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# Protein in *Artemia* and Its Relationship to Stress Tolerance during Development

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**Fertilized oocytes of the brine shrimp *Artemia franciscana* undergo either ovoviviparous or oviparous development, yielding free-swimming larvae (nauplii) or encysted gastrulae (cysts), respectively. Encystment is followed by diapause, wherein metabolism is greatly reduced; the resulting cysts are very resistant to extreme stress, including desiccation and long-term anoxia. The synthesis of p26, a small heat shock/α-crystallin protein produced only in oviparously developing *Artemia,* is shown in this paper to be transcriptionally regulated. A p26 mRNA of about 0.7 kb was detected on Northern blots in the second day after oocyte fertilization. It peaked as embryos encysted and declined rapidly when activated cysts resumed development. The appearance of p26 protein, as indicated by immunoprobing of Western blots, followed mRNA by 1 day; it also increased as encystment occurred but remained constant during postgastrula development of cysts. However, p26 underwent a marked reduction during emergence of nauplii and could not be detected in cell-free extracts of second-instar larvae. p26 entered nuclei of encysting embryos soon after synthesis and was localized therein as late as instar II, when it was restricted to a small set of salt gland nuclei. First-instar larvae derived from cysts were more thermotolerant than larvae that had developed ovoviviparously, but synthesis of p26 was not induced by heat under the experimental conditions employed. Additionally, transformed bacteria synthesizing p26 were more thermotolerant than bacteria that lacked the protein. The results support the proposal that p26, a developmentally regulated protein synthesized during embryo encystment, has chaperone activity *in vivo* and protects the proteins of encysted *Artemia* from stress-induced denaturation.** 

## INTRODUCTION

Alternative developmental processes have evolved in the brine shrimp, *Artemia franciscana* (Fig. 1). In one case, termed ovoviviparous, free-swimming nauplius larvae are released from females, whereas in the other, oviparous development, encysted gastrulae or cysts are produced. The cysts, composed of about 4000 cells enclosed in a complex shell impermeable to nonvolatile molecules, enter diapause, a dormant state with greatly reduced metabolic activity (Clegg, 1997; Jackson and Clegg, 1996; Clegg *et al.*, 1995; Drinkwater and Clegg, 1991; Drinkwater and Crowe, 1987). Diapause terminates upon exposure of cysts to stimuli such as desiccation and if conditions are favorable development resumes. Nauplii then emerge, enclosed in a hatching membrane attached to the cyst shell (Go *et al.*, 1990; Rafiee *et al.*, 1986). Rupture of the membrane, or hatching, releases nauplius larvae. Before emergence, encysted embryos tolerate reversible metabolic arrest (quiescence) in response to adverse environmental factors. Diapause and production of resistant forms occur in many eukaryotes (Marcus, 1996; Fell, 1995; Mead, 1993; Drinkwater and Clegg, 1991), but among them encysted *Artemia* seemingly withstand the greatest physiological stress, including long-term anoxia, temperature extremes, desiccation, γ-irradiation, and exposure to organic solvents (Drinkwater and Clegg, 1991; Clegg and Conte, 1980). For example, hydrated cysts endure anoxia for at least 4 years with low mortality (Clegg, 1997, 1994). Metabolic activity cannot be detected in these anoxic *Artemia* embryos, a finding contrary to the generality that cell maintenance requires a substantial free energy flow.

Because quiescent and diapause *Artemia* embryos survive, cellular components must either be protected or repaired when damaged. In this context, encysted *Artemia*

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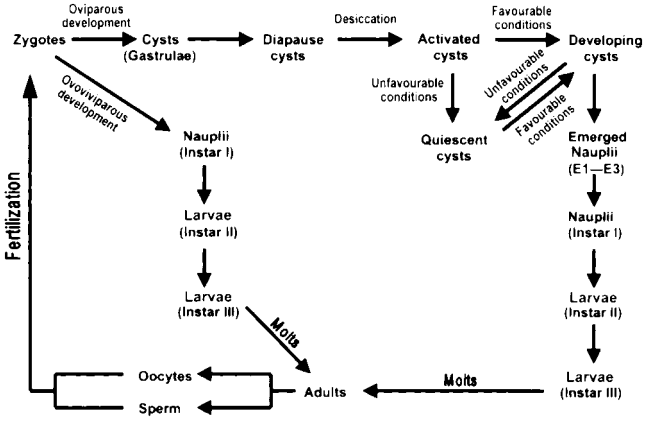


**FIG. 1.** Alternative developmental modes of *A. franciscana*. Fertilized *Artemia* oocytes may undergo ovoviviparous development, wherein free-swimming nauplii (first-instar larvae) are produced (left side of diagram). Alternatively, oviparous development occurs and gastrulae encyst, leave the female, and enter diapause, after which they are often desiccated (top of diagram). Upon activation cysts develop into larvae, but if unfavorable conditions prevail growth is arrested and cysts become quiescent (right side of diagram).

embryos possess an abundant small heat shock/α-crystallin protein, termed p26, which exhibits molecular chaperone activity *in vitro* (Liang *et al.*, 1997a,b; Jackson and Clegg, 1996; Clegg *et al.*, 1995). Molecular chaperones, often acting cooperatively and in concert with associated proteins, facilitate the folding/oligomerization of nascent proteins, prevent aggregation when misfolded, and assist with their distribution in cells (Lu and Cyr, 1998; Bukau and Horwich, 1998; Scheibel *et al.*, 1998; Johnson *et al.*, 1998; Demand *et al.*, 1998; Netzer and Hartl, 1998, 1997; Liang and MacRae, 1997; Nathan *et al.*, 1997; Rassow *et al.*, 1997; Frydman and Höhfeld, 1997; Hartl, 1996; Boston *et al.*, 1996). Of the known molecular chaperones the small heat shock/α-crystallin proteins form a diverse family. They range in molecular mass from about 12 to 30 kDa and assemble into multimers up to 800 kDa, the latter a prerequisite for chaperone function (Liao *et al.*, 1998; Suzuki *et al.*, 1998; Arrigo, 1998; Nover and Scharf, 1997; Graw, 1997; Lee *et al.*, 1997, 1995; Norris *et al.*, 1997; Leroux *et al.*, 1997a,b; Liang *et al.*, 1997a; Chang *et al.*, 1996; Groenen *et al.*, 1994; de Jong *et al.*, 1993). Small heat shock/α-crystallin proteins have an α-crystallin domain, a conserved sequence of 90–100 amino acid residues. This region is preceded by a variable N-terminal domain and followed by a short, poorly conserved C-terminal extension (Liang *et al.*, 1997b; Norris *et al.*, 1997; Caspers *et al.*, 1995; Jakob and Buchner, 1994; Arrigo and Landry, 1994).

The synthesis of small heat shock/α-crystallin proteins increases upon heat shock, suggesting that they convey thermotolerance (Arrigo and Landry, 1994; Groenen *et al.*, 1994; de Jong *et al.*, 1993). For example, NIH-3T3 cells are less susceptible to heat shock when αB-crystallin is induced than when it is absent (Aoyama *et al.*, 1993), and transfected cultured cells constitutively expressing extrinsic HSP27 exhibit greater thermoresistance than their parents, with tolerance proportional to the amount of HSP27 (Lavoie *et al.*, 1993; Landry *et al.*, 1989). Direct evidence for chaperone function comes from *in vitro* experiments, in which the small heat shock/α-crystallin proteins prevent aggregation of proteins during stress and may enhance their refolding (Raman and Rao, 1997; Leroux *et al.*, 1997a; Ehrnsperger *et al.*, 1997; Lee *et al.*, 1997, 1995; Muchowski *et al.*, 1996; Plater *et al.*, 1996; Andley *et al.*, 1996). Unlike HSP90, HSP70, and the chaperonins (Scheibel *et al.*, 1998; Bukau and Horwich, 1998; Grenert *et al.*, 1997; Rassow *et al.*, 1997; Hartl, 1996), small heat shock/α-crystallin proteins function as chaperones in the absence of ATP and are described as chaperone-like (Liang *et al.*, 1997a; Plater *et al.*, 1996). However, the *in vitro* chaperone function of α-B crystallin is increased by ATP (Muchowski and Clark, 1998).

Although the mechanism for chaperone action is unknown, the binding of partially denatured proteins to small heat shock/α-crystallin oligomers may lead to accumulation of folding intermediates. Interaction is probably through exposed hydrophobic regions, preventing irreversible aggregation and allowing proteins to refold, either spontaneously or with the assistance of other chaperones (Ehrnsperger *et al.*, 1997; Leroux *et al.*, 1997a; Lee *et al.*, 1997). In this study, p26 gene expression and the association of p26 with the nucleus were examined, as was the ability of p26 to protect organisms from thermal stress. The results support the conclusion that p26 has a key role during encystment, diapause, and quiescence in developing *Artemia*.

## MATERIALS AND METHODS

***Culture of Artemia and embryo collection.*** Encysted *Artemia*, obtained from Sanders Brine Shrimp Co. (Ogden UT), were hydrated in ice-cold distilled water for 3 h. The cysts that sank were collected by suction in a Buchner funnel, rinsed several times with cold water, and either homogenized immediately (dormant cysts) or incubated in hatch medium (Langdon *et al.*, 1990) at 27°C with shaking at 220 rpm. To obtain synchronous emerged larvae, termed $E_2$ (Go *et al.*, 1990; Rafiee *et al.*, 1986), cultures were removed from the shaker when about 50% emergence had occurred and held stationary at room temperature for 10 min. The $E_2$ larvae, each attached to a buoyant empty cyst shell, were harvested from the top of the liquid, placed in 50-ml plastic centrifuge tubes with cold hatch medium, shaken vigorously for 30 s to separate larvae from shells, and placed on ice for 5 min. The emerged larvae that sank were transferred to a fresh tube containing cold hatch medium, agitated briefly, incubated at room temperature for 5 min, and again collected from the bottom of the tube. If cysts remained the procedure was repeated. The highly synchronized $E_2$ larvae were either homogenized immediately or used for production of instar I, II, and III larvae (Langdon *et al.*, 1991a), except that phototactic collection of nauplii was unnecessary.

Adult *Artemia*, maintained at room temperature in glass aquaria containing filtered, aerated seawater, were fed daily with either *Isochrysis galbana* (clone synonym ISO) or *Isochrysis* sp. (clone synonym: TISO). Alga species were from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). For fertilization, individual females with two oocyte-filled lateral pouches were placed in 10 ml of seawater in covered 12-well culture plates. The females were often coupled with a male, but if not, two to three males were added to each well. The shrimp were examined every 2 h with a dissecting microscope until fertilization occurred, as marked by merging of the lateral pouches, and then at 12-h intervals until either instar I larvae (nauplii) or cysts were released. Females were fixed in 4% paraformaldehyde and photographed with a Tessovar dissecting microscope to document developmental stages. Embryos collected 1, 2, 3, 4, and 5 days postfertilization were either immediately immunostained or processed for RNA and protein.

***Analyses of p26 mRNA and protein during Artemia development.*** Samples containing approximately 200 organisms, from 1 day postfertilization to instar II larvae, were rinsed in hatch medium, transferred to a microtissue grinder (Fisher Scientific, Inc.), and homogenized for 1 min in 0.4 ml of Trizol solution (Life Technologies, Burlington, Ontario). Additional homogenization was achieved by three or four complete passages in a mini Dounce homogenizer and the homogenate was vortexed 1 min with 0.1 ml of chloroform, then centrifuged 5 min in a microcentrifuge. The top RNA-containing layer was mixed with 1 $\mu$l of glycogen (20 mg/ml) and 0.2 ml of isopropanol, incubated at room temperature for 10 min, and centrifuged in a microcentrifuge for 10 min at 4°C. The pellet was washed with 0.5 ml of 70% ethanol, air dried, and dissolved in 15 $\mu$l of DEPC-treated, distilled water supplemented with 0.1% SDS. RNA concentration was determined by measuring absorbance at 260 nm.

The bottom protein-containing layer from the chloroform extraction was mixed with 0.15 ml of ethanol, incubated at room temperature for 5 min, and centrifuged at 2000*g* for 10 min at 4°C. The supernatant was transferred to a fresh tube, 0.6 ml of isopropanol was added, and the mixture was incubated at room temperature, followed by a 10-min spin in a microcentrifuge. The large orange pellet, washed twice with 0.3 M guanidine HCl and once with ethanol, 20 min each time, was dried in a vacuum desiccator and dissolved in 30 $\mu$l of distilled water containing 1% (w/v) SDS. Twelve microliters of 4× treatment buffer (Laemmli, 1970) was added; samples were then placed in a boiling water bath for 5 min, transferred to a fresh tube, and spun briefly in a microcentrifuge. Protein concentrations were determined with the Bio-Rad DC Protein Assay Kit.

Ribosome-associated mRNA was prepared from cysts developed for 0, 3, 6, and 10 h (Langdon *et al.*, 1991b) with slight modifications. Specifically, 5 g of cysts (wet weight) were homogenized in 15 ml of TEMN buffer (20 mM Tris–HCl, 0.2 mM $MgCl_2$, 250 mM NaCl, 2% (v/v) Triton X-100, 0.95% (w/v) EDTA, pH 8.3 at 20°C) with a prechilled mortar and pestle, filtered through one layer of Miracloth (Calbiochem), and centrifuged at 12,000*g* for 15 min at 4°C. The resulting supernatant was applied to a 10-ml, 15% sucrose cushion in Pipes buffer (100 mM [1,4-piperazine-bis(ethane sulfonic acid)] as free acid, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.5 at 20°C) for centrifugation at 113,000*g* in a Beckman SW28 rotor at 4°C for 4 h. The gel-like pellet was dissolved in 2.0 ml of Trizol solution and RNA was prepared as just described, except that glycogen was not added.

Protein samples were electrophoresed in SDS–polyacrylamide gels (Laemmli, 1970) which were either stained with Coomassie blue or blotted to nitrocellulose. Transfer of proteins to nitrocellulose was verified by staining with 0.2% Ponceau-S in 3% TCA. The Western blots were probed by the enhanced chemiluminescence procedure (Renaissance; DuPont NEM) using antibodies to p26 raised in rabbits, as described by Liang *et al.* (1997a), or to purified p26 as part of this work. RNA was electrophoresed in 1.5% agarose gels containing formaldehyde, blotted to Hybond-$N^+$ membranes (Amersham), and hybridized to full-length, $^{32}$P-labeled, p26 cDNA (p26-3-6-3) (Liang *et al.*, 1997b; Langdon *et al.*, 1990). The mRNA was sized by use of a 0.24- to 9.5-kb RNA ladder (Life Technologies, Burlington, Ontario).

***Immunofluorescent staining of Artemia embryos, larvae, and nuclei.*** Immunofluorescent staining of *Artemia* was as described by MacRae *et al.* (1991) with minor modifications. Emerged nauplii and instar I, II, and III larvae were placed in 4% (w/v) paraformaldehyde in PBS at 40°C and left at room temperature for 1 h and overnight at 4°C. Fixed samples were washed three times at room temperature, 20 min per wash, chopped into small pieces, and extracted in two changes of PBSAT (PBS containing 0.5% (w/v) bovine serum albumin and 0.5% (v/v) Triton X-100) over 2–4 h at room temperature. Incubation of larvae in anti-p26 antibody diluted 1:500 in PBSAT for 1 h at room temperature, then overnight at 4°C, was followed by three washes in PBSAT and 2 h at room temperature in FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:200 in PBSAT. Preparations were washed in PBS as just described, rinsed in distilled $H_2O$, and placed in 0.001 $\mu$g/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR) for 5 min, after which they were rinsed in distilled $H_2O$, mounted on glass slides in a drop of Vectashield (Vector Laboratories, Burlingame, CA), covered with a coverslip, and sealed. Fluorescent staining was visualized with either a Leitz Aristoplan fluorescence microscope or a Zeiss confocal laser scanning microscope.

For immunofluorescent staining of nuclei, encysting embryos and cysts were crushed gently on poly-L-lysine-coated slides and fixed immediately, either in methanol at −20°C for 5–10 min or in 4% (w/v) paraformaldehyde, initially at 40°C but allowed to cool at room temperature while fixation was in progress. Incubation with primary and secondary antibodies was at room temperature for 45 min. The remainder of the procedure was as described for larvae.

***Heat shock of Artemia larvae and adults.*** Synchronized first-instar larvae, derived either ovoviviparously or oviparously, were heat shocked 60 min at 0.5°C intervals over the range of 38.5–42.5°C. The larvae were incubated 48 h at room temperature, after which live (motile) and dead (nonmotile) animals were counted. Data from three experiments were pooled and the percentage of live animals was plotted against temperature. To determine if p26 synthesis was induced by heat shock, larval and adult *Artemia* were incubated at 39°C for 60 min, followed by 30 min at room temperature. The animals were homogenized and similar amounts of protein from each treatment were electrophoresed in SDS–polyacrylamide gels, blotted to nitrocellulose, and probed with antibody to p26.

***Thermotolerance in bacteria expressing p26.*** The cDNA p26-3-6-3 (Liang *et al.*, 1997b) was excised from Bluescript SK(−) with *Bam*HI and *Xho*I and ligated into linearized pRSETC (Invitrogen, San Diego, CA). The recombinant construct, termed pRSET-p26-3-6-3, was propagated in XL1-Blue RF′ and its identity confirmed by sequencing. For expression, pRSET-p26-3-6-3 was transformed into BL21(DE3) (Novagen, Madison, WI) (Studier *et al.*, 1990) using a

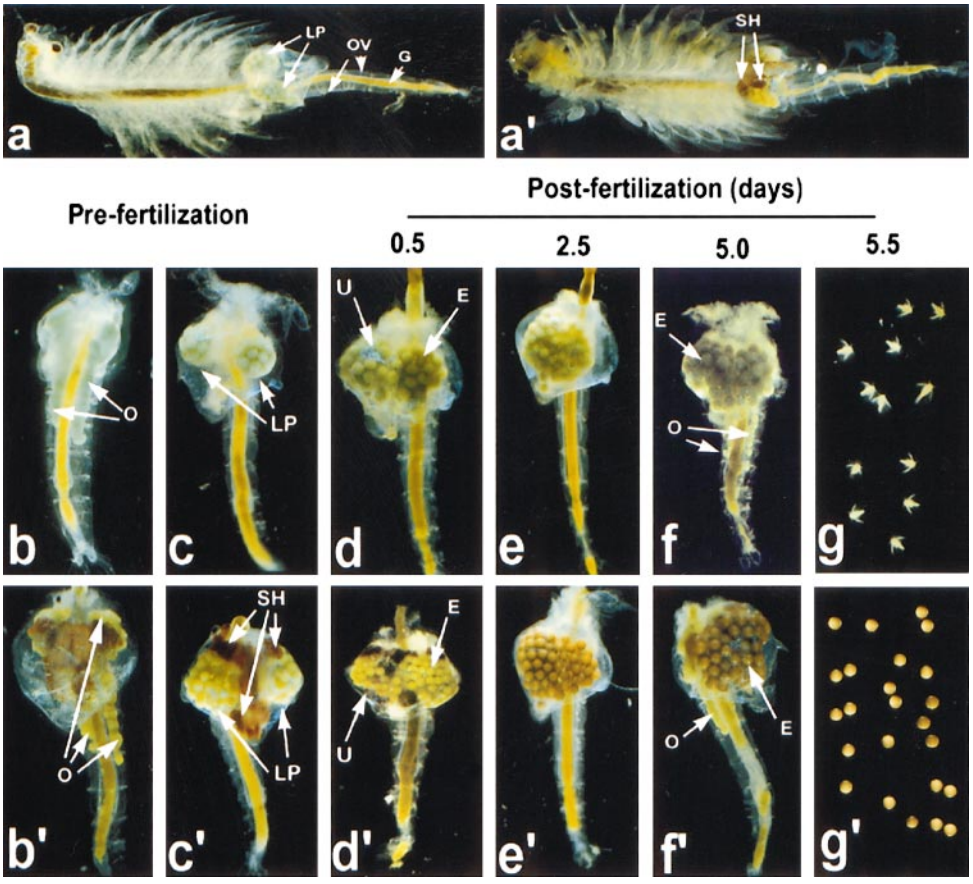


**FIG. 2.** Ovoviviparous and oviparous development in *Artemia.* Samples were fixed in paraformaldehyde and examined by light microscopy. Females with oocytes programmed to develop ovoviviparously (a) and oviparously (a′) were photographed just before fertilization. (b–f and b′–f′) Reproductive organs with either oocytes or embryos destined to become nauplii (g) and cysts (g′). E, embryo; G, gut; O, oocyte; LP, lateral pouch; OV, ovary; SH, shell gland; U, uterus.

standard protocol (Sambrook *et al.*, 1989). Cells from single colonies of the pRSET-p26-3-6-3 and pRSET transformants were cultured overnight at 37°C in 1.0 ml of LB medium supplemented with ampicillin at 50 $\mu$g/ml. A half-milliliter of each culture was diluted 1:5 in fresh LB medium containing 2 mM isopropylthio-$\beta$-galactosidase (IPTG) and incubated at 37°C for 5 h. One milliliter from each culture was centrifuged for 10 s and the pellet was resuspended in 200 $\mu$l of denaturing buffer (8 M urea, 200 mM sodium phosphate, 500 mM NaCl, pH 7.8), placed at room temperature for 30 min, frozen and thawed two or three times, and centrifuged at 12,000*g* for 20 s. Five-microliter samples of the supernatant were electrophoresed in SDS–polyacrylamide gels, blotted to nitrocellulose, and probed with antibodies to p26. To examine thermotolerance, BL21(DE3) carrying either pRSET-p26-3-6-3 or pRSET C was cultured overnight at 37°C in 2.0 ml of LB medium containing ampicillin and IPTG. One-half milliliter of each culture, diluted 1:10 in fresh medium, was transferred to 50°C, after which 50-$\mu$l samples were removed at 0, 15, 30, and 60 min and plated on LB agar in duplicate for overnight incubation at 37°C. The 0-min samples were diluted before plating. Colonies were counted and the data from three experiments were pooled.

## RESULTS

### *Ovoviviparous and Oviparous Development in Artemia*

*Artemia* embryos develop either ovoviviparously (directly into nauplii) or oviparously (into encysted diapause gastrulae) and the pathway that is followed can be determined by microscopic examination of oocytes (Fig. 2). *Artemia* females containing mature oocytes within lateral pouches (Figs. 2a and 2a′), and the changes that occur from a half-day before fertilization until the release of free-swimming nauplii (first-instar larvae) (Figs. 2b–2g) and cysts (Figs. 2b′–2g′) are shown. Ovoviviparous development was signaled by green oocytes, the color discernible before fertilization, and the absence of a visible shell gland (Figs. 2a–2c). In contrast, yellow oocytes and a brown shell gland signified oviparous development (Figs. 2a′–2c′). Fertilization usually occurred within 6 h of the time oocytes moved from the ovaries into the paired lateral pouches (Figs. 2c and

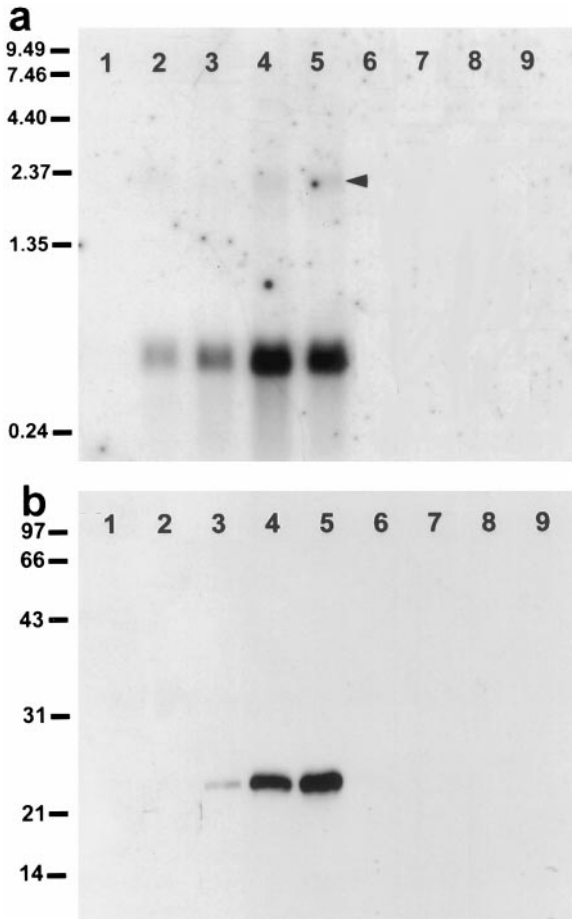


**FIG. 3.** Synthesis of p26 during ovoviviparous and oviparous development. Approximately 200 *Artemia* embryos or larvae, collected at 24-h intervals for 5 days postfertilization, were homogenized in Trizol solution. (a) Northern blot with 2 $\mu$g of total RNA from each developmental stage hybridized to $^{32}$P-labeled p26-3-6-3. (b) Equal volumes of cell-free extract from each developmental stage were transferred to nitrocellulose after electrophoresis in SDS–polyacrylamide gels and probed with antibody to p26 by the ECL procedure. Lanes 1–5 received samples from cyst-destined embryos developed 1–5 days, respectively, while lanes 6–9 contain samples from nauplius-destined embryos 2–5 days postfertilization, respectively. The arrowhead in a indicates an mRNA band of about 1.9 kb. Size markers in kb $\times 10^{-3}$ (a) and molecular weight $\times 10^{-3}$ (b) are to the left.

2c′) and was indicated by formation of one egg mass in the uterus (Figs. 2d and 2d′). Embryos experiencing ovoviviparous development changed color from green to light yellow (Figs. 2c–2f), while those undergoing encystment displayed brown shells, structures that failed to otherwise appear. Clearly, oocytes in each of the alternative developmental modes were easily identified, and progress from fertilization to release of progeny (Figs. 2g and 2g′) could be timed precisely.

### *p26 Gene Expression Occurred Only in Encysting Artemia Embryos*

p26 mRNA, approximately 0.7 kb in size, first appeared in 2-day embryos undergoing oviparous development and increased to maximal levels by day 4 (Fig. 3a, lanes 1–5). A faint band of about 1.9 kb was observed in RNA samples from day 4 and 5 embryos. In contrast, p26 mRNA was never detected during direct development into nauplii (Fig. 3a, lanes 6–9). Immunostaining of Western blots revealed p26 in cell-free extracts prepared from embryos 3 days postfertilization and it increased through to day 5 (Fig. 3b, lanes 1–5). No p26 protein was found in organisms that developed ovoviviparously (Fig. 3b, lanes 6–9).

### *Migration of p26 into Nuclei during Embryo Encystment*

Nuclei from encysting embryos were fluorescently stained with anti-p26-antibody and examined with the confocal microscope in order to determine when p26 first entered. At day 3 postfertilization, when p26 was initially visible on Western blots, many nuclei exhibited a diffuse

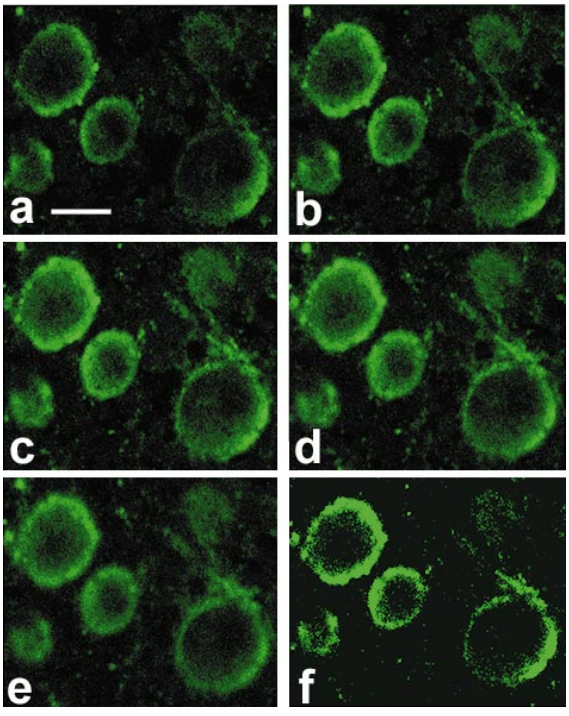


**FIG. 4.** p26 entered nuclei of encysting embryos soon after it was synthesized. Nuclei from cyst-destined embryos 3 days after fertilization were stained with antibody to p26 and examined with the confocal microscope. (a–e) Individual optical sections of stained nuclei; (f) composite image obtained by superimposing images a–e. The bar in a represents 5 $\mu$m and all images are the same magnification.

circumferential ring of fluorescence (Fig. 4), indicating that p26 was translocated across the membranes of these organelles soon after synthesis. By day 5 postfertilization, when cysts were released from females, immunofluorescent staining revealed that p26 was also associated with an unidentified fibrous material located exterior to nuclei (Fig. 5). The fibers were not observed when primary antibody was omitted from staining protocols.

### *p26 mRNA and Protein Disappeared during Postgastrula Development of Artemia*

p26 mRNA and protein were monitored upon reinitiation of cyst development by analyses of cell-free extracts on Northern and Western blots. The amount of p26 mRNA decreased rapidly and had all but disappeared from emerged, membrane-enclosed nauplii (Fig. 6a). On the other hand, p26 protein was unchanged for 10 h (Fig. 6b), then underwent a rapid reduction. As a consequence, cell-free extracts of emerged nauplii and first-instar larvae had less p26 than cysts, although significant amounts of the protein remained. Cell-free extracts of second-instar larvae lacked detectable p26 (Fig. 6b). The decline in p26 was preceded by the loss of ribosome-associated p26 mRNA (Fig. 6c).

### *Nuclear Localization of p26 during Postencystment Development of Artemia*

Immunofluorescent staining revealed that most nuclei in emerged, membrane-enclosed nauplii and first-instar larvae contained p26 (Figs. 7 and 8). Notably, in emerged nauplii, nuclei in the basolateral region of cells neighboring the gut lumen were stained with anti-p26, as was an apical, nucleus-free layer within the same cells (Figs. 7a and 7a′). In contrast, nuclei in portions of gut tubes from first-instar larvae lacked p26 (Figs. 8a and 8a′), and the number of antibody-reactive nuclei declined as larvae progressed to second instar (Fig. 8). Reduced staining coincided with the decrease of p26 in cell-free extracts (Fig. 6). Although p26 was no longer observed on Western blots of protein extracts from late instar II larvae, immunofluorescent staining revealed its presence in five to seven salt gland nuclei (Figs. 8 and 9), and the number further decreased to one or two nuclei by the end of instar II. The salt gland nuclei were the last place p26 was found, and it was not detected in

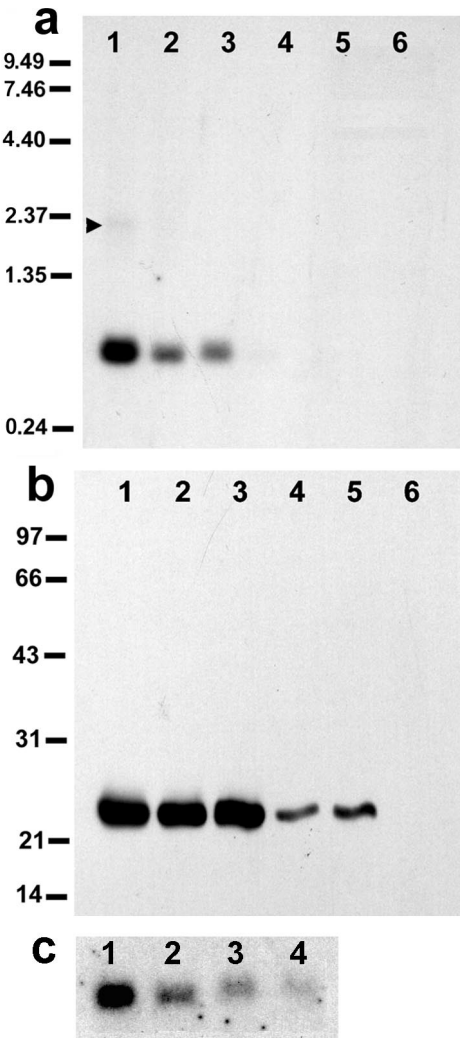


**FIG. 6.** p26 mRNA and protein disappeared during postgastrula development of *Artemia*. Total RNA (a) and protein (b) from encysted *Artemia* incubated for 0 (1), 5 (2) and 10 h (3) and from emerged nauplii (4), instar I larvae (5), and instar II larvae (6). (a) Northern blots with 2 $\mu$g of total RNA from each developmental stage were hybridized to $^{32}$P-labeled p26-3-6-3. (b) Western blots containing equal volumes of cell-free extract from each developmental stage were probed with antibody to p26 by the ECL procedure. (c) Northern blots of ribosome-associated mRNA from *Artemia* cysts incubated 0 (1), 3 (2), 6 (3), and 10 h (4) were hybridized to $^{32}$P-labeled p26-3-6-3. The arrowhead in a indicates an mRNA band of about 1.9 kb. Size markers in kb $\times 10^{-3}$ and molecular weight $\times 10^{-3}$ are to the left of a and b, respectively.

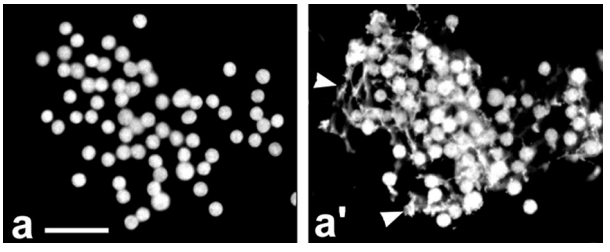


**FIG. 5.** p26 within cysts was associated with extranuclear fibers. Samples obtained by gently squashing *Artemia* cysts on glass slides were double stained with DAPI (a) and antibody to p26 (a′). The arrowheads in a′ indicate fluorescently stained fibrous material. The bar in a represents 50 $\mu$m and both images are the same magnification.

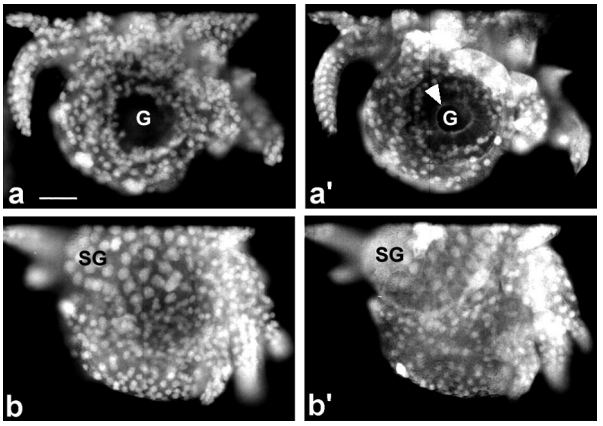


**FIG. 7.** Localization of p26 in emerged, membrane-enclosed nauplii. Emerged nauplii, fixed in paraformaldehyde, were double stained with DAPI (a, b) and antibody to p26 (a′, b′). G, gut; SG, salt gland. The arrowhead in a′ indicates a ring of p26 fluorescence at the apical surface of gut cells. The bar in a represents 20 $\mu$m and both pictures are the same magnification.

third-instar larvae by immunofluorescence microscopy (not shown).

### *Stress Tolerance in Artemia Larvae and Escherichia coli Containing p26*

Oviparously produced, first-instar larvae were more heat resistant than those that developed ovoviviparously and lacked p26 (Fig. 10). Specifically, 50% of first-instar ovoviviparous larvae were killed in 1 h at 40°C, whereas equivalent mortality of p26-endowed larvae was achieved at 41.8°C. However, heat shock neither increased the amount of p26 in cell free extracts of first-instar larvae nor induced its synthesis in instar III larvae and adults under the conditions used in this study (Fig. 11). To further test the relationship between p26 and thermotolerance, *E. coli* BL21(DE3) was transformed with an expression vector containing p26-3-6-3. A fusion protein of approximately 32 kDa (calculated molecular mass of 26 kDa), recognized on Western blots by antibody to p26, was synthesized by bacteria carrying the recombinant plasmid, pRSET-p26-3-6-3 (Figs. 12a and 12b). Compared to cells transformed with pRSET lacking a cDNA insert, these bacteria were significantly more resistant to increased temperature (Fig. 12c), demonstrating that p26 conferred thermotolerance *in vivo*.

## DISCUSSION

Morphological criteria indicate that *Artemia* oocytes commit to encystment and diapause before fertilization, in agreement with those of Jackson and Clegg (1996). Although the signal(s) that triggers oviparous development is unknown, activation of the p26 gene takes place, and synthesis of p26 is restricted to *Artemia* embryos that encyst. Moreover, p26 is not induced by heat shock, at least under the conditions used herein, indicating that its synthesis is controlled in response to developmental cues. In comparison, synthesis of MKBP, a small heat shock/$\alpha$-crystallin protein in mammalian muscle, is not promoted by heat shock (Suzuki *et al.*, 1998) nor are Ha hsp17.6 G1 from sunflower (Carranco *et al.*, 1997) and HSP12.6 from *Caenorhabditis elegans* (Leroux *et al.*, 1997b). Tissue- and stage-specific expression of small heat shock/$\alpha$-crystallin proteins occurs in several organisms other than *Artemia*, including *Drosophila*, nematodes, *Xenopus*, mouse, human, and plants (Michaud *et al.*, 1997a,b; Leroux *et al.*, 1997b; Loones *et al.*, 1997; Heikkila *et al.*, 1997; Carranco *et al.*, 1997; Linder *et al.*, 1996; Heikkila, 1993; de Jong *et al.*, 1993). Within this group, p26 is the only small heat shock/$\alpha$-crystallin protein thought to have a role in embryo

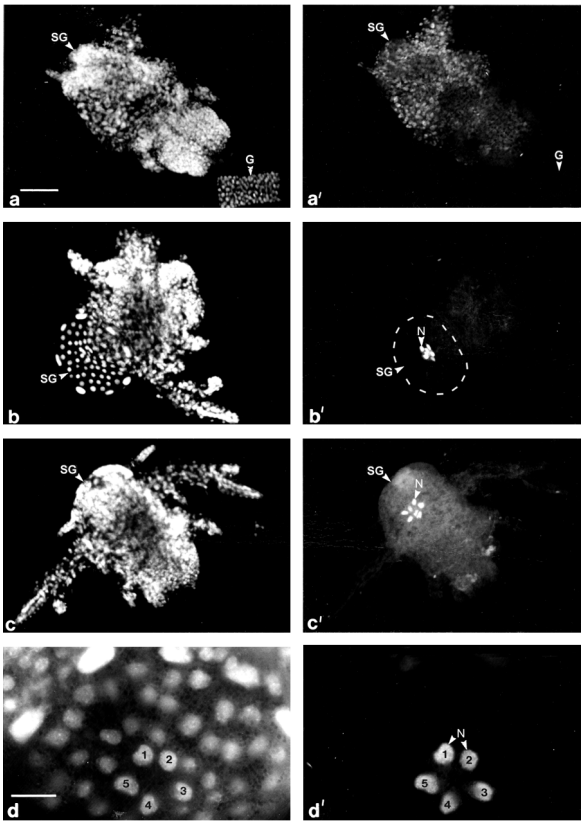


**FIG. 8.** Localization of p26 in *Artemia* larvae. Instar I (a, a′) and late instar II (b, b′–d, d′) *Artemia* larvae, fixed in paraformaldehyde, were double stained with DAPI (a–d) and antibody to p26 (a′–d′). The location of the salt gland in b′ is indicated by the dashed lines. (d and d′) A salt gland with corresponding nuclei indicated by the same number. G, gut; SG, salt gland. The bar in a represents 100 $\mu$m and a, a′–c, c′ are the same magnification. The bar in d represents 15 $\mu$m and it is the same magnification as d′.

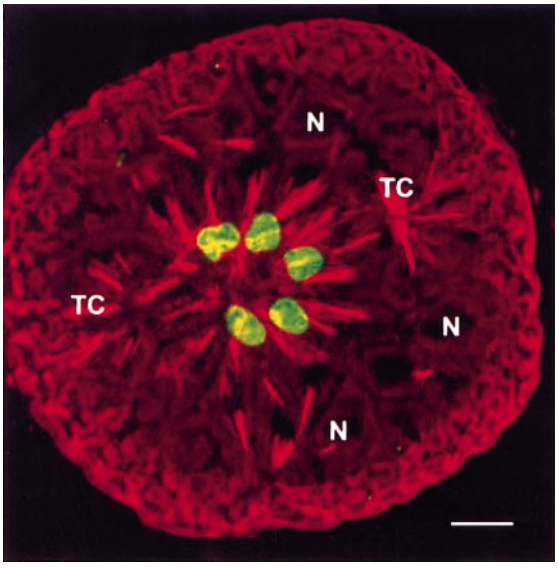


**FIG. 9.** p26 was last observed in a subset of salt gland nuclei in instar II *Artemia* larvae. A salt gland from an instar II larva double stained with rhodamine phalloidin (red) and antibody to p26 (green). The image was constructed by superimposing optical sections obtained by confocal microscopy. N, unstained nucleus; TC, tendinal cell. The bar represents 10 $\mu$m.

encystment and diapause. It therefore provides an interesting opportunity to study the synthesis and function of small heat shock/$\alpha$-crystallin proteins during development.

Probing of Northern blots demonstrated that synthesis of p26 mRNA, first seen 2 days postfertilization, is transcriptionally controlled. p26 protein, analyzed in the same cell-free extracts as those used to study mRNA, appears 3 days after fertilization, supporting work by Jackson and Clegg (1996). Either there is a delay in translation or the relative sensitivities of the methods permit detection of smaller amounts of mRNA than protein. The synthesis of p26 message and protein precedes diapause, both are abundant in activated cysts, and both decline rapidly once postgastrula development begins. These dynamics indicate a role for p26 during *Artemia* encystment and/or diapause. However, because p26 is present as gastrulae form, and encysted embryos containing large amounts of p26 resume development, this protein is seemingly not directly responsible for the cessation of metabolism that accompanies diapause.

Many small heat shock/$\alpha$-crystallin proteins associate with nuclei, either in a developmentally regulated but stress-independent manner or when exposed to stress, returning to the cytoplasm during recovery (Michaud *et al.*, 1997a,b; Loktionova *et al.*, 1996; de Jong *et al.*, 1993; Groenen *et al.*, 1994; Jakob and Buchner, 1994). Previous work demonstrated that p26 translocates into the nuclei of encysted *Artemia* embryos by way of a pH-dependent mechanism (Liang *et al.*, 1997b; Jackson and Clegg, 1996; Clegg *et al.*, 1995, 1994). These observations are extended in this paper and p26 is shown to enter nuclei during encystment, very soon after its synthesis. The localization of p26 within the entire peripheral region indicates that transport occurs over the complete nuclear surface, presumably at the pores. Upon cyst release from females, but prior to diapause and desiccation, p26 distributes throughout the nucleus. Thus, p26 has the potential to modulate transcription, contributing indirectly to the cessation of metabolic activity in encysted gastrulae. Alternatively, p26 may inhibit mitosis and cytokinesis in cysts, both of which resume only at hatching (Olson and Clegg, 1978; Nakanishi *et al.*, 1962), the same time p26 diminishes. Related to these observations, the salt gland (neck organ) is the last repository of p26. The salt gland is a dome-like structure composed of 50–60 cuboidal epithelial cells situated on the cephalothorax (Criel, 1991; Hootman and Conte, 1975). Osmotic balance in larvae is modulated by the salt gland, but the organ is missing from adults, in which the gut assumes this task. Exit of p26 from nuclei is, therefore, slowest in a group of cells that never resume division and eventually disappear. Of interest in this context, during the differentiation of murine embryonic stem cells a transient increase in HSP27 accompanies growth arrest (Mehlen *et al.*, 1997).

First-instar larvae, arising from cysts and containing p26, are more thermotolerant than larvae that develop directly from fertilized oocytes. The heat-induced death of ovovi-

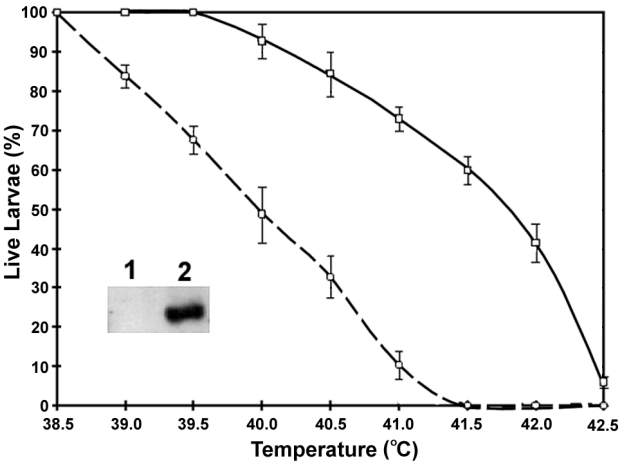


**FIG. 10.** Enhanced thermotolerance of *Artemia* larvae that contain p26. Instar I larvae, either released from females as free-swimming nauplii (dashed line) or derived from cysts (solid line), were incubated in batches of 40–60 organisms for 1 h at temperatures from 38.5 to 42.5°C. The larvae were then incubated at room temperature for 48 h and the percentage survivors (moving organisms) was plotted against temperature of heat shock. (Inset) Immunodetection of p26 on Western blots of cell-free extract protein from larvae used in these experiments and that developed either directly from fertilized oocytes (1) or from cysts (2). Results are the averages of three independent experiments ± the standard deviation.

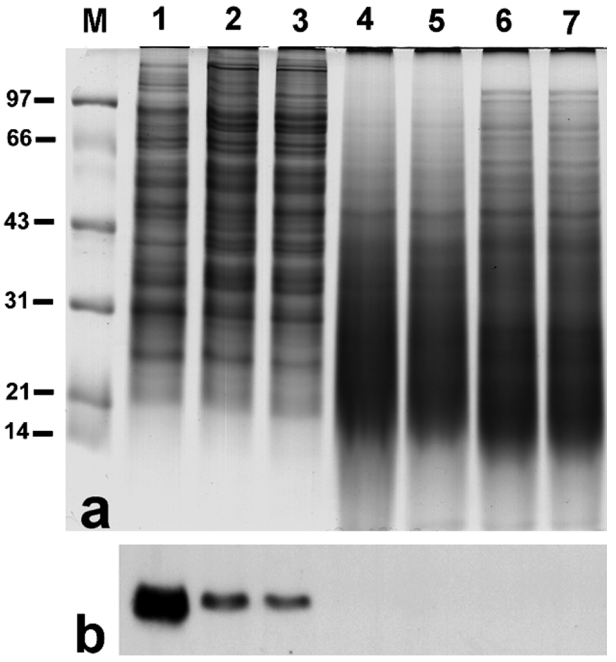


**FIG. 11.** Synthesis of p26 was not induced in *Artemia* by heat shock. Cell-free homogenates from larvae (cyst-derived) and adults, incubated at room temperature (lanes 2, 4, and 6) and heat shocked at 39°C for 60 min (lanes 3, 5, and 7), were electrophoresed in SDS–polyacrylamide gels and either stained with Coomassie blue (a) or blotted to nitrocellulose and stained with antibody to p26 by the ECL procedure (b). Oocytes and developing embryos were removed from adults before homogenization. Lanes: 1, cell free extract from dormant, hydrated cysts; 2 and 3, instar I larvae; 4 and 5, instar III larvae; 6 and 7, adults; M, molecular weight markers $\times 10^{-3}$.

viparously derived nauplii examined in this study was almost identical to the mortality obtained by Miller and McLennan (1987) for cyst-derived nauplii. However, in their experiments, animals were incubated for 24 h before heat shock and would have had very little, if any, p26. Cyst-derived instar II nauplii, with p26 in only a few salt gland nuclei, were no more resistant to heat shock than instar I and II larvae of ovoviviparous origin (data not shown). The results suggest a role for p26 in stress resistance, but instar I larvae arising by alternative pathways undoubtedly have other differences. These include additional heat shock proteins (McLennan and Miller, 1990; Miller and McLennan, 1988a,b) and perhaps organic molecules such as glycerol and trehalose, the latter reported to enhance stress tolerance in yeast (Lee and Goldberg, 1998; McLennan and Miller, 1990).

A direct demonstration of p26-dependent thermotolerance *in vivo* is that bacteria expressing this protein are more heat resistant than those that lack p26. Similarly, the small heat shock/$\alpha$-crystallin proteins, HSP16-2 from *C. elegans* (Leroux *et al.*, 1997a), Oshsp16.9 from rice (Yeh *et al.*, 1997), and human $\alpha$-B crystallin (Muchowski and Clark, 1998), enhance heat resistance of *E. coli.* The extra peptide at the N-terminus of recombinant p26 appears not to affect thermotolerance, reflecting observations by Leroux *et al.* (1997a) for HSP16-2. As one possibility, there may be sufficient space within p26 multimers, thought to be the functional form of the protein, to accommodate extraneous peptide and still permit chaperone activity.

To summarize, data in this paper, when considered with the demonstration of ATP-independent chaperone activity of p26 *in vitro* (Liang *et al.*, 1997a), indicate that p26, a protein synthesized during encystment, protects macromolecules within *Artemia* embryos from irreversible denaturation. As proposed for other small heat shock/$\alpha$-crystallin proteins (Leroux *et al.*, 1997a; Lee *et al.*, 1997, 1995; Ehrnsperger *et al.*,1997), p26 may have chaperone-like activity; it forms oligomers that bind denaturing proteins within oviparous embryos, preventing their aggregation during encystment, diapause, and desiccation. When growth resumes, proteins release from p26, either to other chaperones for assisted folding or for spontaneous renaturation. Thus, embryos tolerate severe stress, and events lethal to most organisms are part of the normal physiological activities of encysted *Artemia.* Moreover, embryos remain primed to exploit favorable environmental conditions even in the face of potentially small amounts of

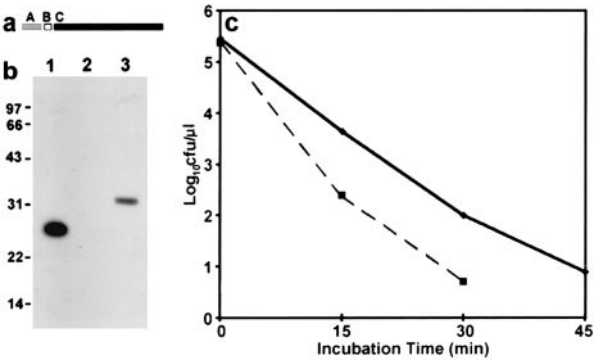


**FIG. 12.** Bacteria expressing recombinant p26 exhibited enhanced thermotolerance. (a) Schematic representation of the fusion protein encoded by pRSET-p26-3-6-3: A, $(His)_6$EK encoded by the plasmid, 3.7 kDa; B, PRAAGIRHELVLK, a product of the 5′-noncoding region of p26-3-6-3 and a short DNA fragment from pRSET, 1.5 kDa; C, full-length p26 encoded by p26-3-6-3, 20.8 kDa. (b) Cell-free protein extract from *Artemia* cysts (lane 1) and lysates from *E. coli* BL21(DE3) carrying pRSET (lane 2) and pRSET-p26-3-6-3 (lane 3) grown in the presence of IPTG were electrophoresed in SDS–polyacrylamide gels, blotted, and immunostained with antibody to p26. Molecular weight markers $\times 10^{-3}$ are on the left. (c) Cultures of *E. coli* BL21(DE3) carrying either pRSET (dashed line) or pRSET-p26-3-6-3 (solid line) were incubated at 53°C for the indicated times, plated on LB agar, and incubated overnight at 37°C. Colonies were counted and $\log_{10}$ values of colony-forming units per microliter (cfu/$\mu$l) were plotted against time of heat shock. Results are the averages of three independent experiments.

intracellular ATP that are the legacy of diapause. As such, p26 would be the first molecular chaperone shown to have a role in diapause, expanding the known functions of these proteins and suggesting a similar role in other organisms.

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