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Developmentally regulated synthesis of p8, a stress-associated transcription cofactor, in diapause-destined embryos of *Artemia franciscana*

Zhijun Qiu and Thomas H. MacRae

Department of Biology, Dalhousie University, Halifax, NS B3H 4J1 Canada

Abstract Diapause-destined embryos of the crustacean *Artemia franciscana* arrest as gastrulae, acquire extreme stress tolerance, and enter profound metabolic dormancy. Among genes upregulated at 2 days postfertilization in these embryos is a homologue of p8, a stress-inducible transcription cofactor. *Artemia* p8 is smaller than vertebrate homologues but shares a basic helix-loop-helix domain and a bipartite nuclear localization signal. Probing of restriction digested DNA on Southern blots indicated a single *Artemia* p8 gene and 5′-RACE specified 2 transcription start sites. Several putative cis-acting regulatory sequences, including two heat shock elements, appeared upstream of the p8 transcription start site. *Artemia* p8 mRNA increased sharply at 1 day postfertilization in diapause-destined embryos and then declined, whereas p8 protein appeared 2 days postfertilization and remained relatively constant throughout development, indicating a stable protein. p8 was not detectable in nauplius-destined (nondiapause) *Artemia* embryos. Immunofluorescent staining revealed p8 within *Artemia* nuclei. The results support the idea that p8, a known stress-responsive transcription cofactor, mediates gene expression in diapause-destined *Artemia* embryos. p8 is the first diapause-related transcription factor identified in crustaceans and 1 of only a small number of such proteins identified in any organism undergoing diapause.

INTRODUCTION

Diapause, which occurs in many different organisms, is characterized by reduced cell growth, inhibition of development, and enhanced stress resistance (Denlinger 2002; Lopes et al 2004; MacRae 2005; Baumeister et al 2006; Koštál 2006), properties that are dependent on differential gene expression. As 1 example, embryos of the crustacean *Artemia franciscana* undergo diapause, arresting as gastrulae, encysting, and entering a deep state of metabolic dormancy (MacRae 2003). The encysted embryos (cysts) are extremely stress tolerant (Clegg 1997; Clegg et al 2000), a property thought to depend on their rigid chitinous walls (Anderson et al 1970), limited consumption of energy stores (Clegg 1997; Clegg and Jackson 1998), trehalose (Viner and Clegg 2001), and molecular chaperones (Sun et al 2004, 2006; Sun and MacRae 2005; Qiu et al 2006; Villeneuve et al 2006; Chen et al 2007). *Artemia* cysts and their postdiapause development are relatively well characterized, but little is known about the molecular events modulating cell growth and metabolism during diapause.

In work to be reported separately, subtractive hybridization was employed to identify upregulated genes in *Artemia* embryos undergoing diapause. Among these was the gene for a homologue of mammalian p8, also called candidate of metastasis-1 (com 1) (Ree et al 1999; Bratland et al 2000), first observed in rat acinar cells because its synthesis is enhanced during acute pancreatitis (Mallo et al 1997). p8 possess a basic helix-loop-helix motif characteristic of DNA binding proteins (Massari and Murre 2000; Jones 2004) and a bipartite, nuclear targeting signal (Mallo et al 1997; Ree et al 1999; Vasseur et al 1999a;

Correspondance to: Thomas H. MacRae, Tel: 902-494-6525; Fax: 902-494-3736; E-mail: tmacrae@dal.ca.

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Igarashi et al 2001; Valacco et al 2006), reflecting its localization within nuclei (Goruppi and Kyriakis 2004; Jiang et al 2005, 2006; Valacco et al 2006). p8 shares properties with the high mobility group (HMG) proteins even though sequence similarity is low and the AT hook motif is absent. Like HMG proteins, p8 is mainly random coil or unstructured in solution and binds DNA in a sequence-independent manner, an activity enhanced by protein kinase A–mediated phosphorylation and resulting in protein structural stabilization (Encinar et al 2001; Malicet et al 2006a). p8 is a transcription cofactor and it influences transforming growth factor β-1 (TGFβ-1) activation of the Smad transcription factor (Garcia-Montero et al 2001). p8 also activates the glucagon gene by promoting Pax2A and Pax2B function, in part by recruitment of p300 (Hoffmeister et al 2002), and it may modulate luteinizing hormone β (LHβ) gene expression in the gonadotrope as a stage-specific transcriptome member with an architectural role (Quirk et al 2003).

To summarize, *Artemia* p8 shares extensive sequence similarity with this protein from other organisms and, although smaller in size due to an amino-terminal deletion, *Artemia* p8 is a basic helix-loop-helix transcription cofactor with a bipartite nuclear localization signal. *Artemia* embryos developing directly into nauplii fail to express p8, whereas diapause-destined embryos exhibit a dramatic postfertilization increase in p8 mRNA, followed by appearance of the corresponding protein. p8 mRNA declines during embryo development, reaching very low levels prior to cyst release, but the protein remains and is found in postdiapause cysts where it localizes to nuclei. This pattern of synthesis, in concert with nuclear localization, suggests p8 influences development of diapause-destined *Artemia* embryos. This is the first indication that p8, a stress-associated transcription cofactor known to modulate cell growth, division, and apoptosis, influences gene expression in organisms negotiating the physiological processes characteristic of diapause.

MATERIALS AND METHODS

Artemia culture

A. franciscana cysts (INVE Aquaculture, Inc., Ogden, UT, USA) were hydrated overnight in distilled water at 4°C, rinsed 3 times with water, and either homogenized or incubated at 27°C for 20 hours with shaking at 200 RPM in hatch medium (Langdon et al 1990). Synchronous emerged nauplii (E_2) were either homogenized or allowed to develop into instar I and II larvae (Liang and MacRae 1999). Adult *Artemia* were maintained at room temperature in filtered, aerated seawater and embryos were collected at 1-day intervals postfertilization (Qiu et al 2006).

p8 cDNA cloning

Full-length p8 cDNA was generated by 5′- and 3′-RACE using primers based on a partial p8 sequence obtained by subtractive hybridization. For 5′-RACE, total RNA from diapause-destined embryos 2 days postfertilization was prepared with TRIzol (Invitrogen, Burlington, ON, Canada). Ten micrograms of RNA were incubated with calf intestine alkaline phosphatase (Ambion, Inc., Austin, TX, USA) at 37°C for 1 hour and purified with acid phenol:chloroform. The RNA then was treated with tobacco acid pyrophosphatase (Ambion) at 37°C for 1 hour, ligated to the 5′-RACE adaptor (5′-GCUGAUGGCGAUGAA CACUGCGUUUGCUGGCUUUGAUGAAA-3′) using T4 RNA ligase (Ambion), and reverse transcribed at 42°C for 1 hour in a mixture containing random decamers, M-MLV reverse transcriptase (Ambion), and dNTP. Nested polymerase chain reaction (PCR) was performed with p8 outer primer (5′-CACCAAGAGCCCTACATGTTGC TA-3′), p8 inner primer (5′-TCGGAAGTCCGGGACCTA TAGAAT-3′), adaptor outer primer (5′-GCTGATGGCGAT-GAATGAACACTG-3′), and adaptor inner primer (5′-CG CGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′). For 3′-RACE, the p8 outer (5′-AGGTAGTAGGATCAGG TGTGACGA-3′) and inner primers (5′-ACCCTAGTG GACACTCAAAGAAAGC-3′), and adaptor outer (5′-GC GAGCACAGAATTAATACGACT-3′) and inner primers (5′-CGCGGATCCGAATTAATACGACTCACTCACTATA GG-3′) were used. RACE PCR conditions were 94°C for 3 minutes then 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by 72°C for 7 minutes. The PCR products were inserted into the TA cloning vector pCR2.1 (Invitrogen) and used to transform TOPO10 competent *Escherichia coli* (Invitrogen) prior to sequencing (DNA Sequencing Facility, Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada).

To obtain full-length p8 cDNA, RNA was prepared from diapause-destined embryos at 2 days postfertilization and genomic DNA was eliminated with the TURBO DNA-free kit (Ambion). RT-PCR was performed with Pfu DNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada); forward primer (5′-GCGGATCCATGTCA GAAGATCATTTTGATA-3′), including a BamHI site; and reverse primer (5′-CGCTGCAGAGTCATTTTTTGTCA GCACG-3′), including a PstI site. PCR was at 95°C for 2 minutes, followed by 30 cycles of 95°C for 1 minute, 56°C for 45 seconds, and 72°C for 1 minute, with extension at 72°C for 5 minutes. After addition of adenosine with the A-Addition kit (Qiagen, Mississauga, ON, Canada), RT-PCR products were inserted into the T/A cloning vector pCR2.1 (Invitrogen), followed by transformation of TOPO10 *E. coli* (Invitrogen). Plasmid DNA was digested with BamHI and PstI. cDNA inserts purified with the

GFX PCR and Gel Band Purification Kit (Amersham Bioscience, Baie d'Urfe, Quebec, Canada), were ligated into the His-tagged pRSET A vector (Invitrogen) and transformed into TOP 10 F′ *E. coli* (Invitrogen).

Artemia p8 gene

p8 cDNA was labeled with digoxigenin-11-dUTP by using the PCR Dig Labeling Mix (Roche, Mannheim, Germany) with the forward primer (5′-GCGGATCCATGTC AGAAGATCATTTTGATA-3′) and the reverse primer (5′-CGCTGCAGAGTCATTTTTTGTCAGCACG-3′). PCR conditions were 95°C for 2 minutes followed by 35 cycles of 95°C for 1 minute, 60°C for 30 seconds, and 72°C for 45 seconds. The products were purified with the GFX PCR and Gel Band Purification Kit (Amersham Bioscience). Fifteen-microgram samples of genomic DNA (Qiu et al 2006) were digested with XhoI, PstI, SalI, BamHI, and HindIII and then precipitated with ethanol, dissolved in 20 μL of TE buffer, and electrophoresed in 0.7% agarose. Before DNA transfer to nylon membranes, the gels were immersed twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes, followed by two 15-minute incubations in neutralization solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) and 10 minutes in 20 × standard saline citrate ([SSC], 0.3 M $C_6H_5Na_3O_7$, 3.0 M NaCl, pH 7.0). Membranes were washed in 2 × SSC, baked at 80°C for 2 hours, prehybridized in Dig Easy Hyb (Roche) at 42°C for 6 hours, and hybridized with probes overnight at 43°C. Membranes were washed twice at room temperature in 2 × SSC containing 0.1% sodium dodecyl sulfate (SDS) and twice at 68°C in 0.5 × SSC containing 0.1% SDS and stained with CDP-Star (Roche).

Genomic DNA digested overnight at 37°C with Sau3AI, SalI, EcoRI, HindIII, PstI, and XbaI, and the fragments were ligated at 16°C for 30 minutes into cassettes designed for LA PCR in vitro cloning (TaKaRa, Otsu, Japan). Nested PCR was performed with primers C1 (5′-GT ACATATTGTCGTTAGAACGCGTAATACGACTCA-3′), S1 (5′-CAGCCTTCCACTTTGCCCGGAGAAGAGATG-3′), C2 (5′-CGTTAGAACGCGTAATACGACTCACTATAGGGAGA-3′), and S2 (5′-TTGTCCATATCAAAGTTAAAGTGTTCAA ATCTATC-3′). C1 and C2 were based on cassette sequence, whereas S1 and S2 were designed on p8 cDNA sequence. Reaction conditions were 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 2 minutes, followed by 72°C for 5 minutes. PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced, and analyzed for cis-acting sites (www.genomatrix.de; Cartharius et al 2005).

p8 mRNA quantification

For quantification of p8 transcripts RNA prepared at daily intervals from *Artemia* embryos was reverse transcribed and quantitative PCR was performed with the iCycler (Bio-Rad, Mississauga, ON, Canada) in 25-μL mixtures containing 0.5 μL of cDNA, 12.5 μL of Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), 0.5 μL of Rox reference dye (Invitrogen), 50 ng of forward primer 5′-AGGTAGTAGGAT CAGGTGTGACGA-3′, and 50 ng of reverse primer 5′-TCGGAAGTCCGGGACCTATAGAAT-3′. Reaction conditions were 50°C for 2 minutes, 95°C for 2 minutes, 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, followed by extension at 72°C for 5 minutes. α-tubulin mRNA was amplified as internal control using forward primer 5′-CTGCATGCTG TACAGA GGAGATGT-3′ and reverse primer 5′-CTCCT TCAAGAGAGTCCATGCCAA-3′ (Qiu et al 2006).

Antibody production and p8 detection

E. coli BL21 (DE3) pLysS (Invitrogen) transformed with p8 cDNA were incubated in 1 mM isopropyl β-D-thiogalactopyranoside ([IPTG], Sigma, Oakville, ON, Canada) for 6 hours. p8 protein was purified with BD TALON™ (BD Biosciences Clontech, Mississauga, ON, Canada; Sun et al 2004) and emulsified with TiterMax™ Gold Adjuvant (Sigma). Rabbits from Charles River Canada (St. Constant, Quebec, Canada), cared for in accordance with Guide to the Care and Use of Experimental Animals, available from the Canadian Council on Animal Care, were injected 3 times at 15-day intervals with purified p8. Serum was harvested 45 days after the first injection.

Artemia embryos collected at daily intervals postfertilization were homogenized in TRIzol (Invitrogen) and the homogenate was incubated for 5 minutes at room temperature. Eighty microliters of chloroform were added and the mixture was centrifuged at 12 000 × *g* for 15 min at 4°C. The upper protein-containing layer was mixed with 120 μL of ethanol, incubated at room temperature for 3 minutes, and centrifuged at 2000 × *g* for 5 minutes at 4°C. The supernatant was incubated with 600 μL of isopropyl alcohol for 10 minutes at room temperature and centrifuged at 12 000 × *g* for 10 minutes at 4°C. The pellet was washed 3 times in 95% ethanol, dissolved in 30 μL of 1% SDS, mixed with 4 × treatment buffer, and electrophoresed in 12.5% SDS polyacrylamide gels prior to staining with Coomassie blue or blotting to nitrocellulose (Bio-Rad, Herculus, CA, USA). Blots were incubated with antibody to p8 followed by horseradish peroxidase– (HRP) conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Mississauga, ON, Canada; Liang et al 1997). Immunoconjugates were detected with Western

Ligthning Enhanced Chemiluminescence (ECL) Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA).

Immunostaining of *Artemia* nuclei

Nuclei purified from cysts and instar II larvae (Sun et al 2004) were placed on poly-L-lysine–coated slides, fixed in 4% (W/V) paraformaldehyde for 20 minutes, hydrated in phosphate-buffered saline ([PBS], 140 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 5 minutes, and incubated with p8 antibody for 30 minutes at room temperature. The samples were washed 3 minutes with PBS containing 0.5% bovine serum albumin (Sigma) and 0.75% Triton X-100 ([PBSAT], Sigma), followed by incubation for 30 minutes with fluorescein isothiocyanate– (FITC) conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch). The samples were washed 3 times with PBS for 3 minutes, incubated with 0.001 μg/mL 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 minutes, washed with water for 5 minutes, and mounted in 0.2 M 1,4-diazabicyclo[2.2.2]octane (DABCO) in 80% glycerol. Slides were examined by epifluorescence and confocal microscopy.

RESULTS

Artemia p8 sequence and domain structure

A partial p8 cDNA sequence was obtained by subtractive hybridization in experiments designed to identify genes upregulated in diapause-destined *Artemia* embryos. The cDNA was extended in both directions, yielding a 480 bp product via 3′-RACE and DNA fragments of 240 and 273 bps for the 5′-reaction, the latter suggesting 2 transcription start sites (Fig 1). Full length p8 cDNA generated by RT-PCR contained an open reading frame of 198 bps and a 5′-noncoding sequence of 43 bps, with transcription start sites 10 and 43 bps upstream of the translation start site. The 336 bp 3′-noncoding region included 2 ATTTA sequences indicative of rapidly degraded mRNAs, a polyadenylation signal of AATAAA, and a poly(A) tail. As determined by the use of GeneRunner software the deduced amino acid sequence of 66 residues has a molecular mass of 7.9 kDa and a pI of 9.90. *Artemia* p8 was most similar to p8 from *Drosophila melanogaster* and *Anopheles gambiae*, although sequence similarity was high, especially in the carboxy half of the protein, for all species examined (Fig 2; Table 1). *Artemia* p8 contained a basic helix-loop-helix DNA binding domain consisting of the conserved peptides, basic, 26-GRLRTK-31, helix I, 32-QEAALHT-38, loop, 39-NRFDPSG-45, and helix II 46-HSRKLVTKM-54 (Fig 2). A bipartate nuclear localization sequence encompassing residues R48 to D64 overlapped with helix II (Fig 2). Only p8 from mosquito was shorter

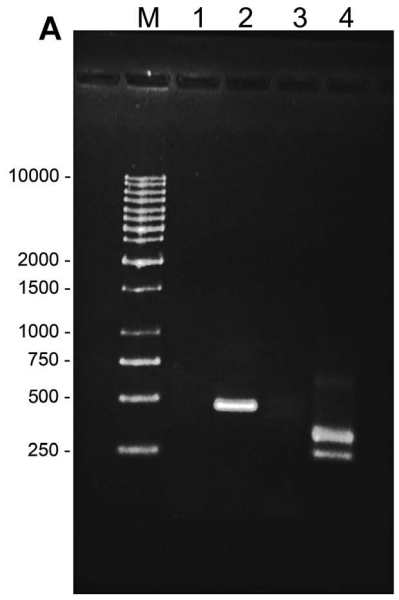


Fig 1. Cloning and sequencing of *Artemia* p8. (A) RNA from diapause destined embryos was extended by 3′- and 5′-RACE. DNA products were electrophoresed in agarose gels and stained with Gel Star. Lane 1: 3′-RACE lacking cDNA; lane 2: 3′-RACE; lane 3: 5′-RACE lacking cDNA; lane 4: 5′-RACE; M, size markers in bp. (B) The complete p8 cDNA and deduced amino acid sequences. Boxed C and A, transcription start sites; boxed ATG, translation start site; boxed TGA, stop codon; boxed AATAAA, polyadenylation signal; shaded ATTTA, sequences indicative of rapidly degraded mRNAs.

than *Artemia* p8 and, in comparison to vertebrates, p8 from invertebrates lacked an amino terminal peptide of 18 or more amino acid residues. The amino-terminal region was more variable than the basic helix-turn-helix domain.

The *Artemia* p8 gene and putative cis-acting regulatory elements

Southern blots probed with full-length p8 cDNA yielded 1 band for each restriction enzyme tested, suggesting a single p8 gene in *Artemia* (Fig 3A). LA PCR in vitro cloning generated 831 bp of DNA sequence upstream of the p8 gene. This region contained a TATA box 57 bp from the transcription start site and putative cis-acting transcription regulatory sites such as GATA2, Sp2, AP-1, CEBP, and heat shock elements (Fig 3B).

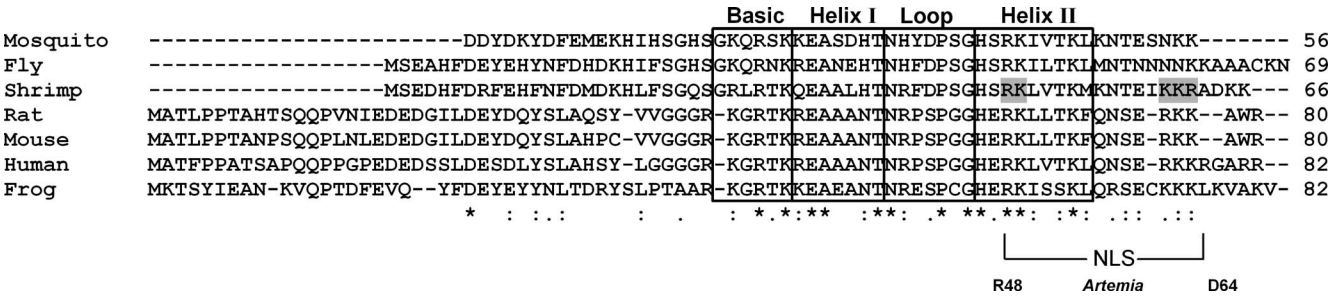


Fig 2. p8 sequence comparison. p8 amino acid sequences from mosquito (XP_315506), fly (NP_609539), rat (AAB94673), mouse (NP_062712), human (AAC19384), and frog (BAB33387) were compared by ClustalW to *Artemia* (shrimp) p8. Asterisk (*), identical residue; colon (:), conserved substitution; period (.), semi-conserved substitution. Basic, helix I, loop, and helix II motifs are boxed; NLS, nuclear localization signal with key basic residues shaded in *Artemia* p8.

p8 mRNA and protein in *Artemia* embryos

p8 mRNA increased markedly by day 1 postfertilization in diapause-destined *Artemia* embryos and then decreased until barely detectable at days 4 and 5, whereas embryos developing directly into larvae exhibited only minor traces of p8 transcript (Fig 4). For example, day 1 diapause-destined embryos contained approximately 60-fold more p8 mRNA than embryos developing into larvae. Probing Western blots of protein extracts from *Artemia* embryos with antibody generated to affinity purified, bacterially produced p8 (Fig 5A,B) revealed p8 initially in diapause-destined embryos 2 days postfertilization and, although staining intensity varied, the protein persisted until day 5 (Fig 5C,D). p8 was absent from embryos undergoing development into nauplii (Fig 5E,F). The immunoprobing of Western blots corroborated the demonstration of differential gene expression obtained by quantitative PCR, although the p8 protein was more stable than mRNA.

Localization of *Artemia* p8

p8 was distributed in a speckled pattern throughout *Artemia* cyst nuclei (Figs 6, 7). Instar II larvae nuclei, which lacked sufficient p8 to be detectable on Western blots, had no staining.

Table 1 p8 sequence comparisons

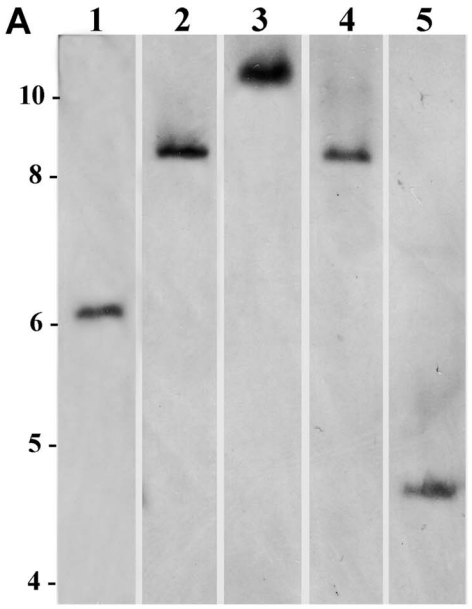
	Nucleotides (ORF)	Amino acids	
Organism	**Identity (%)**	**Similarity (%)**	**Identity (%)**
Mosquito	53	83	57
Fly	60	82	68
Rat	51	63	43
Mouse	53	63	43
Human	51	71	55
Frog	54	65	37

Artemia p8 cDNA and deduced amino acid sequences were compared to p8 cDNA and amino acid sequences from other organisms. Accession numbers for p8 cDNAs are mosquito (*Anopheles gambiae*), XM_315506; fly (*Drosophila melanogaster*), NM_135695; rat, AF014503; mouse, AF131196; human, AF069073 and frog (*Xenopus laevis*), AB056582. Accession numbers for p8-deduced amino acid sequences are in the legend for Fig 2.

DISCUSSION

Molecular analysis of diapause induction and the ensuing events that modulate cell growth and stress resistance in *Artemia* embryos yielded a developmentally regulated basic helix-loop-helix transcription cofactor termed p8 (Igarashi et al 2001; Valacco et al 2006). This protein from *Artemia* and other invertebrates lacks several amino terminal residues constituting a PEST region in vertebrate p8, which is enriched in Pro, Glu, Ser, and Thr and thought to promote ubiquitin-dependent protein degradation by proteasomes (Goruppi and Kyriakis 2004). Susceptibility to ubiquitin-mediated digestion, as indicated for breast cancer cell p8 (Jiang et al 2005), regulates protein longevity and hence activity. That *Artemia* p8 persists into post-diapause development may depend on the absence of the PEST region, allowing the protein to affect gene expression for an extended period. In marked dissimilarity, p8 mRNA half-life is much shorter, perhaps influenced by ATTTA sequences in the 3′-untranslated region, these normally associated with rapidly degraded transcripts (Moribe et al 2001).

Humans and mice each contain a single p8 gene with the 5′-flanking region of the mouse gene displaying a TATA box equivalent, as well as C/EBP, SBE, Myc, Sp1, NFkB, and Ap1 binding sites (Vasseur et al 1999b). Overlapping C/EBP and SBE sites are important cis-acting elements for the mouse p8 gene with the CAAT-enhancer binding proteins C/EBPα and C/EBPβ able to promote p8 gene transcription in NIH 3T3 cells (Vasseur et al 1999b; García-Montero et al 2001). The 5′-untranslated region of the human p8 gene lacks authentic proximal TATA and CAAT boxes (Vasseur et al 1999a), with the CAAT box also missing from the *Artemia* p8 gene. Expression of the LHβ gene in mouse pituitary-derived gonadotrope cell lines may require p8 synthesis, which is proposed to depend on the transcription factor GATA2



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CTTGTGTTTATTATATAACCGATAAGCACACAAAAAAGTG -792
                    GATA
CTATCTTAGCATTATTACAATCAATTTTTTGACTATAGCA -752
  GATA2
TCGAAAATCGCTGTGCAGCACGCAATTATAGCACTTTAGG -712

AACTGACCGTGTCGGCAACCCTAATTCTTCTCCATTGAAC -672

ATAGTCTAGAGCCACCACGCGTACGTCTTCAGGCCTTATT -632

CATGCCTTCTAATCTTTTCATGACCATCATCTTCAGATGG -592
          HSE
AGGGTCCGGCCCTACCGATCCTAGGCCCTTGAATTTGTAG -552
    Sp2
TCCGCTATGGTCGGCTCAACCCACCCTAATAGGATCGAGG -512

CAGCACCCTGAAGAGGCAGCAGTCCATAACTGGTATACCC -472

TGCCGCAGTTGTGCTGCTATAGAGGATCCTCATACGAGGT -432

TATTCTAATAAAAAAAGGTAAAGATCAGGTTGCATTTGAT -392
           Forkhead related
AAATTTTAAGTGGTGTGGGTCTGTGCTTCATTGTTGCTTT -352

TCCTGTGAATTTCTTACCGCTTACCTTCTTAGTCACGAGG -312
                              Ap1
ATCCCTCCCTTTATTCTATGGAGCCCGCTTAATAGCCTCC -272

CTGGAAGCATCCTTGACAGTTCACATGTACACAACATATC -232
         HSE                    GATA
AGCTTTATCTAAAAAATGATGACGAGGTAATTGCATCATG -192
                                C/EBP
TTTAAAGCATTTCGTAAAATGTTTTGAGATTATTTGTGAA -152
      C/EBP
AAAACTGCGCAGTTAAGGTTTTTTTGGCACAGTTGCTAGA -112

TTTTTTTTAACAAAAATGATAAGAGGCTATAACAGCGCCA  -72

TCAGAATCCATATAAGTAACAAAGGTTTTGGATCAGTATC  -32
                                  ↱
AAAACTCGTTTTAGTTTTCTGTTAATGAAGTCATTT       +4
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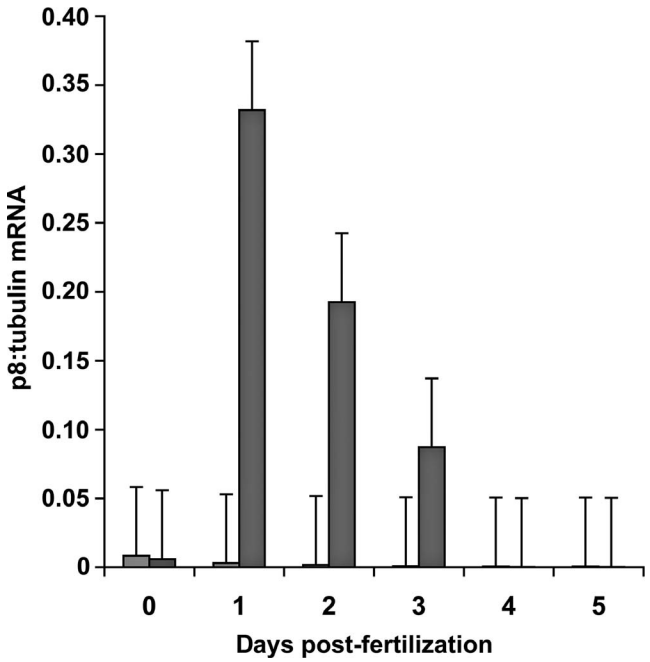

Fig 4. p8 mRNA during embryo development. p8 mRNA from *Artemia* embryos was measured at daily intervals postfertilization by quantitative polymerase chain reaction (PCR) using *Artemia* α-tubulin mRNA as standard. Light and dark shaded bars, p8 mRNA in nauplii and diapause-destined embryos, respectively.

(Quirk et al 2003). In comparison to the situation in mammals, *Artemia* possess 1 p8 gene, although in apparent contrast to mice and humans, there are 2 transcription start sites for the single open reading frame (ORF). The upstream region of the *Artemia* p8 gene contains a TATA box and several putative cis-acting regulatory sequences, including 2 heat shock elements (HSE) of particular interest from a stress induction perspective, and Sp2, Ap1, GATA2, and C/EBP sites, all requiring further analysis to determine their regulatory significance.

Expression patterns and nuclear localization corroborate the proposal that p8 regulates gene expression during *Artemia* diapause, the first example of a crustacean protein with this function. A handful of diapause-related transcription factors are documented with DAF-12 and DAF-16/FOXO, modulators of *Caenorhabditis elegans* diapause (dauer) (Baumeister et al 2006; Rottiers and Antebi 2006), the best studied. Among the insects, a gene similar

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Fig 3. *Artemia* p8 gene. (A) Digested *Artemia* genomic DNA was electrophoresed in agarose, transferred to nylon membranes, and probed with p8 cDNA. Lanes 1–5: loaded with DNA digested, respectively, with XhoI, PstI, SalI, BamHI and HindIII. Size in kbp is indicated on the left. (B) Upstream sequence of the p8 gene obtained by LA polymerase chain reaction (PCR) in vitro cloning. Boxed TATAA, TATA box; bold C with arrow, transcription start site; several putative cis-acting regulatory sites are underlined and labeled.

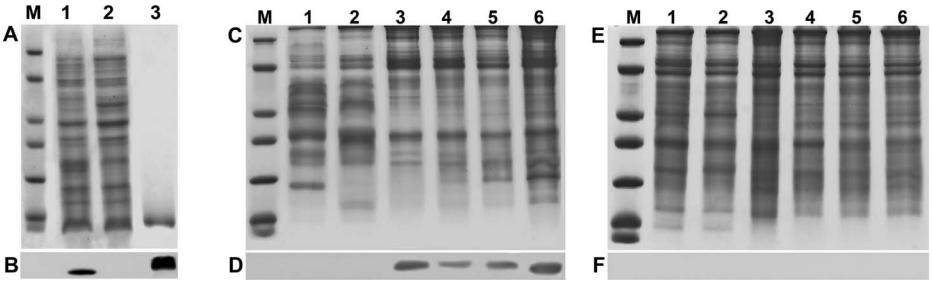

Fig 5. Antibody production and p8 detection. p8 synthesized in transformed *Escherichia coli* was purified on TALON affinity resin for use as antigen. Protein samples were electrophoresed in sodium dodecyl sulfate (SDS) polyacrylamide gels and either stained with Coomassie blue (A) or transferred to nitrocellulose and detected with Omniprobe (B). Lane 1: extract from bacteria transformed with p8 cDNA; lane 2: extract from bacteria transformed with vector only; lane 3: purified p8. Lanes 1 and 2: 30 μg of protein in A and 10 μg in B; lane 3: 3 μg of protein in A and 0.4 μg in B. Protein extracts prepared from diapause-destined (C, D) and nauplii-destined (E, F) *Artemia* embryos at daily intervals postfertilization, were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie blue (C, E) or transferred to nitrocellulose and probed with antibody to p8 (D, F). Lanes 1–6: day 0 (fertilization) to day 5 postfertilization, respectively. All lanes received 30 μg of protein. M, molecular mass markers of 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, and 14.4 kDa.

to the *Drosophila methoprene tolerant protein* gene (Met), possibly a juvenile hormone– (JH) dependent transcription factor, is upregulated early in *Culex pipiens* L diapause (Robich et al 2007), an unexpected observation because this species enters diapause in the absence of JH. mRNA for an ETS transcription factor homologue is enriched during *Bombyx mori* embryonic diapause (Suzuki et al 1999), and a POU factor may affect transcription of the *B. mori* diapause hormone (DH) and pheromone biosynthesis-activating neuropeptide (PBAN) gene (Zhang et al 2004a). *B. mori* DH influences the ovaries of pharate adults, producing diapause eggs in the following generation. Regulation of the DH-PBAN gene in *Helicoverpa armigera*, an insect with pupal diapause, involves multiple *cis*-elements and DNA binding proteins, possibly including a basic helix-loop-helix transcription factor named Har-DHMBP-3 (Hong et al 2006). *H. armigera* DH, in contrast to the situation in *B. mori*, is thought to promote growth rather than diapause (Xu and Denlinger 2003; Zhang et al 2004b), perhaps reflecting mechanistic differences between embryonic and pupal diapauses. In the final insect case 2 *B. mori* transcription factors may function in diapause termination (Moribe et al 2001; Shiomi et al 2005). Analysis by differential gene expression tentatively identified transcriptional regulatory components during delayed implantation (Hamatani et al 2004; Lopes et al 2004). However, in spite of extensive effort, definitive identification of transcription factors promoting diapause is accomplished only for *C. elegans* larvae that, unlike most invertebrates, undergo diapause upon exposure to adverse environmental conditions rather than in their anticipation.

Identification of cell processes regulated by p8 is underway but conclusions remain tenuous because results are contradictory, genetic redundancy is unexplored for the protein, and its function probably is influenced by interaction with different partners. For example, p8 may buttress defense against cell injury (Vasseur et al 2004; Malicet et al 2006b) and promote growth (Vasseur et al 1999a; Garcia-Montero et al 2001; Päth, et al 2006; Malicet et al 2006b). In contrast, p8 also inhibits cell growth and suppresses tumors (Bratland et al 2000; Vasseur et al 2002a, 2002b; Zinke et al 2002; Malicet et al 2003; Jiang et al 2005, 2006) while maintaining tumor phenotype by a mechanism perhaps different from growth promotion per se (Ree et al 1999; Su et al 2001; Vasseur et al 2002b; Mohammad et al 2004). p8 is both proapoptotic (Vasseur et al 2002a; Carracedo et al 2006; Plant et al 2006) and antiapoptotic (Su et al 2001; Giroux et al 2006), with inhibition thought to depend on interaction with prothymosin α (Malicet et al 2006a). p8 may stop growth when cells of *Drosophila* larvae experience nutrient deprivation (Zinke et al 2002), as occurs during diapause. The multiplicity of p8 activities is likely to depend on binding partner diversity (Hoffmeister et al 2002; Malicet et al 2003; Jiang et al 2005), with variation from 1 cell type to another and upon exposure to dissimilar environments. That p8 affects cell growth and apoptosis is especially pertinent, demonstrating this transcription cofactor has

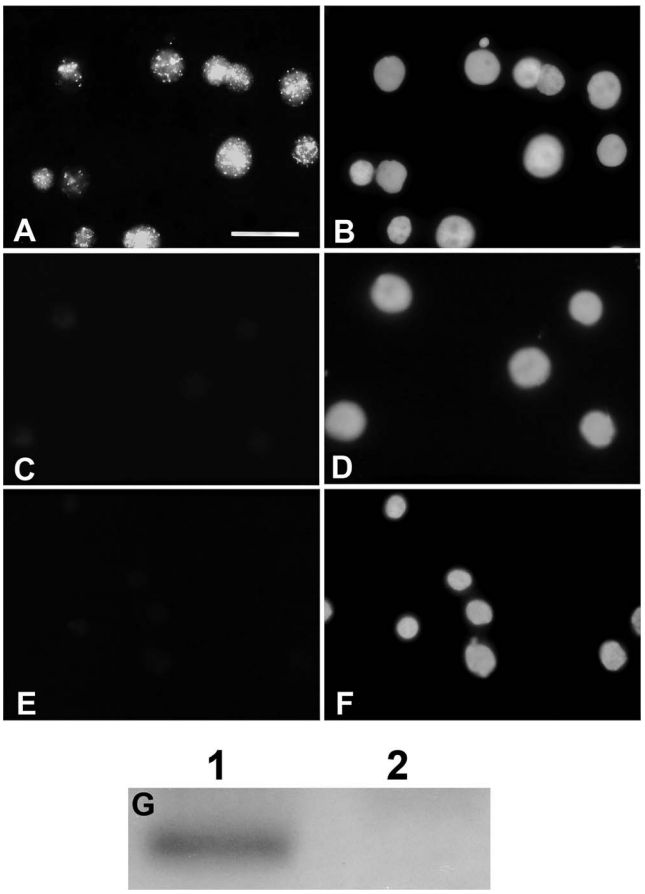

Fig 6. Immunolocalization of *Artemia* p8. Nuclei from *Artemia* cysts (A, B) and instar II larvae (C, D) were incubated with antibody to p8 followed by fluorescein isothiocyanate– (FITC) conjugated goat anti-rabbit IgG and 4′6-diamidino-2-phenylindole dihydrochloride (DAPI). Cyst nuclei (E, F) were incubated in buffer lacking primary antibody followed by secondary antibody and DAPI. (A, C, E) FITC; (B, D, F) DAPI. The bar in A represents 20 μm and all images are the same magnification. (G) Equivalent amounts of protein from cysts (1) and larvae (2) were electrophoresed in SDS polyacrylamide gels, transferred to nitrocellulose, and probed with antibody to p8.

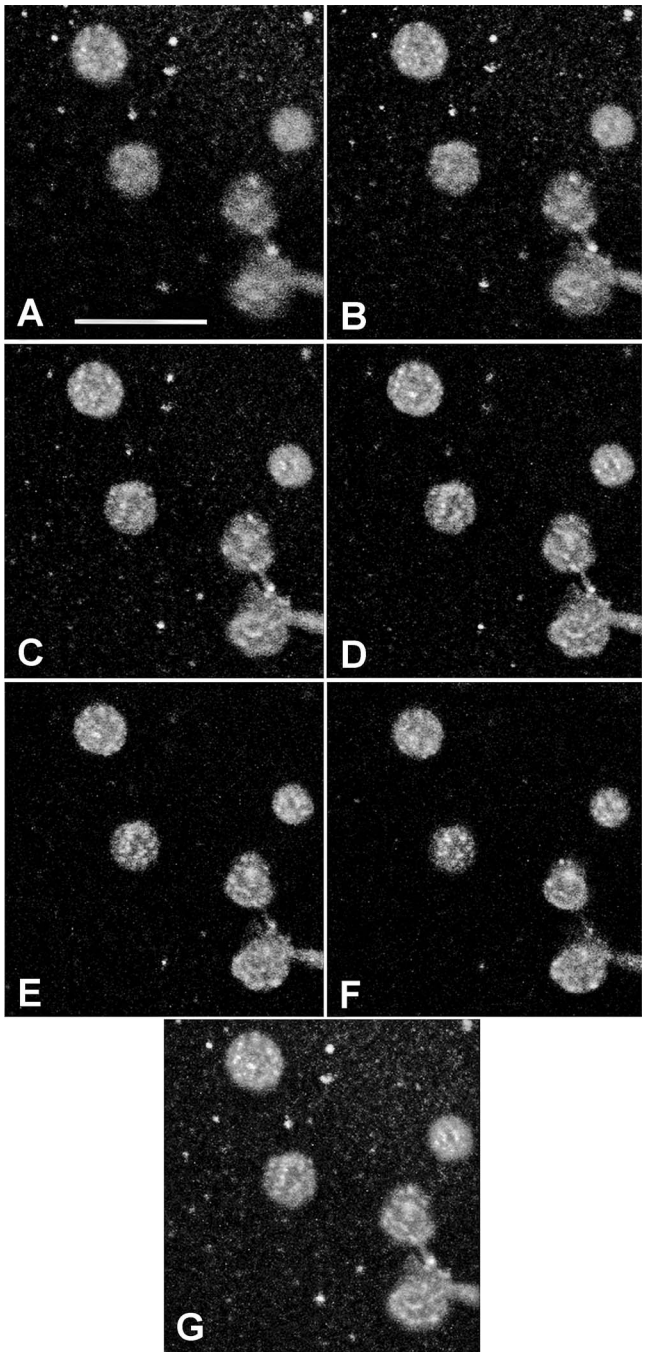

Fig 7. p8 distribution in *Artemia* nuclei. Cyst nuclei stained with antibody to p8 and fluorescein isothiocyanate– (FITC) conjugated goat anti-rabbit IgG were examined by confocal microscopy. (A–F) Serial optical sections of nuclei; (G) reconstruction of sections. The bar in A represents 25 μm and all magnifications are the same.

the capacity to regulate these key processes during *Artemia* embryo diapause.

To summarize, the gene for p8, a stress-inducible transfection cofactor with the ability to either promote or inhibit both cell growth and apoptosis, is synthesized specifically in diapause-destined *Artemia* embryos. *Artemia* p8 mRNA synthesis crests at day 1 postfertilization, prior to undergoing precipitous decline. The p8 protein peaks a day later and persists at about the same level throughout cyst development. As such, and because it resides in *Artemia* nuclei, p8 is an excellent candidate to exert transcriptional regulation during diapause. Pertinent activities include promotion of genes indigenous to the diapause development pathway, including those inhibiting apoptosis, silencing genes that promote cell growth, and differential expression of metabolic enzyme genes. In this context, the 5′-regulatory sequence of the p26 gene, the only other diapause upregulated gene in *Artemia* for which this region is available, contains a basic helix-loop-helix transcription factor binding site at -185-cagctg-180 (Qiu et al 2006; Hong et al 2006). Experiments are underway to determine p8 effects on the expression of p26 and other genes upregulated in diapause-destined *Artemia* embryos.

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