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Molecular Characterization of a Small Heat Shock/α-Crystallin Protein in Encysted *Artemia* Embryos*

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Ping Liang‡, Reinout Amons§, James S. Clegg¶, and Thomas H. MacRae‡‖

From the ‡Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada, the §Department of Medical Biochemistry, University of Leiden, Leiden, The Netherlands, and ¶Bodega Marine Laboratory, University of California, Davis, Bodega Bay, California 94923

Molecular chaperones protect cells during stress by limiting the denaturation/aggregation of proteins and facilitating their renaturation. In this context, brine shrimp embryos can endure a wide variety of stressful conditions, including temperature extremes, prolonged anoxia, and desiccation, thus encountering shortages of both energy (ATP) and water. How the embryos survive these stresses is the subject of continuing study, a situation true for other organisms facing similar physiological challenges. To approach this question we cloned and sequenced a cDNA for p26, a molecular chaperone specific to oviparous *Artemia* embryos. p26 is the first representative of the small heat shock/α-crystallin family from crustaceans to be sequenced, and it possesses the conserved α-crystallin domain characteristic of these proteins. The secondary structure of this domain was predicted to consist predominantly of β-pleated sheet, and it appeared to lack regions of α-helix. Unique properties of the nonconserved amino terminus, which showed weak similarity to nucleolins and fibrillarins, are enrichments in both glycine and arginine. The carboxyl-terminal tail is the longest yet reported for a small heat shock/α-crystallin protein, and it is hydrophilic, a common attribute of this region. Site-specific differences between amino acids from p26 and other small heat shock/α-crystallin proteins bring into question the functions proposed for some of these residues. Probing of Southern blots disclosed a multi-gene family for p26, whereas two size classes of p26 mRNA at 0.7 and 1.9 kilobase pairs were seen on Northern blots, the larger probably representing nonprocessed transcripts. Examination of immunofluorescently stained samples with the confocal microscope revealed that a limited portion of intracellular p26 is found in the nuclei of encysted embryos and that it resides within discrete compartments of this organelle. The results in this paper demonstrate clearly that p26 is a novel member of the small heat shock/α-crystallin family of proteins. These data, in concert with its restriction to embryos undergoing oviparous development, suggest that p26 functions as a molecular chaperone during exposure to stress, perhaps able to limit protein degradation and thus ensure a ready supply of functional proteins when growth is reinitiated.

Molecular chaperones assist the folding of proteins, protect them from denaturation/aggregation, aid in their renaturation, and influence the final intracellular location of mature proteins (1–11). The chaperones, many of which are induced by exposure of cells to stress, are divided into several groups, including HSP100, 90, 70, 60 (chaperonin), and small heat shock/α-crystallin proteins. The functions of chaperones differ, but their activities are interrelated and often dependent on association into macromolecular complexes, sometimes consisting of representatives from more than one family. Of most importance to this work are the small heat shock/α-crystallin proteins, 15–30 kDa in size but with the ability to oligomerize into particles of varying monomer number (12–14). Proteins in this group possess a conserved domain of 90–100 amino acid residues, the α-crystallin signature sequence (11, 15–18). Otherwise, they differ in sequence, although there are conserved residues within variable regions that may be essential to their chaperone-like activity (15, 19, 20). The ATP-independent chaperone-like activity of these proteins is seemingly more directed to preventing protein aggregation early in denaturation, rather than the active refolding of compromised proteins (19, 21–25). This is an especially important characteristic of the α-crystallins, major proteins of the lens which limit aggregation of other eye crystallins and maintain lens transparency (23–25). The α-crystallins, and especially αB-crystallin, are also found in nonlenticular tissues (26–28), where their synthesis is induced by stress (24, 27–31). The small heat shock/α-crystallin proteins are thought to protect cells during stress (27, 32–36), and there is evidence that some of them not only prevent denaturation of proteins but assist in their renaturation as well (13, 37, 38).

The synthesis of small heat shock/α-crystallin proteins is developmentally regulated in many organisms (29, 34, 39–41), and in this context, the brine shrimp *Artemia* is an interesting experimental model. Upon fertilization, *Artemia* oocytes undertake one of two developmental pathways. Either they develop directly into free-swimming larvae and are released from the female. In the alternative mode, development is arrested and embryos are discharged from the female as encysted gastrulae, termed cysts (42). The cysts, composed of about 4000 cells and enclosed in a shell impermeable to most molecules, enter diapause, a condition characterized by an extremely low level of metabolic activity (42–46). They are very resistant to environmental insults including anoxia, temperature extremes, organic solvents, γ-irradiation, and desiccation (42, 43, 47). Desiccation is a normal part of this path of development and may be required to break diapause and reinitiate development. Resistance to environmental insults is not lost immediately upon resumption of cyst development. For example, fully hydrated, post-gastrula cysts survive at least 4 years under anoxic con-

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‖ To whom correspondence should be addressed. Tel.: 902-494-6525; Fax: 902-494-3736; E-mail: tmacrae@is.dal.ca.

ditions in a state of quiescence (48–50).[1] These animals are essentially ametabolic, apparently existing in the absence of a continuous free energy flow (50–52).

The molecular basis for the remarkable stress resistance of *Artemia* cysts is poorly understood. However, we previously purified a low molecular weight protein, termed p26, to apparent homogeneity and obtained evidence that it protects embryo cells during encystment, diapause, and anaerobic quiescence (13, 49, 53). Partial sequence analysis, in concert with other characterization, revealed that p26 is a small heat shock/α-crystallin protein with chaperone activity *in vitro* (13). In this paper we report the isolation and sequencing of a full-length cDNA clone for p26. Comparison to other small heat shock/α-crystallin proteins at both primary and secondary levels disclosed novel aspects of p26 structure. Multiple genes for p26 were observed, as were two size classes of mRNA transcripts, one in much greater abundance than the other. Examination of immunofluorescently stained samples with the confocal microscope revealed that a portion of the p26 in hydrated *Artemia* cysts is found within discrete compartments of their nuclei. We believe that p26 prevents the aggregation of other proteins when these embryos experience stresses of various kinds, thus playing an important role in their growth and development.

MATERIALS AND METHODS

Hydration of Artemia Cysts—Encysted embryos of *Artemia franciscana* (Great Salt Lake) obtained from Sanders Brine Shrimp, Ogden, UT, were hydrated in cold distilled water for at least 3 h. Those cysts that sank to the bottom of the container were collected by suction on a Buchner funnel, rinsed several times with cold distilled water, and used immediately.

Cloning and Sequencing of a p26 cDNA—Poly(A)$^+$ mRNA, prepared from encysted *Artemia* gastrulae (54), was employed as template for the synthesis of first strand cDNA using the First-strand cDNA Synthesis Kit™ (Pharmacia Biotech Inc.) as described in the manufacturer's instructions. PCR[2] was performed against the first strand cDNA using a degenerative primer, based on the p26 peptide sequence of 103-DEYGHVQR-110 and an oligo(dT) primer, *Not*I-$(dT)_{18}$, included in the cDNA synthesis kit. The PCR protocol involved 30 cycles at 92 °C, 30 s; 43 °C, 30 s; 72 °C, 30 s in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA). A DNA fragment of 378 bp, termed p26-3′, was cloned into PUC18 using the SureClone™ Ligation Kit (Pharmacia) and sequenced manually with the T7 Sequencing™ Kit (Pharmacia) to ensure that it encoded a portion of p26. To obtain the full-length p26 clone, a cDNA library from encysted *Artemia* gastrulae in λZAP II (a gift from Dr. L. Sastre, Instituto de Investigaciones Biomédicas del C.S.I.C., Madrid, Spain) was converted to the phagemid, Bluescript SK−, and screened with ^{32}P-labeled p26-3′ according to Sambrook *et al.* (55). Several putative p26 clones were plaque-purified two times, and one of these contained an insert termed p26-3-6-3 shown by sequencing to encode a full-length cDNA for p26.

Peptide Sequencing of p26—Purification of p26, digestion, and separation of peptides by reversed-phase high performance liquid chromatography were as described by Liang *et al.* (13). The three amino-terminal peptides sequenced in this study were generated by BrCN treatment of p26 (13). As the intact protein was blocked amino-terminally (13) at least one of the peptides formed should be blocked. This peptide could therefore be identified in high performance liquid chromatograms of the entire BrCN mixture on the basis of its insensitivity toward aminopeptidase M (not shown). Removal of the blocking group if it is an acetyl moiety, together with the first amino acid, is possible with acyl amino acid peptidase. The isolated peptide was lyophilized and dissolved in 10 μl of 0.2 M ammonium carbonate, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA (pH 8.5). Deblocking was accomplished by adding 10 μl of acylamino acid peptidase (Boehringer Mannheim), containing 2 μg of lyophilized enzyme, and incubating the mixture for 16 h at 37 °C. The deblocked peptide was loaded on a reversed-phase column (1 × 15 mm) which had been preconditioned by rinsing with 0.5 ml of 0.08% (v/v) trifluoroacetic acid containing 75% (v/v) acetonitrile followed by 0.5 ml of 0.1% (w/v) trifluoroacetic acid. After sample application the column was rinsed with 0.5 ml of 0.1% (w/v) trifluoroacetic acid. The peptide was eluted with 75 μl of 75% (v/v) acetonitrile containing 0.1% (w/v) trifluoroacetic acid, applied to a Polybrene-impregnated glass fiber disk, and analyzed in a model 475A Applied Biosystems pulse liquid sequencer, connected on line to a Model 120A phenylthiohydantoin-derivative analyzer.

Because it was not possible to prepare a suitable carboxyl-terminal peptide, the final eight amino acids were determined by pool sequencing tryptic peptides obtained from a larger carboxyl-terminal peptide, in turn produced by exposure of p26 to endoproteinase Lys-C (13). The large peptide, beginning at Thr-144, was treated on a Polybrene-impregnated sequence glass fiber disk with 20 μl of 10 mM $NaHCO_3$, followed by 20 μl of acetic anhydride/methanol (1:4, v/v). After 5 min at room temperature the remaining liquid was removed by lyophilization, and the disk was wetted with 20 μl of 0.2 M ammonium carbonate (pH 8.5) containing 0.1 μg of trypsin. The disk was transferred to an Eppendorf tube to suppress evaporation and incubated at 37 °C for 2 h. The remaining water and ammonium carbonate were removed by lyophilization, and the disk was placed in the sequencer for analysis. Because there were four internal arginine residues in the large peptide, and the amino terminus of the initial peptide was blocked by acetylation, the simultaneous sequencing of four peptides was expected.

Prediction of p26 Secondary Structure and Solvent Accessibility—The secondary structure and solvent accessibility of amino acid residues within p26 were predicted by the methods of Rost and Sander via the Predict Protein E-mail server at the European Molecular Biology Laboratory, Heidelberg, as described by Caspers *et al.* (16).

Analysis of p26 Gene Structure by Southern Blotting—DNA was prepared from *Artemia* larvae as described previously except that ethanol precipitation was avoided (54). The DNA was digested with *Bam*HI, electrophoresed in 0.8% agarose gels with 28 μg of DNA in each lane, and blotted to Hybond™-N+ nylon membranes (Amersham Corp.) in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate (pH 7.0)). The blots were incubated for 12 h at 68 °C in prehybridization solution that contained 6 × SSC, 5 × Denhardt's, 0.5% SDS, and 100 μg/ml salmon DNA which had been sheared and denatured. Hybridization was in the same solution for 12 h at 68 °C with DNA fragments corresponding to the full-length p26 cDNA (p26-3-6-3), 373 bp from the 5′-end (p26-5′), and 378 bp from the 3′-end (p26-3′). The full-length probe was recovered from agarose gels after incubation of the plasmid containing p26-3-6-3 with *Eco*RI and *Xho*I followed by electrophoresis. The probe, p26-5′, was generated by PCR amplification of p26-3-6-3 using a nondegenerate primer based on nucleotides 22–39 of p26-3-6-3 and a degenerate primer based on the peptide sequence, 119-PEHVKPE-126. p26-3′ was produced as described previously in this paper. The 5′- and 3′-probes had a short sequence overlap, but as revealed by the hybridization patterns, this did not appear to affect their specificity. The hybridization probes were labeled with ^{32}P by random priming (T7 Quick Prime™ Kit, Pharmacia) following the manufacturer's instructions. After hybridization the blots were washed with 2 × SSC containing 0.1% SDS at room temperature for 30 min, followed by 1 × SSC containing 0.1% SDS and then 0.1 × SSC with 0.1% SDS, both at 68 °C for 15 min, partly air dried, wrapped in Saran Wrap™ and exposed to Kodak X-OMAT™ AR film (Picker Scientific, Dartmouth, Nova Scotia) at −70 °C.

The number of p26 genes in the *Artemia* genome was determined by hybridizing the ^{32}P-labeled probes just described to Southern blots that contained known amounts of both p26 cDNA and restriction-digested *Artemia* DNA in parallel lanes. For this quantitation the size of the *Artemia* haploid genome was set at 1.37×10^6 kb (1.45 pg) (56), and the size of the Bluescript Sk− plasmid containing p26-3-6-3, which was linearized by digestion with *Xho*I before electrophoresis, was 3.64 kb (3.7×10^{-6} pg). Labeling intensities for bands in each sample were compared by scanning blots with a Bio-Rad GS-670 Imaging Densitometer and quantitated by use of Molecular Analyst software from Bio-Rad.

mRNA Preparation and Northern Blotting—Poly(A)$^+$ mRNA was obtained from hydrated *Artemia* cysts, electrophoresed in 1.5% agarose gels, blotted to Hybond™-N+ membranes, baked at 80 °C for 2 h, and hybridized to ^{32}P-labeled, full-length p26 cDNA as described by Langdon *et al.* (54), except that probes were prepared by random priming. Size determinations were made by comparing migration distances of p26 mRNA with RNA fragments of known size in a 0.24–9.5-kb RNA ladder from Life Technologies (Burlington, Ontario).

Preparation of Artemia Nuclei—Nuclei were prepared from *Artemia*

[1] J. S. Clegg, unpublished data.

[2] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole hydrochloride; BrCN, cyanogen bromide; Pipes, 1,4-piperazinediethanesulfonic acid.

for immunofluorescent staining by two methods. In the first procedure hydrated cysts were crushed gently between two glass slides, one of which was coated with poly-L-lysine. The slides were separated and the nuclei were fixed immediately. Nuclei were also isolated by a modification of the technique of Squires and Acey (57) communicated to us by J. Vaughn (University of Miami, Miami, OH). Briefly, 10 g of hydrated *Artemia* cysts were ground by hand in a chilled mortar and pestle for 2 min in 35 ml of HPC buffer (0.5 M hexylene glycol, 0.05 M Pipes-free acid, 1 mM $CaCl_2$ (pH 7.6)) and then subjected to one passage in a motorized Dounce homogenizer fitted with a size A pestle. The homogenate was passed through one layer of Miracloth (Calbiochem), centrifuged at 2,000 × *g* for 10 min at 4 °C, and the supernatant discarded. The pellet was rinsed once with HPC buffer and then washed two times using 40 ml of HPC buffer for each wash. The large, greenish pellet of nuclei obtained after the second wash was resuspended in 17.5 ml of HPC buffer with a Pasteur pipette. The suspension was layered on a 25-ml cushion of 75% (v/v) Percoll in 0.15 M NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6) and centrifuged at 16,000 × *g* for 30 min at 4 °C in a Beckman JS-13 swinging bucket rotor. The top clear layer was discarded, and the second layer was transferred to a fresh tube and brought to a final volume of 10–15 ml with HPC. The solution was applied to a 25-ml cushion of 75% (v/v) Percoll that had been centrifuged at 16,000 × *g* for 30 min at 4 °C in a Beckman JS-13 rotor, and the centrifugation was repeated. The top layer was discarded and the second layer of 5 to 6 ml was mixed in a fresh tube with an equal volume of HPC buffer before centrifugation at 16,000 × *g* for 30 min at 4 °C. The resulting yellow-brown pellets, containing isolated nuclei, were resuspended in 1.0 ml of HPC buffer. The isolation was also done with all solutions at pH 6.5 to ensure that p26 was not extracted from nuclei during their manipulation under basic conditions (49, 53). Nuclei were mixed with an equal volume of 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (Molecular Probes, Eugene, OR) at 0.001 μg/ml in H_2O for 5 min at room temperature, allowed to settle onto poly-L-lysine-coated slides for 5 min at room temperature, and examined by light and immunofluorescent microscopy to determine purity. Nuclei at different stages of purification were solubilized, electrophoresed in one-dimensional, 12.5% SDS-polyacrylamide gels, and either stained with Coomassie Blue or transferred to nitrocellulose and immunostained by the enhanced chemiluminescence procedure (Renaissance^R, NEN Life Science Products) following manufacturer's instructions. The primary antibody was raised in rabbits to p26 purified as described previously (13).

Immunofluorescent Staining of Artemia Nuclei—Nuclei prepared by crushing of hydrated cysts and by centrifugation on Percoll gradients were fixed in either 4% (w/v) paraformaldehyde at room temperature for 20 min or in methanol at −20 °C for 5 min. The fixed specimens were hydrated in PBS for 5 min at room temperature and exposed for 30 min at room temperature to primary antibody, raised to p26, and diluted 1:500 in phosphate-buffered saline (PBS) (pH 7.4), containing 0.5% bovine serum albumin and 0.5% Triton X-100 (PBSAT). The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:200 in PBSAT. After a 30-min incubation with secondary antibody the nuclei were rinsed with PBS, incubated with DAPI as above, rinsed again, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Purified nuclei were also stained with propidium iodide (Sigma) added directly to mounting medium to achieve a final concentration of 0.1 mg/ml. Slides were examined with either a Leitz Aristoplan epifluorescence microscope or a Zeiss LSM 410 inverted laser scanning confocal microscope equipped with an argon-krypton laser.

RESULTS

Cloning and Sequencing of p26 cDNA—The cloned cDNA, p26-3-6-3, isolated by screening an *Artemia* library contained a single open reading frame that encoded p26, a polypeptide of 192 amino acid residues beginning with methionine and ending in alanine (Fig. 1). The first 21 nucleotides of the cDNA clone were noncoding as were nucleotides 598–709. A termination codon followed the final alanine residue and the 3′-noncoding region contained a typical polyadenylation signal of AATAAA at nucleotides 666–671 and a poly(A) tail (nucleotides 686–709).

The amino acid sequence deduced from the cDNA clone agreed exactly with the partial sequence of p26, starting with Arg-44 and ending with Arg-184, that had been derived previously by Edman digestion (13). To test the remainder of the

```
                    1 M A L N P W Y G G F G G M 13
  1 cggcacgagctcgtgctcaaaatggcacttaacccatggtacggaggatttggtggtatg

                   14 T D P W S D P F G F G G F G G G M D L D 33
 61 actgacccatggtctgatccatttggatttggtggcttcggaggtggcatggaccttgat

                   34 I D R P F R R R M M R R G P D T S R A L 53
121 attgacaggcccttccggagaagaatgatgagaagaggtccagataccagcagggcttta

                   54 K E L A T P G S L R D T A D E F Q V Q L 73
181 aaggagttagctactcctgggtccttgagggacacagctgatgaatttcaagttcagcta

                   74 D V G H F L P N E I T V K T T D D D I L 93
241 gatgttggccacttttttaccaaacgaaattacagtcaagacaaccgacgatgatattctt

                   94 V H G K H D E R S D E Y G H V Q R E F R 113
301 gtccatggcaaacatgacgagcgatctgatgaatatggacacgtacaaagagaatttcga

                  114 R R Y R L P E H V K P E S V S S T L S S 133
361 cgacgatacagactcccagaacatgtcaaaccagaatctgtgtcatctactttgtcatca

                  134 D G V L T I H A P K T A L S S P T E R I 153
421 gatggtgtcttaactatccatgctccgaaaactgctttaagctcaccaacagaacgtatc

                  154 V P I T P A P A V G R I E G G T T G T T 173
481 gtacccatcacaccagcgccagctgttggaaggattgaaggggaactacaggtactact

                  174 T G S T A S S T P A R T T R S G G A A 192
541 acaggcagtacagctagttcaactccagcaagaacaacaagatcaggaggtgcagcttaa

601 tctgcattcaatatatattcaatgaatcttccggtctttttttctttgtacctttatttttt

661 ttgtcaataaatctgtatacgaggcaaaaaaaaaaaaaaaaaaaaaaaa 709
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FIG. 1. **The complete sequence of p26-3-6-3 and the deduced amino acid sequence of p26.** The cDNA clone, which contained the insert p26-3-6-3, was obtained by probing an *Artemia* library with a DNA fragment generated by PCR using primers constructed on the basis of peptide sequence analysis. Both strands of p26-3-6-3 were sequenced manually using the Sanger dideoxy-mediated chain termination method, and the amino acid sequence was deduced from the cDNA. One open reading frame that encoded a polypeptide of 192 amino acids, starting with methionine and ending in alanine, was observed. A stop codon uaa follows alanine and a typical polyadenylation signal aataaa (*underlined*) is followed within 15 nucleotides by a poly(A) tail.

deduced amino acid sequence, selected peptides prepared from purified p26, as described under "Materials and Methods," were analyzed. Three short, nonoverlapping peptides obtained by BrCN digestion agreed exactly in sequence with amino acid residues 3–41 of p26 as deduced from the cDNA clone (Fig. 2*a*). The extreme amino-terminal peptide showed, after deblocking, the sequence LNPWYGGFGG (Fig. 2*a*). Because the acylamino acid peptidase deletes an amino acid from its substrate, in addition to an acetyl group, it was necessary to depend on the cDNA sequence to determine the first residue, which in this case was alanine. Thus, it was revealed that the initiator methionine observed in p26-3-6-3 (Fig. 1) was removed from mature p26, and alanine, originally the penultimate residue, was at the amino terminus. Additionally, methionine residues 13, 30, 42/43 were not observed in the peptides because they were converted to homoserine lactone residues during BrCN cleavage, and these are often not visible during sequencing. The disappearance of these residues was in agreement with their position as revealed by sequencing of p26-3-6-3 cDNA (Fig. 1).

The cDNA-derived sequence indicated that the high performance liquid chromatography-based isolation of the two tryptic peptides containing the carboxyl-terminal eight amino acid residues would be difficult because of their length and composition. Additionally, the peculiar amino acid composition and sequence in this part of the polypeptide chain precluded other digestion possibilities. Therefore, the carboxyl-terminal sequence obtained by analysis of p26-3-6-3 was confirmed by pool sequencing a mixture of tryptic peptides beginning at arginines 152, 164, 184, and 187. The amino terminus of the original peptide was blocked by acetylation and thus not available for sequencing. The amino acid residues obtained in each sequencing step were as follows: step 1, ITS; step 2, VETG; step 3, PGR; step 4, IGA; step 5, TA; step 6, PT; step 7, AG; and it was possible to align these residues with those deduced from the

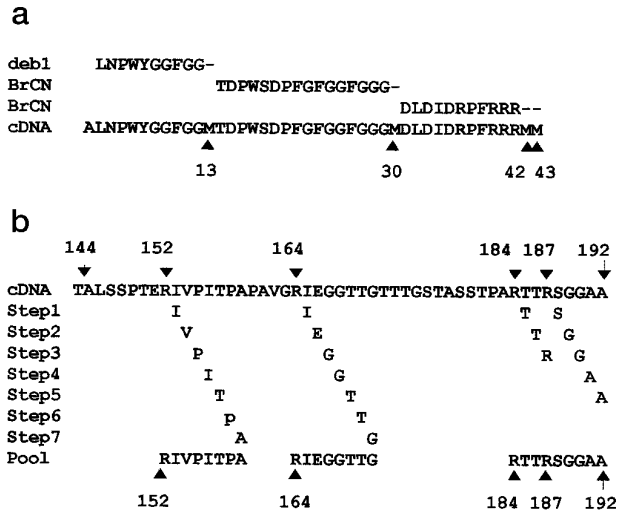

FIG. 2. **Confirmation of the deduced primary structure of p26 by sequencing selected peptides.** The primary structure of two regions of p26, determined initially by analysis of p26-3-6-3, was confirmed by sequencing of selected peptides, obtained as described under "Materials and Methods." *a*, three peptides obtained by cyanogen bromide (*BrCN*) treatment of p26, one of which was deblocked with acylamino acid peptidase (*debl*), were sequenced and found to correspond exactly to residues 3–41 deduced from the cDNA sequence (labeled *cDNA*). The *numbered arrowheads*, *13, 30, 42/43*, indicate internal methionines susceptible to BrCN action. They are converted into homoserine lactone residues that are mostly not visible upon Edman sequencing. *b*, four peptides, obtained by tryptic digestion of a carboxyl-terminal peptide encompassing residues 144–192, were pool sequenced. The residues obtained at each sequencing cycle (steps 1–7) were arranged in relation to the cDNA-derived sequence, and the cumulative results are presented in the line labeled *Pool*. The internal arginines, indicating sites susceptible to tryptic digestion, are labeled with *numbered arrowheads*.

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p26  MAL----------NPWYG----GFGGMTDPWSDPFGFGGF----------
αAC  MD-------VTIQHPWFKRTLGPFY-PSRLFDQFFGEGLFEYDLLPFL--
αBC  MD-------IAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFP-T--
H27  MTERRVPFSLLRGPSW--DPFRDWYPHSRLFDQAFGLPRLPEEWSQWL--
Dro  MSVVPLMF-----RDWWDELDFPM-RTSRLLDQHFGQGLKRDDLMSSVWN
     *            *          .    . **

p26  --GGGMDLDIDRPFRRRMMRRGPDTSRAL-----KELATP-GSLRDTADE
αAC  --SSTISPYY--R------------QSLFRTV---LDSGISEVRSDRDK
αBC  --STSLSPFYLRP------------PSFLRAPSW-FDTGLSEMRLEKDR
H27  --GGSSWPGYVRPLPPAAIESPAVAAPAYSRALSRQLSSGVSEIRHTADR
Dro  SRPTVLRSGYLRPWHTNSLQKQESG----------------STLNIDSEK
                                                .  .    .

p26  FQVQLDVGHFLPNEITVKTTDDDILVHGKHDERSDEYGHVQREFRRRYRL
αAC  FVIFLDVKHFSPEDLTVKVQDDFVEIHGKHNERQDDHGYISREFHRRYRL
αBC  FSVNLNVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRI
H27  WRVSLDVNHFAPDELTVKTKDGVVEITGKHEERQDEHGYISRCFTRKYTL
Dro  FEVILDVQQFSPSEITVKVADKFVIVEGKHEEKQDEHGYVSRQFSRRYQL
       . *.* .* * .. **  .  . .  ***.*. *. * . * * *.* .

p26  PEHVKPESVSSTLSSDGVLTIHAPKTALS--SPTERIVPITPAPAVGRIE
αAC  PSNVDQSALSCSLSADGMLTFCGPKIQTGLDAHTERAIPVS------REE
αBC  PADVDPLAITSSLSSDGVLTVNGPRKQVS---GPERTIPIT------REE
H27  PPGVDPTQVSSSLSPEGTLTVEAPMPKLATQSN-EITIPVT------FES
Dro  PSDVNPDTVTSSLSSDGLLTIKAPMKALP-PPQTERLVQIT------QTG
     *  *    .. .**..* **  .*          *  . ..

p26  GGTTGTTTGSTASSTPARTTRSGGAA
αAC  KP--TSA--------PSS--------
αBC  KPAVTAA--------PKK--------
H27  RAQLGGPEAAKSDETAAK--------
Dro  PS--SKEDNAKKVETSTA--------
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FIG. 3. **Comparison of p26 to other small heat shock/α-crystallin proteins.** The deduced amino acid sequence of p26 was compared by the CLUSTAL V multiple sequence alignment program to the sequences of other small heat shock/α-crystallin proteins, including human αA-crystallin (*αAC*), human αB-crystallin (*αBC*), human small heat shock protein 27 (*H27*), and a *Drosophila* small heat shock protein known as embryonal lethal (2) 13-1 (*Dro*), all obtained from the NCBI data base. −, no residue; *, identical residue; ·, conserved residue.

cDNA sequence (Fig. 2*b*). To summarize, sequencing of both the cDNA and selected peptides revealed that mature p26 is a polypeptide of 191 amino acid residues with a calculated molecular mass of 20.7 kDa and a predicted pI of 6.6. Throughout the paper numbering of amino acid residues is based on the sequence deduced from the cDNA and not on the mature protein.

Comparison of p26 to Other Small Heat Shock/α-Crystallin Proteins—p26 is divided into three regions that differ in their similarity to corresponding segments of other small heat shock/α-crystallin proteins (Figs. 3 and 4). The amino-terminal 59 residues of p26 comprise a variable domain, with limited similarity to other small heat shock/α-crystallin proteins. A unique characteristic of p26 within this family of proteins is that 10 of its first 29 amino acid residues are glycines (34% glycine content), and these are housed in a peptide of 22 residues (45% glycine content). By comparison, other members of the small heat shock/α-crystallin group shown in Fig. 3 have 1–4 glycines in the complementary region, which due to gaps in the sequence alignment are from 43 to 49 residues in length. Also noteworthy is that residues 36–51 of p26 contain 7 arginines (7/16 = 44%) and that 6 of these arginines are in a 10-residue stretch. This domain of p26 contained 3 regions of predicted β-sheet with the first two separated by a predicted α-helix. Although there was no strong homology to other proteins within the first 59 residues of p26, there was a weak similarity, based on the enrichments in glycine, arginine, and phenylalanine, to the fibrillarin and nucleolin families. A superficial similarity with mucins, due to a high serine/threonine content, was found in the carboxyl-terminal 35 residues of p26. However, this resemblance was judged to be meaningless because the mucins are heavily glycosylated and p26 is not.

When data base search programs like FASTA and BLAST were applied to the complete sequence of p26, only small heat shock/α-crystallin proteins appeared in the results. Membership depended on residues 60–157 that form the signature α-crystallin domain of p26, a sequence conserved in all members of the small heat shock/α-crystallin family. We previously reported on the degree of sequence identity between p26 and other small heat shock/α-crystallin proteins (13), and these results are not repeated. In this work we extend study of the p26 α-crystallin domain to include its predicted secondary structure, revealing that this area is divided into discrete regions of β-sheet, apparently with no intervening α-helix. When HSP27, αA-crystallin, and αB-crystallin were analyzed by the methods used for characterization of p26 secondary structure, regions of α-helix within α-crystallin domains were apparent, a result expected from published data (16). Of the β-sheet regions in the α-crystallin domain of p26, the first, beginning at residue 68, and the final three appeared to be buried. It was not possible to predict, based on our current analysis, if the β-sheets beginning at residues 83 and 103 are buried or exposed. Residues 158–192 represented a second variable region, distinguished by a hydrophilic tail longer than that found in any other small heat shock/α-crystallin protein.

Artemia Contain a Multi-gene Family for p26—Southern blots containing *Artemia* DNA digested with *Bam*HI and probed with a ^{32}P-labeled DNA fragment, p26-3-6-3, corresponding to the entire coding region of p26, yielded 4 bands, two strong and two weak (Fig. 5, *lane 5*). When blots were reprobed with PCR-generated DNA fragments, two of the bands hybridized to p26-5′ whereas the other two reacted with p26-3′, corresponding to the 5′- and 3′-ends of p26-3-6-3, respectively (Fig. 5, *lanes 6* and *7*). Comparison of values gener-

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

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

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

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

FIG. 4. **Predicted structural characteristics of p26.** The secondary structure and solvent accessibility of amino acid residues within p26 were predicted as described under "Materials and Methods." The entire sequence (*AA*) of p26 is shown with every 10th residue numbered. The designations within the figure are as follows: *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 line representing the predicted solvent accessibility indicated that it could not be determined if the residue was buried or exposed.

ated by scanning of bands *B1–B4* in Fig. 5, *lane 5*, to those obtained by scanning the bands for increasing amounts of p26-3-6-3 in Fig. 5 (*lanes 1–4*), indicated that there were at least three copies of the p26 gene in *Artemia*.

Multiple mRNAs for p26 in Artemia Embryos—Northern blots of poly(A)$^+$ mRNA from cysts were hybridized to ^{32}P-labeled cDNA consisting of the complete p26 coding region (Fig. 6). Observation of a major band (0.7 kb) and a minor band (1.9 kb) revealed that there were two size classes of p26 mRNA in encysted *Artemia* embryos.

Compartmentalization of p26 in Nuclei of Artemia Embryos—Because pH has been shown to have a marked effect on the intracellular location of p26 (see Ref. 49), nuclei were isolated under acidic (pH 6.5) and basic (pH 7.6) conditions. Both preparations were electrophoresed in SDS-polyacrylamide gels and either stained with Coomassie Blue (Fig. 7*a*) or blotted to nitrocellulose and stained with antibody to p26 (Fig. 7*b*). Comparison of *lanes 1* and *4*, respectively, with *lanes 2* and *5*, in both Fig 7, *a* and *b*, showed that a portion of the p26 within encysted gastrulae cosedimented with nuclei. When nuclei purified on Percoll gradients were examined in SDS-polyacrylamide gels, p26 was again observed, demonstrating that the protein was tightly associated with the nuclei (Fig. 7, *a* and *b, lanes 3* and *6*). To determine if p26 was located internally, purified nuclei prepared under acidic conditions were double-stained with either DAPI or propidium iodide and antibody to p26 and then examined with a fluorescence microscope. All nuclei stained by DAPI/propidium iodide were labeled by antibody to p26 (Fig. 8*a*, *a′*). Examination at a higher magnification indicated that p26 was dispersed throughout the nuclei, with some of the protein concentrated in brightly stained foci (Fig. 8*b*, *b′*). That this is the arrangement of p26 was verified by optical sectioning of nuclei with the confocal microscope (Fig. 9). The brightly stained regions generally appeared in no more than two consecutive sections, indicating they were discrete, spherical objects about 1 μm in diameter, a value approximating the greatest *en face* diameter of stained foci shown in the figure. The staining patterns of nuclei prepared at a basic pH and by crushing cysts between glass slides were identical to those just shown.

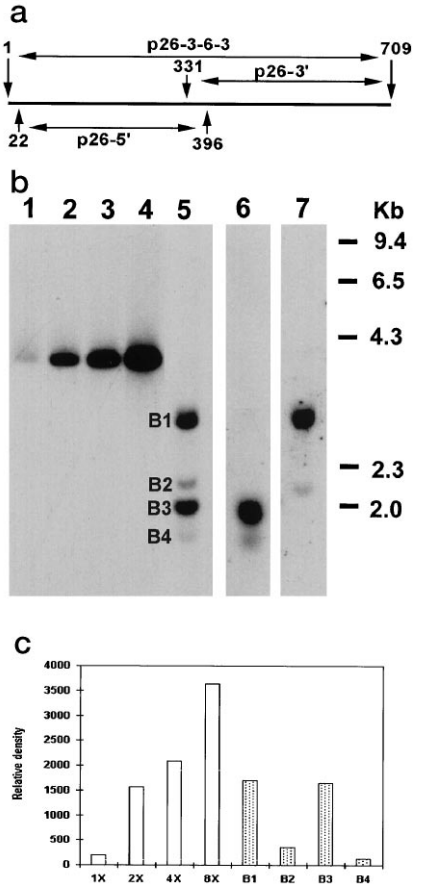

FIG. 5. ***Artemia* contain a multi-gene family for p26.** The number of genes encoding p26 and their arrangement within the genome were examined by hybridization of ^{32}P-labeled probes encoding known regions of p26-3-6-3 to Southern blots. *a*, schematic representation of the probes prepared as described under "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 hybridized to probes prepared from p26-3-6-3. *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 *Bam*HI. 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 1–5* were hybridized to p26-3-6-3, and *lanes 6* and *7* were hybridized to p26-5′ and p26-3′, respectively. The bands obtained by hybridization of p26-3-6-3 to restriction-digested *Artemia* DNA are labeled *B1–B4* in *lane 5*. Size markers in kb are on the *right side* of the figure. *c*, *lanes 1–5* shown in *b* were scanned, and the density of each band was plotted, in arbitrary units, on the histogram: *lane 1*, 1 ×; *lane 2*, 2 ×; *lane 3*, 4 ×; *lane 4*, 8 ×; the values represent densities equivalent to 1, 2, 4, and 8 copies of the p26 gene per haploid genome. *B1–B4* correspond to *bands B1–B4* in *b*.

DISCUSSION

Our previous work revealed that encysted *Artemia* embryos contain very large quantities of a low molecular weight protein termed p26 (13, 49, 53, 58). Partial sequencing of purified p26 indicated that it is a small heat shock/α-crystallin protein, while other experiments demonstrated an *in vitro* chaperone activity (13). Our objectives in this work were to analyze further the properties of p26 and to explore more fully the relationship of this protein to other small heat shock/α-crystallin proteins through study of its molecular structure. Additionally, we hoped to shed light on the role of p26 during diapause and other types of stress, thus contributing to our understanding of small heat shock/α-crystallin proteins in other eukaryotic cells.

Determination of the complete p26 sequence confirmed the presence of an α-crystallin domain and showed that it is positioned toward the carboxyl terminus, as for other small heat shock/α-crystallin proteins (16, 17). The α-crystallin domain has been reported to consist of two hydrophobic motifs enriched

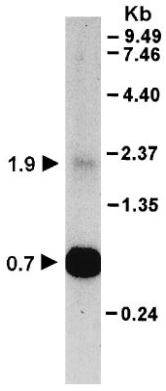

FIG. 6. **Encysted *Artemia* gastrulae contain two size classes of p26 mRNA.** Poly(A)$^+$ mRNA from encysted *Artemia* gastrulae was electrophoresed in 1.5% agarose gels, blotted to Hybond™-N+ membrane, and hybridized to ^{32}P-labeled p26-3-6-3. Two size classes of p26 mRNA, at 1.9 and 0.7 kb, are indicated by the *labeled arrowheads*. Size markers in kb are on the *right side* of the figure.

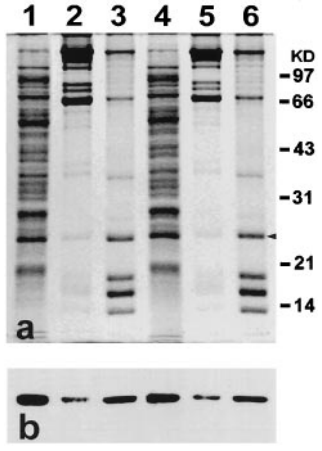

FIG. 7. **p26 is associated with nuclei from encysted *Artemia* embryos.** Protein samples obtained during the preparation of nuclei from *Artemia* cysts were electrophoresed in 12.5% SDS-polyacrylamide gels and either stained with Coomassie Blue (*a*) or blotted to nitrocellulose and stained with antibody to p26 (*b*). *Lanes 1–3* contained samples prepared under basic conditions, and samples in *lanes 4–6* were prepared under acidic conditions. The lanes contained: *M*, molecular mass markers $\times 10^{-3}$; *lanes 1* and *4*, the initial 2000 $\times$ *g* supernatant from a nuclei preparation; *lanes 2* and *5*, the initial 2000 $\times$ *g* pellet from a nuclei preparation suspended in a volume equal to half that of the starting homogenate; *lanes 3* and *6*, purified nuclei. *Lanes 1* and *4* received 10 μl of sample, and *lanes 2* and *5* received 5 μl, allowing direct comparison of band intensities because pellets were resuspended in half of the initial homogenate volume. *Lanes 3* and *6* received 4 μl of sample. The *arrowhead* in *a* indicates the position of p26.

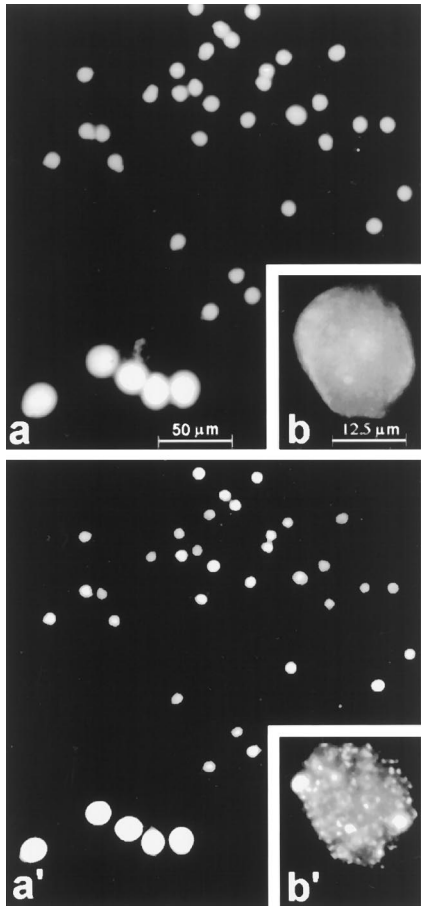

FIG. 8. **Fluorescent staining of nuclei from encysted *Artemia* embryos.** *a, a′*, low magnification image of a field of nuclei purified in Percoll and double-stained with DAPI (*a*) and antibody to p26 (*a′*). The *bar* in *a* represents 50 μm, and both figures are the same magnification. *b*, *b′*, a nucleus purified in Percoll and double-stained with propidium iodide (*b*) and antibody to p26 (*b′*). The *bar* in *b* represents 12.5 μm, and the magnification is the same in both figures.

in β-pleated sheets and separated by an α-helical, hydrophilic region (16, 17). The first motif is composed of three β-pleated sheet regions, preceded by an α-helix ($\alpha\beta\beta\beta$), an arrangement repeated in the second half of the α-crystallin domain. p26 is unusual in that it appears to lack regions of α-helix in the α-crystallin domain, although it is predominantly β-sheet as expected. Similar secondary structure and hydrophobicity profiles among the small heat shock/α-crystallin proteins suggest that α-crystallin domains have the same function. For example, Smulders *et al.* (59) have shown that modification of Asp-69 to Ser within the sequence 63-EVRSDRD-69 disrupts chaperone activity in αA-crystallin, perhaps due to a change in charge distribution. This residue is close to the beginning of the α-crystallin domain; it occurs in several small heat shock/α-crystallin proteins, and Asp-67 within the sequence 61-SLRDTAD-67 may be the equivalent residue in p26.

Site-directed mutagenesis of Phe-24 and Phe-27 indicates that a conserved region (22-RLFDQFFG-29) outside the α-crystallin domain is essential for chaperone-like activity of αB-crystallin (19). Crabbe and Goode (15) also suggest, through comparisons of published data, that the peptide RLFDQFF is important in oligomerization of small heat shock/α-crystallin proteins. The equivalent region in p26, a multimeric protein with chaperone activity, is 21-FGFGGFGGG-29, interestingly in the glycine-rich sequence. There are two phenylalanines in positions comparable to those in αB-crystallin, but the surrounding residues show little similarity. Thus, if the phenylalanines are mechanistically important in chaperone action and oligomerization, their neighboring residues are less critical for these functions. In another case, modification of aspartic acid at the amino terminus, and two juxtaposed lysines at the carboxyl terminus, reduced the chaperone-like action of αB-crystallin (19). Corresponding residues are missing from p26, although its carboxyl-terminal tail contains two arginines in close proximity to one another and near the end of the protein. Moreover, p26 has an extended, hydrophilic tail. Only SEC-1, a developmentally regulated small heat shock protein of 159 amino acids from *Caenorhabditis elegans* possesses a carboxyl-terminal extension with a comparable number of serines and threonines (34). The carboxyl terminus of p26 and other small heat shock/α-crystallin proteins may "capture" unfolded proteins and keep complexes of chaperones and denatured proteins in solution, explaining why its charged/polar characteristics, but not necessarily its sequence, are conserved.

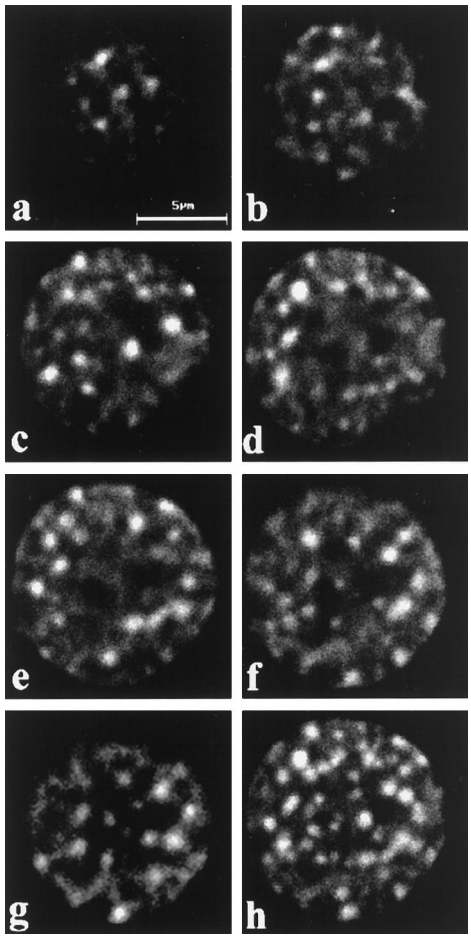

FIG. 9. **p26 is found in discrete compartments throughout nuclei from encysted *Artemia*.** Nuclei purified from *Artemia* cysts were stained with antibody to p26 and examined in the confocal microscope. *a–g* represent a continuous series of 1-μm optical sections through a single nucleus. The *top* and *bottom* sections of the nucleus are not shown as they contained limited information. *h* is a three-dimensional reconstruction of the stained nucleus. The *bar* in *a* represents 5 μm, and the magnification is the same for all figures.

Hybridization of p26-3-6-3 to Southern blots of *Bam*HI-digested DNA yields two strongly labeled bands and two weak bands, each reacting with a probe from either the 5′- or the 3′-end of p26-3-6-3, but not with both. These results, in concert with the relative staining intensities of the various bands, indicate that p26 is encoded by a multi-gene family. The heavily stained bands (B1 and B3) contain opposite halves of at least 2 copies of similar p26 genes, perhaps in tandem repeat, whereas the lighter bands are opposite halves of a separate p26 gene. By comparison, the small heat shock genes of *C. elegans,* except for SEC1 (34), are duplicated and arranged in tandem repeats (61, 62); they contain short introns and their synthesis is induced by stress. Plants exhibit many highly conserved small heat shock/α-crystallin proteins (14, 37, 39), and gene families have been reported for human HSP27 (63) and mouse HSP25 (64). At least a single representative of each family contains two introns, one of 600–700 bp and another of about 120 bp. Because p26-3-6-3 lacks a *Bam*HI site, there must be introns in the p26 genes, and if they exceed approximately 1200 nucleotides, then the weak 1.9-kb band on Northern blots of cyst mRNA probably represents nonprocessed transcripts. In support of this, the larger mRNA is only visible when the 0.7-kb message is most abundant.[3] At this time the transcription rate may be sufficiently rapid that mRNA production exceeds splicing, or as discussed by Head *et al.* (27), processing of mRNA transcripts is compromised during stress. Differential gene transcription may generate, at least in part, the p26 isoform heterogeneity seen previously on Western blots (49).

Small heat shock/α-crystallin proteins enter nuclei during stress (28, 65–67), but their function within the organelle is uncertain. For example, overexpression of human HSP27 in cultured Chinese hamster cells leaves stress-induced formation of aggregates within nuclei unaffected but leads to a faster recovery from damage, suggesting chaperone activity for HSP27 (38). Large amounts of p26 translocate into nuclei of encysted *Artemia* embryos during anoxia, probably due to a reduced intracellular pH, greatly increasing the amount of this protein in the organelle under conditions of stress (49, 53, 58). However, its intranuclear distribution was unknown, this being true for most other small heat shock/α-crystallin proteins. Staining with antibody to p26 is the same for nuclei released directly from cysts onto microscope slides and for those purified on Percoll gradients, under either acidic or basic conditions, regardless of the fixation method. Identical results obtained by several preparative protocols is a strong indication that the observed pattern reproduces the distribution of p26 *in vivo*. p26 may simply aggregate in the nuclei during stress and not interact with a particular structure or protein. Of more interest, the arrangement of p26 into foci suggests its association with specific nuclear compartments, such as protein assemblies required for DNA replication (68), or elements of the structural matrix within the nucleus (69). Sequence analysis failed to reveal a typical nuclear localization signal consisting of either a short stretch of lysines (PKKKRKV) or a bipartite signal wherein a spacer region of 10 to 12 amino acid residues separates a pair of basic clusters (KRPAATKKAGQAKKKK) (70–72). Of note, however, arginine is enriched in the nonconserved amino terminus of p26, and potential thus exists for an unusual nuclear localization signal in p26, a proposal best examined by site-directed mutagenesis and deletion analysis.

Nuclear localization of p26 is also interesting from another perspective. As noted previously, p26 resembles nucleolins and fibrillarins, families of proteins that occur in the nucleus, often in association with the nucleolus. Nucleolin is a 76-kDa protein which, like p26, shuttles between the nucleus and the cytoplasm, and it may be involved in the transport of ribosomal subunits. Fibrillarins are 40-kDa proteins essential for rRNA processing, as are other nucleolar proteins such as NSR1, SSB1, and GAR1, all of which contain the so-called GAR domain (73–75). This domain varies from 15 to 80 amino acid residues in length; it is composed mainly of glycine, arginine, and phenylalanine, and it exhibits, at least in nucleolin (75), repeated β-turns as a major structural motif. In fibrillarin the GAR domain is in the amino terminus and its arginines are largely dimethylated; in nucleolin and NSR1 this domain is in the carboxyl terminus, and it is in the central part of SSB1 and at each end of GAR1 (73–75). Additionally, the GAR domain occurs in nonnucleolar proteins (76–78). By comparison the GAR domain of p26, located in the amino variable region of the protein, is relatively small and its function has yet to be determined.

To conclude, we present, for the first time, the complete sequence of a small heat shock/α-crystallin protein from a crustacean. This protein exhibits a conserved α-crystallin domain, as well as unusual characteristics not found in other members of this group. Equally interesting to the molecular insights of this work are its physiological implications. As *Artemia* embryos enter diapause, deplete their ATP reserves, and undergo desiccation, a mechanism must exist to protect

[3] P. Liang and T. H. MacRae, unpublished data.

macromolecules and subcellular components. The same is true for fully hydrated cysts that survive anoxia for years. p26 is ideally suited for this role. Encysted embryos contain large amounts of p26 that exhibit chaperone activity *in vitro*, and a portion of the protein may engage intranuclear components. We propose that p26 binds to proteins as they denature under stress and expose hydrophobic residues, thereby preventing their aggregation and precipitation. As a consequence, after diapause or quiescence the embryos have a supply of functional proteins available for immediate resumption of growth.

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