteolytic degradation (Craig *et al.* 1994; Parsell & Lindquist 1994; Horwich 1995; Schirmer *et al.* 1996).

1.3 Small heat shock/α-crystallin proteins

1.3.1 The α-crystallin domain and oligomerization, common characteristics of a diverse group of low molecular weight proteins

While much has been learned about the high molecular weight heat shock proteins, the small HSPs were originally neglected. They did not appear to

be universally conserved, as are the other heat shock proteins (Schlesinger 1990), and their function was elusive. The small heat shock/α-crystallin proteins vary from 12 kDa to 30 kDa in mass (Table 1), and their functions differ. Among other activities, they are the major surface antigens in bacteria and parasites (Verbon *et al.* 1992; Nene *et al.* 1986), they prevent the photosynthetic machinery from light-induced damage in plants (Schuster *et al.* 1988; Grimm *et al.* 1989), inhibit the actin polymerization of chicken gizzard *in vitro* (Miron *et al.* 1991), and provide stability and transparency in vertebrate lenses (Groenen *et al.* 1994; Tardieu & Delaye 1988).

Drosophila small HSPs have been studied in detail, and at least four different small HSPs, hsp22, 23, 26 and 27, are found (Arrigo & Landry 1994; McKenzie *et al.* 1975; Pauli & Tonka 1987; Michaud *et al.* 1997; Ayme & Tissières 1985; Arrigo & Tanguay 1991). Plants have the largest number and the most abundantly expressed small HSPs. They comprise products from possibly six different gene families, and they are localized to cellular compartments such as the cytosol, chloroplast, mitochondria, and endoplasmic reticulum (Water *et al.* 1996; Boston *et al.* 1996; Raschke *et al.* 1988). In mammals, at least four groups of small heat shock/α-crystallin proteins are identified, HSP27 (also called HSP25), p20, α-crystallin A, and α-crystallin B. While p20 was found only recently (Kato *et al.* 1994b), α-crystallins had been known as lens-specific structural proteins in vertebrates for almost a century (Mörner 1894) before their recognition as small HSPs. In fact, much of our

knowledge about small HSPs can be attributed to the study of α-crystallins. Constituting up to 50% of the water-soluble protein fraction of the lens, α-crystallin is usually found as large multimers about 800 kDa in mass, consisting of two related polypeptides, αA and αB, with molecular masses of approximately 20 kDa. The two subunits show 57% sequence similarity (Groenen *et al.* 1994). In contrast to the idea that they are specific to lens, αB-crystallin and to a lesser extent αA-crystallin, occur in nonlenticular tissues such as the heart, kidney, striated muscle and various tumors (Bhat & Nagineni 1989; Kato *et al.* 1991; Dubin *et al.* 1989; Dubin & Piatigorsky 1989). The synthesis of αB-crystallin is induced by thermal (Klemenz *et al.* 1991) or hypertonic stress (Dasgupta *et al.* 1992). A milestone in the study of α-crystallin and small HSPs is the discovery by Ingolia and Craig (1982) that α-crystallin is evolutionarily related to the small heat shock proteins from *Drosophila*. It was subsequently shown that all α-crystallins and small HSPs possess a conserved homologous sequence of 90-100 residues, the so called "α-crystallin domain" (Arrigo & Landry 1994; Caspers *et al.* 1995; Wistow 1985). This domain is preceded by an N-terminal region which is highly variable in size and sequence, and followed by a short, poorly conserved C-terminus extension (Caspers *et al.* 1995).

Study of the secondary structure of small heat shock/α-crystallin proteins indicates a predominance of β-sheet in the conserved α-crystallin domain (Casper *et al.* 1995). Notably, it seems that all proteins in this family, with HSP12.6 from *C. elegans* as the only known exception (Leroux *et al.* 1997a),

form large oligomers/aggregates ranging from 150 kDa to 800 kDa in mass, a property which may be required for chaperone activity (Groenen *et al.* 1994; Arrigo & Landry 1994; Leroux *et al.* 1997b). A simple, well characterized small HSP oligomer is the 150 kDa trimer formed by HSP13.6 from *M. tubuerculosis* (Chang *et al.* 1996). Plant small HSPs assemble into complexes of 200 to 300 kDa, while α-crystallin is usually in aggregates of 800 kDa, although complexes ranging from 280 kDa to 10 mDa are observed (Groenen *et al.* 1994; Crabbe & Goode 1994). Small HSPs from animals form multimers ranging in size from 180 kDa in chicken to 400-800 kDa in mammals (Groenen *et al.* 1994). Multimer size varies with protein concentration and it increases upon heat shock (Jakob & Buchner 1994). For example, α-crystallin aggregates greater than 50,000 kDa are observed at protein concentrations above 15%. In the lens, the size of α-crystallin multimers increases upon aging, and during stress the size of αB-crystallin oligomers in the heart increases (de Jong at al. 1993). Mixed aggregates of αB-crystallin and small HSPs occur naturally in adenovirus transformed cells and they disassociate during stress (Zantema *et al.* 1989). Electron microscopic analysis has shown that small HSPs and α-crystallins form similar granule-like structures, generally ranging from 10 – 15 nm in diameter. Although several models are proposed (Leroux *et al.* 1997b; Bindels *et al.* 1979; Augusteyn & Koretz 1987; Groenen *et al.* 1994), the detailed structure of these particles remains obscure, in part because these proteins have resisted crystallization.

1.3.2 Function of small heat shock/α-crystallin proteins in stressed cells

Many small heat shock/α-crystallin proteins are expressed in response to heat shock suggesting that they convey thermotolerance with other HSPs. This has now been proven with evidence from both *in vivo* and *in vitro* experiments (Arrigo & Landry 1994; Jakcob & Hartl 1994; Groenen *et al.* 1994; de Jong *et al.* 1993). For instance, *Dictyostelium* mutants defective in small HSPs, but not other HSPs, fail to develop thermotolerance (Loomis & Wheeler 1982), while NIH-3T3 cells show increased resistance to heat shock when αB-crystallin is selectively induced (Aoyama *et al.* 1993). Disruption of the HSP30 gene in *Neurospora crassa* reduces the survival of the organism to combined stresses of high temperature and carbohydrate limitation by 90%, although there is no observable effect on its growth at high temperature (Plesofsky-Vig & Brambl 1990, 1995). Other *in vivo* evidence comes from transfection studies with a number of small heat shock genes. For example, cultured cells that constitutively express high levels of extrinsic human HSP27 are much more thermoresistant than the parental cells, with the extent of thermotolerance being proportional to the amount of expressed hsp27 (Landry *et al.* 1989; Lavoie *et al.* 1993a). Bacteria expressing recombinant SEC-1, an 18 kDa small HSP from *C. elegans* (Linder *et al.* 1996) or αB-crystallin (Plater *et al.* 1996) also gain increased tolerance to heat shock.

The most direct evidence for the chaperone function of small heat shock/α-crystallin proteins is from recent *in vitro* experiments which

demonstrate that they prevent the heat- or chemical-induced aggregation/denaturation of other proteins and enhance their refolding after denaturation (Horwitz 1992; Jakob *et al.* 1993; Merck *et al.* 1993; Raman *et al.* 1995; Andley *et al.* 1996; Plater *et al.* 1996; Muchowski *et al.* 1996; Richarme & Caldas 1997; Lee *et al.* 1995, 1997; Leroux *et al.* 1997b; Ehrnsperger *et al.* 1997). A very interesting observation is that authentic lens extracts do not aggregate significantly when heated, while α-crystallin-depleted extracts rapidly become turbid (Horwitz 1992). In contrast to β/γ-crystallins, α-crystallin unfolded by 8 M urea refolds rapidly by itself under renaturing conditions, and its presence significantly increases the refolding of β/γ-crystallins denatured with 8 M urea *in vitro* (Raman *et al.* 1995). These results have led to the proposal that α-crystallins serve primarily as *bona fide* molecular chaperones, even in the lens, preventing the irreversible aggregation of other lens proteins and preserving the lens transparency, although a possible structural role in the lens is not excluded (Horwitz 1992; Marin *et al.* 1996; Wang & Spector 1994; Rao *et al.* 1994). This new concept may elucidate the factors leading to cataract. For example, factors that affect the chaperone activity of α-crystallin, such as posttranslational modifications which occur naturally during aging, may be the major cause of aggregation of soluble lens proteins, the ultimate physical/biochemical change in the cataractous lens (Kamei *et al.* 1997; Koretz *et al.* 1997).

Unlike other HSPs, such as HSP70 and chaperonin, small heat shock/α-

crystallin proteins do not require ATP for chaperone activity. Therefore, their activity is more often described as "chaperone-like" (Horwitz 1992; Jakob *et al.* 1993; Merck *et al.* 1993; Plater *et al.* 1996). Although the mechanism for this activity is enigmatic, recent data concerning the interaction of small heat shock/α-crystallin proteins with target proteins *in vitro* has provided some clues. Ehrnsperger *et al.* (1997) have shown that the stable binding of heat-denatured citrate synthase (CS) to HSP25 oligomers leads to accumulation of CS unfolding intermediates which are protected from further aggregation. Under appropriate folding conditions, CS is released from HSP25 oligomers and the native state is restored in cooperation with HSP70. Similarly, Lee *et al.* (1997), using a photoincorporation technique with a hydrophobic probe, demonstrated that HSP18.1 from pea stably binds heat-denatured substrates via a hydrophobic interaction. Moreover, heat-denatured luciferase bound to HSP18.1, in contrast to aggregated luciferase, is reactivated in the presence of either rabbit reticulocyte or wheat germ extract in an ATP-dependent manner. The results suggest that the major task of small HSPs is to bind a large number of unfolding proteins through hydrophobic regions. This prevents the irreversible aggregation of denatured proteins and creates a reservoir of proteins that refold, either spontaneously or through interaction with other chaperones such as HSP70 and chaperonin.

1.3.3 Function of small heat shock/α-crystallin proteins under non-stress conditions

Not only do the small HSPs confer stress resistance and thermal tolerance, but they play a role during development and differentiation of cells (Arrigo & Landry 1994; Gernold *et al.* 1993). Constitutive and tissue-specific expression of small heat shock/α-crystallin proteins in the absence of stress during normal development of plants, *Drosophila,* nematodes, *Xenopus*, mouse and human are well documented (Mason *et al.* 1984; Kurtz *et al.* 1986; Arrigo & Tanguay 1991; Michaud *et al.* 1997; Linder *et al.* 1996; Leroux *et al.* 1997a; Coca *et al.* 1994; Loones *et al.* 1997; Adnan *et al.* 1993; Heikkila 1993a,b; de Jong *et al.* 1993; Dubin *et al.* 1989; Tanguay *et al.* 1993). The small HSPs synthesized under non-stress conditions have similar or identical molecular characteristics to those induced by stress (Arrigo 1987; Haass *et al.* 1990), indicating that their functions are similar under both conditions. The synthesis of *Drosophila* hsp27 during embryonic and larval development occurs mainly in tissues with rapidly proliferating cells. However, HSP27 also accumulates in tissues of third instar larvae where differentiation has begun and cellular proliferation has ceased or slowed (Pauli *et al.* 1990). This suggests that the protein has both proliferative and differentiative functions in the developing insect. In comparison to the situation in *Drosophila,* HSP27 seems to play an equally important role in unstressed mouse tissues, as well as during differentiation and development of the animal (Gernold *et al.* 1993). Transcription block of SEC-1, an embryonic small HSP in *C. elegans,* by *in vivo* injection of oocytes with antisense oligo nucleotides arrests nematode

development at an early stage after fertilization, indicating an essential function of SEC-1 in early embryogenesis (Linder *et al.* 1996). Lastly, small HSPs may function in meiosis and gametogenesis in *Zea mays* (Atkinson *et al.* 1993) and during embryogenesis in sunflower (Coca *et al.* 1994).

The role of small HSPs and α-crystallins in events associated with growth and development remains unclear. However, they appear to influence cytomorphological rearrangements by interaction with cytoskeletal elements, preferentially actin/microfilaments and intermediate filaments (Arrigo & Landry 1994; Huot *et al.* 1996; Lavoie *et al.* 1993a,b, 1995; Liang & MacRae 1997; Jakob & Buchner 1994). Small HSPs from chicken, mouse and yeast inhibit actin polymerization (Miron *et al.* 1988, 1991; Rahman *et al.* 1995; Benndorf *et al.* 1994), while α-crystallin prevents depolymerization of microfilaments by cytochalasin D and their heat-induced aggregation, both in a phosphorylation-dependent manner (Wang & Spector 1996). Mammalian HSP27 participates in signal transduction to microfilaments via a pp45-54 HSP27 kinase-regulated mechanism (Huot *et al.* 1995, 1996; Lavoie *et al.* 1993a, b, 1995). In addition to its interaction with microfilaments, αB-crystallin, and perhaps other small HSPs, may influence intermediate filament turnover and remodeling during normal development and differentiation, as well as under stress (Haynes *et al.* 1996; Nicholl & Quinlan 1994; Liang & MacRae 1997).

A function for small HSPs in apoptosis has been proposed by Mehlen *et al.* (1996b) showing that constitutive expression of either human and *Drosophila*

HSP27, or human αB-crystallin, protects mouse L929 cells against death induced by tumor necrosis factor (TNFα), hydrogen peroxide and menadione.

1.3.4 Cellular localization of small heat shock/α-crystallin proteins

Small heat shock/α-crystallin proteins occur in the cytosol, nucleus, mitochondria, chloroplasts, and endoplasmic reticulum, the site depending on the specific protein, whether it is expressed under non-stress or stress condition and the stage of the stress response (Arrigo & Landry 1994; Boston *et al.* 1996; Michaud *et al.* 1997). Most small heat shock/α-crystallin proteins redistribute inside or around the nucleus during heat shock or other forms of stress, and they gradually return to the cytoplasm during recovery (de Jong *et al.* 1993; Groenen *et al.* 1994; Jakob & Buchner 1994). Constitutively expressed human HSP27 and αB-crystallin are cytoplasmic, but usually concentrated in the perinuclear region (Arrigo *et al.* 1988; Arrigo 1990; Mehlen *et al.* 1993), while HSP25 from chicken is more evenly distributed in the cytoplasm (Collier & Schlesinger 1986). The four small HSPs of *Drosophila* localize differently. HSP27 is a nuclear protein in all cases (Beaulieu *et al.* 1989; Arrigo & Pauli 1988; Pauli *et al.* 1990), even when expressed in COS cells (Mehlen *et al.* 1993), while HSP23 and HSP26 concentrate mainly in the cytoplasm, although in different structures (Tanguay at al. 1985). *Drosophila* HSP22 (Michaud *et al.* 1997), like HSP30 from *N. crassa* (Plesofsky-Vig & Brambl 1990) and HSP22 from pea (Lenne *et al.* 1995), reside mainly in mitochondria, indicating that their function is organelle-specific (Plesofsky-Vig & Brambl 1995). ER-localized small heat shock/α-crystallin

proteins are found in plants. These proteins possess a ER-retention signal (Helm *et al.* 1993; Boston *et al.* 1996).

1.3.5 Multiple isoforms of small heat shock/α-crystallin proteins

Two dimensional analysis of the small heat shock proteins from different organisms reveals that many of them exist as multiple isoforms. In a majority of cases, the isoforms arise from posttranslational phosphorylation on specific serine residues in response to various stimuli (Arrigo & Landry 1994; Landry *et al.* 1992; de Jong *et al.* 1993). The number and position of phosphorylation sites differ from protein to protein and among species. αA-crystallin has a single site at Ser-122 while αB-crystallin has three, Ser-19, -45, and -59 (Voorter *et al.* 1986, 1989; Chiesa *et al.* 1987). Human and mouse HSP27 have a major phosphorylation site at Ser-82 and a second site at Ser-15, but human HSP27 has a third site at Ser-78, not found in mouse HSP27 (Gaestel *et al.* 1991; Landry *et al.* 1992). All these sites are in the "Arg-X-X-Ser" sequence motif (Landry *et al.* 1992; Arrigo & Landry 1994).

It appears that the amount of phosphorylation correlates with the intracellular localization and translocation of isoforms into the nucleus during heat shock (Marin *et al.* 1996). *Drosophila* HSP27 exists in pupae and third instar larvae in four stable isoforms, all of which are constitutively present in the nervous system and in testes, but only two are found in ovaries (Marin *et al.* 1996). Three of these isoforms are phosphoproteins and the fourth is not phosphorylated (Marin *et al.* 1996; Arrigo & Pauli 1988; Pauli *et al.* 1990). Two

isoforms of HSP 23 occur in the head and testes of unstressed flies, but only one of them exists in ovaries while the other appears during heat shock (Marin *et al.* 1996). The results suggest that small HSP function is regulated by a tissue-specific posttranslational modification system. Phosphorylation affects the size of HSP27 (Kato *et al.* 1994a), and it is required for HSP27 to protect the heat- or chemical-induced aggregation of microfilaments (Huot *et al.* 1995, 1996; Lavoie *et al.* 1993a,b, 1995). However, it seems that phosphorylation is not essential for the chaperone activity and cellular thermoresistance of HSP25 (Knauf *et al.* 1994).

In addition to phosphorylation, several other posttranslational modifications are known, such as glycosylation (O-linked N-acetyl-glucosamine), acetylation and glycation of α-crystallin A and B, and glutamination of HSP27 and αB-crystallin (de Jong *et al.* 1993). However, their role in relation to the function of these proteins is largely unknown.

1.3.6 Regulation of sHSP synthesis

When cells are exposed to stress, the small heat shock/α-crystallin proteins are among the most strongly induced heat shock proteins (Arrigo & Landry 1994). For example, when mouse Swiss-3T3 and Chinese hamster CCL39 cells are heat shocked, HSP27 increases by more than 10-fold, accounting for 1% of the total cellular protein, while αB-crystallin increases 25 fold from 0.002% to 0.05% of the total protein (Landry *et al.* 1991). The stress-induced regulation of small heat shock/α-crystallin genes is similar to that of the

other heat shock genes. That is, transcription is controlled through the binding of activated heat shock factors (HSFs) to specific regions of heat shock genes characterized by inverted repeats of a 5-bp sequence, 5'-nGAAn-3', known as heat shock elements (HSEs) (Craig *et al.* 1994; Fernandes *et al.* 1994; Schlesinger 1990). HSEs are located in the upstream region of almost all characterized small HSP genes, including genes for the four *Drosophila* small HSPs, the human and mouse HSP27, αB-crystallin and HSPs16-2/41 from *C. elegans* (Hickey *et al.* 1986; Gaestel *et al.* 1993; Klemenz & Gehring 1986; Klemenz *et al.* 1991; Jones *et al.* 1986; Quivy & Becker 1996; Oesterreich *et al.* 1996; Michaud *et al.* 1997). The αA-crystallin gene is the only characterized small heat shock/α-crystallin gene that lacks an HSE (Quax-Jeuken *et al.* 1985), explaining its inability to response to heat shock. In contrast, αB-crystallin is inducible by several forms of stress including thermal and hypertonic stress and exposure to sodium arsenite (Klemenz *et al.* 1991; Dasgupta *et al.* 1992; Kato *et al.* 1993).

As described previously, many small heat shock/α-crystallin proteins are synthesized in the absence of stress, either constitutively or during specific stages of normal growth and development, a phenomenon not often observed for other HSPs. Therefore, these genes must be subject to various regulatory mechanisms. Other upstream regulatory elements, in addition to the HSEs, are found in several small heat shock/α-crystallin genes. In mammals, an estrogen-responsive element has been identified in genes for mouse HSP25 and human

hsp27, explaining their hormone-induced expression (Gaestel *et al.* 1993; Oesterreich *et al.* 1996; Hayward *et al.* 1990; Mendelsohn *et al.* 1991). Very likely a similar situation applies to *Drosophila* HSP27 which is inducible by the moulting hormone, β-ecdysterone (Ireland & Berger 1982).

The synthesis of α-crystallins provides a good example for mechanistic aspects of tissue-specific regulation of small heat shock/α-crystallin proteins. The αA-crystallin gene expression is strongly lens-preferred, with extralenticular expression restricted to very low levels in the spleen and thymus (Kato *et al.* 1991; Srinivasan *et al.* 1992). However, αB-crystallin mRNA and protein are found at varying but significant levels in a number of nonlens tissues, including heart, skeletal muscle, kidney, and lung, but excluding spleen (Bhat & Nagineni 1989; Dubin *et al.* 1989). Much of the complex expression pattern of αB-crystallin genes is attributed to shared and tissue-specific control elements located within the 500 bp region upstream from the transcription initiation site. Among the four known *cis*-acting regulatory elements, αBE-1, αBE-2, αBE-3, and MRF, αBE-1 and αBE-2 are utilized for expression in the lens, skeletal muscle, and lung (Dubin *et al.* 1991; Gopal-Srivastava & Piatigorsky 1993; Haynes *et al.* 1995, 1996). An additional element, αBE-4, which resembles a control sequence shared by other genes expressed in heart, is required for synthesis of αB-crystallin in cardiac muscle (Gopal-Srivastava *et al.* 1995). High expression of the αB-crystallin gene in lens is attributed to the activation of two lens-specific promoters, LSR1 and LSR2, by Pax-6, a lens-specific transcription

factor (Gopal-Srivastava *et al.* 1994, 1996).

1.4 The brine shrimp, *Artemia*

The brine shrimp, *Artemia,* typically inhabits salt water ponds or lakes where only few species live due to high salinity and temperatures, low oxygen content and quickly changing water levels and food resources (Lenz & Browne 1991). To adapt to these harsh and unstable environmental conditions, *Artemia* have evolved two methods of development, termed ovoviviparous and oviparous. While ovoviviparous embryos develop directly into free-swimming nauplius larvae within the female, those undergoing oviparity are arrested at the gastrula stage and released from the female as cysts. The cysts, composed of about 4000 cells and enclosed in a complex shell impermeable to all nonvolatile molecules, enter a dormant state known as diapause in which their metabolic activity is so reduced that it becomes difficult to detect (Clegg *et al.* 1995; Drinkwater & Clegg 1987, 1991; Jackson & Clegg 1996; MacRae *et al.* 1989; Warner *et al.* 1989; Clutter 1978). Diapause, maintained even under favorable conditions by an endogenous mechanism, is terminated by exposure of cysts to environmental stimuli such as desiccation. After activation, the cysts resume development if conditions are favourable (Drinkwater & Clegg 1987, 1991). Cysts complete postgastrular development in 12-15 hr at 28°C when well aerated, emerging from the cracked shell as larvae enclosed in hatching membranes. Rupture of the hatching membrance releases free swimming larvae (Go *et al.* 1990; Rafiee *et al.* 1986). Before emergence, the embryos tolerate

return to a metabolic standstill (quiescence) in response to adverse environmental factors; development resumes once favorable conditions are restored (Fig.1) (Drinkwater & Clegg 1991; Bagshaw et al. 1986).

Diapause occurs in the life cycle of many organisms, and it is usually associated with resistance to environmental stress (Mead 1993; Marcus 1996; Fell 1995; Drinkwater & Clegg 1991; Yamashita & Hasegawa 1985; McNamara 1994). However, among these diapause organisms, *Artemia* (cysts) seemingly possesses the most striking ability to tolerate environmental insults including anoxia, temperature extremes, desiccation, γ-irradiation and organic solvents (Clegg & Conte 1980; Drinkwater & Clegg 1991). For example, cysts, even after hydration, can tolerate up to 4 years of anoxia with low loss of viability (Clegg 1994, 1997). The metabolism of anoxic embryos is reduced to a point where it cannot be detected, a condition contrary to the generality that, under normal hydration and temperature, cell maintenance requires a constant and substantial free energy flow. In order for the embryos to survive, they must protect macromolecules and organelles from damage and/or repair damaged macromolecules. The heat shock proteins, which have molecular chaperone function and are induced in response to stress, are ideal candidates for this role.

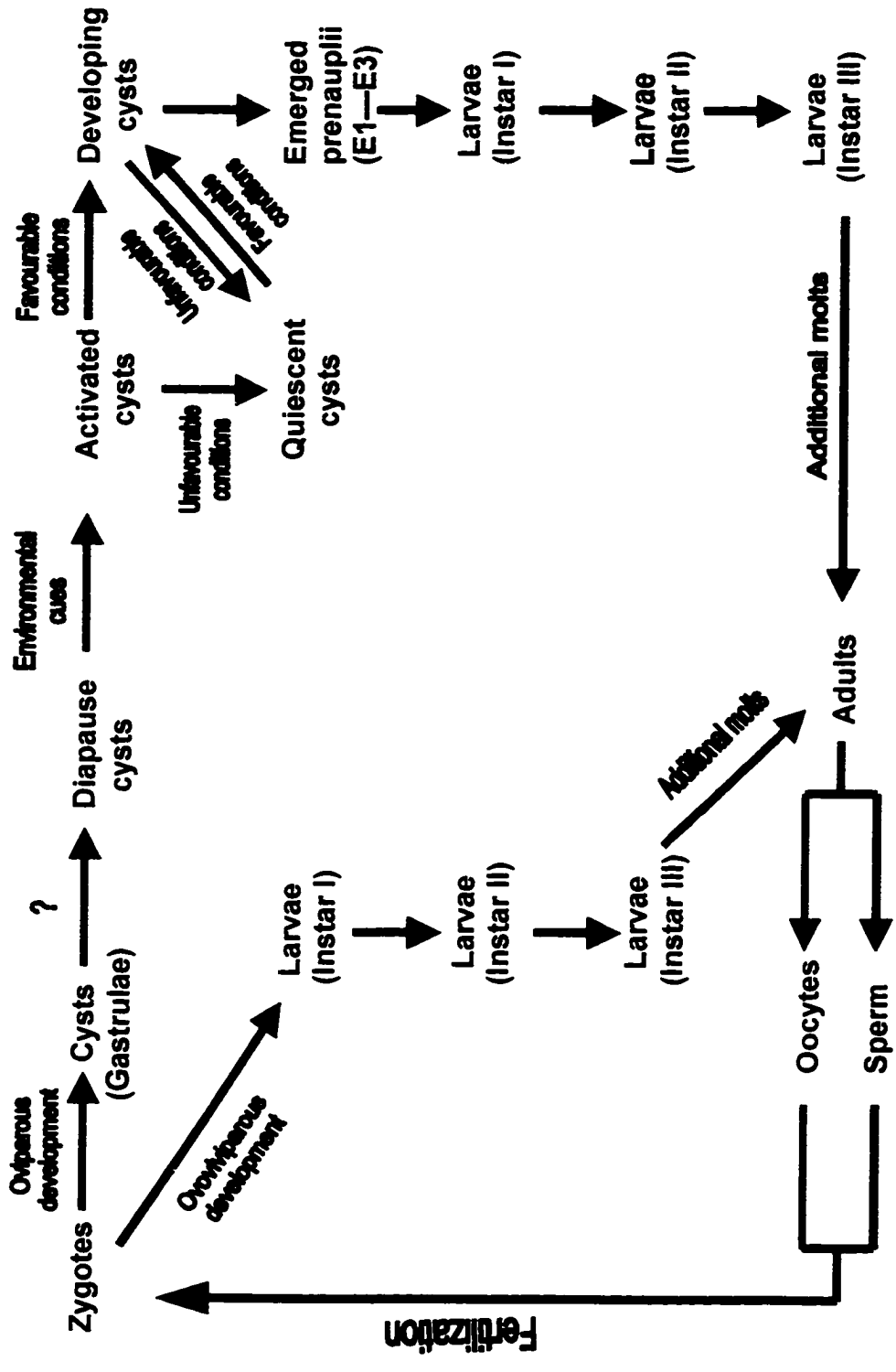
1.5 p26, an abundant low molecular weight protein found in *Artemia* cysts

A prominent low molecular weight protein in cell-free extracts of *Artemia* cysts was previously observed in our laboratory to aggregate upon incubation at 37°C and form particles visible in the electron microscope (unpublished). An

antibody was made to this protein. At about the same time, Dr. Jim Clegg (Bodega Marine Laboratory, University of California, Davis) was studying the same protein, named it p26 and also made an antibody to it (Clegg *et al.* 1994). p26 exists as large complexes in cell-free supernatants from cysts and it translocates reversibly into the nucleus (Clegg *et al.* 1994, 1995). These features of p26, as well as peptide sequence obtained by Dr. Reinout Amons (Department of Medical Biochemistry, University of Leiden) suggested that p26 is a member of the small heat shock/α-crystallin protein family. The objective of my work was to determine if p26 is a small heat shock protein, to further characterize its molecular properties and investigate its role in *Artemia* encystment and diapause.

p26 was purified to apparent homogeneity from cell-free extracts of *Artemia*, allowing measurement of its native molecular mass and observation of its multimer structure, determination of chaperone activity *in vitro*, and sequencing of the entire protein. The cloning and sequencing of p26 cDNA confirmed that p26 is a small heat shock/α-crystallin protein. *Artemia* has a multiple gene family for p26 as shown by analysis on Southern blots. Probing of northern and western blots revealed that p26 gene expression is cyst-specific. The results support the conclusion that p26 plays an important protective role during encystment and diapause of *Artemia*.

Fig. 1. Life cycle of *Artemia franciscana*



II. Materials and Methods

2.1 Hydration of *Artemia* cysts and synchronization of larvae

Artemia cysts (Sanders Brine Shrimp Co. Ogden, UT) were hydrated in distilled H_2O for at least 3 hrs at 0°C and washed under suction on a Buchner funnel with cold distilled water. Hydrated cysts were used to produce synchronized larvae and to prepare cell-free extracts.

The procedure of Langdon *et al.* (1990) slightly modified, was used to obtain large amounts of synchronized *Artemia* larvae. After hydration, those cysts that sank to the bottom of the hydration beaker were collected, washed several times to remove residual floating cysts, and cultured until about 50% emergence was obtained. The floating (emerged) cysts were collected and transferred into 40 ml of cold hatch media in a 50 ml plastic tube. The capped tube was shaken vigorously for 30 seconds to separate the emerged larvae from the cyst shells, after which the tube was placed on ice for 5 min. The larvae sink to the bottom and they were collected and transferred to another tube which contained cold hatch medium. After a brief agitation, the tube was incubated at RT for 5 min and the larvae at the bottom were collected. This procedure was repeated once if any cysts remained. Larvae collected this way were well synchronized at emergence, and they were used for production of synchronized nauplii at Instar I, II and III as described by Langdon *et al.* (1990).

2.2 Laboratory culture of *Artemia* and collection of embryos

A laboratory population of *Artemia,* maintained in 10 L of filtered, aerated sea water in a glass tank (20 X 35 X 20 cm^3), was fed daily with algae (*Isochrysis sp.*) according to a procedure developed by Jackson and Clegg (1996). Under these conditions, animals were mature in about 4 weeks, which was indicated by the formation of visible ovaries and ovasacs along the sides of females and the appearance of mating pairs in the population. Females which had two ovasacs containing oocytes were collected and cultured individually in 10 ml of sea water in covered 12-well petri dishes. Any males mating with the collected females were also placed in the wells, otherwise two to three males were added to each well. The animals were fed with algae, and the development of the oocytes/embryos was monitored every 2 hr within the first 24 hr and every 12 hr after that, until cysts or nauplii were released from the female. To record developmental stages, females were fixed in 4% paraformaldehyde, and photographed with a Zeiss Tessovar dissecting microscope. Embryos at 1, 2, 3, 4, and 5 days post-fertilization, undergoing either ovoviviparous or oviparous development, were collected and immediately processed, either for immunostaining or for preparation of RNA and protein.

2.3 Isolation of nuclei from encysted *Artemia* embryos

To purify nuclei from *Artemia*, a protocol originally developed by Squires and Acey (1989) and modified by J. Vaughn (University of Miami, Miami, OH; personal communication) was used with some adjustments. To exclude the possibility of p26 translocation into nuclei (Clegg *et al.* 1994, 1995) during

purification, preparations of nuclei were made at either pH 6.5 or 7.6, except for the final step, when purified nuclei were suspended in 1 X HPC (Appendix I) at pH 7.6. All the procedures were performed on ice unless otherwise indicated. Ten grams of hydrated *Artemia* cysts were ground by hand in a chilled mortar and pestle in 35 ml of cold 1 X HPC for two minutes and then transferred to a Dounce homogenizer and subjected to one complete passage. The homogenate was passed through one layer of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 2,000 g for 10 min. The supernatant was discarded, the tube was rinsed once with 1 X HPC and the large greenish pellet was washed twice with 40 ml of 1 X HPC before resuspension in 30 ml of the same buffer. The suspension was layered onto a 25 ml cushion of 75% Percoll (Appendix I) and centrifuged in a Beckman JS-13 swinging bucket rotor at 10,000 rpm (16,000 g) for 30 min. The top clear layer was discarded while the adjacent cloudy layer was transferred to a fresh tube and brought to a final volume of 15 ml with 1 X HPC, after which it was applied to another 25 ml Percoll cushion, pre-spun during the previous step, and re-centrifuged. The top layer with orange oil and debris was discarded, while the second layer of about 5 to 6 ml was transferred to a fresh centrifuge tube, mixed with an equal volume of 1 X HPC and re-centrifuged. The resulting yellow-brownish pellet of purified nuclei was suspended in 1.0 ml of 1 X HPC, pH 7.6. Purity of the nuclei was determined by mixing samples with an equal volume of 0.4% DAPI (Molecular Probes, Eugene, OR) and examining in a fluorescent microscope equipped with a UV filter.

2.4 Preparation of *Artemia* cell-free extracts (S1)

Hydrated cysts were washed once with Pipes buffer (100 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.5) and homogenized in 25 ml of Pipes buffer per 50 g (wet weight) of cysts with a Retsch motorized mortar and pestle (Brinkman Instruments Canada, Rexdale, ON). The homogenate was centrifuged at 16,000 X g for 10 min and the supernatant, after passage through two layers of Miracloth, was centrifuged at 40,000 X g for 30 min at 4°C. The upper two thirds of the supernatant was transferred to a fresh tube, re-centrifuged under the same conditions for 20 min and either used immediately or frozen at -70°C. The cell-free extract was termed S1.

2.5 Preparation of *Artemia* RNA and protein in Trizol solution

Samples of 150 to 200 embryos or larvae at developmental stages, ranging from 1-day post-fertilization to instar II larvae, were used for preparation of total RNA and protein, both from the same specimens. The samples were rinsed in hatch medium (Appendix I), transferred to a micro tissue-grinder (Fisher Scientific Inc.) with minimal carry over of medium, and ground in 0.4 ml of Trizol solution (Life Technologies, Burlington, ON) for 1 min. The material was further ground in a mini Dounce homogenizer by three or four complete passages. The homogenate, transferred to a 1.5 ml plastic tube to which 0.1 ml of chloroform was added, was vortexed for 1 min, followed by a 5 min full speed spin in a microcentrifuge. The top clear layer was used for preparation of RNA while the bottom layer was used for protein. The top layer was transferred to a

fresh tube, 1 µl of glycogen (Boehringer Mannheim) at 20 mg/ml and 0.2 ml of isopropanol were added and the mixture was incubated at RT for 10 min, followed by centrifugation in a microcentrifuge for 10 min at 4°C. The pellet was washed once with 0.5 ml of 70% ethanol, air-dried, and dissolved in 15 µl of DEPC-treated dH_2O supplemented with 0.1% sodium dodecylsulphate (SDS). The concentration of RNA was determined by measuring absorbance at 260 nm.

For preparation of protein, the remaining aqueous solution was removed from the lower part of the chloroform extract, and 0.15 ml of 100% ethanol was added. The mixture was inverted several times, incubated at RT for 5 min, and centrifuged at 2,000 g for 10 min at 4°C. The supernatant was transferred to a fresh tube and 0.6 ml of isopropanol was added, after which the mixture was incubated at RT for 10 min followed by a 10 min centrifugation in a microcentrifuge. The large orange pellet was washed twice with 1.0 ml of 0.3 M guanidine HCl and once with 100% ethanol, with a 20 min incubation at RT during each wash. The pellet was dried in a vacuum desiccator and dissolved in 30 µl of dH_2O containing 1% SDS. Twelve µl of 4 X gel treatment buffer (Appendix I) was added and the samples were placed in boiling water bath for 5 min to dissolve the pellet. The supernatant was transferred to a fresh tube after a brief spin in the microcentrifuge. The protein concentration of these samples was determined by use of the DC Protein Assay Kit from Bio-Rad.

Polysomal RNA from 0, 3, 6, and 10 hr postgastrula cysts was prepared according to a slightly modified procedure developed by Langdon *et al.* (1990). Five grams of cysts (wet weight) were homogenized in 15 ml of TEMN (Appendix

l) with a pre-chilled mortar and pestle. After filtration through one layer of Miracloth, the homogenate was centrifugated at 12,000 g for 15 min, and the resulting supernatant was applied to a 10 ml, 15% sucrose cushion and centrifuged at 113,000 g (Beckman SW28 rotor) at 4°C for 4 hr. The supernant was discarded and the gel-like pellet was dissolved in 2.0 ml of Trizol solution. The preparation of RNA from this suspension was performed as described for the precyst embryos, except that glycogen was not used during precipitation by isopropanol.

2.6 Preparation of *Artemia* DNA

A procedure developed by Jack Vaughn (University of Miami, OH) and modified slightly by Joe Bagshaw (Worcester Polytechnic Institute, MA, personal communication) was used to extract DNA from *Artemia*. Instar I nauplii, collected as described previously, were homogenized in a Dounce homogenizer in ice-cold 1 X SSC (Appendix I) at a ratio of 4 ml of buffer to 1 g of larvae. The homogenate was filtered through 1 layer of Miracloth and centrifuged at 2,000 x g for 5 min to pellet the nuclei. With the supernatant carefully removed, the lower whitish layer and the packed lightly colored layer of the pellet were re-suspended in 100 ml of 1 x SSC, and transferred to a flask without disturbing the dark bottom layer of the pellet. SDS was added to 1% at RT with swirling for 2-3 min. Proteinase K (Sigma) was then added to 50 µg/ml and the solution was incubated at RT for 2-3 hr, followed by stirring at RT for 30 min with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1). After a 10 min

centrifugation at 10,000 g, the aqueous phase was collected and extracted twice more, after which it was dialyzed against two changes of 400 ml of 1 x SSC at 4°C for at least 24 hr. The solution was transferred to a flask, heat-treated RNase I was added to 50 µg/ml, and the mixture was incubated for 1 hr at 37°C. Then Proteinase K was added to 50 µg/ml and the solution was incubated for 1 hr at 37°C, followed by three extractions with chloroform/isoamyl alcohol as for the phenol/chloroform extraction. The final aqueous phase (purified genomic DNA) was dialyzed against 2-3 changes of TE buffer (pH 7.4) over a period of 2 to 3 days, and stored at 4°C.

2.7 Preparation of antibody to p26

Either purified p26 (described later) or denatured p26 recovered from SDS-polyacrylamide gels, and homogenized in a motor-driven Dounce homogenizer (Zhang & MacRae 1992) was used for immunizations. Rabbits were injected subcutaneously with approximately 500 µg of antigen in Freunds complete adjuvant, followed by three boosters consisting of 100 µg of protein in Freunds incomplete adjuvant administered at two week intervals. Antisera were monitored for p26-specific antibody production on western blots containing S1 from cysts.

2.8 Preparation of immuno-affinity columns

2.8.1 Purification of IgG from rabbit serum

The IgG fraction of anti-p26 serum was obtained as described by Harlow

and Lane (1988). Briefly, 5 ml of rabbit serum was centrifuged at 4,000 g for 10 min., ammonium sulfate was added to the supernatant to 50% saturation (1.45 g in 5.0 ml) and stirred for 20 min at 4°C, followed by centrifugation as described above. The resulting pellet (IgG fraction) was dissolved in 1 ml of 10 mM Tris buffer and dialyzed overnight in the same buffer before loading onto a 10 ml DE52 column. The protein was eluted from the column with 10 mM Tris buffer containing 50 mM NaCl, pH 8.5, and the peak fractions (absorbance at 280 nm) containing purified IgG were pooled and dialyzed overnight against PBS buffer at 4°C. The protein concentration was determined by the method of Lowry *et al.* (1951).

2.8.2 Coupling of IgG to Protein A-Sepharose beads

Direct coupling of anti-p26 IgG to protein-A-Sepharose (Sigma) was performed according to Harlow and Lane (1988). Briefly, 0.3 g of protein-A-Sepharose hydrated in 5.0 ml of PBS was mixed with 2.0 mg of purified IgG in a total volume of 5.0 ml. After incubation at RT with gentle mixing for 2 hr, the mixture was transferred into a 10 ml plastic column and washed with 50 ml of 0.2 M sodium borate (pH 9.0). The beads were resuspended in 10 ml of the same buffer, 10 μl of the suspension was removed for assay, dimethylpimidate (powder) (Sigma) was added to 20 mM, and the column was gently shaken at RT for 30 min to allow conjugation. At this point, 10 μl of the suspension was again removed for assay. To stop the coupling reaction, the beads were washed with 10 ml of 0.2 M ethanolamine (pH 8.0) and then resuspended in 10 ml of the same solution for 2 hr at RT with gentle shaking, followed by a wash with 50 ml

of PBS. The efficiency of coupling was determined by electrophoresing the samples of beads taken before and after the coupling in 10% SDS-polyacrylamide gels and staining with Coomassie blue. Good coupling was indicated by the presence of IgG heavy chain in gel lanes containing the "before" sample but not the "after" sample.

2.9 Purification of p26

Twenty ml samples of S1 were applied to columns (2.5 cm X 7 cm) of DE-52 (Whatman, Maidstone, England) prepared according to the manufacturer's instruction and washed in Pipes buffer containing 1 M NaCl and equilibrated in Pipes buffer. Flow through fractions with peak absorbance at 280 nm were pooled and applied to a P11 (Whatman, Maidstone, England) column (2.5 cm X 7 cm) prepared as described for DE-52. The P11 column was washed with Pipes buffer and then eluted sequentially with Pipes buffers containing 0.2 and 0.4 M NaCl. Protein containing fractions obtained with 0.4 M NaCl were pooled and the protein was precipitated by addition of $(NH_4)_2SO_4$ to 40% (6.3 g of $(NH_4)_2SO_4$ in 28 ml of sample at 4°C) followed by centrifugation at 10,000 X g for 10 min. The precipitated protein was re-dissolved in 3.0 ml of Pipes buffer, 1.0 ml was mixed with 1.0 ml of antibody conjugated-Protein-A-Sepharose CL-4B in a 10 ml plastic column and the mixture was incubated at RT overnight with gentle shaking. The column was washed with 10 ml of Tris-glycine (0.1 M for both Tris and glycine, pH 7.4), and protein was then eluted three times with 0.5 ml of 0.1 M glycine (pH 2.3). The eluates were collected separately and neutralized

immediately with 50 μl of 1.0 M Tris (pH 8.0). The purity of p26 in these samples was examined by SDS polyacrylamide gel electrophoresis (described in section 2.12).

2.10 Sizing of purified p26

2.10.1 Sepharose CL-6B gel filtration chromatography

A 40 ml (1.02 X 48 cm) Sepharose CL-6B (Sigma) column equilibrated with 0.1 M Tris-glycine (pH 7.4) was standardized with gel filtration molecular weight makers (Sigma) which were chromatographed individually (carbonic anhydrase, 29 kDa; albumin, 66 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa; thyroglobulin, 699 kDa). The void bed volume of the column was determined with dextran blue (2000 kDa). Five hundred mg of purified p26 was applied to the column, and the eluate was collected as 1.0 ml fractions. To examine the distribution of p26 among the fractions, a 75 μl sample from each fraction was electrophoresed in a 15% SDS-polyacrylamide gel, blotted to nitrocellulose and probed with antibody to p26. The resulting protein bands were scanned on ThunderScan and quantitated.

2.10.2 Sedimentation on sucrose gradients

Two hundred microgram of purified p26 was loaded onto a 10 ml, 17-40% continuous sucrose gradient in the same buffer as for the filtration gel, and centrifuged at 40,000 g for 21 h in a Beckman Ti41 rotor. Molecular weight markers (same as above) were centrifuged individually as for p26. After centrifugation, the gradients were fractionated as ten 1.0 ml aliquots and with

one additional sample for a pellet at the bottoms of tubes. The positions of molecular weight markers were determined by absorbance measurements at 280 nm. Quantitation was performed as described above for samples from the filtration gels.

2.10.3 Examination of p26 oligomers by electron microscopy

For electron microscopy, preparations of purified p26 were centrifuged at 40, 000 g for 30 min at 4°C. The supernatant was removed and the tube was rinsed with Pipes buffer. The pellet was re-suspended in Pipes buffer to one tenth of the starting volume, applied to Formvar-coated, carbon-stabilized grids, and negatively stained with 1% uranyl acetate. Samples were examined in a Philips 201 electron microscope as described by Zhang and MacRae (1992).

2.11 Immunofluorescent staining of *Artemia* embryos and larvae

Immunofluorescent staining of *Artemia* larvae was performed essentially as described by Langdon *et al.* (1991a) with minor modifications. Emerged prenauplii and larvae at instar I, II and III were fixed in prewarmed (40°C) 4% paraformaldehyde in PBS (pH 7.4) for 1 hr at RT, then overnight at 4°C. Fixed larvae were washed 3 times in PBS, 20 min each wash with shaking at RT, and chopped into small pieces on a glass slide with a razor blade. Dissected larvae were transferred to a 1.5 ml Eppendorf tube and extracted by incubation in 2 changes of PBSAT (Appendix 1) over a 2 - 4 hr period with shaking at RT. Following extraction, the larvae were incubated in anti-p26 antibody diluted 1:500 in PBSAT for 1 hr at RT, then overnight at 4°C. After three washes in

PBSAT, larvae were incubated for 2 hr at RT in FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) diluted 1:200 in PBSAT and washed in PBS as described above, followed by a quick rinse in dH_2O and incubation in 0.4% DAPI for 2 min. Animals were rinsed in dH_2O, mounted on a glass slide in a drop of Vectashield (Vector Laboratories, Burlingame, CA), covered with a coverslip and sealed with nail polish. Fluorescent staining was visualized with either a Leitz Aristoplan fluorescence microscope or a Zeiss confocal laser scanning microscope.

For immunofluorescent staining before emergence, embryos or cysts were gently crushed on poly-L-lysine coated slides with another slide, and fixed immediately, either in methanol for 5 to 10 min at -20°C or prewarmed (40°C) 4% paraformaldehyde at RT for 1 hr. Incubation with both primary and second antibodies (same dilutions as described above) was for 45 min at RT. The rest of the procedure was as described for the larvae.

2.12 SDS-polyacrylamide gel electrophoresis, western blotting and immunodetection of proteins

One dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze proteins as described by Laemmli (1979). The gels (12.5% or 15%) were either stained with Coomassie blue (Appendix 1) or protein was electrophoresitically transferred to nitrocellulose (Protran, Mandel Scientific Co. Ltd., Guelph, ON) as described by Towbin *et al.* (1979). For immunodetection of proteins, the nitrocellulose membranes were rinsed briefly with TBS (Appendix

I), and then stained with 0.2% Ponceau-S in 3% TCA to check transfer efficiency and to mark the position of protein lanes. The membranes were blocked in 5% milk powder in TBS-Tween (Appendix 1) for 30 min at RT before incubating with primary antibody. Immuno-conjugates were detected by either alkaline phosphatase or the enhanced-chemiluminescence (ECL) procedure as recommended by the manufacturer (DuPont Canada). In the former procedure the primary antibody was diluted 1: 1000 whereas for ECL the p26 antibody was used at dilutions of 1:5000 to 1:10,000. Incubation with primary and secondary antibodies was at RT for 45 min in both methods.

2.13 Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing (IEF) and two dimensional (2D) gel electrophoresis were performed essentially as described by MacRae and Ludueña (1984) and Langdon *et al.* (1991b). pH gradients of approximately 6.0 to 8.0 were established in IEF tube gels composed of 4% polyacrylamide and 8 M urea (Appendix I), using a combination of Biolyte 3-10, 5-7, and 6-8 ampholytes (Bio-Rad) at a ratio of 1:2:2. IEF gels were either stained with Coomassie blue or loaded onto 12.5% SDS polyacrylamide slab gels, after incubation in treatment buffer for 30 min at RT, for second-dimensional gel analysis. Two-dimensional gels were either stained by Coomassie blue or processed for western blotting. To judge the position of p26 isoforms on 2-dimensional gels, samples of S1 and molecular markers (Bio-Rad) were added to the slab gels for concurrent one-dimensional electrophoresis.

2.14 First-strand cDNA synthesis

Poly $(A)^+$ RNA from 0 hr *Artemia* cysts was prepared as described by Langdon *et al.* (1990) and first-strand cDNA was synthesized with a "First-strand cDNA Synthesis Kit" (Pharmacia) according to the manufacturer's instructions. Briefly, 5 μg (20 μl) of heat denatured poly $(A)^+$ mRNA was mixed with 11 μl of Bulk First-Strand Reaction Mix, 1μl of Not I-$d(T)_{18}$ (0.6 μg/μl) and 1 μl of DTT solution, and the mixture was incubated at 37°C for 1 hr.

2.15 Amplification of *Artemia* p26 cDNA by the polymerase chain reaction (PCR)

In order to amplify p26 cDNA, a degenerative primer, p26-S (5'-AYGARTAYGGHGAYGTNCA-3') (N=A+T+G+C; Y=C+T; R=A+G; H=A+T+C), based on the peptide EYGHVQR was used as the sense primer, while an oligo dT, Not I-$d(T)_{18}$, included in the cDNA synthesis kit, was used as the antisense primer. For a 50 μl PCR reaction, the following reagents were mixed in a 500 μl plastic tube (Bio-Rad Micro Test tube, "EZ"): 3 μl of the first-strand cDNA; 5 μl of 10X PCR buffer; 5 μl of dNTP (1 mM); 3 μl of Mg^{2+} (50mM); 5μl of p26-S and Not I-$d(T)_{18}$ (both at 0.2 μg/μl); 24 μl of dH_2O, and 2 μl of Taq polymerase (1 U/μl) (Life Technologies, Burlington, ON). The mixture was topped with 50 μl of mineral oil, and PCR was performed under the following conditions: 30 sec at 92°C, 43°C and 72°C, sequentially for 30 cycles, on a PTC-100 programmable thermal controller (MJ Research, Inc, Watertown, Mass. USA) with a hot start at 94°C for 2 min. The PCR products were analyzed in 1.5% agarose gels in TAE

buffer (Appendix I) with a 100 bp size marker from Pharmacia.

2.16 Cloning and sequencing of PCR products

A DNA fragment of 401 bp obtained by PCR amplification was cloned into pUC18 (Smal I/BAP) using the SureClone™ ligation kit from Pharmacia according to the manufacturer's instructions. In this procedure, the PCR fragment was first blunt-ended to remove the 3' d(A) overhangs and then phosphorylated at the 3' end. It was then ligated into the blunt-ended Sma I site of dephosophorylated PUC18 vector using T4 DNA ligase. The recombinant DNA was transformed into DH5α *E. coli* host cells using standard protocols described by Sambrook *et al.* (1989). Positive clones were selected and propagated for DNA mini-preparation, and both strands of the 401 bp cloned fragment, named p26-3', were sequenced manually using the T^7 sequencing kit from Pharmacia which was based on the method of Sanger *et al.* (1977).

2.17 Screening of an *Artemia* cDNA library

To obtain a full length p26 cDNA, a cDNA library from 0 hr *Artemia* cysts in pBluescript SK- was screened with ^{32}P-labelled p26-3'. This library was converted from a cDNA library originally constructed in λZAP II (see Appendix II for the conversion procedure) which was kindly provided by Dr. L. Sastre (Instituto Investigaciones Biomedicas, Madrid, Spain). The probe was labelled by use of the T^7 Quick Prime Kit from Pharmacia. Briefly, 25 μl of p26-3' (25 to 50 ng of DNA) in low melt agarose gel was placed in boiling water bath for 7 min, then immediately transferred to a 37°C water bath and incubated for 10

min. This solution was mixed with 9 μl of dH_2O, 10 μl of labeling mix, 1 μl of T^7 polymerase, and 5 μl of ^{32}P-α-dCTP (3000 μCu/mmol) and incubated at 37°C for 30 min. The unincorporated nucleotides were removed by passing the reaction mixture through a Sephacryl S200 HR MicroSpin™ column (Pharmacia). The screening of the library was performed according to Sambrook *et al.* (1989) with sequential washes of 2 X SSC containing 0.1% SDS for 30 min at RT, followed by 1X SSC containing 0.1% SDS and 0.1 X SSC containing 0.1% SDS, both for 30 min.

2.18 Sequence analysis

Analysis of DNA and protein sequences, including sequence editing, reading frame and translation, restriction enzyme mapping, and calculation of molecular mass and pI, were done with Gene Runner (Hastings software, Inc.). Sequences were send to the Internet-based databases at the National Center of Biological Information (NCBI) for the analysis of significant matches with known sequences using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) including blastn and blastx (for DNA) and blastp (for protein). Protein and DNA motif searches were performed through web based databases in addition to the analysis by Gene Runner.

To do multiple alignments, protein sequences for other small heat shock/α-crystallin proteins were retrieved from NCBI protein databases using the Web browser based Entrez program, and analyzed with "Clustal" version V and W (Higgins *et al.* 1992; Thompson *et al.* 1994). The output files of Clustal were

viewed and edited in Microsoft™ word.

The secondary structure and solvent accessibility of amino acid residues within p26 were predicted by the methods of Rost and Sander (Rost, 1996; Rost & Sander 1994, 1993) via the Predict Protein E-mail server at the European Molecular Biology Laboratory, Heidelberg, as described by Casper *et al.* (1995).

For construction of phylogenetic trees, all protein sequences, except p26, were retrieved from the NCBI protein databases and aligned with the Clustal W program. Phylogenetic trees were constructed with the computer program, Mega (Kumar *et al.* 1993).

2.19 Analysis of p26 gene number and structure

Twenty µg of *Artemia* genomic DNA was completely digested with restriction enzyme(s) at a ratio of 5 U per µg of DNA, using conditions recommended by the manufacturer. The digested DNA was collected by ethanol precipitation, re-suspended in 20 µl of TE buffer (pH 8.0), and electrophoresed overnight in 0.7% agarose gels at 30 V. After electrophoresis, the gel was placed in denaturation buffer (Appendix I) for 30 min at RT with shaking. The gel was then rinsed with distilled water for 15 min, incubated in neutralization buffer for 45 min with shaking, with one change of buffer, and blotted overnight with 10 X SSC to Hybond-N nylon membrane using the capillary method. The filters were pre-hybridized in DNA hybridization medium (Appendix I) at least 3 hr at 68°C, followed by addition of ^{32}P-labelled p26 cDNA fragments and overnight incubation at the same temperature. After hybridization, the filter was washed

twice with 2 x SSC/0.1%(w/v) SDS at RT, once with 1x SSC/0.1%(w/v) SDS and once with 0.1x SSC/0.1%(w/v) SDS, both at 68°C, before overnight exposure to X-ray film (Kodak Co.) at -70°C. The number of p26 gene copies in the *Artemia* genome was determined by hybridizing the ^{32}P-labeled p26-3-6-3 to blots that contained known amounts of BamH I digested *Artemia* DNA and p26-3-6-3 in linerized Bluescript SK-. The size of the *Artemia* haploid genome is 1.37 X 10^6 kb (1.45 pg) (Roberts & Vaughn 1982), while the size of the plasmid containing p26-3-6-3 is 3.64 kb (3.7 X 10^{-6} pg). Therefore, the copy numbers of p26 cDNA in 73.6, 147.2, 294.4, and 588 pg of Bluescript SK-containing p26-3-6-3 were 1, 2, 4, and 8 times the copy number of the *Artemia* haploid genome in 28 μg of *Artemia*, respectively.

2.20 Analysis of p26 gene expression

For northern blots, equivalent amounts of total RNA from each developmental stage were resolved in 1.5% formaldehyde denaturing agarose gels, transferred to Hybond-N nylon membrane (Amersham) as described by Langdon *et al.* (1990), and hybridized to ^{32}P-labelled p26-3-6-3. The labeling and hybridization were performed as described in section 2.17. The size of mRNA was determined by use of a 0.24–9.5 kb RNA ladder (Life Technologies, Burlington, ON), transferred to membrane and stained with 0.04% methylene blue.

2.21 Expression of p26 in bacteria

2.21.1 Insertion of p26 cDNA into the expression vector, pRSET

p26-3-6-3 was excised from bluescript SK- by digestion with BamH I and Xho I and ligated into pRSET C (Invitrogen, San Diego, CA), which was linarized with the same enzymes. The recombinant construct, termed pRSET-p26-3-6-3, was propagated in XL1-blue RF' and its identity was confirmed by DNA sequencing.

2.21.2 Expression of p26 in *E. coli*

For expression, pRSET-p26-3-6-3 was transformed into BL21(DE3) (Novagen, Madison, WI) (Studier *et al.* 1990) using a standard transformation protocol (Sambrook *et al.* 1989). Five colonies from the transformation were selected and cultured in 1.0 ml of LB medium (Appendix I) supplemented with ampicillin (50 μg/ml) at 37°C overnight. One half ml of the overnight culture was transferred into 2.0 ml of fresh medium, incubated at 37°C with shaking for 30 min, and IPTG was added to a final concentration of 2 mM before further incubation for 5 hr. One ml from each culture was transferred into Eppendorf tubes and centrifuged in a microcentrifuge for 10 sec. The cell pellet was resuspended in 200 μl of denaturing binding buffer (Appendix I), incubated at RT for 30 min, frozen and thawed two or three times, and centrifuged at 12,000 g for 20 sec. The supernatant was transferred to a fresh tube and 5 μl samples were used for SDS-PAGE and western blot analysis with anti-p26 antibody to confirm the synthesis of recombinant proteins, and to reveal their sizes.

2.22 Heat shock of bacteria expressing p26

BL21 (DE3) carrying pRSET-p26-3-6-3 and pRSET C was cultured in 2.0

ml of LB medium containing ampicillin and 2 mM IPTG at 37°C, overnight. A half milliliter of these cultures after a 1:10 dilution were transferred to a 50°C circulating water bath and 50 μl samples were removed at 0, 15, 30, and 60 min, and plated on LB agar plates for incubation at 37°C overnight (the 0 min samples were further diluted before plating). The number of colonies on each plate was recorded and the data from three experiments were pooled for analysis.

2.23 Heat shock of *Artemia* larvae and adults

First instar nauplii, either directly released from females (direct-nauplii) or hatched from cysts (cyst-nauplii) were collected as described in section 2, and heat shocked at single temperature ranging from 38.5°C to 42.5°C, at 0.5°C intervals, for 60 min. Heat treated animals were transferred into 12-well petri dishes for a 48 hr incubation, and the number of dead and live larvae were recorded. The data from three separate experiments were pooled and the percentage of live animals after the treatment was plotted against the temperatures of the treatment.

To determine if the synthesis of p26 was induced by heat shock, cyst-derived *Artemia* larvae at instar I and III, as well as adults, were incubated at 39°C for 60 min, and transferred to RT for 30 min before homogenization for protein preparation. Approximately equal amounts of protein from each sample were analyzed by SDS-PAGE and western immunoblotting with anti-p26 antibody.

III. Results

3.1 Purification and biochemical characterization of p26

3.1.1 Purification of p26 to apparent homogeneity

As a prerequisite for biochemical characterization, p26 was purified to apparent homogeneity from cell-free extracts (S1) of *Artemia* cysts by a 4-step procedure (Fig. 2). p26 was recovered in the flow-through fractions from DE-52 Cellulose, and in the 0.2 to 0.4 M NaCl eluates from phosphocellulose P11, after which it was precipitated by 40% $(NH_4)_2SO_4$. Although p26 was greatly enriched at this stage, there were several other proteins in this sample which were subsequently eliminated by use of p26-specific affinity-chromatography. When 20 μg of affinity-purified p26 was electrophoresed in a 12.5% SDS-polyacrylamide gel and stained with Commassie blue, only one band was visible (Fig. 2a). A lower molecular mass polypeptide of uncertain origin was present in Phosphocellulose P11 fractions, but it was almost completely removed by the $(NH_4)_2SO_4$ fractionation procedure (Fig. 2). The protein recoveries during purification are summarized in Table 2, revealing that 3.6 mg of p26 was obtained from 900 mg of S1.

3.1.2 p26 consists of multiple isoforms

Two-dimensional gel electrophoresis of cyst cell-free extract resolved several p26 isoforms detectable with anti-p26 antibody; visible were one major isoform which had a pI of 7.1, and several minor isoforms which were for the most part more basic than the major isoform (Fig. 3).

Figure 2. Purification of p26 to apparent homogeneity.

Fractions obtained during the purification of p26 were electrophoresed in 12.5% SDS-polyacrylamide gels and either visualized with Commassie blue (a) or blotted to nitrocellulose, immunostained with anti-p26 antibody, and visualized with alkaline phosphatase-conjugated secondary antibody (b). Lane 1, 220 μg of cell-free protein extract; lane 2, 150 μg of the flow-through protein fraction from DE-52; lane 3, 70 μg of the 0.2 to 0.4 M NaCl elution fraction from phosphocellulose P11; lane 4, 100 μg of the 0 to 40% $(NH_4)_2SO_4$ precipitation fraction; lane 5, 20 μg of affinity purified p26; lane 6, molecular mass markers of 97, 66, 43, 31, 22 and 14 kDa. The arrow indicates the position of p26.

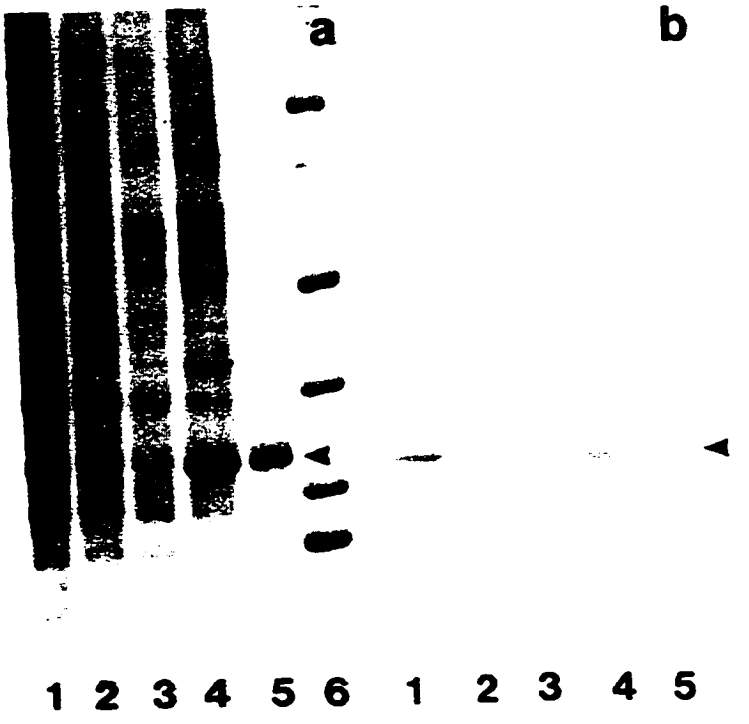


Figure 2.

Table 2. Recovery of p26 during purification

Fraction	Volume (ml)	Protein concentration (mg/ml)	Total protein mg	Protein Recovery (%)
S1	20	45±3	900±60	100
DE-52	36	16±1	576±36	64±4
P11	28	2.3±0.2	64±5	7.1±0.5
$(NH_4)_2SO_4$	3.0	8.0±0.3	24±1	2.7±0.1
Affinity Column	4.5	0.8±0.1	3.6±0.5	0.4±0.1

The yield of protein at each stage during the purification of p26 from 20 g of hydrated cysts is reported. The values shown represent the average of three preparations and include the standard deviations.

Figure 3. Isoelectric point determination and isoform composition of p26.

Cell-free extracts were electrophoresed in IEF gels (a), followed by electrophoresis in 12.5% SDS-polyacrylamide gels which were either stained with Commassie blue (a, b), or blotted to nitrocellulose and stained with antibody to p26 (c). Lane 1, cell-free extract samples were applied to gels to permit concurrent one and two dimensional gel electrophoresis; lane 2, molecular mass markers. The unlabelled arrowheads indicate p26.

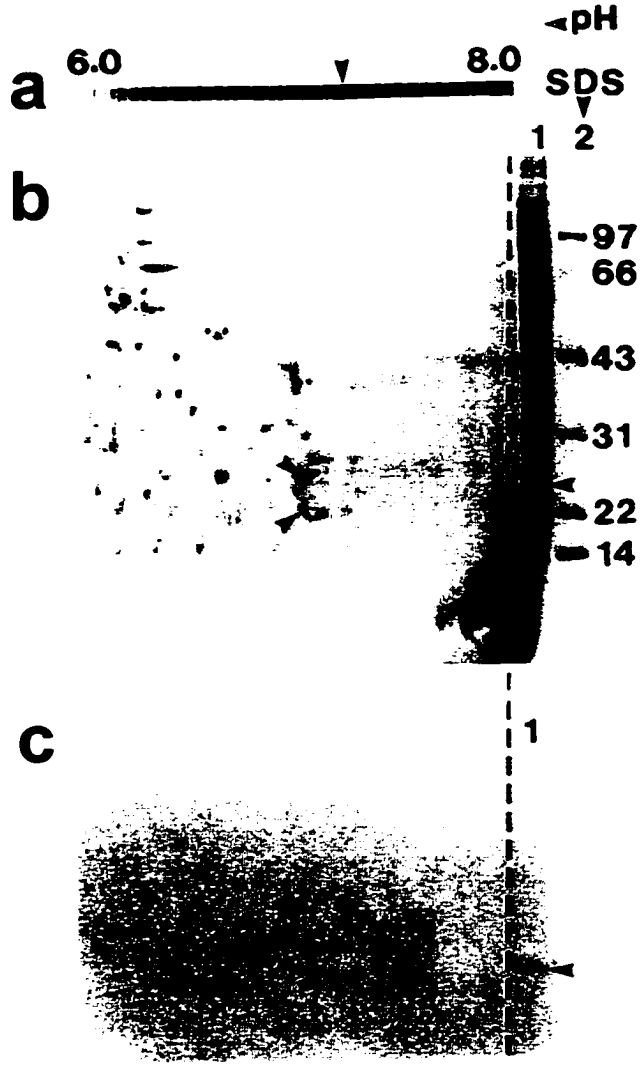


Figure 3.

3.1.3 Oligomer formation by p26 *in vitro*

Analysis by molecular-sieve chromatography on Sepharose CL-6B (Fig. 4a, b) and sucrose-density gradient centrifugation (Fig. 4c, d) demonstrated that purified p26 exists as oligomers. Although its size varied, the largest number of oligomers has a mass of about 700 kDa. A preliminary study of the morphology of these oligomers by electron microscopy revealed particles about 10-12 nm in size and of different morphologies (Fig. 5). Some of the particles were spherical and had little in the way of distinguishing characteristics. Other spherical particles possessed, in a more or less central location, an area of dense staining, indicating a depression or hole in the particle. Less common, but easily found, were particles with short projections radiating outward from a central structure which often has a densely stained region. Finally, some particles appeared to consist of closely apposed structures, usually three in number, stacked one on the other.

3.2 Molecular characterization of p26

3.2.1 PCR amplification of p26 cDNA

PCR amplification of first-strand cDNA from 0 hr *Artemia* yielded a 401 bp DNA fragment, which was termed p26-3'. p26-3' was cloned into pUC18 and sequenced, revealing one open reading frame encoding 89 amino acids and a typical 3' noncoding region of eukaryotic cDNA which included a poly (A) signal, AATAAA, and a poly(A) tail (Fig. 6). The deduced protein sequence covered more than half of the partial peptide sequences obtained previously from peptide analysis done by Drs. J. Clegg and R. Amons using p26 purified by

Figure 4. Oligomer formation by native p26 *in vitro*.

Purified p26 was applied to Sepharose CL-6B columns previously standardized with markers of 29, 66, 200, 443, and 699 kDa, which are labeled in Fig. 4b. Samples of equal volume were taken from each column fraction, electrophoresed in 15% SDS-polyacrylamide gels, blotted to nitrocellulose and stained with antibody to p26 (a). Some of the samples, which failed to yield a band after reaction with anti-p26 antibody, are not shown. The molecular mass of p26 was also determined by centrifugation on continuous sucrose gradients standardized with the same molecular mass markers as described above (c, d). The density determinations of the p26 bands in panels a and c, in arbitrary units (closed circles), were plotted in concert with the eluate volumes (b) or the fraction numbers (d) for the molecular mass markers (open circles). Vo, void volume (ml); Ve, eluate volume (ml); Vt, total volume of the column (ml); p, pellet.

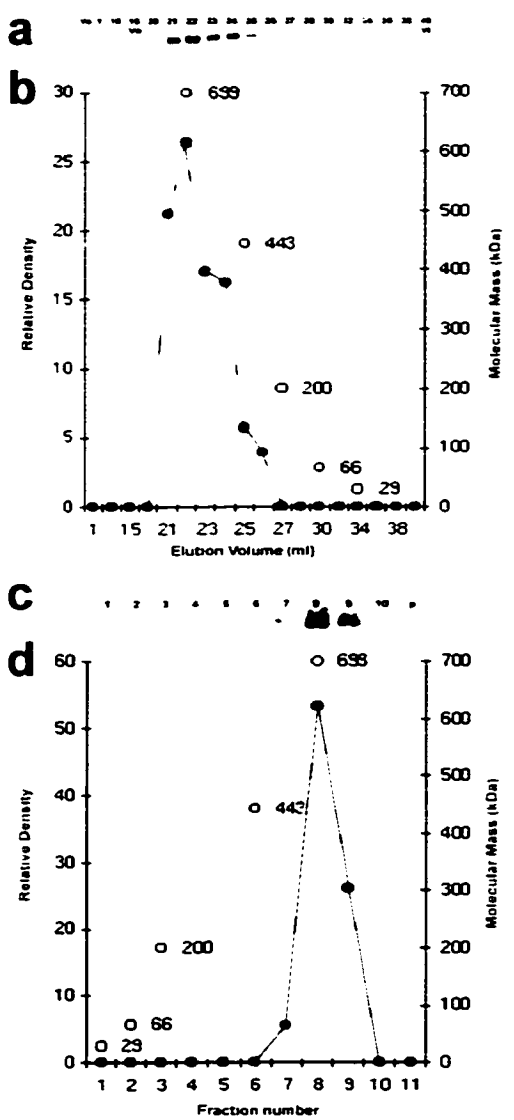

a
b
c
d
Relative Density
Molecular Mass (kDa)
Elution Volume (ml)
Fraction number
669
443
200
66
29
698

Figure 4.

Figure 5. Morphology of p26 oligomers.

Purified samples of p26 were placed on grids, negatively stained with uranyl acetate and examined in the electron microscope. Two representative fields of view are shown and they contain particles of different appearances. Unlabelled arrowheads, spherical particles; arrowheads labelled 1, spherical particles with an electron dense region; arrowheads labelled 2, particles with short projections, arrowheads labelled 3, particles which appear to consist of closely apposed structures stacked one on another. The bars in a and b represent 40 nm.

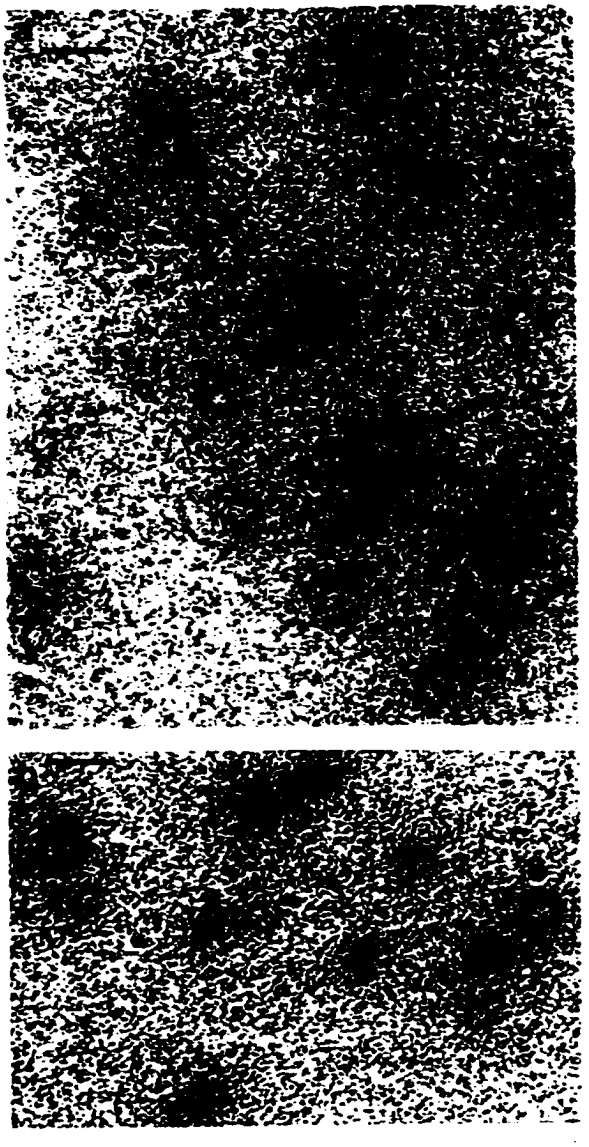
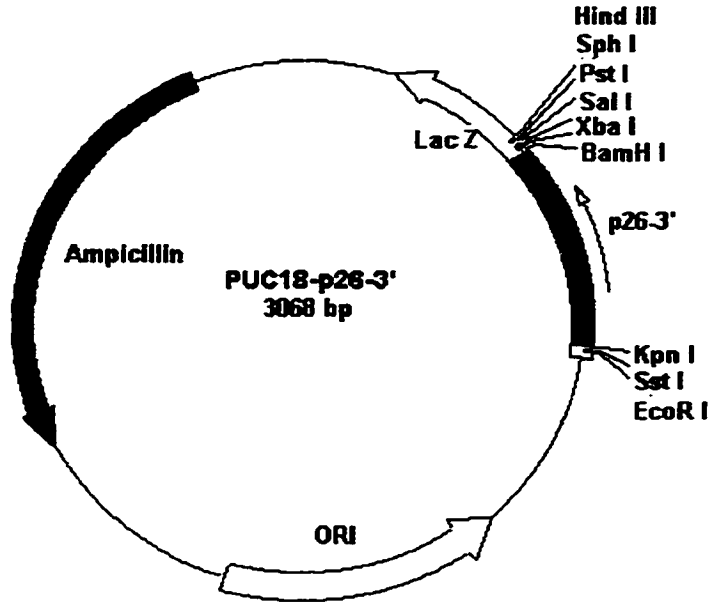


Figure 5.

Figure 6. The nucleotide sequence of p26-3' and the deduced amino acid sequence.

A 401 bp PCR product was cloned into PUC18 (a) and sequenced manually by the Sanger dideoxy-mediated chain termination method. The 401 bp insert included one open reading frame encoding a polypeptide of 89 amino acids and a typical eukaryotic mRNA 3' noncoding region which had a poly(A) signal sequence, aataaa (single underlined), followed by a poly(A) tail (double underlined) (b). Amino acids in bold represent sequence obtained by peptide analysis. The regions used for design of primers are indicated by arrows under the sequence.

a



b

```
1 gaatatggac acgtacaaag agaatttcga cgacgataca gactcccaga acatgtcaaa
  E  Y  G  H  V  Q  R  E  F  R  R  R  Y  R  L  P  E  H  V  K
            P26-s
61 ccagaatctg tgtcatctac tttgtcatca gatggtgtct taactatcca tgctccgaaa
    P  E  S  V  S  S  T  L  S  S  D  G  V  L  T  I  H  A  P  K

121 actgctttaa gctcaccaac agaacgtatc gtacccatca caccagcgcc agctgttgga
     T  A  L  S  S  P  T  E  R  I  V  P  I  T  P  A  P  A  V  G

181 aggattgaag ggggaactac aggtactact acaggcagta cagctagttc aactccagca
     R  I  E  G  G  T  T  G  T  T  T  G  S  T  A  S  S  T  P  A

241 agaacaacaa gatcaggagg tgcagcttaa tctgcattca atatatattc aatgaatctt
     R  T  T  R  S  G  G  A  A  #

301 ccggtctttt ttctttgtac ctttattttt ttgtcaataa atctgtatac gaggcaaaaa

361 aaaaaaaaaa aaaattcctg cggccgcgaa ttcttcctgt t401
    <------------------------------------------
                Not I (dT)18
```

Figure 6.

centrifugation and gel electrophoresis (Fig. 6, sequences in bold print; personal communication) indicating that p26-3' represented the 3' portion of the cDNA for p26.

3.2.2 Cloning and sequencing of a complete p26 cDNA

A cDNA clone, termed p26-3-6-3, was isolated by screening an *Artemia* library with p26-3'. It had a 709 bp insert and was ligated into the bluescript SK- plasmid at the EcoR I and Xho I restriction enzyme sites (Appendix III). P26-3-6-3 contained a single open reading frame, a 21 bp 5' non-coding region, an 85 bp 3' non-coding region and a poly(A) tail (Fig. 7). The entire sequence of the PCR fragment, p26-3', (excluding the sequence for the Not I site) was located the 3'-end of p26-3-6-3 and the start of p26-3' is indicated by ⌐→ (Fig. 7). A polypeptide of 192 amino acid residues, beginning with methionine and ending in alanine, was deduced from the coding region of p26-3-6-3 and it included the remainder of the peptides obtained previously (amino acid sequence in bold), in addition to those found in p26-3'. The sequence of p26-3-6-3 and it's deduced amino acid sequence was submitted to the NCBI GenBank under acession number, AF031367 (Appendix V).

3.2.3 Biochemical and molecular properties of p26 DNA and protein

The restriction enzyme map and codon usage for p26 cDNA, and the amino acid composition of p26 are listed in Appendix IV. Properties of p26 that were determined include a calculated molecular mass of 20.8 KD, a calculated pI of 6.61, protein motifs for recognition by casine kinase II, protein kinase C, and a site for myristylation. No "Arg-X-X-Ser" phosphorylation motifs were found.

Figure 7. The complete sequence of p26-3-6-3 cDNA and the deduced amino acid sequence of p26.

The cDNA clone, which contained the insert p26-3-6-3, was obtained by screening an *Artemia* library with ^{32}P-labelled p26-3'. Both strands of p26-3-6-3 were sequenced manually using the Sanger dideoxy-mediated chain termination method. One open reading frame that encoded a polypeptide of 192 amino acids, starting with methionine and ending in alanine, was observed. The start of the sequence of p26-3' (nucleotide 331) is indicated by ⌜⟶ . Protein sequences in bold were those also obtained from previous peptide sequencing by R. Amons.

```
                          1M  A  L   N  P  W   Y  G  G  F   G  G  M
  1 cggcacgagc tcgtgctcaa aatggcactt aacccatggt acggaggatt tggtggtatg
    gccgtgctcg agcacgagtt ttaccgtgaa ttgggtacca tgcctcctaa accaccatac

     T  D  P   W  S  D  P   F  G  F   G  G  F   G  G  G  M   D  L  D
 61 actgacccat ggtctgatcc atttggattt ggtggcttcg gaggtggcat ggaccttgat
    tgactgggta ccagactagg taaacctaaa ccaccgaagc ctccaccgta cctggaacta

     I  D  R   P  F  R  R   R  M  M   R  R  G   P  D  T  S   R  A  L
121 attgacaggc ccttccggag aagaatgatg agaagaggtc cagataccag cagggcttta
    taactgtccg ggaaggcctc ttcttactac tcttctccag gtctatggtc gtcccgaaat

     K  E  L   A  T  P  G   S  L  R   D  T  A   D  E  F  Q   V  Q  L
181 aaggagttag ctactcctgg gtccttgagg gacacagctg atgaatttca agttcagcta
    ttcctcaatc gatgaggacc caggaactcc ctgtgtcgac tacttaaagt tcaagtcgat

     D  V  G   H  F  L  P   N  E  I   T  V  K   T  T  D  D   D  I  L
241 gatgttggcc acttttttacc aaacgaaatt acagtcaaga caaccgacga tgatattctt
    ctacaaccgg tgaaaaatgg tttgctttaa tgtcagttct gttggctgct actataagaa

                                          p26-3'
     V  H  G   K  H  D  E   R  S  D   E  Y  G   H  V  Q  R   E  F  R
301 gtccatggca aacatgacga gcgatctgat gaatatggac acgtacaaag agaatttcga
    caggtaccgt ttgtactgct cgctagacta cttatacctg tgcatgtttc tcttaaagct

     R  R  Y   R  L  P  E   H  V  K   P  E  S   V  S  S  T   L  S  S
361 cgacgataca gactcccaga acatgtcaaa ccagaatctg tgtcatctac tttgtcatca
    gctgctatgt ctgagggtct tgtacagttt ggtcttagac acagtagatg aaacagtagt

     D  G  V   L  T  I  H   A  P  K   T  A  L   S  S  P  T   E  R  I
421 gatggtgtct taactatcca tgctccgaaa actgctttaa gctcaccaac agaacgtatc
    ctaccacaga attgataggt acgaggcttt tgacgaaatt cgagtggttg tcttgcatag

     V  P  I   T  P  A  P   A  V  G   R  I  E   G  G  T  T   G  T  T
481 gtacccatca caccagcgcc agctgttgga aggattgaag ggggaactac aggtactact
    catgggtagt gtggtcgcgg tcgacaacct tcctaacttc ccccttgatg tccatgatga

     T  G  S   T  A  S  S   T  P  A   R  T  T   R  S  G  G   A  A192#
541 acaggcagta cagctagttc aactccagca agaacaacaa gatcaggagg tgcagcttaa
    tgtccgtcat gtcgatcaag ttgaggtcgt tcttgttgtt ctagtcctcc acgtcgaatt

601 tctgcattca atatatattc aatgaatctt ccggtctttt ttctttgtac ctttatttttt
    agacgtaagt tatatataag ttacttagaa ggccagaaaa aagaaacatg gaaataaaaa

661 ttgtcaataa atctgtatac gaggcaaaaa aaaaaaaaaa aaaaaaaaa709
    aacagttatt tagacatatg ctccgttttt ttttttttt ttttttttt
```

Figure 7.

3.2.4 p26 is a small heat shock/α-crystallin protein

All standard database searching such as blastn and blastX (DNA) and blastp (protein) clearly indicated that p26 is a small heat shock/α-crystallin protein. A multiple-sequence alignment of p26 with representatives from other species showed that p26 possesses three domains: a variable amino-terminal domain (M1 to G60), a highly conserved "α-crystallin domain" (S61 to R152) and a short, less conserved carboxyl terminal extension (I153 to A192) (Fig. 8). When pairwise comparison was performed the numbers of identical residues in the "α-crystallin" domain were as high as 50%, while conserved residues reached 73%. In the phylogenetic tree based on the primary structure of proteins, p26 located at the expected position, showing the closest relationship with the small HSPs from *Drosophila* (Fig. 9). On the other hand, p26 has several unique characteristics within the small heat shock/α-crystallin family. Its amino-terminal domain has glycine-rich and arginine-rich regions. Ten of its first 29 residues are glycines (34% glycine content), and these are allocated in a peptide of 22 residues (45% glycine content). By comparison, other members of this family, such as those listed in Fig. 8, have only 1- 3 glycines in the corresponding regions. Residues 36-51 of p26 contain 7 arginines (7/16=44%), 6 of which are in a 10 residue stretch (60%) with three arranged consecutively (residues 39 to 41). Moreover, p26 has the longest carboxyl-terminal extension among the known small heat shock/α-crystallin proteins, and the conserved α-crystallin domain of p26, based on the PHD secondary structure prediction, apparently lacks intervening α-helix between the discrete regions of β-sheet (Fig. 10).

Figure 8. Comparison of p26 to other small heat shock/α-crystallin proteins.

The deduced amino acid sequence of p26 was compared by the Clustal W program to the sequences of other small heat shock/α-crystallin proteins: human αA-crystallin (CRAA_H, P02489); human αB-crystallin (CRAB_H, P02511); human small heat shock protein 27 (Hsp27_H, P04792); *Drosophila* HSP27 (HSP27_D, P02518); and a small heat shock protein from a nematode, Sec-1 (Sec-1_N, Z35640). The first block of aligned sequences represents the N-terminal domain, the second block corresponds to the α-crystallin domain, and the third block shows the C-terminal extension. The number of residues in each domain and the total number of residues in each protein are listed at the end of the third block. -, no residue (gap); *, identical residue; ., conserved residue.

```
          N-terminal domain (N)
p26       --------MALNPW----YGGFGG-MTDPWSDPFGFGGFGGGMDLDIDRPFRRRMMRRGP-D-----------TSRAL--KELATPG
CRAA_H    -----MDVTIQHPWFKRTLGPFY--PSRLFDQFFGEGLFEYDLLPFLS-STISPYYRQS-------------LFR---TVLDSGIS
CRAB_H    -----MDIAIHHPWIRRPFFPFHS-PSRLFDQFFGEHLLESDLFPTST-SLSPFYLRPP-------------SFLRAPSWFDTGLS
HSP27_H   MTERRVPFSLLRGPSWDPFRDWYP-HSRLFDQAFGLPRLPEEWSQWLGGSSWPGYVRPLPPAAIESPAVAAPAYSRALSRQLSSGVS
HSP25_C   MAERRVPFTFLTSPSWEPFRDWYH-GSRLFDQSFGMPHIPEDWYKWPSGSAWPGYFRLLPSESALLPAPGSP-YGRALS-ELSSGIS
Hsp27_D   -----MSIIPLLHLARELDHDYRTDWGHLLEDDFGFGVHAHDLFNPRR-LLLPNTLGLGRRRYSPYERSHGPHNQMSRRASGGPNAL
sec-1_N   -------MSSLCPYTGRPTGLFR-----DFEDMMPYWAQRHSMLNNFN-NIVPQQL----------------------------N

          α-crystallin domain (α)
p26       SLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDERSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTA-----LSSPTER
CRAA_H    EVRSDRDKFVIFLDVKHFSPEDLTVKVQDDFVEIHGKHNERQDDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFCGPKIQ--TGLDATHAER
CRAB_H    EMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQ------VSGPER
HSP27_H   EIRHTADRWRVSLDVNHFAPDELTVKTKDGVVEITGKHEERQDEHGYISRCFTRKYTLPPGVDPTQVSSSLSPEGTLTVEAPMPK----LATQSNEI
HS25_C    EIRQSADSWKVTLDVNHFAPEELVVKTKDNIVEITGKHEEKQDEHGFISRCFTRKYTLPPGVEATAVRSSLSPDGMLTVEAPLPK----PAIQSSEI
Hsp27_D   LPAVGKDGFQVCIDVSQFKPNELTVKVVDNTVVVEGKHEEREDGHGMIQRHFVRKYTLPKGLTPTKVVSTVSSEGVLTLRAPPPPGRERARSERIVR
sec-1_N   EVENTAQKFCVKLDVAAFKPEELKVNLEGHVLTIEGHHEVKETEHGFSKRSFTR-QTLPKDVDLAHIHTVINKEGQMTIDAPKTG------SNTTVR
                    . .**  * * .. *      . . *.*  .    *   * * *   .*  .    . . .  .* .*   *              .

          C-terminal region (C)                          N     α     C     Total
p26       IVPITPAPAVGRIEGGTTGTTTGSTASSTPARTTRSGGAA       60    92    40    192
CRAA_H    AIPVS----------------REEKP--TSAPSS------       62    95    15    173
CRAB_H    TIPIT----------------REEKPAVTAAPKK------       68    89    18    175
HSP27_H   TIPVT----------------FESRAQ-LGGRSCKIR---       86    93    20    199
HS25_C    TIPVT----------------VEAKKE-EPAKK-------       84    93    15    193
Hsp27_D   IQQTGPAHLSVKAPAPEAGDGKAENGSGEKMETSK-----       81    97    35    213
sec-1_N   ALPTH----------TSAQHAVTQKPSSTTTTGKH-----       44    90    25    159
```

Figure 8.

Figure 9. A phylogenetic tree of small heat shock/α-crystallin proteins

The tree was constructed according to the neighbor-joining method, using the program MEGA. The pairwise gap sequences were deleted and the Poisson corrections for multiple substitutions were made according to Fitch and Margoliash (1967). The numbers are the relative bootstrap values from 1000 replicates. Branch lengths are proportional to the minimal number of mutations per residue. The scale bar represents approximately 0.1 substitutions/amino acid. Only representatives from each group of organisms are used for construction of the tree, with the biological source of the protein following the protein name. Proteins from plants were not included in the tree. CRAA, αA-crystallin; CRAB, αB-crystallin.

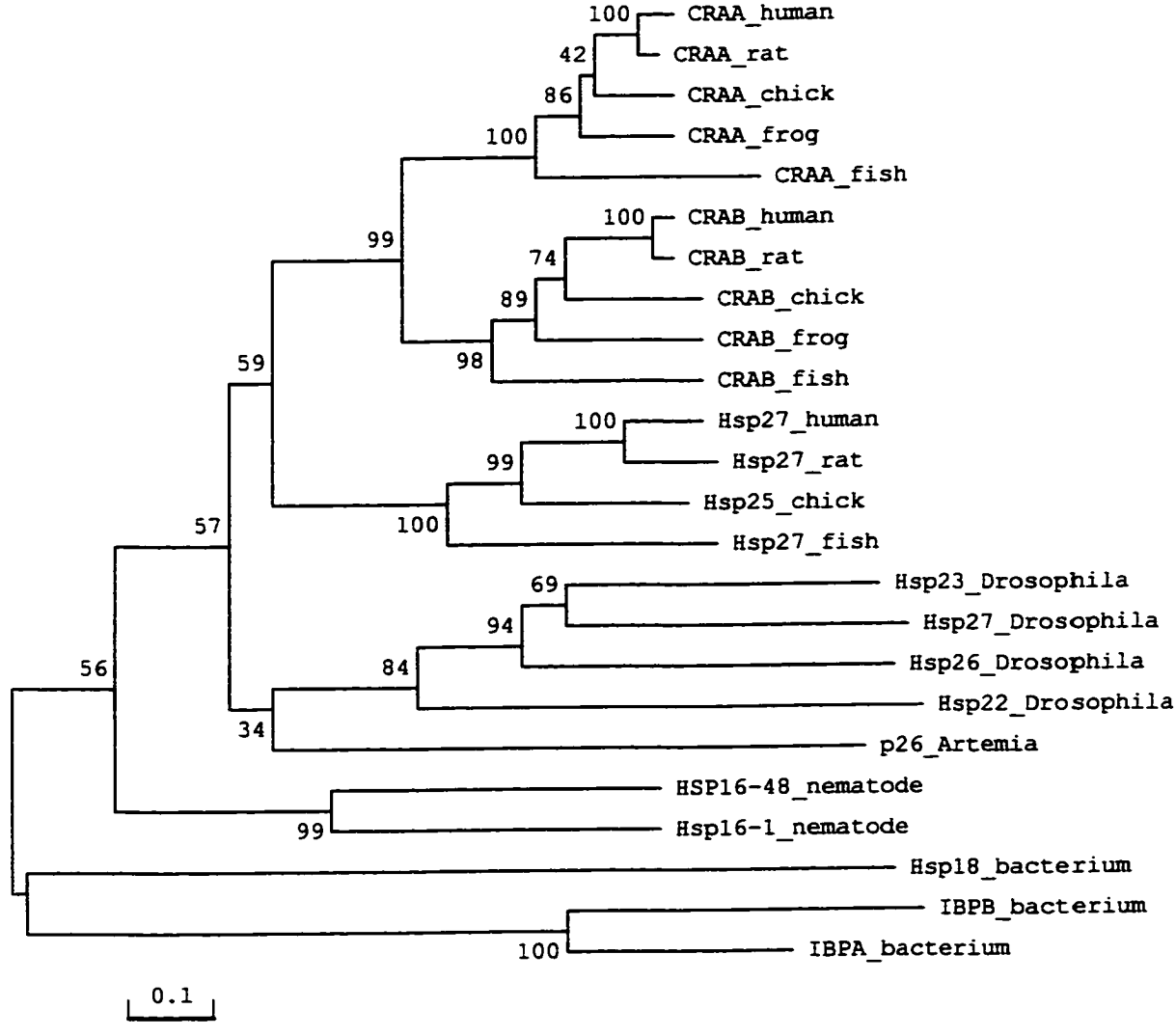

100
CRAA_human
42
CRAA_rat
86
CRAA_chick
100
CRAA_frog
CRAA_fish
100
CRAB_human
99
74
CRAB_rat
89
CRAB_chick
CRAB_frog
59
98
CRAB_fish
100
Hsp27_human
99
Hsp27_rat
Hsp25_chick
57
100
Hsp27_fish
69
Hsp23_Drosophila
94
Hsp27_Drosophila
56
84
Hsp26_Drosophila
Hsp22_Drosophila
34
p26_Artemia
HSP16-48_nematode
99
Hsp16-1_nematode
Hsp18_bacterium
IBPB_bacterium
100
IBPA_bacterium
0.1

Figure 9.

Figure 10. Predicted secondary structure of p26.

The secondary structure and solvent accessibility of amino acid residues within p26 were predicted as described in "Material and Methods." The entire sequence (AA) of p26 is shown with every 10th residue numbered. The designations within the figure are: PHD sec, secondary structure prediction; E, β-sheet; H, α-helix; P_3 acc, predicted solvent accessibility; e, exposed residue; b, buried residue. A blank in the solvent accessibility line indicates that it could not be determined if the residue was buried or exposed.

```
         ....,....1....,....2....,....3....,....4....,....5
AA       |MALNPWYGGFGGMTDPWSDPFGFGGFGGGMDLDIDRPFRRRMMRRGPDTS|
PHD sec  |  EEEE                 HHHH    EEEE     EEEEEE     |
P_3 acc  |bebbbe e beebbeeb beebbbebbbeb beeeeeee  ebbbeebeee|

         ....,....6....,....7....,....8....,....9....,....10
AA       |RALKELATPGSLRDTADEFQVQLDVGHFLPNEITVKTTDDDILVHGKHDE|
PHD sec  |             EE   EEEEEEEE       EEEEEEEEEEEEE     |
P_3 acc  |ebeeeeeeeeeb eeeeebbbbbbbe b eeebebebbeebbebbbe ee|

         ....,....11...,....12...,....13...,....14...,....15
AA       |RSDEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPT|
PHD sec  |        EEEEEEEEEE         EEEEEE    EEEEE          |
P_3 acc  | eeeeb bbbebb ebebbeebeebbbbbbbbbbbebbbbbbebeeeeeeeee|

         ....,....16...,....17...,....18...,....19.
AA       |ERIVPITPAPAVGRIEGGTTGTTTGSTASSTPARTTRSGGAA|
PHD sec  |   EEE                                    |
P_3 acc  |eebbebbeeeeeeeeeeeeeeeeeeeeeebebeeeeeeeeeeee|
```

Figure 10.

3.3 *Artemia* may contain multiple genes for p26

Southern blots containing *Artemia* DNA digested with BamH I and probed with ^{32}P-labelled p26-3-6-3, which corresponds to the entire p26cDNA, yielded 4 bands, two strong and two weak (Fig. 11b). When blots were reprobed with PCR generated DNA fragments, B1 and B2 hybridized to p26-3' whereas B3 and B4 reacted with p26-5', corresponding respectively to the 3'- and 5'-ends of p26-3-6-3 (Fig.11). Comparison of values generated by scanning of bands, B1 to B4, to those bands which represented increasing amounts of p26-3-6-3 suggested that there were more than one copy of the p26 gene in *Artemia* (Fig. 11c).

3.4 Developmental regulation and cellular localization of p26

3.4.1 *Artemia* exhibits two developmental modes: ovoviviparity and oviparity

Artemia franciscana embryos develop either ovoviviparously or oviparously, and both developmental processes were recorded microscopically from a half day before fertilization to the release of either nauplii or cysts (Fig. 12). These observations demonstrate that the fate of oocytes in a given female can be predicted by the color of the oocytes and the shell gland at least half a day before fertilization. Ovoviviparous development is signaled by greenish oocytes and a clear (invisible) shell gland (Fig. 12a-c), while oviparous development is indicated by yellowish oocytes and a brown shell gland (Fig. 12a'-c'). Fertilization occurs within 6 hrs after all oocytes move into ovasacs and is marked by the merging of the two ovasacs (Fig. 12d, d'). Embryos that undergo encystment develop a brown shell which is easily visible from one

Figure 11. Analysis of p26 gene number and structure.

The number of genes encoding p26, and their arrangement within the genome of *Artemia*, were examined by Southern blotting using ^{32}P-labelled probes corresponding to known regions of p26 cDNA. a, schematic representation of the probes prepared as described in "Materials and Methods" and corresponding to the full-length p26 cDNA (p26-3-6-3), its 5'-end (p26-5') and its 3'-end (p26-3'). Numbered arrows represent the terminal nucleotides in each probe. b, Southern blot. Lanes, 1-4 were loaded, respectively, with 73.6, 147.2, 294.4, and 588 pg of linearized plasmid containing p26-3-6-3, whereas lanes 5-7 contained 28 μg of *Artemia* DNA digested with BamH I. The amounts of DNA were chosen such that the intensity of hybridization signals in lanes 1 and 5 would be equal if the haploid *Artemia* genome contained one copy of the gene for p26. Lanes 1 to 5 were hybridized to p26-3-6-3, and lanes 6 and 7 were hybridized to p26-5' and p26-3' respectively. The four bands obtained by probing with p26-3-6-3 were labelled as B1 to B4 in lane 5. Size markers in kb are on the right side of the figure. c, Quantification. Bands in lanes 1 to 5, panel b, were scanned, and the density of each band was plotted, in arbitrary units, on the histogram: lane 1, 1X, lane 2, 2X, lane 3, 4X, lane 4, 8X; the values represent, respectively, densities equivalent to 1, 2, 4, and 8 copies of the p26 gene per haploid genome. B1- B4 correspond to bands B1 - B4 in panel b.

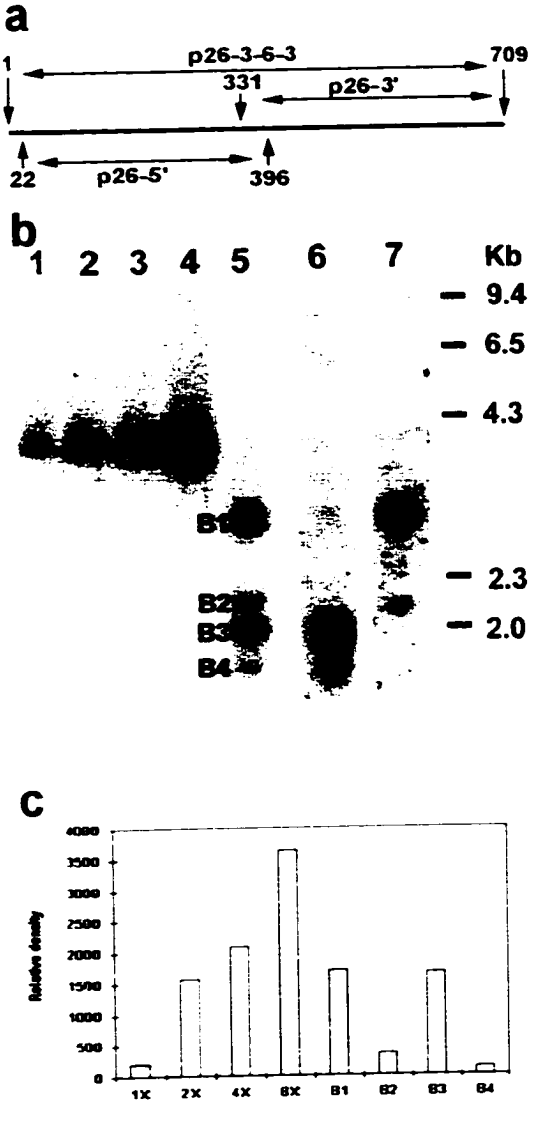

a
1
p26-3-6-3
709
331
p26-3'
22
p26-5'
396
b
1 2 3 4 5 6 7
Kb
9.4
6.5
4.3
2.3
2.0
B1
B2
B3
B4
c
Relative density
4000
3500
3000
2500
2000
1500
1000
500
0
1X
2X
4X
8X
B1
B2
B3
B4

Figure 11.

Figure 12. Ovoviviparous and oviparous development of *Artemia*.

Female *Artemia* at different reproductive stages were fixed in paraformaldehyde and examined by light microscopy. a, a': intact females containing, respectively, nauplii- and cyst-destined oocytes immediately before fertilization. b-f, b'-f': reproductive organs from females which contain, respectively, nauplii- and cyst-destined oocytes or embryos. g, first instar nauplii; g', newly released cysts. Oviparous development is signaled by the yellowish oocytes and brown shell gland, and it leads to cyst production, while ovoviviparous development, which leads to release of nauplii, is indicated by greenish oocytes and a colorless shell gland. SH, shell gland; OS, ovasac; OV, ovary; O, oocyte; E, embryo; G, gut.

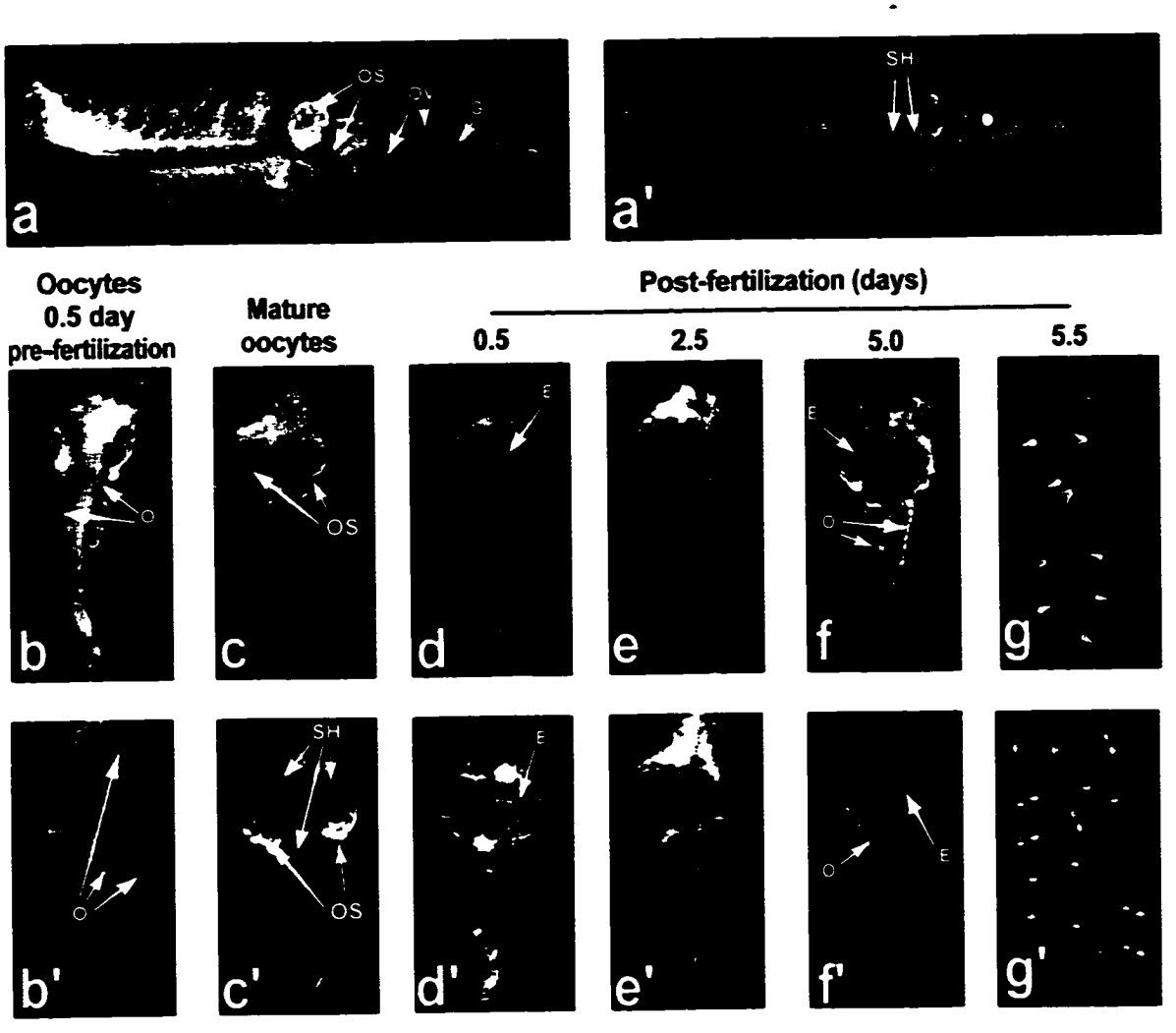

OS
SH
a
a'
Oocytes
0.5 day
pre-fertilization
Mature
oocytes
Post-fertilization (days)
0.5
2.5
5.0
5.5
E
O
OS
b
c
d
e
f
g
SH
b'
c'
d'
e'
f'
g'

Figure 12.

day post-fertilization onward (Fig.12d'-g'), while those that develop directly into nauplii maintain a green (earlier stages) to light yellow (later stages) color (Fig. 12d-g).

3.4.2 p26 is specifically synthesized in encysting *Artemia* embryos

Although cysts contain large amounts of p26, it was not clear when gene expression begins, when the protein is synthesized during encystment, and whether or not it occurs in embryos experiencing ovoviviparous development. Therefore, embryos undertaking both developmental routes were collected at 24 hr intervals after fertilization until their release as nauplii or cysts, for preparation of total RNA and protein. p26 mRNA, approximately 0.7 kb in size, was first detected in 2-day embryos undergoing encystment but was never found in those developing directly into nauplii (Fig. 13a). p26 protein was first detected in 3-day cyst-destined embryos, but never in nauplii-destined embryos (Fig. 13b). The amount of mRNA and protein increased as embryos developed, with a significant change in 4-day embryos. An mRNA band with an estimated size of 1.9 kb and which reacted with p26 cDNA was present in four and five day cyst-destined embryos (Fig. 13a).

3.4.3 p26 localizes to both the nuclei and the cytoplasm of encysted *Artemia* embryos

To examine the subcellular distribution of p26, nuclear and cytoplasmic samples were prepared from hydrated 0 hr cysts. To exclude the effect of pH on the location of p26, nuclei were prepared under basic and acid conditions. Protein samples from crude homogenates, the first nuclear pellet and purified nuclei were resolved by SDS-PAGE and either stained by Commassie blue or

Figure 13. p26 in cyst-destined versus nauplii-destined *Artemia* embryos.

Two hundred *Artemia* embryos were collected at 24 hr intervals for 5 days post-fertilization, and homogenized in 0.4 ml of Trizol solution for preparation of total RNA and protein. a, northern blot of *Artemia* total RNA. Two μg of total RNA were electrophoresed in 1.4% agarose denaturing gels, transferred to Hybond N and hybridized with ^{32}P-labelled p26-3-6-3. b, equal amounts of protein from each developmental stage were resolved in 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibody to p26 using the chemiluminescent procedure. Lanes 1 to 5 contained samples from cyst-destined embryos developed for 1 to 5 days respectively, while lanes 6 to 9 contained samples from nauplii-destined embryos developed 2-5 days respectively. The arrowhead in panel a indicates an extra mRNA band of about 1.9 kb that hybridizes to the p26 cDNA probe.


a
9.49
7.46
4.40
2.37
1.35
0.24
1 2 3 4 5 6 7 8 9
b
97
66
43
31
21
14
1 2 3 4 5 6 7 8 9

Figure 13.

blotted to nitrocellulose and stained with anti-p26 antibody (Fig. 14). p26 in the nuclear fractions represented approximately 10% of the total p26 in these embryos and was one of the major proteins in purified nuclei (Fig. 14). The results were the same for the basic and acid conditions. Immunofluorescent staining of purified nuclei with anti-p26 antibody revealed that p26 occurred within the nuclei and that it was present in specific compartments of the organelle, while the presence of p26 in the cytoplasm was revealed by the staining of cysts crushed onto slides (Fig. 15). Confocal microscopy revealed that p26 was often distributed in patches, approximately 1 nm in diameter, throughout the entire nucleus (Fig. 16), suggesting that it associated with specific compartments in this organelle.

3.4.4 p26 migrates into nuclei during *Artemia* encystment

To examine the cellular localization of p26 during encystment, embryos at different stages of encystment were immunofluorescently stained with anti-p26 antibody. In 3-day embryos undergoing encystment, wherein p26 was initially synthesized (Fig. 13b), the protein was found in the peripheral region of nuclei (Fig. 17). Staining was more complete when the embryos had developed further, as shown by the results obtained with nuclei from 5-day embryos (Fig. 17).

3.4.5 p26 disappeared during post-gastrula development of *Artemia*

To follow the fate of p26 mRNA and protein during development, samples prepared from post-gastrula embryos up to third instar larvae were analyzed on northern and western blots. p26 mRNA decreased rapidly once post-gastrula

Figure 14. Localization of p26 in encysted *Artemia* embryos.

Protein samples obtained during the preparation of nuclei from *Artemia* cysts were resolved by electrophoresis in 12.5% SDS-polyacrylamide gels and either stained with Commassie blue (panel a) or blotted to nitrocellulose and stained with antibody to p26 (panel b). Lanes 1-3 contained samples prepared under basic conditions (pH7.6) while samples in lanes 4-6 were prepared under acidic conditions (pH 6.5). The lanes contained: 1 and 4, the initial supernatant obtained during nuclei preparation; lanes 2 and 5, the initial pellet from a nuclei preparation resuspended in a volume equal to half that of the starting homogenate; lanes 3 to 6, concentrated samples of purified nuclei. The arrowhead in panel a indicates the position of p26. KD, molecular mass markers.

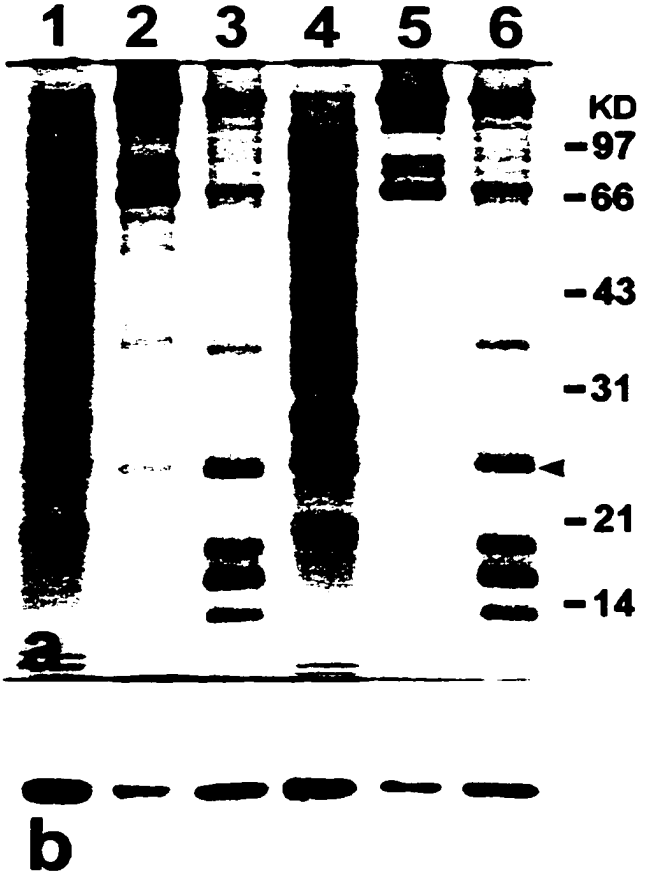


Figure 14.

Figure 15. Immunofluorescent staining of nuclei from encysted *Artemia* embryos.

Purified nuclei (a, a', b and b') and crushed 0 hr cysts (c and c') were fixed on poly-L-lysine coated slides and double stained. a, b and c, nuclei stained with DAPI. a', b' and c', same fields as in a, b and c, respectively, but immunofluorescently stained with antibody to p26. Arrowheads in c' indicate p26 located outside nuclei. The bar in a represents 50 μm and figures a', c and c' are the same magnification. The bar in b represents 12.5 μm and it is the same magnification as b'.

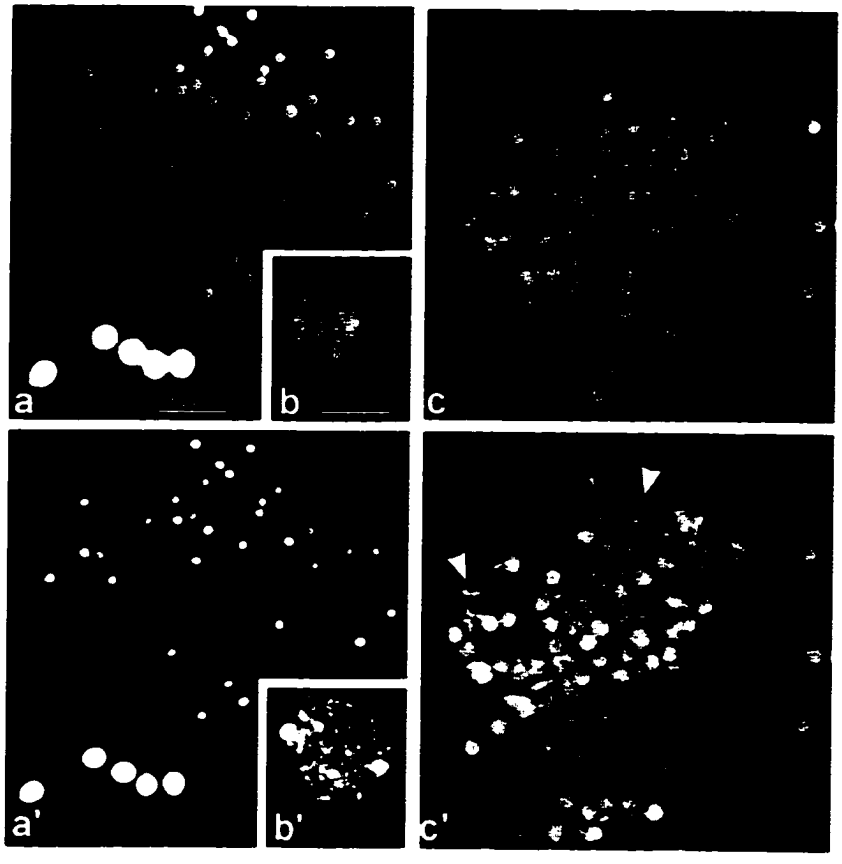


Figure 15.

Figure 16. p26 localizes to discrete compartments throughout cyst nuclei.

Nuclei purified from *Artemia* cysts were stained with antibody to p26 and examined with the confocal microscope. Panels a to g represent a continuous series of 1 μm optical sections through a single nucleus. The top and bottom sections of the nucleus are not shown as they contained limited information. Panel h is a three-dimensional reconstruction of the stained nucleus. The bar in a represents 5 μm and the magnification was the same for all figures.

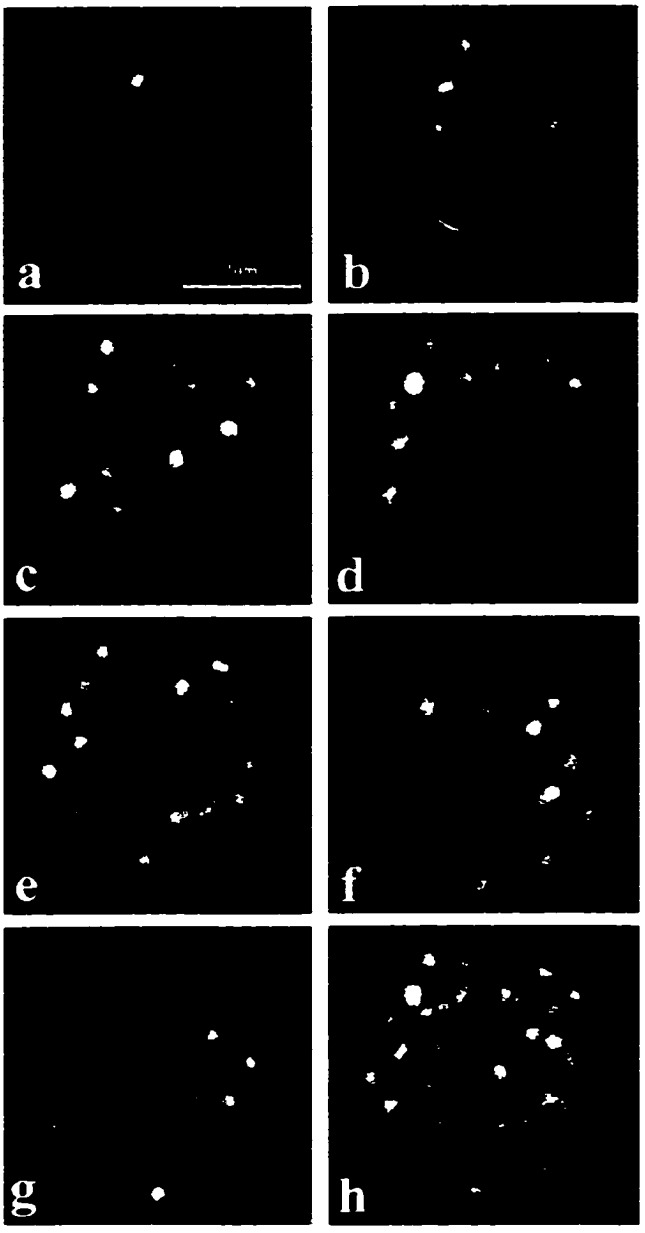


Figure 16.

Figure 17. p26 migrated into nuclei of encysting *Artemia* embryos.

Nuclei from developing cyst-destined embryos, three days (a, a') and five days (b, b') post-fertilization were crushed onto poly-L-lysine coated slides and double stained. a and b, nuclei stained by DAPI; a' and b', nuclei immunofluorescently stained with antibody to p26. The bar in a represents 5 μm and all pictures are the same magnification.

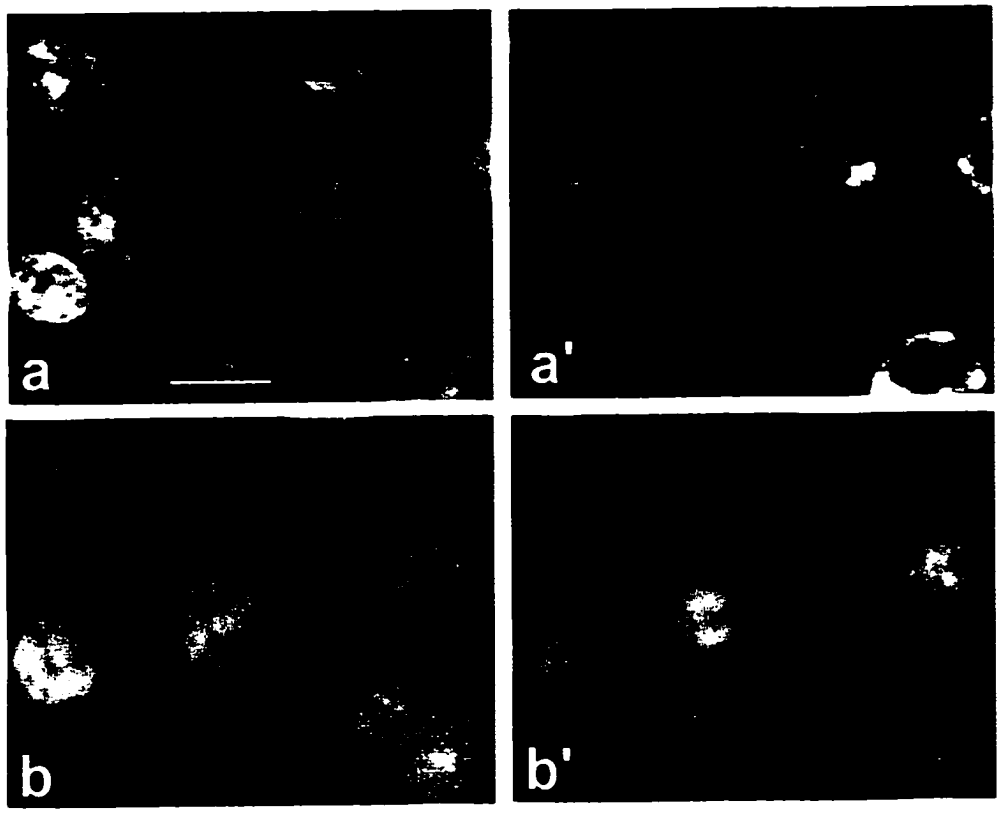


Figure 17.

development began and it had all but disappeared when emergence occurred (Fig. 18a). p26 protein, on the other hand persisted at the same level up to emergence, after which it decreased rapidly and had disappeared from instar II larvae (Fig. 18b). Northern blot analysis also showed that p26 mRNA was incorporated into polysomes early in postgastrula development and that it disappeared at about the same time as the remainder of the message (Fig. 18c).

Cellular localization of p26 in postgrastrula embryos or larvae was examined by immunofluorescent staining, indicating that it was mainly in nuclei. Almost all the nuclei in emerged prenauplii and first instar larvae were stained by the anti-p26 antibody (Figs. 19, 20). Notably, in the emerged prenauplii, the inner region of the gut was stained by anti-p26 antibody (Fig 19a'), while nuclei in the small portion of gut from first instar larvae shown in Fig. 20a' had no staining. Both the amount of p26 in nuclei and the percentage of nuclei stained by anti-p26 antibody dropped quickly as the animals progressed from first to second instar (Fig. 20). The reduction in staining was coincident with the decline in the amount of p26 in cell-free extracts of these larvae (Fig. 18b). Interestingly, p26 in instar II larvae was exclusively limited to a subset of up to 7 nuclei in the center of salt glands (Figs. 20, 21). The number of stained nuclei in the salt gland decreased gradually until there were no nuclei stained in third instar larvae.

3.5 Relationship bewteen thermoresistance and p26 synthesis *in vivo*

3.5.1 Recombinant p26 confers thermoresistance upon *E. coli*

To determine if p26 confers thermoresistance *in vivo*, p26 cDNA was

Figure 18. Disappearance of p26 during *Artemia* post-gastrula development.

Total RNA (a) and proteins (b) were obtained from encysted *Artemia* embryos developed 0 h (lane 1), 5 h (lane 2), and 10 h (lane 3), from emerged prenauplii (lane 4), and from larvae at instar I (lane 5) and instar II (lane 6). c, Northern blots of polysomal RNA from *Artemia* cysts developed 0 h (lane 1), 3 h (lane 2), 6 h (lane 3) and 10 h (lane 4). Northern blots (a, c) were probed with ^{32}P-labelled p26-3-6-3, while western blots (b) were stained with antibody to p26 by the ECL procedure.

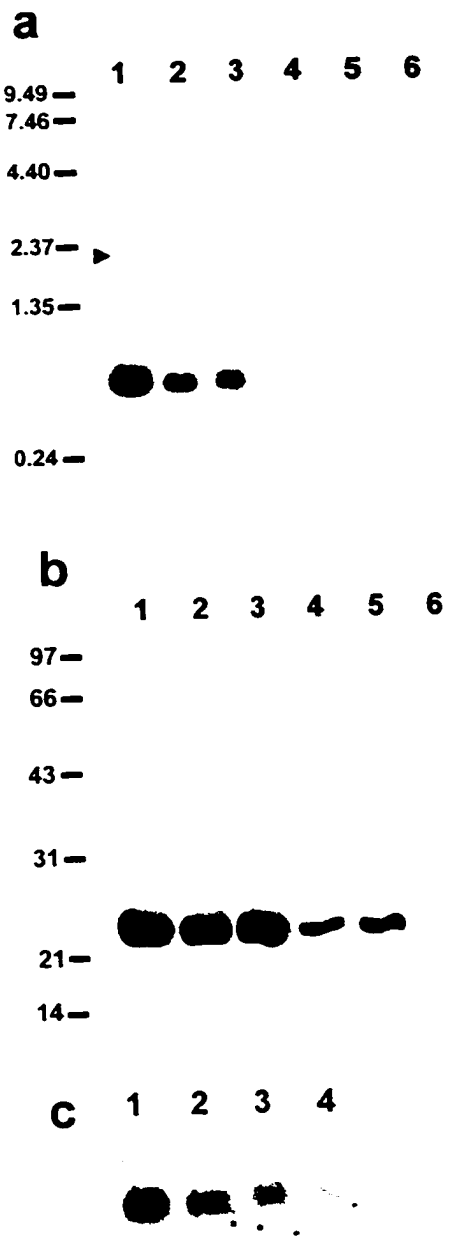


Figure 18.

Figure 19. Immunofluorescent localization of p26 in emerged *Artemia* prenauplii.

Emerged prenauplii were fixed and double stained with DAPI and antibody to p26 as described in "Material and Methods". a and b, prenauplii stained with DAPI; a' and b', the same fields as in a and b respectively, but immunofluorescently stained with anti-p26 antibody. sg, salt gland; g, gut. The bar in a represents 20 μm and all the pictures are at the same magnification.

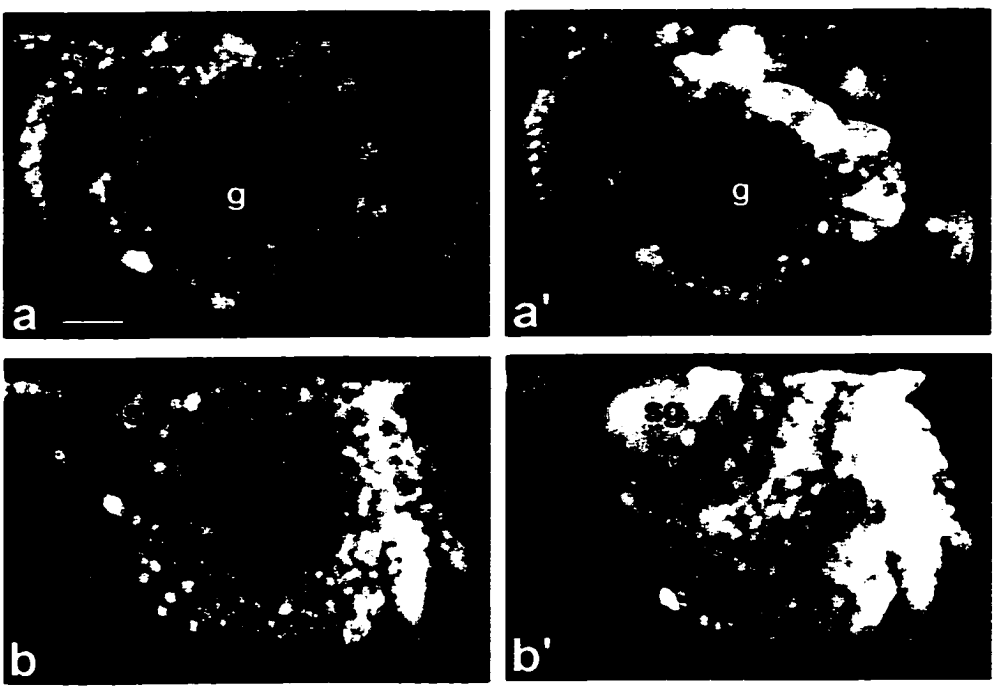


Figure 19.

Figure 20. Immunofluorescent localization of p26 in *Artemia* early larvae.

Artemia larvae at instar I (a, a') and late instar II (b, b', c, c', d, d') were chemically fixed and double stained with DAPI (a to d) and antibody to p26 (a' to d'). Figs. d, d' show an enlarged portion of a salt gland in which corresponding nuclei are labelled with the same number. g, gut; n, nuclei; sg, salt gland. The bar in a represents 100 μm and figures a', b, b', c and c' are the smae magnification. The bar in d represents 15 μm and it is the same magnification as d'.

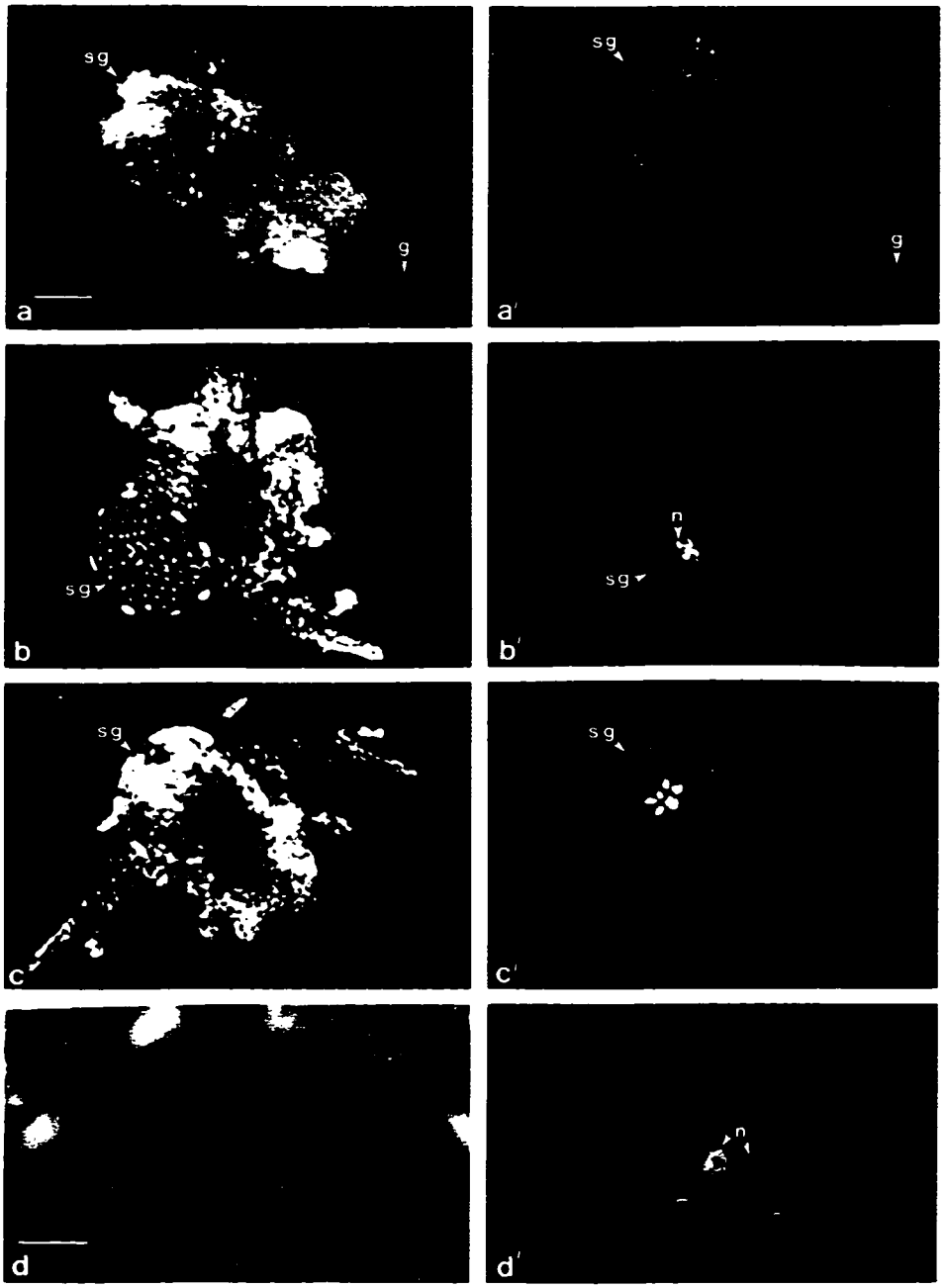


Figure 20.

Figure 21. p26 localizes to a subset of nuclei in the salt glands of instar II larvae.

An isolated salt gland from an instar II *Artemia* larva, stained with antibody to p26 (green) and rhodamine phalloidin (red), was examined by confocal microscopy. The picture is a three-dimensional reconstruction of several serial sections. N, unstained nucleus.

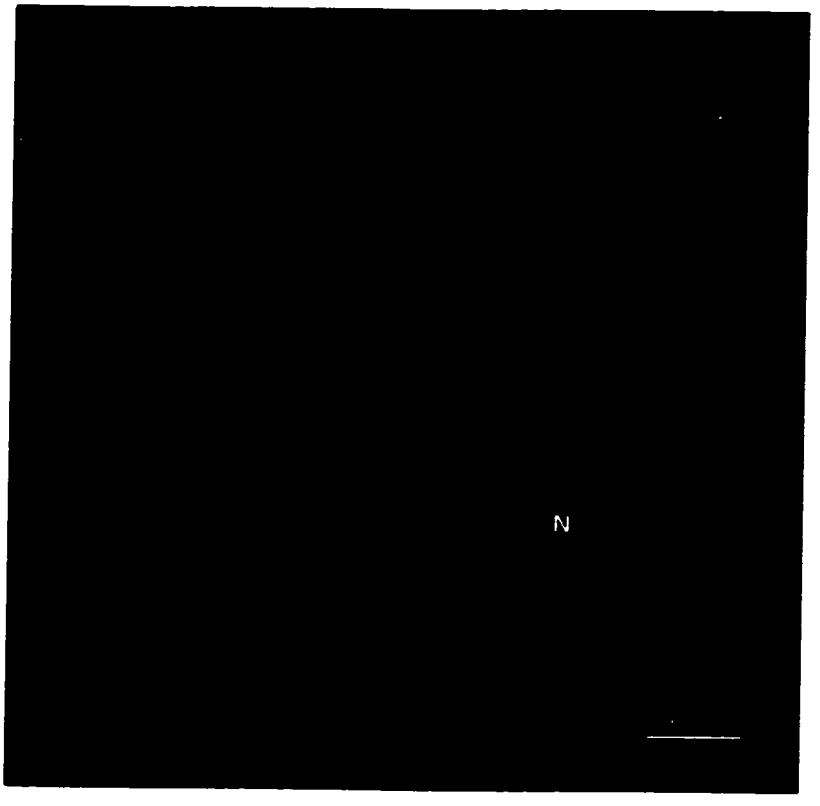


Figure 21.

inserted into a vector for expression in bacteria. As shown in Fig. 22, a fusion protein of approximately 32 kDa (calculated size, 26 kDa), detected by anti-p26 antibody, was synthesized in bacteria carrying the recombinant DNA, pRSETC-p26-3-6-3. When subjected to heat treatment at 53°C, significantly higher numbers ($P<0.01$) of bacteria carrying pRSETC-p26-3-6 survived when compared to bacteria carrying the plasmid pRSETC (Table 3, Fig. 23).

3.5.2 Heat resistance of cyst-derived larvae versus ovoviviparously developed larvae

The availability of two kinds of instar I larvae, direct instar I (ovoviviparous development) with no p26 and cyst-derived instar I (oviparous development) which contain p26, allowed comparison of their ability to resist heat shock. As indicated in Fig. 24, cyst-derived instar I larvae (cyst larvae) were significantly more resistant to heat treatment than were the direct-instar I larvae (direct larvae) ($P<0.01$). The 50% lethal dose of cyst larvae (~41.8°C) was almost 2°C higher than that of direct larvae (~40°C).

3.5.3 Heat shock does not induce the synthesis of p26 in *Artemia*

Although the presence of p26 correlated with the enhanced thermoresistance of *Artemia* larvae, it was not clear if the synthesis of p26 was induced by heat shock. Therefore, instar I and instar III *Artemia* larvae and adults were heat shocked. Cell-free protein extracts from heat-shocked, as well as from nonheat-shocked animals, were analyzed by SDS-PAGE and by immunoprobing of western blots with anti-p26 antibody. While approximately the same amounts of p26 were detected in heat-treated and untreated instar I

Figure 22. Expression of p26 in *E. coli*

P26-3-6-3 was ligated into the expression vector, PRSETC, and recombinant p26 was synthesized by transforming the constructs into the bacterium, BL21(DE3), and culturing the bacteria in LB which contained IPTG. BL21(DE3) carrying PRSETC was used as a control. Panel a, schematic representation of the fusion protein encoded by pRSETC-p26-3-6-3. A, $(His)_6EK$ domain encoded by the plasmid, pRSETC (3.7 kDa); B, a peptide, PRAAGIRHELVLK, encoded by the 5'-noncoding region of p26-3-6-3 and the partial DNA sequence from bluecript SK- (1.5 kDa); C, p26, the peptide of 192 amino acids encoded by the coding region of p26-3-6-3 (20.8 kDa). Panel b. bacterial lysates were analyzed by SDS-PAGE and p26 was immunodetected on western blots. Lane 1, cell-free extract from 0 hr *Artemia* cysts; lanes 2 and 3, cell lysates of BL21 (DE3) carrying respectively PRSETC and p26-3-6-3-PRSETC.

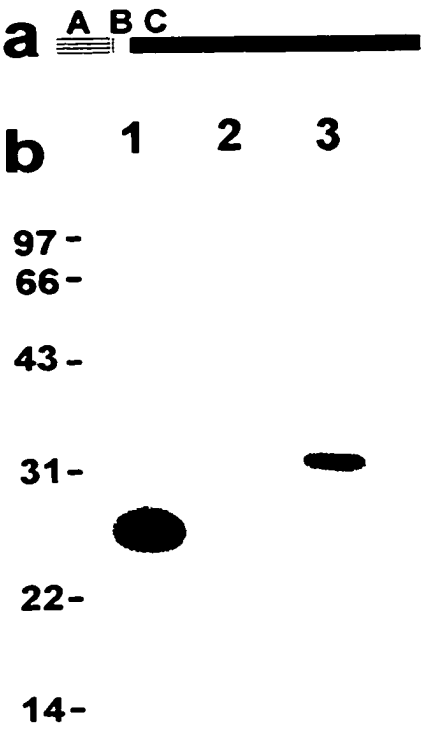


Figure 22

Table 3. Survival of transformed bacteria during heat shock

	Incubation time at 53°C (min)				
	0	15	30	45	60
PRSETC (cfu/μl)	(2.4±0.5) X 10^5	250±50	5±2	0	0
P26-pRSETC (cfu/μl)	(3.0±0.7) X 10^5	4500±500	100±15	8±2	0

The table represents the colony-forming units (cfu) in 1 μl samples of BL21(DE3) carrying either the plasmid, pRSETC, or pRSETC-p26-3-6-3 (p26-pRSETC). The preparation of constructs, bacterial culture, and heat shock treatments were described in "Materials and Methods". The data in this table represent the average of results from three separate experiments.

Figure 23. Heat resistance of *E. coli* expressing recombinant p26

Cultures of BL21(DE3) carrying, either p26-3-6-pRSETC (p26-pRSETC) or the plasmid (pRSETC), were incubated at 53°C for 0, 15, 30, 45 and 60 min, after which samples were plated on LB-agar plates and incubated at 37°C overnight. The number of colonies was recorded for each sample and converted into colony-forming units per μl (cfu/μl) (Table 3). Log_{10} values of cfu/μl of pRSETC and p26-pRSETC were plotted against incubation time at 53°C. No data point was shown for p26-pRSETC at 60 min and for pRSETC at 45 and 60 min because the number of cfu/μl was zero.

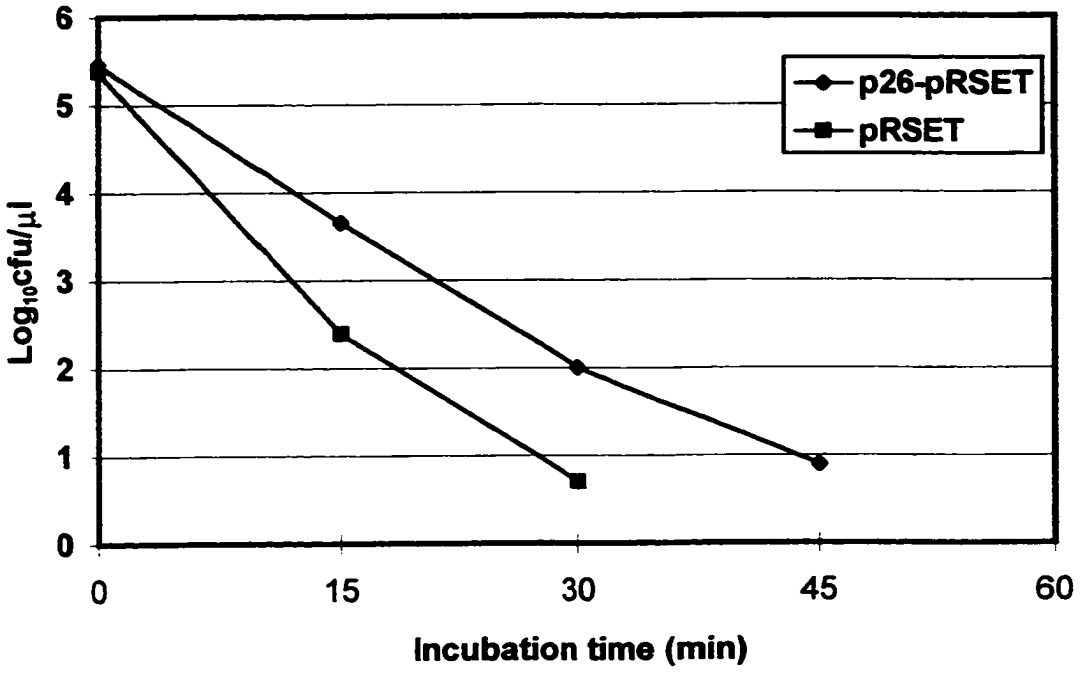

6
5
4
3
2
1
0
Log10cfu/µl
p26-pRSET
pRSET
0
15
30
45
60
Incubation time (min)

Figure 23.

Figure 24. Heat shock resistance of *Artemia* larvae

Instar I larvae which developed directly within females and were released as free-swimming larvae (direct larvae) or those developed from cysts (cyst larvae) were incubated at temperatures ranging from 38.5°C to 42.5°C, at 0.5°C intervals, for 60 min and then cultured at RT for 48 hrs. The percentage of larvae that survived the heat shock in each batch of 40 to 60 larvae was recorded. A minimum of three repetitions was performed at each temperature. The average percentage of surviving direct larvae and cyst larvae were plotted against the incubation temperatures.

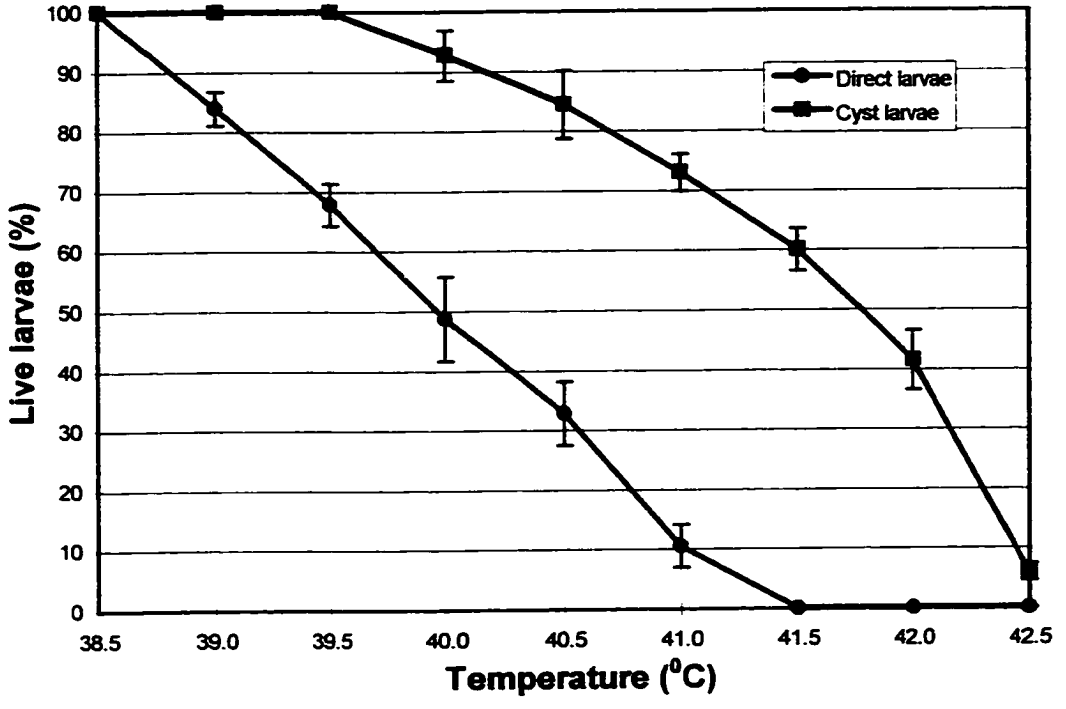

Live larvae (%)
100
90
80
70
60
50
40
30
20
10
0
38.5
39.0
39.5
40.0
40.5
41.0
41.5
42.0
42.5
Temperature (°C)
Direct larvae
Cyst larvae

Figure 24.

larvae, no p26 was detected in samples from instar III larvae and adults, regardless of the heat-treatment (Fig. 25). Heat shock appeared to induce the synthesis of neither p26 nor other similar small heat shock/α-crystallin proteins in *Artemia*.

Figure 25. Synthesis of p26 was not induced in *Artemia* by heat shock

Approximately equivalent amounts of protein from heat shocked and nonheat shocked larvae and adults (excluding ovasacs), were analyzed by in 12.5% SDS-polyacrylamide gels. The gels were either stained by Commassie blue (a) or transferred to nitrocellulose and stained with anti-p26 antibodies using the ECL protocol (b). Lane 1, cell-free extract from 0 hr cysts; lanes 2, 4 and 6, nonheat shocked instar I larvae, instar III larvae and adults, respectively; lanes 3, 5 and 7, heat shocked instar I larvae, instar III larvae and adults, respectively; lane M, molecular mass markers.

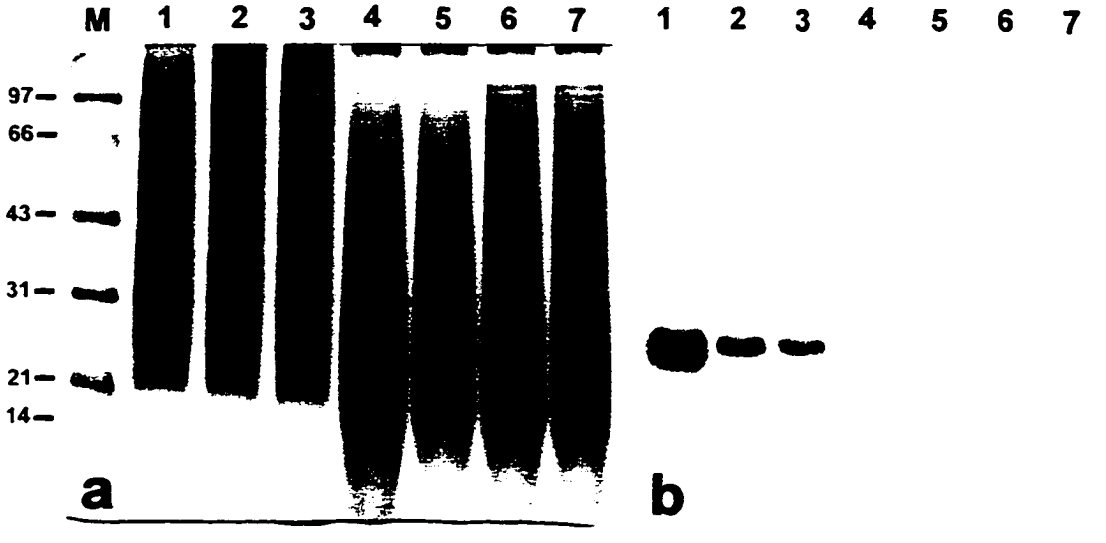


Figure 25.

IV. Discussion

4.1 Purification of p26 from *Artemia* cysts

Previous work suggested that p26, a low molecular mass protein existing in very large amount in *Artemia* encysted embryos, is a small heat shock/α-crystallin protein and that it plays a key role in protecting the macromolecules in these embryos during periods of environmental stress (Clegg *et al.* 1994, 1995; Clegg 1994). The purification of p26 was undertaken to clarify its biochemical characteristics, including oligomer formation, chaperone activity, and its amino acid sequence. A four step-protocol including ion-exchange chromatography, ammonium sulfate precipitation, and immuno-affinity chromatography was established for the preparation of p26 from cell-free extracts of encysted embryos. The use of two ion-exchange columns was based on my unpublished observation that during the purification of tubulin, p26 was retained by Phosphocellulose P11 at pH 6.5, indicating an overall positive charge at this pH (i.e. a pI above pH 6.5). The final contaminating proteins were eliminated by use of a p26 affinity column, and the apparent homogeneity of purified p26 was shown on Commassie blue and silver stained gels. A single step purification using the immuno-affinity column was tried, but the efficiency and purity were not as good as obtained with the four-step protocol (data not shown). One unusual observation during the purification procedure was the appearance of an immunoreactive polypeptide smaller than p26 in samples from Phosphocellulose

P11 which was removed by ammonium-sulfate fractionation. This result suggests proteolytic digestion of p26, perhaps by an enzyme normally sequestered in an inactivated form, but which is activated during purification.

4.2 p26 occurs as multiple isoforms

The existence of multiple isoforms, either as primary gene products or more often from post-translational protein modifications, is a common characteristic of small heat shock/α-crystallin proteins (Arrigo & Landry 1994; de Jong *et al.* 1993). IEF and 2D gel analysis of *Artemia* cell-free extracts indicated numerous isoforms of p26, with the major isoform having a pI of 7.1, a value close to the pH at which translocation of p26 into nuclei is initiated (Clegg *et al.* 1995). A pI of 7.1 or higher is indicative of p26 behavior in the ion-exchange columns employed for its purification. Purified p26 samples also gave a major band on IEF gels at a pH of 7.1 and a major spot on 2D gels, although less isoforms were detected (Data not shown), perhaps resulting from the loss of some isoforms during purification. An unusual result from the western blot of the 2D gels is that two series of spots arranged in families of slightly different size and composition were detected by anti-p26 antibody, while one-dimensional gel analysis of the same samples usually showed only one band. To limit the possibility that the lower-molecular weight isoforms originated from the larger isoforms by proteolysis, the experiments were repeated in the presence of a proteolytic enzyme inhibitor cocktail (Campbell *et al.* 1989), yielding the same result. Also, when tubulin, a protein very sensitive to

proteolytic enzymes (Zhang & MacRae 1992) was subjected to the same treatment, no degradation was observed (data not shown). However, proteolytic degradation to yield the small family of p26 isoforms still cannot be completely excluded.

That at least some of the p26 isoforms are the products of multiple genes is favored by the existence of several p26 genes in *Artemia*. In further support of this proposal, p26 lacks the "Arg-X-X-Ser" motif, which is the phosphorylation site commonly found in α-crystallins, mammalian HSP27 (Landry *et al.* 1992; Arrigo & Landry 1994; de Jong *et al.* 1993) and most other small HSPs from *Drosophila*, nematodes, plants, and bacteria. Coincidentally, several attempts have failed to reveal phosphorylated isoforms (data not shown). Nevertheless, the presence of phosphorylated isoforms and other posttranslational modifications of p26 cannot yet be ruled out. In all likelihood, the major isoform of p26 corresponds to the protein encoded by the cloned cDNA, because the same amino acid sequence was obtained by sequencing purified p26, the cloned cDNA and the PCR product generated from first-strand cDNA.

4.3 Oligomerization of p26

The formation of oligomers is a characteristic of small heat shock/α-crystallin proteins and it seems to be necessary for their chaperone activity. As reported by Leroux *et al.* (1997a), deletion of only approximately one third (15 residues) of the N-terminal domain of HSP16-2 from *C. elegans* dramatically decreases oligomers size as well as its chaperone activity *in vitro*. In agreement

with this, another small HSP from this animal, HSP12.6, which has very short N- and C-terminal regions, does not form large oligomers nor does it have molecular chaperone activity *in vitro* (Leroux *et al.* 1997b).

Previous work (Clegg, 1994) indicated that p26 forms multimers, but because these experiments were done with cell-free extracts, the contribution of other proteins to the oligomers, and thus the number of p26 monomers within a oligomer, were uncertain. Purified p26 was used to examine the native molecular mass. As indicated by both gel filtration chromatography and density gradient centrifugation, purified p26 exists as oligomers, some as large as 700 kDa. This represents about 27 monomers based on a 26 kDa unit molecular mass for p26 as indicated by SDS-PAGE, or 33 monomers based on the calculated molecular mass of 21 kDa. Thus, the oligomer mass of p26 falls within the range expected for a small heat shock/α-crystallin protein. Electron microscopy revealed that p26 multimers are 10-12 nm in size, and that they exhibit a range of morphologies somewhat more complex than that reported for other small heat shock/α-crystallin proteins (Arrigo & Landry 1994; Lee *et al.* 1995). The relationship between the various types of structures I observed is uncertain but the most obvious possibility is that the spherical particles represent end on views of short stacks which, in turn, consist of disks layered one on another. These particles may normally have an opening or depression in one or both ends, demarcated by the accumulation of stain. It is more difficult to understand how the particles with the short projections are related to the other structures

seen in the micrographs and this awaits a more detailed ultrastructural study of the oligomers.

4.4 Cloning and sequencing of p26 cDNA

The cDNA, p26-3-6-3, isolated by screening an *Artemia* cDNA library, contains a single open reading frame that encodes a polypeptide of 192 amino acid residues, beginning with methionine. A termination codon, UAA, follows the final residue and the 3'-noncoding region contains a typical polyadenylation signal of AATAAA and a poly (A) tail. The deduced amino acid sequence from Arg44 to Arg184 agrees exactly with that derived from Edman digestion of purified p26 (Liang *et al.* 1997a). The remainder of the deduced sequence, except for the first two residues, was confirmed by sequencing of selected peptides from BrCN digestion of purified p26 (Liang *et al.* 1997b). The extreme amino-terminal peptide from the BrCN digestion showed the sequence, LNPWYGGFGG, missing the first two residues of the deduced sequence. Because the acylamino acid peptidase used to digest p26 deletes one amino acid from the substrate, it can be concluded that the second deduced residue, alanine, is the first residue in mature p26, while the initiator methionine is cleaved during posttranslational protein processing, a phenomenon often observed for eukaryotic proteins. It can be further concluded, based on peptide analysis of p26 purified during my work and recognition of recombinant p26 by the specific antibody, that the deduced polypeptide corresponds the purified p26 and that the cloned cDNA encodes the complete sequence of p26. The

correspondence between the cDNA, p26-3-6-3, and the purified p26 is also indicated by the fact that antibody to p26 recognizes the recombinant p26.

4.5 Comparison of p26 with other small heat shock/α-crystallin proteins

P26 is a small heat shock/α-crystallin protein as indicated by its sequence similarity to other proteins from this family. Within the phylogenetic tree of this family constructed on the basis of primary protein sequences, p26 locates at the expected position, showing a close evolutionary relationship to the small HSPs from *Drosophila*. Moreover, sequence alignment of p26 with other small heat shock/α-crystallin proteins clearly demonstrates the presence of three domains, a characteristic of all members of this protein family. Residues S61 to R152 constitute the conserved "α-crystallin domain". The N-terminal region, which covers residues M1-G60, has little similarity to the corresponding region in other proteins from this family, an observation also true for the C-terminal extension which comprises residues I153 to A192.

Several unusual aspects of p26 were observed. The N-terminal domain of p26 is enriched in glycine, arginine and phenylalanine, and this region shows a weak similarity to fibrillarin and nucleolin, both of which localize to the nucleus. However, the significance of this similarity, such as its contribution to the cellular localization of p26, remains to be examined (discussed later). As indicated by a comprehensive comparison with all available small heat shock/α-crystallin protein sequences (data not shown), p26 has the longest C-terminal extension among the small heat shock/α-crystallin proteins. Recent data suggest

that the C-terminal extension of small heat shock/α-crystallin proteins is partly responsible for maintaining their solubility in cells and thus is important for chaperone activity (Smulders *et al.* 1996; Leroux *et al.* 1997b). In support of this conclusion, the last 8-10 C-terminal amino acid residues of α-crystallins and HSP25 form flexible extensions, as shown by ^{1}H NMR analysis (Carver *et al.* 1992, 1995). Since differences in chaperone activity exist among small heat shock/α-crystallin proteins (Arrigo & Landry 1994; Sun *et al.* 1997), it is possible that the long C-terminal region endows p26 with an unusually effective protective activity, matching the ability of encysted *Artemia* embryos to resist extreme environmental stress. Therefore it will be very interesting to compare the chaperone activity of p26 with other small heat shock/α-crystallin proteins and examine how the removal and/or modification of the long C-terminal extension by *in vitro* DNA mutagenesis affects its function.

4.6 *Artemia* has multiple p26 genes

Hybridization of the entire p26 cDNA, p26-3-6-3, to *Artemia* genomic DNA digested with BamH I yields two strong and two weak bands, each reacting with a probe corresponding to either the 5' or 3'-end of the cDNA. A similar result was obtained with DNA digested by Hind III (data not shown). In concert with the staining densities of the various bands, these results suggest that *Artemia* has a multi-gene family for p26. The two heavier bands (B1 and B3 in Fig. 11) may represent the opposite halves of at least two copies of very similar or identical p26 genes, which perhaps are arranged in a tandem repeat. The lighter bands

(B2 and B4 in Fig. 11), on the other hand, may be opposite halves of a separate p26 gene. To substantiate these tentative conclusions, further restriction analysis of *Artemia* DNA is required. Additionally, attempts to obtain genome clones encoding p26 may shed light on this question. Moreover, because p26 cDNA lacks a BamH I site, there must be at least one intron with a BamH I recognition site in the middle of the gene. If the total length of introns exceeds 1200 bp, then the weak 1.9 kb band seen on northern blots of mRNA samples from cysts probably represents unprocessed transcripts. In support of this, the larger mRNA is only visible at the developmental stages where the 0.7 kb message is most abundant, when the transcription rate may exceed the splicing rate. Or as discussed by Head *et al.* (1994), processing of mRNA transcripts may be compromised during stress. Differential gene transcription may, therefore, be responsible for some of the p26 isoform heterogeneity seen on 2D gels, while the presence of multiple p26 genes in *Artemia* may partially account for the large amount of p26 in cysts.

Many small heat shock proteins have multiple-gene families. For example, In *C. elegans,* four genes encode two slightly different HSP16s. HSP16-1 and HSP16-48 are a pair of genes duplicated from HSP16-2 and HSP16-41 respectively. The two sets of duplicates are arranged in inverted repeats (Russnak & Candido 1985; Jones *et al.* 1986). HSP27 in human is encoded by a single copy active gene on chromosome 8, but two pseudogenes locate to chromosomes 3 and X respectively (Hickey *et al.* 1986). Although the small HSP

genes from yeast, plants and most invertebrates are intronless, the four small HSP genes, HSP16-1/2 and HSP41/18, in *C. elegans* have one intron, while α-crystallins and HSP27 from mammals have two introns, one of 600-700 bp and another of about 120 bp (Russnak & Candido 1985; Casper *et al.* 1995). The estimated position of the intron in the p26 genes falls within the region corresponding to the position of the intron in the *C. elegans* small HSP genes, and of the first intron in the α-crystallin genes.

4.7 Cellular localization of p26 in encysted *Artemia*

Small heat shock/α-crystallin proteins reside in various cellular locations, including the cytosol, nucleus, mitochondrion, chloroplast and endoplasmic reticulum, and they move between these organelles during times of stress and development (de Jong *et al.* 1993; Groenen *et al.* 1994; Jakob & Buchner 1994). Previous work has shown that large amounts of p26 translocate into nuclei of encysted *Artemia* embryos, greatly increasing the amount of this protein in the organelle, possibly through a pH-dependent mechanism (Clegg *et al.* 1994, 1995). However, it was not clear if p26 is always found in nuclei, and if so, when it enters during encystment and when it disappears during postgastrula development. Moreover, the intranuclear distribution was unknown, which is the case for other small heat shock/α-crystallin proteins. Nuclear localization of p26 was demonstrated in samples of nuclei from cysts that were crushed on slides, and in purified nuclei prepared under either acidic or basic conditions, regardless of the fixation method. During encystment, p26 appears to enter

nuclei upon its synthesis, as indicated by the localization of p26 in the peripheral region of nuclei in day 3 embryos. During postgastrula development, p26 gradually disappears from nuclei until a subset of nuclei in the center of salt glands is its only remaining location. Interestingly, p26 in the nuclei is often located in discrete foci, suggesting its association with specific nuclear components. Cytoplasmic localization of p26 was demonstrated by SDS-PAGE of postnuclear fractions, as well as by immunofluorescent staining of embryos in which cytoplasmic components were stained.

The mechanism for nuclear localization of p26 is uncertain, as sequence analysis fails to reveal a typical nuclear localization signals (NLS). NLSs usually consist of either a short stretch of lysines in a sequence of RKKKRKV or as a bipartite signal wherein a spacer region of 10 to 20 residues separates a pair of basic clusters KRPAATKKAGQAKKK (Jans 1995; Dingwall & Laskey 1991; Gerace 1995). However, the N-terminal region of p26 is enriched in glycine, arginine and phenylalanine, a property not shared by other small heat shock/α-crystallin proteins, but which resembles proteins from the fibrillarin and nucleolin families. Nucleolin, a 76 kDa protein, shuttles between the nucleus and the cytoplasm, and it may transport ribosomal subunits (Ghisolfi *et al.* 1992). Fibrillarins are 40 kDa proteins essential for rRNA processing (Girard *et al.* 1992; Osborne & Silver 1993; Ghisolfi *et al.* 1992). The weak similarity may suggests that p26 localizes to nuclei through a mechanism employed by nucleolin and fibrillarin, a proposal that can best be examined by site-directed

mutagenesis and expression in eukaryotic cells of the p26 cDNA.

4.8 Molecular chaperone activity of p26

Like other small heat shock/α-crystallin protein, p26 has chaperone-like activity *in vitro* (Liang *et al.* 1997a). The addition of p26 results in a twofold increase in the recovery of heat-treated citrate synthase over spontaneous levels, and a 25-fold increase in recovery when citrate synthase is heated after freeze/thaw in the presence versus the absence of p26. Moreover, a protective role for p26 *in vivo* is indicated by the increased thermoresistance of bacteria expressing recombinant p26 to heat shock. The extra peptide at the N-terminus of p26 appears not to affect the chaperone function of p26 as shown by Leroux *et al.* (1997a) for HSP16-2 from *C. elegans*. It is suggested that the N-terminus is located inside the oligomer where there is sufficient capacity to host peptides of various length.

In agreement with the proposal that p26 confers thermoresistance *in vivo*, cyst-derived instar I larvae containing a small amount of p26 are more resistant to heat shock than are the directly developed instar larvae containing no p26. Additionally, cyst-derived instar II nauplii which have lost all of their p26 except for a residual amount in a few salt gland nuclei are no more resistant to heat shock than the direct instar II larvae (data not shown). However, the contribution of other HSPs and organic materials, such as glycerol and trehalose, to the enhanced thermoresistance of cyst-derived instar I larvae cannot be excluded (Drinkwater & Clegg 1991; Clegg & Jackson 1992).

Therefore, the indication of *in vivo* chaperone activity of p26 needs to be further examined by other approaches, such as transfection experiments, protein microinjection and gene disruption.

4.9 Developmental regulation of p26

Many small heat shock/α-crystallin proteins are developmentally regulated, and a few are expressed specifically in embryos. For example, SEC-1, an 18 kDa small HSP from *C. elegans,* is synthesized only in unstressed oocytes and embryos (Linder *et al.* 1996). However, p26 is the only small heat shock/α-crystallin protein yet reported to be diapause-related. By probing of northern and western blots, it was demonstrated that p26 is exclusively expressed in encysted *Artemia* embryos. p26 mRNA appears in embryos two days post-fertilization while protein is synthesized from 3 days post-fertilization, onward. The message and protein reach their highest level before the cysts are released from the female. Once post-gastrula development starts, the amount of message drops quickly and it disappears completely after hatching. The polysomal mRNA for p26 shows developmental patterns similar to that for the main population of mRNA, and p26 protein remains high before hatching, after which it gradually disappears from western blots of cell-free extracts. Therefore, the synthesis of p26 appears to be regulated mainly at the level of transcription, although changes in mRNA stability and degradation cannot be excluded.

Although the presence of p26 in the post-cyst larvae correlates with enhanced thermoresistance, it is not clear if the protein in these larvae has a

function. In other words, is p26 in emerged prenauplii and early larvae simply leftover from cysts or is it still required in animals at these stages? Interestingly, p26 remains in a subset of nuclei in the salt gland of instar II larvae and the number of stained nuclei decreases from 7 in early instar II larvae to 0 in late instar II. The salt gland, also called the neck organ, is a dome-like gland situated atop the cepholothorax of larvae and it is composed of 50 to 60 cuboidal epithelium cells (Hootman & Conte 1975; Criel 1991). It is present in emerged prenauplii but is not found in adults. The salt gland regulates the osmotic balance in larvae until the gut takes over this role (Hootman & Conte 1975; Criel 1991). The prolonged presence of p26 may indicate that these cells are under stress, especially of an osmotic nature.

The cyst-specific synthesis of the p26, and its absence from heat shocked larvae and adults, indicate that p26 gene expression is developmentally regulated and that it is not induced by heat shock. Therefore, p26 genes may lack heat shock elements, but contain other regulatory components, a proposal to be examined by sequencing the upstream sequence of p26 genes. It is noteworthy that *Artemia* cysts have the highest amount of small heat shock/α-crystallin protein of any known organism (Clegg et al. 1994, 1995). Only the vertebrate lens appears to have a similar level of this type of protein, but it represents only a small portion of the whole organism. Therefore, p26 genes must be expressed very efficiently and/or p26 mRNA and protein must have long half-lives. The existence of multiple copies of similar or identical p26 genes

may ensure an efficient synthesis of mRNA, and thus of p26.

The production of diapause *Artemia* embryos entails at least two major discrete physiological events: determination of the reproductive mode in the female and induction of diapause in the embryos. What is the factor(s) or mechanism(s) that controls the switch in development between ovoviviparity and oviparity in females? How is the expression of the p26 genes regulated? What is the factor that induces diapause and when does diapause initiate? Based on our observations, it seems that the fate of embryos within a female is determined before fertilization, with oviparous development and encystment most obviously indicated by the brown color of the shell gland. In other words, the preparation for encystment in females starts before fertilization. A genetic predisposition to a particular mode is excluded, because the same female can alternate between production of nauplii and cysts. Also, a single predominate environmental factor is unlikely to be important, because the two developmental pathways can exist side by side in the same population, although the ratio between nauplii and cyst production varies during the history of one population and between populations. It is possible that the effect of the controlling factor(s) accumulates, rather than works in an on/off mechanism. Indeed, the influence of environmental factors including the oxygen level, population density, photoperiod, salinity, and temperature on the reproductive mode of *Artemia* has been observed (Drinkwater & Clegg 1991). For example, decreasing levels of oxygen that parallel increasing salinities in the natural habitat induce females to

switch to cyst production (Drinkwater & Clegg 1991). In addition, intrinsic factors such as the brood number, which is roughly equivalent to maternal age, also affect the reproductive mode. As Brown (1980) reported, and we observe in the laboratory, female *Artemia* have a much higher tendency to produce nauplii in their first reproductive cycle than in other cycles.

The activation of the p26 gene, is one of the events associated with encystment, as are the formation of shell and accumulation of trehalose (Drinkwater & Clegg 1991), and all are likely to be up-regulated by the factor(s) that triggers oviparous development. The synthesis of large amounts of p26 during encystment brings into question its role in diapause. Although no experiment has been designed to answer this question, the fact that encysted *Artemia* embryos containing large amount of p26 can resume development suggests that p26 does not prevent normal cell activities, thus it is not responsible for cessation of metabolism during diapause.

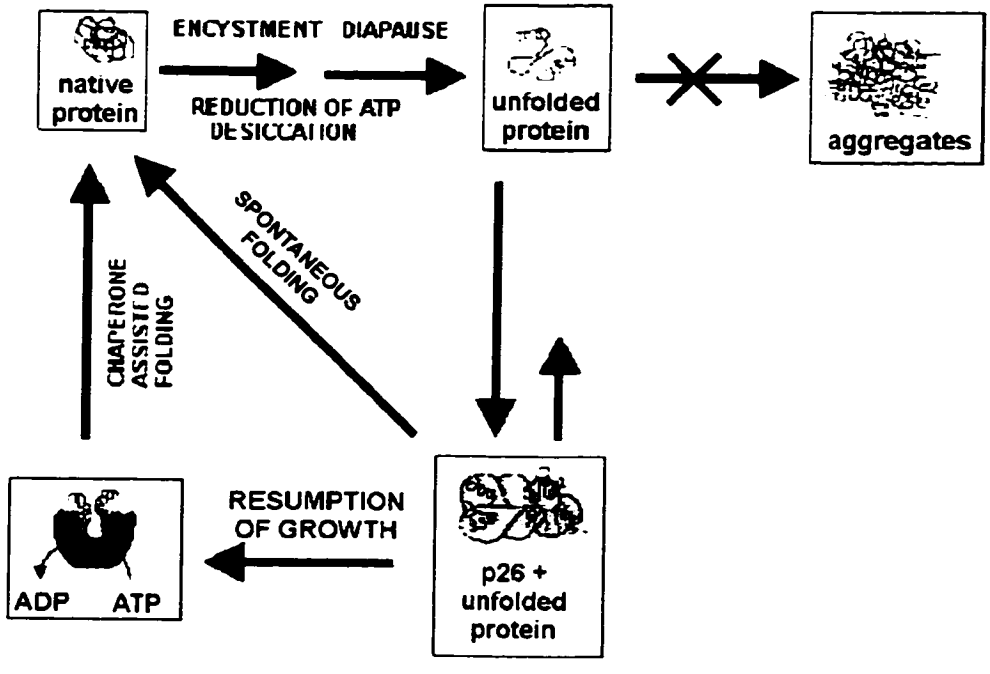
It is interesting that anti-p26 antibodies do not detect small heat shock/α-crystallin proteins at other stages of *Artemia* development examined, even when animals are heat shocked. This suggests that p26 is the only member of this family in *Artemia*, because these antibodies crossreact with a heat inducible protein with approximately the same molecular weight in corals (Brenton, M., M.Sc. thesis, Dalhousie University), although they appear not to react with bovine α-crystallins (data not shown). It was reported that a low molecular mass protein of 31 kD is induced when larvae are heat-treated (McLennan & Miller

1990; Miller & McLennan 1988, 1987), but this protein was not characterized. Other organisms, such as *Drosophila* and *C. elegans*, have several small heat shock proteins, some of which are developmentally regulated and some heat-inducible (Leroux *et al.* 1997a, b; Linder *et al.* 1996; Sirotkin & Davidson 1982; Marin *et al.* 1993; Arrigo 1987; Haas *et al.* 1990; Pauli *et al.* 1988, 1989, 1990).

4.10 Conclusions

p26, an abundant low molecular weight protein in encysted *Artemia* embryos, possesses an α-crystallin domain. It oligomerizes *in vitro*, and it has chaperone-like activity. p26 is interesting because it represents the only characterized small heat shock/α-crystallin protein from a crustacean, and it is the only protein in this family thought to function during diapause. As *Artemia* embryos enter diapause, deplete their ATP reserve and undergo desiccation, a mechanism must exist to protect macromolecules and subcellular components. An equivalent mechanism is required for fully hydrated cysts to survive long term anoxia. p26, a small heat shock/α-crystallin protein synthesized in large amounts in these embryos, is an ideal candidate for this role, either in isolation or as part of a protein team. We propose that p26 binds to proteins and perhaps other macromolecules as they denature under stress, the stress of diapause and desiccation, preventing their irreversible aggregation and/or degradation. This allows spontaneous and/or assisted folding of proteins, permitting rapid resumption of cyst metabolism and growth when conditions are favourable (Fig. 26).

Fig. 26. Proposed function of p26 during *Artemia* encystment



Adapted from Ehrnsperger et al. (1997) EMBO J.16: 221-229.

Appendix I. Solutions and Recipes

Media/solutions for *Artemia* culture and protein preparation

Hatch Medium (6 L)

148.2 g	NaCl
37.6 g	$MgSO_4.7H_2O$
27.7 g	$MgCl_2.6H_2O$
4.20 g	KCl
1.30 g	$CaCl_2.2H_2O$
0.24 g	$NaHCO_3$
6.00 g	Na borate

Pipes

100 mM	Pipes
1 mM	$MgCl_2.6H_2O$
1 mM	EGTA
pH 6.5	with 10 N NaOH

Solutions for SDS-polyacrylamide gel electrophoresis

1A: 39 g acrylamide
1 g Bis
20 ml glycerol
Add dH2O to 100 ml, stir and filter through Miracloth.

1B: 18.3 g Tris
2.5 ml 20% SDS stock solution
Add dH_2O to 100 ml, stir and filter through Miracloth.

1C: 0.1 ml TEMED
Add dH_2O to 50 ml.

1D: 50 mg Ammonium persulfate
Add dH_20 to 10 ml.

2B: 6.0 g Tris
2.5 ml 20 % SDS stock solution
Add dH2O to 100 ml, stir and filter through Miracloth.

2C: 0.2 ml TEMED
Add dH_2O to 10 ml.

Electrode buffer (4 L)

12.0 g	Tris
57.6 g	glycine
8.0 ml	20 % SDS stock solution

pH 8.1 - 8.3 as mixed.

Coomassie blue staining solution (2 L)

10 g	Coommassie Blue R250
140 ml	acetic acid
800 ml	methanol

Stir for 1 hr and filter through miracloth.

Destaining solution (2 L)

400 ml	methanol
140 ml	acetic acid
100 ml	glycerol

Filter through miracloth.

4 X Treatment buffer (20 ml)

0.6 g	Tris
1.6 g	SDS
8.0 ml	glycerol
4.0 ml	2-mercaptoethanol

Adjust pH to 6.8 with HCl.

<u>Isoelectric focusing (IEF) electrophoresis</u>

IEF gel solution

5.5 g	Urea
1.0 ml	Monomer solution (30% acrylamide)
2.0 ml	10% NP-40
2.4 ml	dH_2O
100.0 ml	Ampholyte 3/10
200.0 ml	Ampholyte 5/7
200.0 ml	Ampholyte 6/8
7.0 ml	TEMED
100.0 ml	1% ammonium persulphate

IEF Sample Overlay Solution

2.75 g	Urea
0.05 ml	Ampholyte 3/10
0. 1 ml	Ampholyte 5/7
0.1 ml	Ampholyte 6/8

Add dH_2O to 5 ml

IEF anode solution

2. 04 ml	Phosphoric acid
Add dH_2O to 3 L	

IEF cathode solution

20 ml	1 N NaOH
Add	dH_2O to 1 L

IEF Fixative

4 g	Sulfosalicylic acid
100 ml	TCA

IEF gel staining solution

27 ml	Isopropanol
10 ml	Acetic acid
49 ml	dH_2O
4 ml	1% Coomasie Blue R-250
10 ml	5% $CuSO_4$

IEF gel destaining solution I

120 ml	Isopropanol
70 ml	Acetic acid
5 g	$CuSO_4$
812 ml	dH_2O

IEF destaining solution II

280 ml	Acetic acid
200 ml	Methanol
Add dH_2O to 4 L	

Reagents for the Lowry Method

Solution A:	100 ml	2% Na_2CO_3 stock solution
	1 ml	2% Na K tatrate stock solution
	1 ml	1% cupric sulfate stock solution
	Mix just before use.	
Solution B:	Folin (phenol) reagent diluted 1:2 in dH_2O.	

Western blotting and immunodection

Blot Electrode buffer (4 L)

12.0 g	Tris
57.6 g	glycine
800 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 ml	Tween 20

Add TBS Buffer to 1 L.

HST buffer (1 L)

1.21 g	Tris
58.4 g	NaCl
5.0 ml	Tween 20

Adjust pH to 7.4 with HCl.

2% Ponceau S solution

2.0 g	Ponceau S
30 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

<u>Immunofluorescence staining</u>

PBS buffer (1 L)

8.0 g	NaCl
0.2 g	KCl
1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4

pH 7.4 as mixed.

PBSAT buffer

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

Add PBS Buffer to 50 ml.

Isolation of Nuclei

10X HPC buffer (500 ml)

0.735 g	$CaCl_2.2H_2O$
0.075	PIPES

pH to 6.5 with NaOH. Autoclave.

1X HPC solution

50 ml	10X HPC
32 ml	Hexylene glycol
418 ml	dH_2O

10X NaCl/$MgCl_2$/Tris solution (100 ml)

8.7 g	NaCl
2.8 g	$MgCl_2.6H_2O$
1.9 g	Tris

pH to 6.5 with HCl, autoclave.

Percoll solution

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

Media/solutions for bacteria and phage

LB/2XYT medium (1 L)

10/16 g	Bacto-tryptone
5/10 g	Bacto-yest extract
10/5 g	NaCl

Adjust pH to 7.0 with NaOH, add 1.5% agar for bottom agar, 0.7% agar for top agar. Autoclave.

DNA mini-preparation solutions

Solution I

100 mM	Tris-HCl pH 7.5
10 mM	EDTA
400 ug	heat-treated Rnase I /ml

Solution II

0.2 M	NaOH
1%(w/v)	SDS

Solution III

60 ml	5 M KAoC
11.5 ml	Glacial acetic acid

Add dH_2O to 100 ml

TE

10 mM	Tris-HCl pH 8.0/7.5/4.3
1 mM	EDTA

Solutions for DNA gel electrophoresis and Southern blot

6X DNA loading buffer (type II)

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

5X TBE

108 g	Tris
55 g	Boric acid
40 ml	0.5 M EDTA (pH 8.0)

Add dH_2O to 1 L. Use 0.5X for working solution

50X TAE

242 g	Tris
57.1 ml	Glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

Add dH_2O to 1 L.

Denaturing solution (per liter)

87.66 g	NaCl
20 g	NaOH

Neutralizing solution (per liter)

87.66 g	NaCl
60.55 g	Tris
0.372 g	EDTA

Adjust pH to 7.2 with HCl

20X SSC (1 L)

88.2 g	Trisodium citrate
176.3 g	NaCl

Adjust pH to 7.2 with HCl.

DNA pre-hybridization solution

6.25 ml	20X SSC
2.26 ml	Denhardt's solution
2.27 ml	20% SDS (w/v)

Add dH_2O to 25 ml

Solutions for RNA preparation and Northern blot

10X MOPs

0.2 M	MOPs (3-N-morpholino propanessulfonic acid)
50 mM	NaAoC
10 mM	EDTA

Adjust pH to 7.2 with NaOH, Autoclave. Use DEPC treated dH_2O for dilution.

5 X RNA loading buffer

50%	Glycerol
1 mM	Disodium EDTA
0.4%	Bromophenol blue
0.4%	Xylene cynaol

Use DEPC treated dH_2O

Solutions for expression of p26 in bacteria

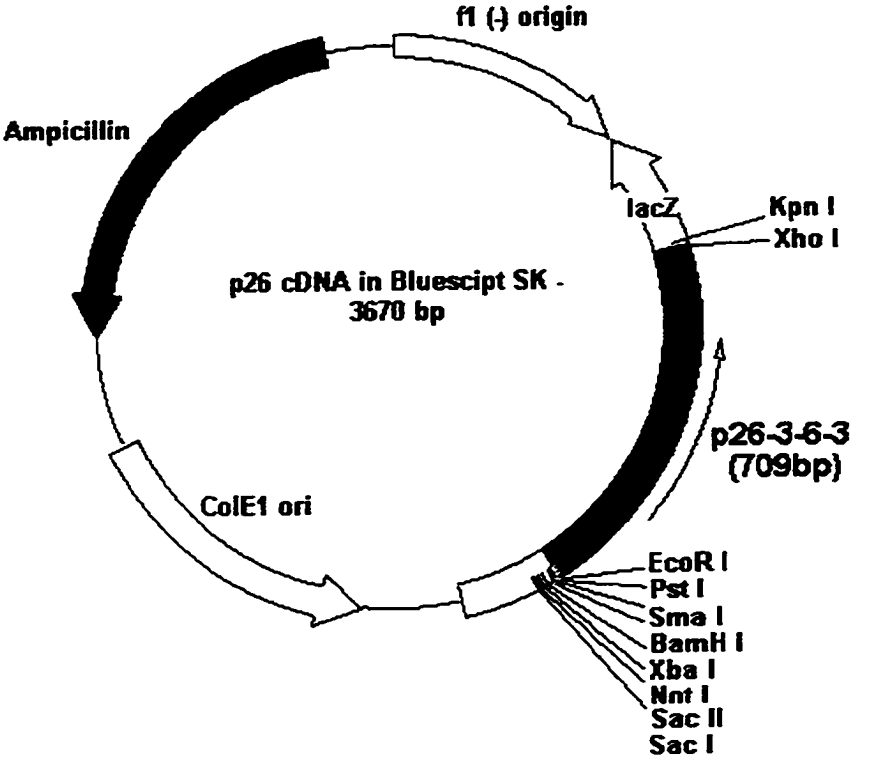
Denaturing binding buffer:

8 M Urea
200 mM Sodium phosphate, pH 7.8
500 mM Sodium chloride

Appendix II. Protocol for conversion the *Artemia* cDNA library in λZAP II into a library in Bluescript SK-

A preparation of XL1-blue SF' cells was made by inoculating 50 ml of 2X YT medium supplemented with 0.2 % maltose with a single fresh XL1-blue colony, and culture it at 37°C overnight. The cells was pelleted by centrifugation at 2000 g for 10 min, and resuspended in appropriate volume of 10 mM Mg_2SO_4 such that the OD_{600} reading was around 1.0. 0.2 ml of above XL1-blue suspension was mixed with 0.2 ml of the diluted *Artemia* 0 hr cDNA library in λZAP II (1.0 X 10^7 pfu/ml, provided by Dr. L. Sastre, Instituto Investigaciones Biomedicas, Madrid, Spain) and 10 μl of R408 helper phage. The mixture was incubated at 37°C for 15 min after which 2.0 ml of 2 X YT was added and incubated at 37°C for additional 6 hrs followed by an incubation at 70°C for 20 min and centrifugation at 4000 g for 5 min. The supernatant containing the bluescript plasmids which were packaged as f1 phage particles was transferred into a fresh tube and stored at 4°C. 0.2 ml of the supernatant was mixed with 2.0 ml of XL1-blue cells as described above (the ratio between the phage particle and bacteria was optimized for this specific preparation) and the mixture was incubated at 37°C for 30 min after which 2.0 ml of 2 X YT and 500 mg of ampicillin were added and cultured overnight. The resulted library which was in bluescript SK- was tittered and either freshly plated on ampicillin media for library screening or stored at -70°C for later use.

Appendix III. Schematic representation of p26 cDNA clone



Appendix IV. Properties of p26 cDNA and protein

1. Restriction map of p26-3-6-3

A. List of enzymes (N>=6) by name

Mame	Cut #	Specificity (5'-3')
AccIII	1	T/CCGGA 134
AhaIII	1	TTT/AAA 179
AlwNI	1	CAGNNN/CTG 397
BalI	1	TGG/CCA 248
BbvI	1	GCAGCNNNNNNNN/ 604
BsiI	1	C/TCGTG 10
BsmI	1	GAATGCN/ 604
BspMII	1	T/CCGGA 134
Bst71I	1	GCAGCNNNNNNNN/ 604
DraI	1	TTT/AAA 179
Eam1105I	1	GACNNN/NNGTC 69
Ecl136II	1	GAG/CTC 9
EcoICRI	1	GAG/CTC 9
HphI	1	GGTGANNNNNNNN/ 455
MboII	3	GAAGANNNNNNNN/ 152 164 619
MscI	1	TGG/CCA 248
NcoI	3	C/CATGG 34 67 303
PflMI	1	CCANNNN/NTGG 505
PleI	1	GAGTCNNNN/ 365
PvuII	2	CAG/CTG 217 502
SacI	1	GAGCT/C 11
SstI	1	GAGCT/C 11
Tth111II	1	CAARCANNNNNNNNNN/ 325

B. List by site order

9	Ecl136II	69	Eam1105I	217	PvuII	455	HphI
9	EcoICRI	134	BspMII	248	BalI	502	PvuII
10	BsiI	134	AccIII	248	MscI	505	PflMI
11	SacI	152	MboII	303	NcoI	604	BbvI
11	SstI	164	MboII	325	Tth111II	604	Bst71I
34	NcoI	179	DraI	365	PleI	604	BsmI
67	NcoI	179	AhaIII	397	AlwNI	619	MboII

C. Non cut enzymes

AatII	Acc65I	AclI	AflII	AgeI	Alw26I
Alw44I	ApaBI	ApaI	ApaLI	AscI	Asp718I
AsuII	AvrII	BamHI	BbeI	BbvII	BclI
BglI	BglII	Bpu1102I	Bsc91I	Bsp1407I	BspHI
BspMI	BssHII	BstD102I	BstEII	BstXI	Bsu36I
ClaI	Csp45I	CspI	CvnI	DraIII	DrdI
EagI	Eco31I	Eco47III	Eco52I	Eco56I	Eco57I
Eco72I	EcoNI	EcoRI	EcoRV	EheI	EspI
FokI	FseI	HgaI	HindIII	HpaI	I-PpoI
KpnI	MfeI	Mlu113I	MluI	MstI	MstII
NaeI	NarI	NdeI	NheI	NotI	NruI
NsiI	PacI	PinAI	PmaCI	PmeI	PstI
PvuI	RleAI	SacII	SalI	SapI	SauI
ScaI	SciI	SfaNI	SfiI	SgrAI	SmaI
SnaBI	SpeI	SphI	SplI	SpoI	SrfI
SspI	SstII	StuI	SunI	SwaI	Tth111I
VspI	XbaI	XcmI	XhoI	XmaI	XmaIII
XmnI	XorII				

D. Codon usage of p26-3-6-3

AA	Codon	Usage (%)	AA	Codon	Usage(%)	AA	Codon	Usage(%)
F	UUU	75	V	GUU	30	Y	UAU	13
	UUC	25		GUC	40		UAC	67
				GUA	20			
L	UUA	42		GUG	10	P	CCU	7
	UUG	17					CCC	13
	CUU	25	S	UCU	27		CCA	73
	CUC	8		UCC	7		CCG	7
	CUA	8		UCA	40			
	CUG	0		UCG	0	A	GCU	67
				AGU	13		GCC	0
I	AUU	50		AGC	13		GCA	25
	AUC	38		GCG	8			
	AUA	13	ST	UAA	100			
				UGA	0	H	CAU	67
R	CGU	6		UAG	0		CAC	33
	CGC	0						
	CGA	24	T	ACU	43	Q	CAA	67
	CGG	6		ACC	10		CAG	33
	AGA	41		ACA	48			
	AGG	24		ACG	0	N	AAU	0
							AAC	100
E	GAA	80	K	AAA	60			
	GAG	20		AAG	40	D	GAU	60
							GAC	40
W	UGG	100	M	AUG	100			

2. Amino acid composition of p26

AA	Count	% By #	% By Wt
Ala A	12	6.25%	5.14%
Xxx X	0	0.00%	0.00%
Arg R	18	9.38%	15.07%
Asn N	2	1.04%	1.27%
Asx B	0	0.00%	0.00%
Asp D	15	7.81%	9.59%
Cys C	0	0.00%	0.00%
Glu E	10	5.21%	7.07%
Gln Q	3	1.56%	2.11%
Glx Z	0	0.00%	0.00%
Gly G	23	11.98%	8.30%
His H	6	3.12%	4.47%
Ile I	7	3.65%	4.41%
Leu L	12	6.25%	7.56%
Lys K	5	2.60%	3.51%
*** *	0	0.00%	0.00%
Met M	5	2.60%	3.58%
Phe F	8	4.17%	6.35%
Pro P	15	7.81%	8.30%
Ser S	15	7.81%	7.57%
Amb @	0	0.00%	0.00%
Och #	0	0.00%	0.00%
Umb &	0	0.00%	0.00%
Thr T	21	10.94%	12.02%
Trp W	2	1.04%	1.96%
Tyr Y	3	1.56%	2.61%
Val V	10	5.21%	5.63%

Acidic = 31 Basic = 26
Non-Polar = 94 Polar = 98
MW = 20813.8
From 1 to 192, total = 192

Appendix V. NCBI GenBank record for p26-3-6-3

```
LOCUS       AF031367      709 bp    mRNA            INV       01-DEC-1997
DEFINITION  Artemia franciscana small heat shock/alpha-crystallin protein
            precursor (p26-3-6-3) mRNA, complete cds.
ACCESSION   AF031367
KEYWORDS    .
SOURCE      Artemia franciscana.
  ORGANISM  Artemia franciscana
            Eukaryotae; Metazoa; Arthropoda; Crustacea; Branchiopoda;
            Sarsostraca; Anostraca; Artemiidae; Artemia.
REFERENCE   1  (bases 1 to 709)
  AUTHORS   Liang,P., Amons,R., Macrae,T.H. and Clegg,J.S.
  TITLE     Purification, structure and in vitro molecular-chaperone activity
            of Artemia p26, a small heat-shock/alpha-crystallin protein
  JOURNAL   Eur. J. Biochem. 243 (1-2), 225-232 (1997)
  MEDLINE   97182604
REFERENCE   2  (bases 1 to 709)
  AUTHORS   Liang,P., Amons,R., Clegg,J.S. and MacRae,T.H.
  TITLE     Molecular characterization of a small heat shock/alpha-crystallin
            protein in encysted Artemia embryos
  JOURNAL   J. Biol. Chem. 272 (30), 19051-19058 (1997)
  MEDLINE   97373614
REFERENCE   3  (bases 1 to 709)
  AUTHORS   Liang,P., MacRae,T.H., Amons,R. and Clegg,J.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-OCT-1997) Biology, Dalhousie University, Halifax,
            NS, B3H 4J1, Canada
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                     /dev_stage="embryos (encysted)"
     gene            1..709
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     CDS             22..600
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                     /codon_start=1
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                      DEYGHVQREFRRRYRLPEHVKPESVSSTLSSDGVLTIHAPKTALSSPTERIVPITPAP
                      AVGRIEGGTTGTTTGSTASSTPARTTRSGGAA"

BASE COUNT      222 a    150 c    157 g    180 t
ORIGIN
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       61 actgacccat ggtctgatcc atttggattt ggtggcttcg gaggtggcat ggaccttgat
      121 attgacaggc ccttccggag aagaatgatg agaagaggtc cagataccag cagggcttta
      181 aaggagttag ctactcctgg gtccttgagg gacacagctg atgaatttca agttcagcta
      241 gatgttggcc acttttacc aaacgaaatt acagtcaaga caaccgacga tgatattctt
      301 gtccatggca aacatgacga gcgatctgat gaatatggac acgtacaaag agaatttcga
      361 cgacgataca gactcccaga acatgtcaaa ccagaatctg tgtcatctac tttgtcatca
      421 gatggtgtct taactatcca tgctccgaaa actgctttaa gctcaccaac agaacgtatc
      481 gtacccatca caccagcgcc agctgttgga aggattgaag ggggaactac aggtactact
      541 acaggcagta cagctagttc aactccagca agaacaacaa gatcaggagg tgcagcttaa
      601 tctgcattca atatatattc aatgaatctt ccggtctttt ttctttgtac ctttatttt
      661 ttgtcaataa atctgtatac gaggcaaaaa aaaaaaaaaa aaaaaaaaa
//
```

References

Adnan, A., P. H. Krone, and J. J. Heikkila. 1993. Expression of endogenous and microinjected hsp 30 genes in early *Xenopus laevis* embryos. Dev. Gen. 14, 42-50.

Allen, S. P., J. O. Polazzi, J. K. Gierse, and A. M. Easton. 1992. Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. J. Bacteriol. 174, 6938-1947.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Andley, U. P., S. Mathur, T. A. Griest, and J. M. Petrash. 1996. Cloning, expression, and chaperone-like activity of human αA-crystallin. J. Biol. Chem. 271, 31973-31980.

Aoyama, A., E. Fröhli, R. Schafer, and R. Klemenz. 1993. αB-crystallin expression in mouse NIH 3T3 fibroblasts: Glucocorticoid responsiveness and involvement in the thermal protection. Mol. Cell Biol. 13, 1824-1835.

Arrigo, A.-P. 1987. Cellular localization of HSP23 during *Drosophila* development and following subsequent heat shock. Dev. Biol. 122,39-48.

Arrigo, A.-P. 1990. The monovalent ionophore monesin maitains the nuclear localization of the human stress protein hsp28 during heat shock recovery. Dev. Biol. 122, 39-48.

Arrigo, A.-P. and J. Landry. 1994. Expression and function of the low-molecular-weight heat shock proteins. In *The biology of heat shock proteins and molecular chaperones*. Morimoto, R. I., A. Tissières, and C. Georgoupoulos (eds.). pp. 335-374. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Arrigo, A.-P. and D. Pauli. 1988. Charaterization of hsp27 and three immunologically related polypeptides during *Drosophila* development. Exp. Cell Res. 175,169-183.

Arrigo, A.-P. and W. J. Welch. 1987. Characterization and purification of the small 28,000-dalton mammalian heat shock protein. J. Biol. Chem. 262, 15359-15369.

Arrigo, A.-P. and R.M. Tanguay. 1991. Expression of heat shock proteins during

development in *Drosophila*. In *Heat Shock and Development*. Hightower, L. and L. Nover (eds). pp106-306. Springer-Verlag, Berlin.

Arrigo, A.-P., J. Suhan, and W. J. Welch. 1988. Dynamic changes in the structure and locale of the mammalian low molecular weight heat shock protein. Mol. Cell. Biol. 8, 5059-5071.

Atkinson, B. G., M. Raizada, R. A. Bouchard, J. R. H. Frappier, and D. B. Walden. 1993. The independent stage-specific expression of the 18-kDa heat shock protein genes during microsporogenesis in *Zea mays* L. Dev. Gen. 14,15-26.

Augusteyn, R.C. and J. F. Koretz. 1987. A possible structure for α-crystallin. FEBS Lett. 222, 1-5.

Ayme, A. and A. Tissières. 1985. Locus 67B of *Drosophila melanogaster* contains seven, not four, closely related heat shock genes. EMBO J. 4, 2949-2954.

Bagshaw, J. C. and A. H. Warner. 1979. Biochemistry of *Artemia* development. University Microfilms International, Ann Arbor, Michigan.

Beaulieu, J. F., A.-P. Arrigo, and R.M. Tanguay. 1989. Interaction of *Drosophila* 27Kd heat shock protein with the nucleus of heat-shocked and ecdysterone-stimulated cultured cells. J. Cell Sci. 92, 29-36.

Beckmann, R. P., L. A. Mizzen, W. J. Welch. 1990. Interaction of Hsp70 with newly synthesized proteins: implication for protein folding and assembly. Science 248, 850-854.

Benndorf, R., K. Hayess, S. Ryazantsev, M. Wieske, J. Behlke, and G. Lutsch. 1994. Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. J. Biol. Chem. 269, 20780-20784.

Bhat, S. P. and C. N. Nagineni. 1989. αB subunit of lens-specific protein a-crystallins present in other ocular and non-ocular tissues. Biochem. Biophys. Res. Commun. 158, 319-325.

Bindels, J. G., R. J. Siezen, and H.J. Hoenders. 1979. A model for the architecture of α-crystallin. Ophthalmic Res. 11, 441-452.

Blond-Elguindi, S., S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Sprang, J. F. Sambrook, M.-J. H. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of Bip. Cell 75,

717-728.

Bohen, S. P., A. Kralli, and K. R. Yamamoto. 1995. Hold'em and Fold'em: chaperones and signal transduction. Science 268, 1303-1304.

Boston, R. S., P. V. Viitanen, and E. Virling. 1996. Molecular chaperone and protein folding in plants. Plant Mol. Biol. 32, 191-222.

Brodsky, J. L. 1996. Post-translational protein translocation: not all hsc70s are created equal. Trends Biochem. Sci. 21, 122-126.

Browne, R. A. 1980. Reproductive pattern and mode in the brine shrimp. Ecology 61, 466-477.

Campbell, J. C., S. A. MacKinlay, and T. H. MacRae. 1989. Crosslinking of microtubule-associated proteins (MAPs) from the brine shrimp, *Artemia*. J. Cell Sci. 93, 29-39.

Carver, J.A., J. A. Aquilina, R. J. Truscott and G. B. Ralston. 1992. Identification by ^{1}H NMR spectroscopy of flexible C-terminal extenstions in bovine lens α-crystallin. FEBS Lett. 311, 143-149.

Carver, J.A., G. Esposito, G. Schwedersky, and M. Gaestel. 1995. ^{1}H NMR spectroscopy reveals that mouse hsp25 has a flexible C-terminal extension of 18 amino acids. FEBS Lett. 369, 305-310.

Caspers, G-J, J. A. M. Leunissen, and W. W. de Jong. 1995. The expanding small heat-shock protein family, and structure predictions of the conserved "α-crystallin domain". J. Mol. Evol. 40, 238-248.

Chang, Z., T. P. Primm, J. Jakana, I. H. Lee, I. Serysheva, H.F. Gilbert, and F.A. Quiocho. 1996. *Myobacterium tuberculosis* 16-kDa antigen (Hsp16.3) functions as an oligomeric structure *in vitro* to supress thermal aggregation. J. Biol. Chem. 271, 7218-7223.

Chernoff, Y. O., S. L. Lindquest, B. Ono, S. G. Inge-Vechtomov, and S. W. Liebman. 1995. Role of the chaperone protein 104 in propagation of the yeast prion-like factor [*psi+*]. Science 268, 880-884.

Chiesa, R., M. A. Gawinowwicz-Kolks, N. J. Kleiman, and A. Spector. 1987. The phosphrylation sites of the B2 chain of bovine α-crystallin. Biochem. Biophys. Res. Commun. 144, 1340-1347.

Chrétien, P. and J. Landry. 1988. Enhanced constitutive expression of the 27-kDa heat shock proteins in heat-resistant variants from Chinese hamster

cells. J. Cell Physiol. 137, 157-166.

Clegg, J. S. 1997. Embryos of Artemia franciscana survive four years of continuous anoxia: the case for complete metabolic rate depression. J. Exp. Biol. 200, 467-475.

Clegg, J. S. 1994. Unusual response of *Artemia franciscana* embryos to prolonged anoxia. J. Expl. Zool. 270, 332-334.

Clegg, J. S. and S. A. Jackson. 1992. Aerobic heat shock activates trehalose synthesis in embryos of *Artemia franciscana*. FEBS Lett. 303, 45-47.

Clegg, J.S. and F. P. Conte. 1980. A review of the cellular & developmental biology of *Artemia*. In *Brine Shrimp Artemia. Physiology, Biochemistry, Molecular Biology*, Vol. 2. Persoone, G., Sorgeloos, P., Roels, O., and Jaspers, E. (eds.) pp 11-54. Universa Press, Wetteren, Belgium.

Clegg, J.S., S.A. Jackson, and A.H. Warner. 1994. Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*. Exp. Cell Res. 212, 77-83.

Clegg, J.S., S. A. Jackson, P. Liang, and T. H. MacRae. 1995. Nuclear-cytoplasmic translocations of protein p26 during aerobic-anoxic transitions in embryos of *Artemia franciscana*. Exp. Cell Res. 219, 1-7.

Clutter, M. E. 1978. (Eds.) *Dormancy and Developmental Arrest*. Academic press, New York, NY.

Coca, M.A., C. Almoguera, and J. Jordano. 1994. Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant. Mol. Biol. 25, 479-492.

Collier, N. C. and M. Schlesinger. 1986. The dynamic state of heat shock proteins in chicken embryo fibroblasts. J. Cell Biol. 103, 1495-1507.

Crabbe, J. C. and D. Goode. 1994. α-Crystallin: chaperoning and aggregation. Biochem. J. 297, 653-654.

Craig, E. A., J. S. Weissman, and A. L. Horwich. 1994. Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell 78, 365-372.

Creighton, T.E. 1997. How important is the molten globule for correct protein folding? Trends Biochem. Sci. 22, 6-11.

Criel, G. L. 1991. Ontogeny of *Artemia*. In *Artemia Biology*, Browne, R.A., Sorgeloos, P., and Trotman, C.N.A. (eds.) pp 155-185. CRC Press, Boca Raton, FL.

Dasgupta, S., T. C. Honman, and D. Carper. 1992. Hypertonic stress induces αB-crystallin expression. Exp. Cell Res. 54, 461-470.

de Jong, W.W., J.A.M. Leunissen, and C.E.M. Voorter. 1993. Evolution of the alpha-crystallin/small heat-shock protein family. Mol. Biol. Evol. 10, 103-126.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. A. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332, 800-805.

Dingwall, C. and R. A. Laskey. 1991. Nuclear targeting sequences--a consensus? Trends Biochem. Sci. 16, 478-481.

Drinkwater, L. E. and J. H. Crowe. 1987. Regulation of embryonic diapause in *Artemia:* Environmental and physiological signals. J. Exp. Zool. 241, 297-307.

Drinkwater, L.E., and J.S. Clegg. 1991. Experimental biology of cyst diapause. In: *Artemia Biology*, Browne, R.A., Sorgeloos, P., and C.N.A. Trotman (eds.). pp 93-117. CRC Press, Boca Raton, Florida.

Dubin, R. A., and J. Piatigorsky. 1989. Expression of the murine alphaB-crystallin gene is not restricted to the lens. Mol. Cell. Biol. 9, 1083- 1091.

Dubin, R. A., R. Gopal-Srivastava, E.F. Wawrousek, and J. Piatigorsky. 1991. Expression of the murine αB-crystallin gene in lens and skeletal muscle: identification of a muscle-preferred enhancer. Mol. Cell. Biol. 11, 4340-4349.

Ehrnsperger, M., S. Gräber, M. Gaetel and J. Buchner. 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J. 16, 221-229.

Ellis, R. J. 1987. Proteins as molecular chaperones. Nature 328, 378-379.

Ellis, R. J. and S. M. van der Vies. 1991. Molecular chaperones. Annu. Rev. Biochem. 60, 321-347.

Fell, P. E. 1995. Deep diapause and the influence of low temperature on

hatching of gemonules of *Spongilla lacustris* and *Eunapius fragilis*. Invert. Biol. 114, 3-8.

Fernandes, M., T. O'brien, and J. T. Lis. 1994. Structure and regulation of heat shock gene promoters. In: *The biology of heat shock proteins and molecular chaperones*. R. I. Morimoto, A. Tissières, and C. Georgoupoulos (eds.) pp. 375-393. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Fenton, W.A., J. S. Weissman, A. L. Horwich. 1996. Putting a lid on protein folding: structure and function of the co-chaperonin, GroES. Chem. Biol. 3, 157-161.

Fitch, W. M. and E. Margoliash. 1967. Construction of phylogenetic tree. Science 155, 279-284.

Flaherty, K. M., C. DeLuca-Flaherty, and D. B. McKay. 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. Nature 346, 623-628.

Flynn, G.C., C.J. Beckers, W. A. Baase, and F. W. Dahlquist. 1993. Individual subunits of bacterial luciferase are molten globules and interact with molecular chaperones. Proc. Natl. Acad. Sci. 90. 10826-30.

Frydman, J. and F. -U. Hartl. 1996. Principles of chaperone-assisted protein folding: differences between *in vitro* and *in vivo* mechanisms. Science 272, 1497 - 1502.

Frydman, J. and J. Höhfeild. 1997. Chaperone get in touch: the Hip-Hop connection. Trends Biochem. Sci. 22, 87-92.

Frydman, J., E. Nimmesgern, K. Ohtsuka, and F.-U. Hartl. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature 370, 111-117.

Gaestel, M., R. Gotthardt, and T. Muller. 1993. Structure and organization of a murine gene encoding small heat shock protein HSP25. Gene 128, 279-283.

Gaestel, M., W. Schröder, R. Benndorf, C. Lippman, K. Buchner, F. Huchot, V. A. Ermann, and H. Bielka. 1991. Identification of the phosphorylation sites of the murine small heat shock protein 25. J. Biol. Chem. 266, 14721-14727.

Georgopoulos, C. and W. J. Welch. 1993. Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. 9, 601-634.

Gerace, L. 1995. Nuclear export signals and the fast track to the cytoplasm. Cell 82, 341-4.

Gernold, M., U. Knuf, M. Gaestel, J. Stahl, and P.-M. Kloetzel. 1993. Development and tissue-specific distribution of mouse small heat shock protein hsp25. Dev. Gen. 14,103-111.

Ghisolfi, L., G. Joseph, F. Amalric, and M. Erard. 1992. The glycine-rich domain of nucleolin has an unusual supersecondary structure responsible for its RNA-helix-destabilizing properties. J. Biol. Chem. 15, 267, 2955-2959.

Girard, J. P., H. Lehtonen, M. Caizergues-Ferrer, F. Amalric, D. Tollervey, and B. Lapeyre. 1992. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. EMBO J. 11, 673-82.

Go, E. C., A. S. Pandey, and T. H. MacRae. 1990. Effect of inorganic mercury on the emergence and hatching of the brine shrimp *Artemia franciscana.* Mar. Biol. 107,93-102.

Gopal-Srivastava, R. and J. Piatigorsky. 1993. The murine αB-crystallin/small heat shock protein enhancer: Identification of αBE-1, αBE-2, αBE-3, and MRF control elements. Mol. Cell. Biol. 13, 7144-7152.

Gopal-Srivastava, R. and J. Piatigorsky. 1994. Identification of a lens-specific regulatory region (LSR) of the murine αB-crystallin gene. Nucleic Acids Res. 22, 1281-1286.

Gopal-Srivastava, R., J. I. Hayners II, and J. Piatigorsky. 1995. Regulation of the murine αB-crystallin/small heat shock protein gene in cardiac muscle. Mol. Cell. Biol. 15, 7081-7090.

Gopal-Srivastava, R., A. Cvekl, and J. Piatigorsky. 1996. Pax-6 and αB-crystallin/small heat shock protein gene regulation in the murine lens, interaction with the len-specific regions, LSR1 and LSR2. J. Biol. Chem. 271, 23029-23036.

Grimm, B., D. Ish-Shalom, D. Even, H. Glaczinski, P. Ottersbach, K. Kloppstech, and I. Ohad. 1989. The nuclear-coded chloroplast 22 kDa heat-shock protein of *Chlamydomonas.* Eur. J. Biochem. 182, 539-546.

Groenen, P.J.T.A., K.B. Merck, W.W. de Jong, and H. Bloemendal. 1994. Structure and modifications of the junior chaperone alpha-crystallin. From lens transparency to molecular pathology. Eur. J. Biochem. 225, 1-19.

Gupta, R. S. 1995. Evolution of the chaperonin families (Hsp60, Hsp10, and Tcp-1) of proteins and the origin of eukaryotic cells. Mol. Microbiol. 15, 1-11.

Haass, C., U. Klein, and P.-M. Kloetzel. 1990. Developmental expression of *Drosophila melanogaster* small heat-shock proteins. J. Cell. Sci. 96, 413-418.

Hand, S. C. and I. Hardewig. 1996. Downregulation of cellular metabolism during environmental stress: mechanisms and implication. Annu. Rev. Physiol. 58, 539-563.

Harlow, E. and D. Lane. 1988. (Eds.) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. Nature 381, 571-580.

Hartl, F. U. and J. Martin. 1992. Protein folding in the cell: The role of molecular chaperones hsp70 and hsp60. Annu. Rev. Biophys. Biomol. Struct. 21, 293-322.

Haynes, J. I., M. K. Duncan, and J. Piatigorsky. 1996. Spatial and temporal activity of the αB-crystallin/small heat shock protein gene promoter in transgenic mice. Dev. Dynamics 207, 75-88.

Haynes II, J. I., R. Gopal-Srivastava, P. H. Frederikse, and J. Piatigorsky. 1995. Differential use of the regulatory elements of the αB-crystallin enhancer in cultured murine lung (MLg), lens (αTN4-1) and muscle (C2C12) cells. Gene 155, 151-158.

Hayward, J. R., A. I. Coffer, and R. J. B. King. 1990. Immunoaffinity purification and characterization of p29 – an estrogen receptor related protein. J. Steroid. Biochem. Mol. Biol. 37, 513-519.

Head, M.W., E. Corbin, and J.E. Goldman. 1994. Coordinate and independent regulation of alpha B-crystallin and hsp27 expression in response to physiological stress. J. Cell. Physiol. 159, 41-50.

Heikkila, J. J. 1993a. Review article: Heat shock gene expression and development. I. An overview of fungal, plant, and poikilothermic animal development sysstems. Dev. Gen. 14, 1-5.

Heikkila, J. J. 1993b. Review article: Heat shock gene expression and

development. II. An overview of mammalian and avian developmental systems. Dev. Gen. 14, 87-91.

Heikkila, J. J., N. Ohna, Y. Tam, and A. Ali. 1997. Heat shock protein gene expression during Xenopus development. CMLS. Cell. Mol. Lif. Sci. 53, 114-121.

Helbing, C., C. Gallimore, and B. G. Atkinson. 1996. Charaterization of a *Rana catesbeiana* hsp30 gene and its expression in the liver of this amphibian during both spontaneous and thyroid hormone-induced metamorphosis. Dev. Genet. 18, 223-233.

Helm, K. W., P. R. LaFayette, R. T. Nagao, J. L. Key, and E. Vierling. 1993. Localization of small heat shock proteins to the higher plant endomembrane system. Mol. Cell. Biol. 13, 238-247.

Hendrick, J. P. and F. -U. Hartl. 1995. The role of chaperones in protein folding. FASEB J. 9, 1559-1569.

Hendrick, J. P. and F.-U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. 62, 349-384.

Hickey, E., S. E. Brandon, R. Potter. G. Sterin, J. Stein, and L. A. Weber. 1986. Sequence and organization of genes encoding the human 27 kDa heat shock protein. Nucleic Acids Res. 14, 4127-4144.

Hightower, L.E., S.E. Sadis, and I.M. Takenaka. 1994. Interactions of vertebrate hsc70 and hsp70 with unfolded proteins and peptides. In *The biology of heat shock proteins and molecular chaperones.* Morimoto, R. I., A. Tissières, and C. Georgoupoulos (eds.). pp. 179-208. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Higgins, D.G., A.J. Bleasby, and R. Fuchs. 1992. CLUSTAL V: improved software for multiple sequence alignment. Computer Applicat. Biosci. (CABIOS) 8, 189-191.

Höhfeld, J. and F.-U. Hartl. 1994. Post-translational protein import and folding. Curr. Opin. Cell Biol. 6, 499-509.

Höhfeld, J., Y. Minami, and F.-U. Hartl. 1995. Hip, a novel cochaperone invovled in the eukaryotic Hsc70/Hsp40 rection cycle. Cell 83, 589-598.

Hootman, S. R. and F. P. Conte. 1975. Functional morphology of the neck organ in *Artemia* salina nauplii. J. Morph. 145, 371-386.

Horwich, A.L. 1995. Molecular chaperones: resurrection or destruction. Curr. Biol. 5, 455-458.

Horwitz, J. 1992. α-Crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. USA 89, 10449-10453.

Huot, J., F. Houle, D. R. Spitz, and J. Landry. 1996. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res. 56, 173-179.

Huot, J., G. Roy, H. Lambert, P. Chrétien, and J. Landry. 1991. Increased srvival after treatments with anticancer agents of Chinese hamster cell expressing the human Mr 27,000 heat shock protein. Cancer Res. 51, 5245-5252.

Huot, J., H. Lambert, J. N. Lavoie, A. Guimond, F. Houle, and J. Landry. 1995. Characterization of 45-54 kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27 kDa heat shock protein HSP27. Eur. J. Biochem. 227, 416-427.

Ingolia, T. D. and E. Craig. 1982. Four small *Drosophila* heat shock proteins are related to each other and to mammalian a-crystallin. Proc. Natl. Acad. Sci. 79, 2360-2364.

Ireland, R. C. and E. M. Berger. 1982. Synthesis of the low molecular weight heat shock proteins stimulated by ecdysterone in a cultured *Drosophila* cell line. Proc. Natl. Acad. Sci. 79, 855-859.

Ireland, R. C., E. M. Berger, K. Sirotkin, M. A. Yund, D. Osterburg, and J. Fristom. 1982. Ecdysterone induces the transcription of four heat shock genes in *Drosophila* S3 cells and imaginal discs. Dev. Biol. 93, 498-507.

Jackson, S. A. and J. S. Clegg. 1996. The ontogeny of low molecular weight stress protein p26 during early development of the brine shrimp *Artemia franciscana*. Develop. Growth Different. 38,153-160.

Jacob, U. And J. Buchner. 1994. Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. Trends Biochem. Sci. 19, 205-211

Jaenicke, R. and P. Zavodszky. 1990. Proteins under extreme physical conditions. FEBS Lett. 268, 344-349.

Jakob, U., M. Gaestel, K. Engel, and J. Buchner. 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517-1520.

Jakob, U., H. Lilie, I. Meyer, and J. Buchner. 1995. Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. J. Biol. Chem. 270, 7288-7294.

Jans, D.A. 1995. The regulation of protein transport to the nucleus by phosphorylation. Biochem. J. 311, 705-716.

Joly, E. C., E. Tremblay, R. M. Tanguay, Y. Wu, and V. Bibor-Hardy. 1994. TriC-P5, a novel TCP-1-related protein, is localized in the cytoplasm and in the nuclear matrix. J. Cell Sci. 107, 2851-2859.

Jones, D., R. H. Rassnak, R. J. Kay, and E.P.M. Candido. 1986. Structure, expression, and evolution of a heat shock gene locus in *Caenorhabditis elegans* that is flanked by repetitive elements. J. Biol. Chem. 261, 12006-12015.

Kamei, A., H. Iwase, and K. Masuda. 1997. Cleavage of amino acid residue(s) from the N-teminal region of αA- and αB-crystallins in human crystalline lens during aging. Biochem. Biophys. Res. Comm. 231, 373-378.

Kato, K., S. Goto, K. Hasegawa, and Y. Inaguma. 1993. Coinduction of two low-molecular-weight stress proteins, alpha B crystallin and HSP28, by heat or arsenite stress in human glioma cells. J. Biochem. 114, 640-647.

Kato, K., K. Hasegawa, S. Goto, and Y. Inaguma. 1994a. Dissociation as a result of phosphorylation of an aggregated form of the small stress protein, J. Biol. Chem. 269, 11274-11278.

Kato, K., S. Goto, Y. Inaguma, K. Hasegawa, R. Morishita, and T. Asano. 1994b. Purification and characterization of a 20-kDa protein that is highly homologous to alpha B crystallin, hsp27. J. Biol. Chem. 269, 15302-15309.

Kato, K., H. Shinohara, N. Kurobe, S. Goto, Y. Inaguma, K. Ohshima. 1991. Immunoreactive αA-crystallin in rat non-lenticular tissues detected with a sensitive immunoassay method. Biochem. Biophys. Acta 10180, 173-180.

Klemenz, R. and W. J. Gehring. 1986. Sequence requirement for expression of the Drosophila melanogaster heat shock protein hsp22 gene during heat shock and normal development. Mol. Cell Biol. 120, 639-645.

Klemenz, R., E. Frohli, R. H. Steiger, R. Schafer, and A. Aoyama. 1991. α-Crystallin is a small heat shock protein. Proc. Natl. Acad. Sci. 88, 3652-3656.

Klemenz, R., A. C. Andres, E. Froehli, R. Schaefer, and A. Aoyama. 1993. Expression of the murine a small heat shock protein hsp25 and alpha B crystallin in the absence of stress. J. Cell Biol. 120, 639-645.

Knauf, U., U. Jakob, K. Engel, J. Buchner, and M. Gaestel. 1994. Stress- and mitogen-induced phosphorylation of the small heat shock protein Hsp25 by MAPKAP kinase 2 is not essential for chaperone properties and cellular thermoresistance. EMBO J. 13, 54-60.

Koretz, J. F., E. W. Doss, and G. H. Reid. 1997. Analysis of the factors involved in the loss and restoration of the chaperone-like function of α-crystallin. Biochem. Biophys. Res. Comm. 231, 270-276.

Koyasu, S., E. Nishida, Y. Miyata, H. Sakai, and I. Yahara. 1989. HSP100, a 100-kDa heat shock protein, is a Ca2+-calmodulin-regulated actin-binding protein. J. Biol. Chem. 264, 15083-15087.

Kumar, S., K. Tamura, and M. Nei. 1993. MEGA: Molecular evolutionary genetics analysis, version 1.0. The Pennsylvania State University, University Park, PA 16820.

Kurtz, S., J. Rossi, L. Petko, and S. Lindquist. 1986. An ancient developmental induction: heat-shock protein induced in sporulation and oogenesis. Science 231, 1154-1157.

Laemmli, U. K. 1979. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680-685.

Landry, J., P. Chrétien, L. M. Nicole, R. M. Tanguay, and N. Marcaeu. 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. 42, 2457-2461.

Landry, J., P. Chrétien, A. Laszlo, and H. Lambert. 1991. Phosphorylation of HSP27 during development and decay of thermotolerance in Chinese hamster cells. J. Cell. Physiol. 147, 93-101.

Landry, J., P. Chrétien, H. Lambert, E. Hickey, and L. A. Weber. 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J. Cell. Biol. 109, 7-15.

Landry, J., H. Lambert, M. Zhou, J. N. Lavoie, E. Hickey, L. A. Weber, and C. W. Anderson. 1992. Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. J. Biol. Chem. 267, 794-803.

Langdon, C. M., J. C. Bagshaw, and T.H. MacRae. 1990. Tubulin isoforms in the brine shrimp, *Artemia*: primary gene products and their posttranslational modification. Eur. J. Cell Biol. 52, 17-26.

Langdon, C. M., J. H. Freeman, and T.H. MacRae. 1991a. Post-translationally modified tubulins in *Artemia:* prelarval development in the absence of detyrosinated tubulin. Dev. Biol. 148, 147-155.

Langdon, C.M., P. Rafiee, and T.H. MacRae. 1991b. Synthesis of tubulin during early postgastrula development of Artemia: isotubulin generation and translational regulation. Develop. Biol. 148, 138-146.

Laskey, R. A., B. M. Honda, A. D. Mills, and J. T. Finch. 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature 275, 416-420.

Lavoie, J. N., G. Gingras-Breton, R. M. Tanguay, and J. Landry. 1993a. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. J. Biol. Chem. 267, 3420-3429.

Lavoie, J. N., E. Hickey, L. A. Weber, and J. Landry. 1993b. Modulation of actin filament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J. Biol. Chem. 268, 24210-24214.

Lavoie, J. N., H. Lambert, E. Hickey, L. A. Weber, and J. Landry. 1995. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol. Cell. Biol. 15, 505-516.

Lee, G. J., A. M. Roseman, H. R. Saibil, and E. Vierling. 1997. A small heat shock protein stably binds heat-denatured in a folding-competent state. EMBO J. 16, 659-671.

Lee, G.J., N. Pokala, and E. Vierling. 1995. Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock proteins from pea. J. Biol. Chem. 270, 10432-10438.

Lenne, C., M. A. Block, J. Garin, and R. Douce. 1995. Sequence and expression of the mRNA encoding HSP22, the mitochondria small heat-shock protein in pea leaves. Biochem. J. 311, 805-813.

Lenz, P. H. and R. A. Browne. 1991. Ecology of *Artemia*. In: *Artemia Biology*, Browne, R.A., Sorgeloos, P., and C.N.A. Trotman (eds.). pp 237-254. CRC Press, Boca Raton, Florida.

Leroux, M. R., B. J. Ma, G. Batelier, R. Melki, and E.P.M. Candido. 1997a. Unique structural features of a novel class of small heat shock proteins. J. Biol. Chem. 272, 12847-12853.

Leroux, M. R., R. Melki, B. Gordon, G. Batelier, and E.P.M. Candido. 1997b. Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. EMBO J. (in press)

Liang, P., R. Amons, T. H. MacRae. and J. S. Clegg. 1997a. Purification, structure and molecular chaperone activity *in vitro* of *Artemia* p26, a small heat shock/a-crystallin protein. Eur. J. Biochem. 243, 225-232.

Liang, P., R. Amons, J. S. Clegg, and T. H. MacRae. 1997b. Molecular characterization of a small heat shock/a-crystallin protein in encysted *Artemia* embryos. J. Biol. Chem. 272, 19051-19058.

Liang, P. and T. H. MacRae. 1997. Heat shock proteins and the cytoskeleton. J. Cell Sci. 110, 1431-1440.

Linder, B., Z. Jin, J. H. Freedman, and C. S. Rubin. 1996. Molecular characterization of a novel, developmentally regulated small embryonic chaperone from *Caenorhabditis elegans*. J. Biol. Chem. 271, 30158-30166.

Lindquist, S. and G. Kim. 1996. Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. Proc. Natl. Acad. Sci. 93, 5301-5306.

Loomis, W.F. and S. Wheeler. 1982. Chromotin-associated heat shock proteins in *Dictyostelium*. Dev. Biol. 90, 412-418.

Loones, M. T., M. Rallu, V. Mezger, and M. Morange. 1997. HSP gene expression and HSF2 in mouse development. CMLS Cell. Mol. Lif. Sci. 53, 179-190.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 835-841.

MacRae, T. H. and R. F. Ludueña. 1984. Developmental and comparative aspects of brine shrimp tubulin. Biochem. J. 219, 137-148.

MacRae, T.H., J.C Bagshaw, and A.H. Warner. 1989. (Eds.) *Biochemistry & Cell Biology of Artemia*, CRC Press, Boca Raton, FL.

Marin, R., J. Landry, and R. M. Tanguay. 1996. Tissue-specific posttranslational modification of the small heat shock protein HSP27 in *Drosophila*. Exp. Cell. Res. 223, 1-8.

Marin, R., J. P. Valet, and R.M. Tanguay. 1993. Hsp23 and hsp26 exhibit distinct spatial and temporal patterns of constitutive expression in *Drosophila* adults. Dev. Gen. 14, 69-77.

Martin, J., A. L. Horwich, and F. U. Hartl. 1992. Prevention of protein denaturation under heat stress by the chaperonin Hsp60. Science 258, 995-998.

Martin, J. and F. U. Hartl. 1994. Molecular chaperones in cellular protein folding. BioEssay 16, 689-692.

Mason, P. J., L. M. C. Hall, and J. Gausz. 1984. The expression of heat shock genes during normal development in *Drosophila melanogaster*. Mol. Gen. Genet. 194, 73-78.

Marcus, N. H. 1996. Ecological and evolutionary significance of resting eggs in marine copepods: Past, present, and future studies. Hydrobiologia 320, 141-152.

McKenzie, S. L., S. Henikoff, and M. Melseson. 1975. Localization of RNA from heat-induced polysomes at puff sites in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 72, 1117-1121.

McLennan, A. G. and D. Miller. 1990. A biological role for the heat shock response in crustaceans. J. Therm. Biol. 15, 61-66.

McNamara, J. M. 1994. Time of entry into diapause. J. Theoret. Biol. 168, 201-209.

Mead, R. A. 1993. Embryonic diapause in vertebrate. J. Exp. Zool. 266, 629-641.

Mehlen, P., J, Briolay, L. Smith, C, Diaz-Latoud, N. Fabre, D. Pauli, and A.-P. Arrigo. 1993. Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat shock protein. Proc. Natl. Acad. Sci. 88, 11212-11216.

Mehlen, D., C. Kretz-Remy, X. Préville, and A.-P. Arrigo. 1996a. Human hsp27, Drosophila hsp27 and human αB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFα-induced cell death. EMBO J. 15, 2695-2706.

Mehlen, D., K. Schulze-Usthoff, and A.-P. Arrigo. 1996b. Small stress proteins

as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J. Biol. Chem. 271, 16510-16514.

Melki, R. and N. Cowan. 1994. Facilitated folding of actins and tubulins occurs via nucleotide-dependent interaction between cytoplasmic chaperonin and distinctive folding intermediates. Mol. Cell. Biol. 14, 2895-2904.

Melnick, J., J.L. Dul, and Y. Argon. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature 370, 373-375.

Mendelsohn, M. E., Y. Zhu, and S. O'neill. 1991. The 29-kDa proteins phosphorylated in thrombin-activated human platelets are forms of the estrogen receptor-related 27 kDa heat shock protein. Proc. Natl. Acad. Sci. 88, 11212-11216.

Merck, K.B., P.J.T.A. Groenen, C.E.M. Voorter, W.A. de Haard-Hoekman, J. Horwitz, H. Bloemendal, and W.W. de Jong. 1993. Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein, a family of chaperones. J. Biol. Chem. 268, 1046-1052.

Michaud, S., R. Marin, and R. M. Tanguay. 1997. Regulation of heat shock gene induction and expression during *Drosophila* development. CMLS. Cell. Mol. Life Sci. 53, 104-113.

Miller, D. and A. G. McLennan. 1987. Synthesis of heat shock proteins and thermotolerance in *Artemia* cysts and larvae. Phys. Bioc. Mol. Biol. 2, 433-449.

Miller, D. and A. G. McLennan. 1988. The heat shock response of the cryptobiotic shrimp *Artemia* - I. A comparison of the thermotolerance of cysts and larvae. J. Therm. Biol. 13, 119-123.

Miron, T., M. Wilchek, and B. Geiger. 1988. Characterization of an inhibitor of actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle. Eur. J. Biochem. 178, 543-553.

Miron, T., K. Vancompernolle, J. Vandekerchhove, M. Wilchek, and B. Geiger. 1991. A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J. Cell Biol. 114, 255-261.

Morimoto, R. I., A. Tissières, and C. Georgoupoulos. 1991. (Eds.) *Stress proteins in biology and medicine.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Morimoto, R. I., A. Tissières, and C. Georgoupoulos. 1994. (Eds.) *The Biology of Heat shock proteins and molecular chaperone.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Mörner, C. T. 1894. Untersuchung der Proteinsubstanzen in den leichtbrechenden Medien des Auges. Hoppe Seler's Z. Phyiol. Chem. 18, 61-106.

Morris, J.E. and B.A. Afzelius. 1967. The structure of the shell and outer membranes in encysted Artemia salina embryos during cryptbiosis and development. J. Ultrastruct. Res. 20, 244-259.

Muchowski, P. J., J. A. Bassuk, N. H. Lubsen, and J. I. Clark. 1996. Human αB-crystallin - small heat shock protein and molecular chaperone. J. Biol. Chem. 272, 2578-2582.

Murakami, H., D. Pain, G. Blobel. 1988. The 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. 107, 2051-2057.

Nene, V., D. V. Dunne, K. S. Johnson, D. W. Taylor, and J. S. Cordingley. 1986. Sequence and expression of a major egg antigen from Schistosoma mansoni: Homologies to heat shock proteins and α-crystallins. Mol. Biochem. Parasitol. 21, 179-188.

Nicholl, I. D. and R. A. Quinlan. 1994. Chaperone activity of α-crystallins modulates intermediate filament assembly. EMBO J. 13, 945-953.

Oesterreich, S., E. Hickey, L. A. Weber, and S. A. W. Fuqua. 1996. Basal regulatory promotor elements of the hsp27 gene in human breast cancer cells. Biochem. Biophys. Res. Comm. 222, 155-163.

Osborne, M. A. and P. A. Silver. Nucleocytoplasmic transport in the yeast Saccharomyces cerevisiae. Annu. Rev. Biochem. 62, 219-254.

Parsell, D. A. and S. Lindquist. 1994. Heat shock proteins and stress tolerance. In *The biology of heat shock proteins and molecular chaperones.* Morimoto, R. I., A. Tissières, and C. Georgoupoulos (eds.). pp. 457-494. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Patino, M. M., J. J. Liu, J. R. Glover, and S. Lindquist. 1996. Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science 273, 622-626.

Pauli, D. and C.-H. Tonka. 1987. A *Drosophila* heat shock gene from locus 67B

is expressed during embryogenesis and pupation. J. Mol. Biol. 198, 235-240.

Pauli, D., A.-P. Arrigo, J. Vasquez, C.H. Tonka, and A. Tissières. 1989. Tissue specific expression of the heat shock genes during *Drosophila* development: comparison of the accumulation of HSP23 and HSP27 mRNA and polypeptides. Genome 31, 671-676.

Pauli, D., C.-H. Tonka, and A. Ayme-Southgate. 1988. An unusual split *Drosophila* heat shock gene expressed during embryogenesis, pupation and in testis. J. Mol. Biol. 200, 47-53.

Pauli, D., C.-H. Tonka, A. Tissières, and A.-P. Arrigo. 1990. Tissue specific expression of the heat shock protein hsp27 during *Drosophila melanogaster* development. J. Cell Biol. 111, 817-828.

Plater, M. L., D. Goode, and M. J. C. Crabbe. 1996. Effects of site-directed mutuations on the chaperone-like activity of αB-crystallin. J. Biol. Chem. 271, 28558-28566.

Plesofsky-Vig, N. and R. Brambl. 1995. Disruption of the gene for hsp30, an α-crystallin-related heat shock protein of *Neurospora crassa*, causes defects in thermotolerance. Proc. Natl. Acad. Sci. USA 92, 5032-5036.

Plesofsky-Vig, N. and R. Brambl. 1990. Gene sequence analysis of hsp30, a small heat shock protein of *Neurospora crassa* which associates with mitochondria. J. Biol. Chem. 265, 15432-15440.

Quax-Jeuken, Y., W. Quax, G. L. N. van Rens, P. Meera Khan, and H. Bloemendal. 1985. Assignment of the human αA-crystallin gene (CRYA1) to chromosome 21. Cytogenet. Cell Genet. 40, 727-728.

Quivy, J. P. and P.B. Becker. 1996. The architecture of the heat-inducible Drosophila hsp27 promoter in nuclei. J. Mol. Biol. 256, 249-263.

Rafiee, P., C. O.,Matthews, J. C. Bagshaw, and T. H. MacRae. 1986. Reversible arrest of *Artemia* development by cadmium. Can. J. Zool. 64, 1633-1641.

Rahman, D. R. J., N. J. Bentley and M. F. Tuite. 1995. The *Saccharomyces cerevisiae* small heat shock protein Hsp26 inhibits actin polymerization. Biochem. Soc. Trans. 23, 77s.

Raman, B., T. Tangirala, and C. M. Rao. 1995. Rapid refolding studies on the chaperone-like α-crystallin, effect of α-crystallin on refolding of β- and γ-crystallins. J. Biol. Chem. 270, 19888-19892.

Rao, P.V., J. Horwitz, and J. S. Zigler, Jr. 1994. Chaperone-like activity of alpha-crystallin. The effect of NADPH on its interaction with zeta-crystallin. J. Biol. Chem. 269, 13266-13272.

Raschke, E. G., G. Baumann, and F. Schöffl. 1988. Nucleotide sequence analysis of soybean small heat shock protein genes belonging to two different multigenic families. J. Mol. Biol. 199, 549-557.

Rassow, J. and N. Pfanner. 1996. Protein biogenesis: chaperones for nascent polypeptides. Curr. Biol. 6, 115-118.

Rassow, J., O. Von Ahsen, U. Bömer, and N. Pfanner. 1997. Molecular chaperones: towards a characterization of the heat-shock protein 70 family. Trends Cell Biol. 7,129-133.

Redmond, T., E. R. Sanchez, E. H. Bresnick, M. J. Schlesinger, D. O. Toft, W. B. Pratt, and M. J. Welsh. 1989. Immunofluorescence colocalization of the 90-kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. Eur. J. Cell Biol. 50, 66-75.

Richarme, G. and T. D. Caldas. 1997. Chaperone properties of the bacterial periplasmic substrate-binding proteins. J. Biol. Chem. 272, 15607-15612.

Roberts, M. P. and J. C. Vaughn. 1982. ribosomal RNA sequences conservation and gene number in the larval brine shrimp. Biochem. Biophy. Acta 697, 148-155.

Robinson, J.B., L.G. Brent, B. Sumegi, and P.A. Srere. 1987. Enzymatic approach to the study of the Krebs tricarboxylic acid cycle. In *Mitochondria - A Practical Approach*, Darley-Usmar, V.M., Rickwood, D., & Wilson, M.T. (eds.) pp 160-161. IRL Press, Oxford.

Rossi, J. M. and S. Lindquist. 1989. The intracellular location of yeast heat-shock protein 26 varies with metabolism. J. Cell Biol. 108, 425-439.

Rost, B. 1996. PHD: predicting one-dimensional protein structure by pro-file based neural networks. Meth. Enzym. 266, 525-539.

Rost, B. and C. Sander. 1993. Prediction of protein structure at better than 70% accuracy. J. Mol. Biol. 232, 584-599.

Rost, B. and C. Sander. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. Proteins. 19, 55-72.

Russnak, R. H. and E. P. M. Candido. 1985. Locus encoding a family of small heat shock genes in *Caenorhabditis elegans*: two genes duplicated to form a 3.8 kilobase inverted repeat. Mol. Cell. Biol. 5, 1268-1278.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning – a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sanger, F., S. N. Nicklen, and A. R. coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74, 5463-71.

Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist. 1996. Hsp100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. 21, 289-296.

Schlesinger, M. J. 1990. Heat shock proteins. J. Biol. Chem. 265, 12111-12114.

Schuster, G., D. Even, K. Kloppstech, and I. Ohad. 1988. Evidence for protection by heat shock proteins against photoinhibition during heat shock. EMBO J. 7, 1-16.

Sirotkin, K. and N. Davidson. 1982. Developmentally regulated transcription from *Drosophila melanogaster* chromosomal site 67B. Dev. Biol. 89, 196-210.

Smulders, R.H.P.H., J. A. Carver, R. A. Lindner, M. A. van Boekel, H. Bloemendal, and W.W. de Jong. 1996. Immobilization of the C-teminal extension of bovine α-crystallin reduces chaperone-like activity. J. Biol. Chem. 271, 29060-29066.

Spence, J. and C. Georgopoulos. 1989. Purification and properties of the *Escherichia coli* heat shock protein, HtpG. J. Biol. Chem. 264, 4398-4403.

Squires, S. L. and R. A. Acey. 1989. A new method for the purification of nuclei from *Artemia*. In *Cell & Molecular Biology of Artemia Development*. Warner, A.H., MacRae, T.H., and J.C. Bagshaw. (Eds.) pp 221-222. Plenum Press, NY.

Squires, C. and C. L. Squires. 1992. The Clp proteins: proteolysis regulators or molecular chaperones. J. Bacteriol. 174, 1081-1085.

Srinivasan, A. N., C. N. Nagineni, and S.P. Bhat. 1992. αA-crystallin is expressed in non-ocular tissues. J. Biol. Chem. 267, 23337-23341.

Sternlicht, H., G. W. Farr, M.L. Sternlicht, J. K. Driscoll, K. Willison, and M. B. Yaffe. 1993. The t-complex polypeptide 1 complex is a chaperonin for

tubulin and actin *in vivo*. Proc. Natl. Acad. Sci. 90, 9422-9426.

Stoldt, V., F. Rademacher, V. Kehren, J. F. Ernst, D. A. Pearce, and F. Sherman. 1996. The Cct eukaryotic chaperonin subunits of Saccharomyces cerevisiae and other yeast. Yeast 12, 523-529.

Studier, F. W., A. H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60-89.

Stuart, R.A., D.M. Cyr, E.A. Craig, and W. Neupert. 1994. Mitochondrial molecular chaperones: their role in protein translocation. Trends Biochem. Sci. 19, 87-92.

Susek, R. E. and S. Lindquist. 1989. Hsp26 of *Saccharomyces cerevisiae* is related to the superfamily of small heat shock proteins but is without a demonstrable function. Mol. Cell. Biol. 9, 5265-5271.

Sun, T. B. K. Das, and J. J. -N. Liang. 1997. Conformational and functional differences betwwen recombinant human lens αA- and αB-crystallin. J. Biol. Chem. 272, 6220-6225.

Tam, Y. and J. J. Heikkila. 1995. Identification of members of the HSP30 small heat shock protein family and characterization of their developmental regulation in heat-shocked *Xenopus laevis* embryos. Dev. Gen. 17, 331-339.

Tanguay, R. M., J.-L. Duband. F. Lettre, J.-P. Valet, A.-P. Arrigo, and L. Nicole. 1985. Biochemical and immunocytochemical localization of heat shock proteins *Drosophila* culture cells. Ann. N. Y. Acad. Sci. 445, 711-714.

Tanguay, R. M., Y. Wu, and E. W. Khandjian. 1993. Tissue-specific expression of heat shock proteins of the mouse in then absence of stress. Dev. Gen. 14, 112-118.

Tardieu, A. and M. Delaye. 1988. Eye lens proteins and transparency: from light transmission theory to solution X-ray structural analysis. Annu. Rev. Biophys. Chem. 17, 47-70.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Tissières, A., H. K. Mitchel, and U. M. Tracy. 1974. Protein synthesis in salivary

gland of Drosophila melanogaster: relation to chromosome puffs. J. Mol. Biol. 84, 389-398.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. 76, 4350-4354.

Trent, J. D., E. Nimmesgern, J. S. Wall, F. -U. Hartl, and A. L. Horich. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. Nature 354, 490-493.

Verbon, A., A. Hartskeerl, A. Schuitma, A. H. J. Kolk, D. B. Young, and R. Lathigra. 1992. The 14,000-molecular-weight antigen of *Mycobacterium tuberculosis* is related to the alpha-crystallin family of low molecular weight heat shock proteins. J. Bacteriol. 174, 1352-1359.

Voorter, C. E. M., W. A. de Haard-Hoekman, E. S. Roesrma, H. E. Meyer, H. Bloemendal, and W.W. de Jong. 1989. The in vivo phosphorylation sites of bovine αB-crystallin. FEBS Lett. 259, 50-52.

Voorter, C. E. M., J. W. M. Mulders, H. Bloemendal, and W.W. de Jong. 1986. some aspects of the phosophorylation of α-crystallin A. Eur. J. Biochem. 160, 203-210.

Warner, A.H., MacRae, T.H., and J.C. Bagshaw. 1989. (Eds.) *Cell & Molecular Biology of Artemia Development.* Plenum Press, NY.

Wang, K. and A. Spector. 1996. α-Crystallin stabilizes actin filaments and prevents cytochalasin-induced depolymerization in a phosphorylation-dependent manner. Eur. J. Biochem. 242, 56-60.

Water, E. R., G. J. Lee, and E. Vierling. 1996. Evolution, structure and function of the small heat shock proteins in plants. J. Exp. Bot. 47, 325-338.

Willison K. R. and H. Kubota. 1994. The structure, function, and genetics of the chaperone containing TCP-1 (CCT) in eukaryotic cytosol. In *The biology of heat shock proteins and molecular chaperones.* Morimoto, R. I., A. Tissières, and C. Georgoupoulos (eds.). pp. 299-312. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Wistow, G. 1985. Domain structure and evolution in α-crystallins and small heat shock proteins. FEBS Lett. 181, 1-6.

Wotton, D., K. Freeman, and D. Shore. 1996. Multimerization of Hsp42p, a novel heat shock protein of *Saccharomyces cerevisiae*, is dependent on a

conserved carboxyl-terminal sequence. J. Biol. Chem. 271, 2717-2713.

Yamashita, O. and K. Hasegawa. 1985. Embryonic diapause. In *Comprehensive Insect Phyiology, Biochemistry and Phacology*. Kerkut, G. A. and L. I. Gilbert (Eds.), Vol. 1. pp. 407-434. Pergamon Press, New York, New York.

Zantema, A., W. W. de Jong, R. Lardenoije, and A.J. van der Eb. 1989. The expression of heat shock protein hsp27 and a complexed 22-kilodalton protein is inversely correlated with oncogenicity of adenovirus-transformed cells. J. Virol. 63, 3368-3375.

Zhang, J. and T.H. MacRae. 1992. A novel 49-kilodalton protein from *Artemia* cross-links microtubules *in vitro*. Biochem. Cell Biol. 70, 1055-1063.

IMAGE EVALUATION
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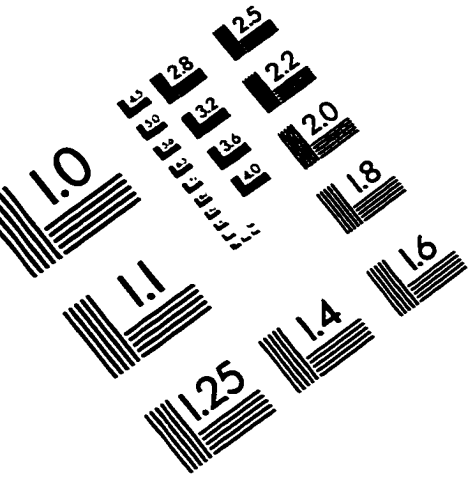

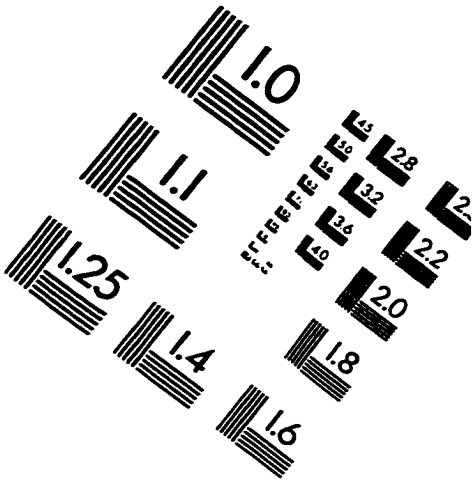

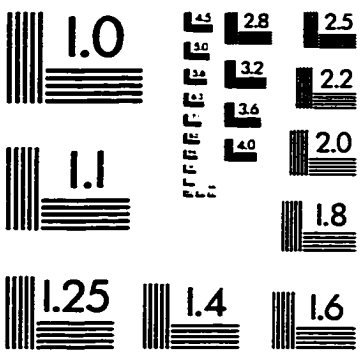

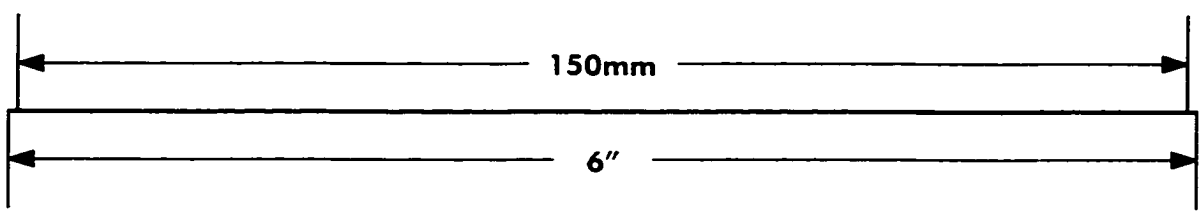

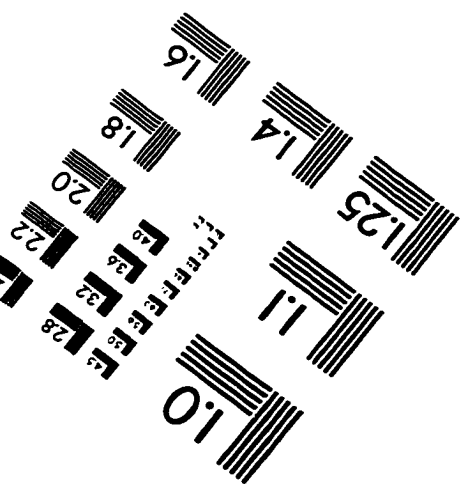

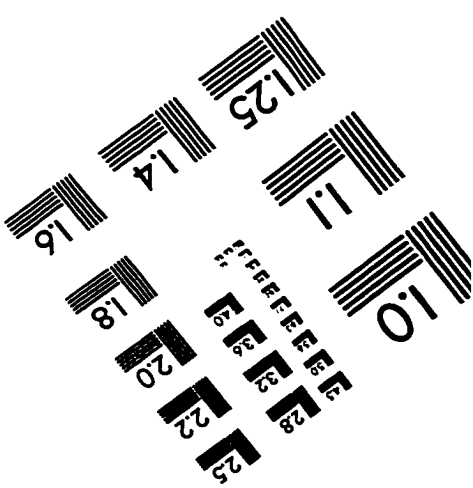

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