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Small Heat Shock Protein p26 Associates With Nuclear Lamins and HSP70 in Nuclei and Nuclear Matrix Fractions From Stressed Cells

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Abstract The small heat shock/α-crystallin protein p26 undergoes nuclear translocation in response to stress in encysted embryos of the brine shrimp *Artemia franciscana*. About 50% of total p26 translocates to nuclei in embryos treated with heat shock or anoxia, and in embryo homogenates incubated at low pH. Nuclear fractionation shows that the majority of nuclear p26 and a nuclear lamin are associated with the nuclear matrix fraction. To further explore the roles of p26 and other HSPs in stabilizing nuclear matrix proteins (NMPs), nuclear matrices from control, and heat-shocked embryos were disassembled in urea and evaluated by one and two-dimensional (2-D) gel electrophoresis and Western immunoblotting after reassembling. Nuclear lamins were present only in reassembled fractions and, in the case of heat shock, p26 and HSP70 were also present. HSP90 was not detected in any nuclear fraction. Confocal microscopy on isolated nuclei and nuclear matrix preparations from control and heat-shocked embryos showed that the majority of p26 and a nuclear lamin share similar nuclear distributions. The combination of microscopy and fractionation results suggests that p26 and HSP70 play a role in the protection of nuclear lamins within the nuclear matrix. J. Cell. Biochem. 84: 601–614, 2002.

Key words: small HSP; HSP90; anoxia; thermal stress; *Artemia*; 2-D gel electrophoresis

The small heat shock/α-crystallin proteins are a family of molecular chaperones sometimes referred to as α-HSPs [de Jong et al., 1998]. These α-HSP proteins range in size from about 10 to 40 kDa monomer molecular mass but oligomerize into particles ranging from 400–800 kDa [Vanhoudt et al., 1998; Ehrnsperger et al., 1999; reviewed in MacRae, 2000]. As with many other heat shock proteins, α-HSPs protect cells during stress by preventing aggregation of unfolded proteins [Horwitz, 1992; Horwitz et al., 1998; Lindner et al., 1998], in some cases assisting in their renaturation [Jakob et al., 1993; Liang et al., 1997; Ganea and Harding, 2000; Viner and Clegg, 2001], and undergoing nuclear translocation [Arrigo et al., 1988; van de Klundert et al., 1998; van de Klundert and de Jong, 1999].

Encysted embryos of the primitive crustacean *Artemia franciscana* contain very large amounts of the α-HSP known as p26 (12–15% of total non-yolk protein). The genus *Artemia* is found in hypersaline environments world-wide, and its biology is well known [Persoone et al., 1980; Decleir et al., 1987; MacRae et al., 1989; Warner et al., 1989; Browne et al., 1991]. Reproduction occurs along two different pathways: fertilized eggs either develop directly and are released from females as free-swimming nauplii, or development stops at the gastrula stage (about 4,000 nuclei) and the embryos, surrounded by a complex shell, are released into the environment. These "encysted embryos" enter diapause [Drinkwater and Clegg, 1991], a condition of obligate developmental and metabolic arrest [Clegg et al., 1996]. It is only in these diapause-destined embryos that p26 is expressed. Synthesis is developmentally regulated and about 50% of p26 enters nuclei during diapause, exiting back into the cytoplasm when

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diapause is terminated [Jackson and Clegg, 1996; Liang and MacRae, 1999]. These embryos are among the most resistant of all multicellular eukaryotes to environmental insults, surviving years of continuous anoxia [Clegg, 1997; Clegg et al., 1999; Clegg et al., 2000], temperature extremes [Clegg and Conte, 1980; Clegg and Jackson, 1992; Clegg et al., 1999], exposure to various forms of radiation [see Persoone et al., 1980; Decleir et al., 1987], dehydration/rehydration cycles [Morris, 1971], and virtually complete desiccation [Clegg and Drost-Hansen, 1990; Crowe et al., 1992; Crowe et al., 1997]. p26 is one essential component of the adaptive mechanisms underlying these impressive abilities, but its role in nuclei of diapause and stressed embryos remains unclear.

Previous work revealed that after diapause ends, about 50% of the total p26 translocated back into nuclei in response to stresses such as heat shock and anoxia in vivo, and low pH in vitro [Clegg et al., 1994; Clegg et al., 1995; Clegg et al., 1999; Willsie and Clegg, 2001]. α-HSPs are involved in the stabilization of proteins that can be reactivated later by other chaperones [Rao et al., 1993; Ehrnsperger et al., 1997; Lee and Vierling, 2000; Wang and Spector, 2000], and recent studies show that they play a role in the regulation and protection of the cytoskeleton [Nicholl and Quinlan, 1994; Djabali et al., 1997; Muchowski et al., 1999; Perng et al., 1999; Head et al., 2000]. Much less is known about specific targets in the nucleus. One potential target is the nuclear matrix (NM), a thermally labile structure [Kampinga et al., 1985; Fisher et al., 1989; Roti Roti et al., 1998; Lepock et al., 2001; see also Berezney and Jeon, 1995] containing lamins, a type of intermediate filament (IF) protein [Nakayasu and Berezney, 1991; reviewed in Moir et al., 1995; Stuurman et al., 1998]. HSP70 has been associated with the nuclear matrix (NM) [Pouchelet et al., 1983; Kampinga et al., 1993; Xu et al., 1998] and with specific nuclear matrix proteins (NMPs) including lamins [Gerner et al., 1999]. These findings led us to explore interactions between p26, HSP70, and NMPs in encysted *A. franciscana* embryos. Nuclear fractionation showed that the majority of nuclear p26 in embryos treated with heat shock or anoxia, and in homogenates incubated at low pH, was present in the NM fraction, as was nuclear lamin. We next examined the involvement of p26, HSP70, and HSP90 in the reassembly of NMPs in vitro, finding that both p26 and one molecular weight isoform of HSP70 associated with the fraction containing reassembled lamin complexes. 2-D gel electrophoresis showed that all detectable isoforms of nuclear p26 and HSP70 were present in these reassembled fractions. Finally, nuclei and nuclear matrices were double-labeled for confocal microscopy which supported co-localization of nuclear p26 and lamin.

MATERIALS AND METHODS

Sources of *A. franciscana* Embryos and Their Decapsulation

A. franciscana from salterns in the San Francisco Bay (SFB) were purchased from San Francisco Bay Brand, Hayward, California, as dried (activated) embryos, and stored at about −10°C under 100% N_2. Embryos were placed at room temperature for 5 days before use and had a final hatching percentage of 87%. Dried embryos were hydrated in seawater overnight at 2°C to suppress metabolism, and the outer shell removed (decapsulation) prior to heat shock, anoxia, or homogenizing. Briefly, 5 g hydrated embryos were washed well with ice-cold distilled deionized (dd) H_2O, blotted, and placed in 200 ml of antiformin (7% NaOH, 3% Na_2CO_3, and 1% NaCl in 50% v/v Clorox™ bleach) on ice for 20 min, with frequent stirring. When the outer shell dissolved and embryos appeared orange, 200 ml of ddH_2O were added to dilute the antiformin which was decanted. These embryos were washed twice with 400 ml ice-cold 0.5 M NaCl, followed by 5 min of gentle stirring in 300 ml 1% sodium thiosulfate, rinsing with ddH_2O, then 5 min of gentle stirring in 300 ml 0.1 M HCl, and a final wash in ddH_2O. These steps neutralized and removed adsorbed hypochlorite. Decapsulated embryos were used immediately or stored at 2°C in 0.5 M NaCl overnight and then used. Hatching was monitored after each decapsulation to ensure that hatch level was not affected.

Hatching Assays

Groups of at least 200 embryos were placed into 20-welled plastic depression plates, each well containing 10–20 embryos in 400 μl 0.2 μm-filtered seawater. The plates were covered, sealed with tape to prevent evaporation, and incubated in constant light at 21–24°C until hatching was complete. Adequate O_2 is present in these sealed plates since controls

(activated embryos) exhibited 87% hatching within 2 days.

Nuclear Translocation of p26

Three treatments were used to transfer p26 into nuclei of activated embryos: heat shock, anoxia in vivo, and acidic pH in vitro [Clegg et al., 1994; Clegg et al., 1995; Clegg et al., 1999]. For heat shock, embryos were heated in aerated seawater in a Lauda RM40 waterbath (accurate to $\pm 0.05^{\circ}$C), at a rate of 0.7°C/min, from 22°C to 50°C, then used for nuclear isolation immediately. The temperature of 50°C was selected because this translocates the maximum amount of p26 into nuclei without killing any embryos [Clegg et al., 1999]. Embryos were made anoxic using 100% N_2 [Clegg, 1997] and subjected to 2 weeks of anoxia. Acid pH also acts as a translocation "switch" for p26, and was used to move p26 into nuclei in vitro [Clegg et al., 1995]. Control embryos were homogenized in buffer K (5 mM $MgSO_4$, 5 mM NaH_2PO_4, 40 mM HEPES, 70 mM potassium gluconate, and 150 mM sorbitol, pH 6.5), incubated at 22°C for 15 min, and the homogenates were then used for nuclear isolation.

Nuclear Preparations and Immunoblotting

Nuclei were isolated essentially according to the methodology of van Breukelen et al. [2000]. Decapsulated embryos were rinsed with ddH_2O on a cloth filter, drained and blotted, then homogenized in a Dounce homogenizer (loose pestle) on ice at 100 mg/ml in homogenizing medium (10 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, and 0.1% Nonidet P-40) and filtered through a single layer of Durawipe® (Johnson and Johnson). Filtrates were centrifuged at 800*g* for 10 min at 4°C, supernatants discarded, and carotenoids wiped from the tube walls. Pellets were resuspended in 2.2 ml buffer K (pH 8.0 for control, pH 6.5 for anoxia and heat shock) with a chilled glass rod. The resuspended pellet was layered onto a 75% (v/v) Percoll solution (9 ml Percoll; 3 ml 4 × buffer K, pH 8.0 or 6.5) and centrifuged (15,000*g*, 15 min, 4°C). This centrifugation pelleted most yolk platelets, while the nuclei formed a buff-colored layer at the Percoll/buffer K interface where they were collected and washed with buffer K (850*g*, 10 min, 4°C). The buff-colored nuclear pellet was resuspended in 1.5 ml buffer K and an aliquot stained with 0.1% bromophenol blue in buffer K for counting on a hemocytometer. Nuclear preparations consistently contained small to moderate numbers of yolk platelets. Nuclei were spun down (75 s at 2,000*g*) and resuspended at a concentration of 2×10^8/ml in the appropriate pH buffer K and used immediately for nuclear fractionation, NM preparation or confocal microscopy.

For SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting, a known number of nuclei was resuspended in a small volume of buffer K, followed by addition of an equal volume of 2 × sample buffer [Laemmli, 1970]. The suspension of nuclei was vortexed and heated at 100°C for 5 min. Supernatants were electrophoresed in 12% polyacrylamide gels, and proteins detected by Coomassie blue-G. Proteins from SDS–PAGE were also transferred to nitrocellulose sheets and prepared for immunodetection using a polyclonal anti-p26 (1:10,000; 1 h) [Clegg et al., 1994] or polyclonal anti-lamin (1:500; 12 h) [Dessev and Goldman, 1988] as the primary antibody, and horseradish peroxidase conjugated anti-rabbit IgG (1:5,000; 1 h) as secondary. The lamin antibody (rabbit anti-*Spisula* (clam)) was a gift from A.E. Goldman. For detection of HSP70 and HSP90, primary antibodies were purchased from Affinity BioReagents, Inc. (HSP70 MA3-001; 1:1,250; 2 h) and Stressgen, Inc. (anti-HSP90 SPA 840; 1:5,000; 1 h) and the secondary used was horseradish peroxidase conjugated anti-rat IgG (1:5,000; 1 h). Chemiluminescence was detected on X-ray film with Super Signal® West Pico (Pierce, Rockford, Illinois).

Nuclear Fractionation and Nuclear Matrix Isolation

Nuclear fractionation was carried out as follows [modified from Fisher, 1998 and Fey et al., 1984]. Approximately 2×10^8 nuclei (from control, heat-shocked or anoxic embryos, or homogenates incubated at pH 6.5) were transferred to 1.5 ml Eppendorf tubes and spun 75 s at 2,000*g* at 4°C. The supernatant was removed and the nuclei resuspended in 1 ml permeabilization buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, 0.5% Triton X-100, and 1.2 mM phenylmethyl sulfonylfluoride (PMSF)). Nuclei were incubated for 10 min at 0°C and spun 5 min at 2,000*g* at 4°C. Supernatants were removed and labeled "soluble fractions" (S). Pellets were resuspended in 1 ml nuclease digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM

$MgCl_2$, 0.5% Triton X-100, and 1.2 mM PMSF). RNase A (8 μg/ml) was added and the tube vortexed, and then DNase I (10 μg/ml) was added and the tube mixed by gentle inversion. Digestion was carried out at 23°C for 20 min and the samples spun at 10,000*g* for 10 min at 4°C. Supernatants were removed and labeled "nuclease digest" (ND). Pellets were extracted in 1 ml extraction buffer (250 mM ammonium sulfate, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, 0.5% Triton X-100, and, 1.2 mM PMSF) for 10 min at 0°C then spun 5 min at 2,000*g* at 4°C. Supernatants contained material extractable by "ammonium sulfate" (AS) and pellets were the "nuclear matrix" (NM) fraction. The S, ND, AS, and NM protein fractions were prepared for SDS–PAGE and immunoblotting as described above. Immunoblots were scanned and analyzed by Scion Image (www.scioncorp.com) to calculate pixel intensity and area of p26 bands. Calculations were repeated three times and the means were used to determine the relative % p26 present in each fraction.

Further study of NM fractions was carried out using the NM isolation method of Gerner et al. [1998] with modifications. This method was developed and optimized for the preparation of intact nuclear matrices as opposed to the preparation of sequential nuclear fractions described previously. All steps were performed at 0°C or 4°C, and all buffers contained a protease inhibitor cocktail (Complete™, Mini, Boehringer Mannheim). Isolated nuclei were suspended in 2 mM vanadyl ribonucleoside complex (VRC) with 1 M sucrose in buffer B (50 mM HEPES/NaOH, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA), incubated 3 min on ice and centrifuged 700*g*, 5 min, 4°C. Nuclei were resuspended in 0.2% Na-deoxycholate and 0.4% Tween, 2 mM VRC and 3 mM $MgCl_2$ in buffer A (10 mM HEPES/NaOH, pH 7.4, 10 mM NaCl, 1 mM EGTA) and incubated for 5 min on ice. Nuclei were collected by centrifugation (400*g*, 5 min) through a 300 mM sucrose cushion in buffer B. The pelleted nuclei were resuspended in 1.6 M sucrose in buffer B and sheared in a tightly fitting glass-Teflon Potter homogenizer. The suspension was placed on top of a sucrose step gradient (2.0/2.3/2.5 M sucrose in buffer B) and centrifuged in a swingout rotor at 77,000*g* at 4°C for 1 h. The pellet was carefully resuspended (gently using glass pipettes) in 1 M sucrose in buffer B and the nuclei pelleted (400*g*, 5 min, 4°C).

Nuclei were suspended in buffer C with 100 U/ml deoxyribonuclease I (DNase I from bovine pancreas, EC 3.1.21.1: Sigma) and incubated on a slowly tilting rotator at 22°C for 30 min. After centrifugation (400*g*, 5 min, 4°C), the residual nuclear pellet was resuspended in 250 mM ammonium sulfate in buffer C and incubated at 4°C for 5 min. The pelleted NM fraction was washed with 3 mM $MgCl_2$ in buffer A and used for reassembly assays or confocal microscopy.

Reassembly of NMPs

NMPs were dissolved in 8 M urea, 20 mM 4-morpholineethanesulfonic acid (pH 6.6), 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM PMSF, and 1% 2-mercaptoethanol (disassembly buffer) and separated from insoluble material by centrifugation for 2.5 h at 4°C and 111,000*g*. The supernatant was dialyzed for 12 h at 25°C against 2,000–4,000 volumes of 0.15 M KCl, 25 mM imidazole.HCl (pH 7.1), 5 mM $MgSO_4$, 2 mM DTT, 125 μM EGTA, and 0.2 mM PMSF (reassembly buffer). Reassembled (insoluble) proteins were pelleted by centrifugation at 111,000*g* for 2.5 h at 25°C while proteins remaining soluble (unassembled) were acetone precipitated (0.2% HCl in acetone, −20°C, overnight). All samples were then prepared for 1-D or 2-D gel electrophoresis.

2-D PAGE

IEF of NMPs used 7 cm non-linear pH gradient (3–10, 5–7 expanded) IPG strips on a Multiphor II apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. NMPs were solubilized in rehydration buffer (8M urea, 2% CHAPS, 20 mM DTT, 2% IPG Buffer, trace amounts of bromophenol blue) and incubated for 1 h at room temperature, then centrifuged at 5,000*g* for 15 min prior to loading. IPG strips were rehydrated overnight in a reswelling cassette with 130 μl of sample. The protocol for IEF was as follows: 200 V for 1 min, rising from 200 V to 3,500 V over the next 90 min and 3,500 V for 90 min for a total of 8,000 V-h. Following IEF, the strips were loaded with SDS in two 15 min equilibration steps at room temperature with continuous shaking then loaded onto the second dimension 12% polyacrylamide gels and sealed in place with 1% Agar. SDS–PAGE and Western immunoblotting were carried out as for 1-D gels.

Confocal Microscopy

Isolated nuclei and nuclear matrices were placed on poly-L-lysine coated slides (3 slides/isolate, 2 isolates/treatment) and fixed in 2% paraformaldehyde in 0.22 μm-filtered phosphate-buffered saline, pH 7.4 (PBS) for 30 min. After three 10 min washes in PBS, samples were permeabilized in 0.5% Triton X-100 in PBS for 2×15 min, washed 3×5 min in PBS, blocked in 0.1% Tween-20 in PBS for 2×15 min, washed 3×5 min in PBS, incubated in anti-p26 (polyclonal, goat, 1:500 in PBS) for 1 h, washed 3×5 min in PBS, blocked in normal goat serum (1:50 in PBS) for 30 min, incubated in Texas Red goat anti-rabbit (1:80 in PBS, Molecular Probes, Inc., Eugene, OR) for 50 min, and washed 3×5 min in PBS. Nuclear lamin was visualized by repeating the incubation procedure just described, but with a monoclonal rat anti-*Spisula* lamin (nwr2, 1:200, 12 h; gift from A.E. Goldman) as the primary and goat-anti-rat Alexa Fluor™ 488 (1:500, 1 h, gift from A.E. Goldman) as the secondary. The rat monoclonal lamin antibody was prepared by cutting out the 67 kDa band from a polyacrylamide gel and injecting the incomplete adjuvent into rats [A.E. Goldman, personal communication]. Slides were either stored at −20°C or cover-slipped with anti-fade medium (90% glycerol, 10% PBS, 0.2% n-propyl galate), sealed, and viewed on a BioRad MRC 600 scanning confocal microscope equipped with a krypton/argon laser. Dual channel 568/488 excitations used a 568 nm filter block with < 600 nm cut-off to ensure no signal bleed-through occurred from the 488 channel and this was regularly checked at a gain level $10 \times$ above that used to collect images. Secondary antibody controls were carried out, confirming that no signal was present in the absence of primary antibodies (negative controls not shown). Fifteen fields of view were examined per slide ($20 \times$) of isolated nuclei, with the number of nuclei per field of view ranging from 20–200. Images were collected at $60 \times$, pseudocolor added in Adobe Photoshop 5.0 and the "difference" function used to overlay signals.

RESULTS

Fractionation of Nuclei From Control and Stressed Embryos

Nuclei were isolated from control, heat-shocked or anoxic embryos, and from embryo homogenates incubated at low pH. Sequential fractionation of nuclei resulted in four fractions: soluble material (S), material released by nuclease digestion (ND), material extracted by ammonium sulfate (AS), and the insoluble NM fraction (NM). Fractions were analyzed by SDS–PAGE and Western immunoblotting with 700,000 nuclei (or fraction equivalents) loaded per lane. Figure 1A–D shows gels stained with Coomassie blue-G, and panels E–H show the corresponding Western immunoblots probed for p26 and nuclear lamin. Little p26 is present in control nuclei (Fig. 1A,E), and what is detected is limited to a small percentage of embryos that appear to remain in diapause [Willsie and Clegg, 2001]. In all treatments other than controls, nuclear p26 is present in significant amounts and found predominantly in NM fractions (Fig. 1B–D,F–H; lane NM). Nuclei from heat-shocked embryos (Fig. 1B,F) contain a trace of soluble p26 (lane S), and also some p26 that is extractable by ammonium sulfate (lane AS), but 72% of nuclear p26 is associated with the NM fraction (Fig. 1, lane NM; Table I). The effects of anoxia are similar to those of heat shock, with 69% of nuclear p26 in the NM fraction, but no soluble p26 is detectable (Fig. 1G; Table I). Anoxic samples had to be prepared on a different day than C, HS, and pH samples, but otherwise were treated identically. Nuclei isolated from embryo homogenates incubated at pH 6.5 contain p26 in all fractions

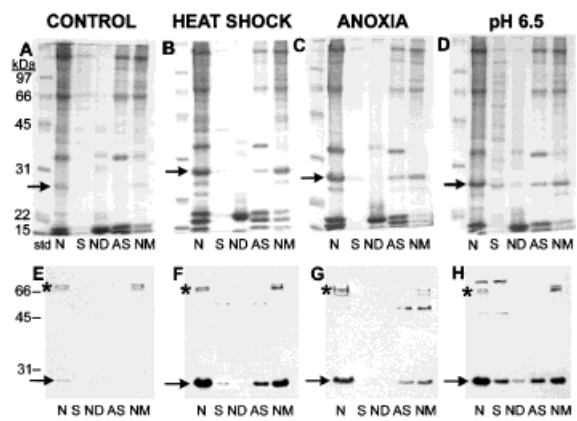

Fig. 1. Fractionation of nuclei from control and treated embryos. Nuclei were isolated from control, heat-shocked, and anoxic embryos, and embryos homogenized and incubated at pH 6.5. Isolated nuclei (N) were processed into soluble (S), nuclease-digested (ND), ammonium sulfate-extracted (AS), and nuclear matrix (NM) fractions. These fractions were analyzed by SDS–PAGE and Coomassie blue-G staining (**A–D**) and by Western immunoblotting (**E–H**). Arrows indicate p26 and asterisks indicate nuclear lamin.

TABLE I. Relative Amounts of p26 in Nuclear Fractions

Treatment	S	ND	AS	NM
Heat Shock	1.8	—	26.2	72.0
Anoxia	—	—	30.5	69.5
pH 6.5	20.0	2.9	26.5	50.6

Amounts of p26 in soluble (S), nuclease-digest (ND), ammonium sulfate extracted (AS), and nuclear matrix (NM) fractions. Not detected (—). Numbers are relative percent of total p26 in fractions as quantified using Scion Image (www.scioncorp.com, Materials and Methods).

(Fig. 1H). This difference in p26 distribution may be related to the in vitro technique used to translocate p26 into nuclei (homogenization and incubation at low pH at 22°C). However, most nuclear p26 (50%) is still associated with the NM fraction (Fig. 1D,H; Table I). The lamin antibody appears to recognize two proteins other than lamins around 50 and 90 kDa (Fig. 1B–D). This antibody normally detects two bands close to 67 kDa in *Spisula* [A.E. Goldman, personal communication] indicating our additional bands are probably *A. franciscana* non-lamin proteins with epitopes similar to *Spisula* lamin. P26 bands from nuclear fractions in Figure 1F–H were analyzed by Scion Image (www.scioncorp.com) to determine the relative % nuclear p26 associated with each fraction (Table I). Nuclei from all three treatments contain 26–30% of p26 in AS fractions. Anoxic and heat-shocked embryos have the remaining approximately 70% in the NM fraction, while nuclei from homogenates incubated at low pH contain only 50% in the NM fraction with the remaining 20% present in the S fraction. Small amounts (1–2%) of p26 were also detected in the heat shock S and pH 6.5 ND fractions (Table I).

Reassembly of NMPs and Chaperones

To further explore the interaction of p26 and lamin in the NM, the protocol of Gerner et al. [1999] was used (Materials and Methods). NMPs from control (C) and heat-shocked (HS) embryos were dissolved in disassembly buffer containing urea, and insoluble aggregated material was removed by centrifugation. The supernatant was dialyzed overnight in reassembly buffer, allowing NM proteins to reassemble. Reassembled protein complexes were spun down by centrifugation (INSOL) and unassembled proteins remaining soluble were collected by acetone precipitation (SOL). Samples were analyzed by SDS–PAGE and Western immunoblotting for the presence of HSP70, HSP90 (Fig. 2A), p26 and lamin (Fig. 2B). Control lanes contained either control supernatants (con sup) or nuclei from HS embryos (hs nuc). HSP90 was not detected in any of the NM preparations, control or HS, soluble or insoluble, while HSP70 was primarily in the insoluble heat shock fraction with a trace amount in the soluble fraction (Fig. 2A). Note that only one of the two molecular weight isoforms of HSP70 present in the supernatant (con sup) is detected in nuclei. Lamin was almost exclusively present in the insoluble (reassembled) fraction of both control and heat shock NM preparations, although small amounts remained soluble, mainly in the heat shock sample (Fig. 2B). p26 was detected only in the reassembled insoluble heat shock fraction.

2-D Gel Electrophoresis of NMPs After Reassembly

Soluble and insoluble fractions of post-dialysis NM preparations from control and heat-shocked embryos were analyzed by 2-D gel electrophoresis and Western immunoblotting to determine which isoforms of p26 and HSP70

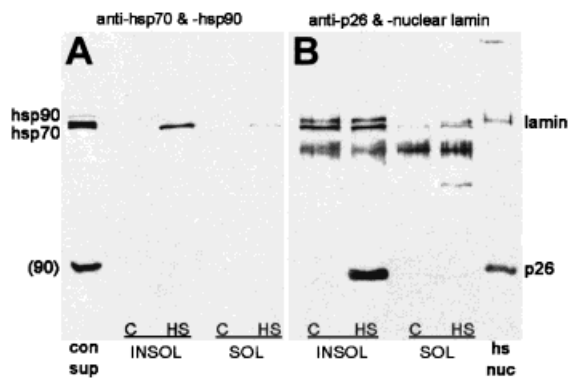

Fig. 2. Nuclear matrix preparations from nuclei isolated from control and heat-shocked embryos. Nuclear matrix proteins (NMPs) were prepared according to Gerner et al. [1999], solubilized in urea and dialyzed over night. Reassembled protein complexes were spun down as insoluble protein (INSOL), while proteins remaining unassembled were collected in the soluble fraction (SOL). These fractions were analyzed by Western immunoblotting for HSP70 and HSP90 (**A**) and p26 and nuclear lamin (**B**). Control lanes contained either a low-speed supernatant (known to contain HSP70 and 90) from control embryos (A), or intact nuclei from heat-shocked embryos (known to contain p26 and nuclear lamins) (B). (90) indicates a cytoplasmic protein recognized by the HSP90 antibody.

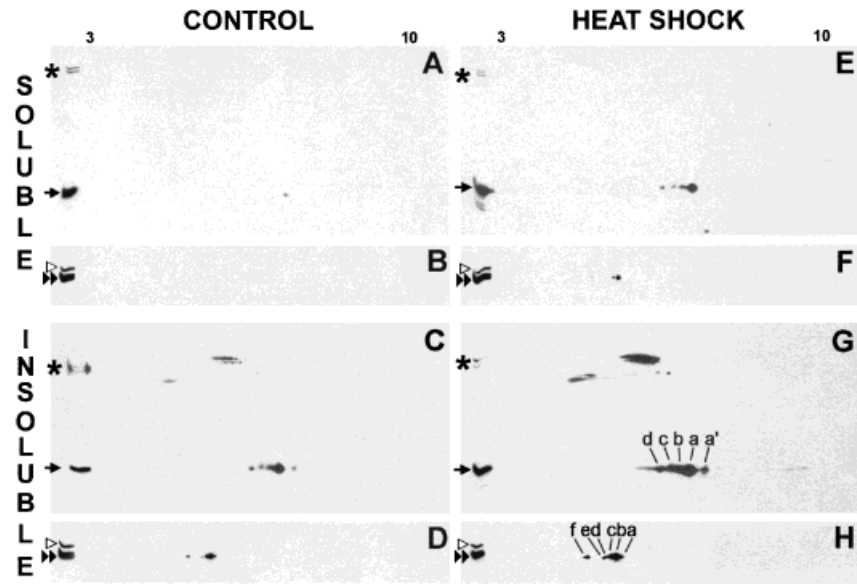

Fig. 3. Reassembly of NMPs p26, HSP70, and lamin. Samples, prepared as in Figure 2, were separated by two-dimensional electrophoresis (control soluble: **A,B**; control insoluble: **C,D**; heat shock soluble: **E,F**; heat shock insoluble: **G,H**) and analyzed by Western immunoblotting for p26, lamin (A,C,E,G), HSP70, and 90 (B,D,F,G). p26 isoforms are labeled a–d, and as the most basic p26 isoform was not observed previously, it has been labeled a′. HSP70 isoforms are labeled a–f, starting with the most basic isoform. Isoform labeling in G and H applies to all parts of the figure. (arrows: p26; asterisk: lamin; double arrow head: HSP70, open triangle: HSP 90). This figure is representative of two separate experiments.

were associated with reassembled NMPs (Materials and Methods). Figure 3 shows these results for control soluble (Fig. 3A,B), control insoluble (Fig. 3C,D), heat shock soluble (Fig. 3E,F), and heat shock insoluble (Fig. 3G,H) fractions. Isoform labels in G and H apply to all parts of the figure. HSP90 was not detected in any sample. The control soluble blots show only a small amount of p26 isoform a, no lamin, and no HSP70 (Fig. 3A,B) while in the heat shock soluble fraction five p26 isoforms (a′,a,b,c,d) and three HSP70 isoforms (b,c,f) remain associated with unfolded proteins (Fig. 3E,F). p26 isoforms a–d are all present in p26 purified from embryo cytoplasm and are currently being described [Viner et al., unpublished observations] and a′ (named because it is more basic than isoforms a–d) appears to be an isoform unique to NM preparations. In the control insoluble (reassembled) fraction, five isoforms of p26 (a′,a,b, c,d), four isoforms of HSP70 (a,b,c,f) and two MW groups of lamin isoforms (pI range 5.3–5.8) are detected. Individual lamin isoforms are difficult to distinguish when they blend together as in Figure 3C,G. In the heat shock insoluble fraction, we see again five p26 isoforms (a′,a,b, c,d), two molecular weight groups of lamin isoforms (Fig. 3G) and six HSP70 isoforms (a,b,c,d,e,f) (Fig. 3H). Control lanes contain nuclei from heat-shocked embryos for panels A,C,E,G and control supernatants for panels B,D,F,H. As in Figure 2, the lamin antibody recognizes additional unknown proteins around 50 kDa.

Confocal Microscopy of Nuclei and NM Preparations

Nuclei and nuclear matrices from control and heat-shocked embryos were placed on poly-L-lysine coated slides and prepared for confocal microscopy. Figure 4 shows representative nuclei and nuclear matrices from control and heat-shocked embryos double-labeled with Alexa Flour 488^{TM} (lamin, red) and Texas Red^{TM} (p26, yellow). The two separately labeled images are followed by a third, showing signal overlap (green). Control nuclei show homogeneous lamin distribution with little or no signal from p26 (CON NUC). Lamin staining of control NM preparations gives slightly less homogeneous results, indicating the extraction protocol had an effect on nuclear lamin organization (CON NM). It also appears that heat shock somewhat disrupts lamin organization in nuclei (HS NUC), as the lamin signal here is less homogeneous than in control nuclei. We have previously shown that nuclei from heat-shocked embryos contain large amounts of p26, [Willsie

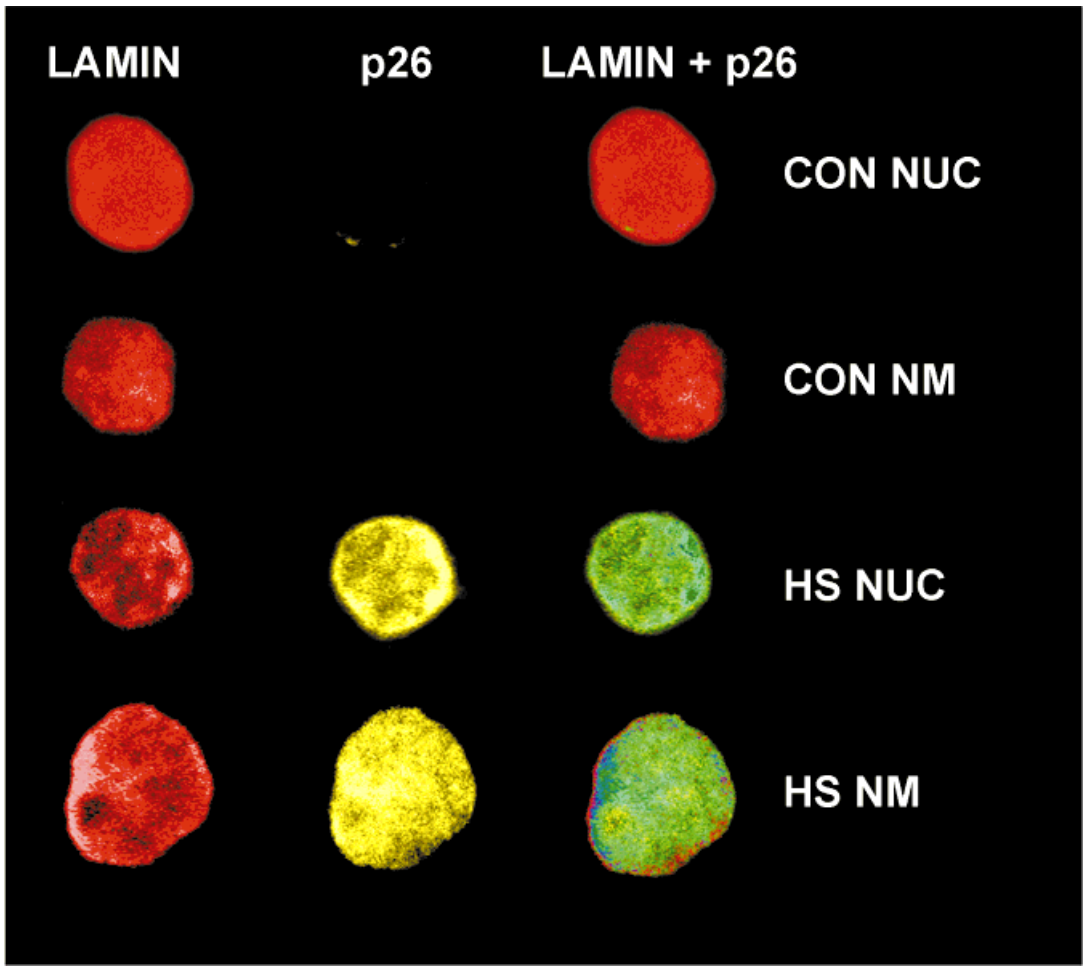

Fig. 4. Nuclear distribution of p26 and lamin in control and heat-shocked embryos. Nuclei (NUC) and nuclear matrices (NM) from control (CON) and heat-shocked (HS) embryos were double-labeled for lamin (Alexa Flour™ 488, red; lighter where saturated) and p26 (Texas Red™, yellow; lighter where saturated) and examined using confocal microscopy. Secondary labeling was consistent with both antigens when viewed separately as well as together, and saturation is due to elevated antigen levels and not artifact. Pseudocolor was added in Photoshop 5.0, and the difference function used to show overlap of signals (green, blue where saturated). No signal bleed-through occurred (Materials and Methods). Nuclei shown are representative of three replicates prepared per treatment from each of two nuclear isolations and nuclear matrix preparations (bar = 10 μm).

and Clegg, 2001] and this is supported in Figure 4 (HS NUC). Figure 4 also shows that p26 remains associated with nuclear matrices extracted from nuclei of heat-shocked embryos (HS NM), supporting the immunoblot data in Figures 1 and 2. There is also a difference in texture between nuclei and nuclear matrices from heat-shocked embryos, and the HS NM overlay image contains regions of red and yellow, demonstrating that p26 and lamin do not always overlap completely. In signal hot spots where fluorescence approaches saturation, red (lamin) and yellow (p26) signals become lighter and green becomes blue (overlap). In all cases, overlap of fluorescent signal was not due to channel bleed-through as this was regularly checked at a gain level 10 × above that used to collect images. Samples prepared for detection of p26 or lamin alone (not shown) resulted in images similar to samples that were double-labeled. Signal patterns for lamin and p26 are very similar and the overlaid images demonstrate that these proteins largely overlap as would be expected if p26 were chaperoning lamins as part of the NM.

DISCUSSION

We suggested previously that one potential target of p26 and its chaperone function is the

NM [Willsie and Clegg, 2001]. The term "nuclear matrix" was first introduced by Zbarskii and Debov [1948] and then re-introduced by Berezney and Coffey [1974]. Initially described as "a residual framework structure that maintains the spherical shape of the nucleus ... essentially free of the DNA, RNA and phospholipids", the NM initially appeared to be involved in DNA synthesis [Berezney and Coffey, 1975]. What we now call the NM remains controversial; there are strongly held opinions both for and against its existence [reviewed by Cook, 1988; Jack and Eggert, 1992; Martelli et al., 1996; Pederson, 1998; Pederson, 2000]. However, the consistent presence of a non-chromatin scaffolding network in the nucleus after various chromatin removal techniques [Jackson et al., 1988; Wan et al., 1999], extraction protocols [Fey et al., 1984; Nickerson et al., 1997; Fisher, 1998; Gerner et al., 1998], and visualization methods [Fey et al., 1986; Hendzel et al., 1999] supports the existence of a structural and functional nuclear filamentous network. For the purposes of this discussion, we refer to this extrachromosomal scaffolding as the nuclear matrix, or NM.

Nuclear lamins are among the core NMPs [Nakayasu and Berezney, 1991]: they are type V IF proteins and are closely related to cytoplasmic IFs [reviewed in Stewart, 1993; Moir et al., 1995; Stuurman et al., 1998]. Nuclear lamins are a major constituent of the nuclear lamina, but are also present in the nucleoplasm [Jackson et al., 1988; Goldman et al., 1992; Moir et al., 1994; Hozák et al., 1995] and undergo dynamic changes throughout the cell cycle [reviewed in Stuurman et al., 1998; Moir et al., 2000b]. Destabilzation of this nuclear lamin network using immunoprecipitation [Newport et al., 1990; Meier et al., 1991] or introduction of mutant lamins [Goldberg et al., 1995; Spann et al., 1997; Moir et al., 2000a] creates brittle and unstable nuclei in which DNA synthesis is blocked. These results suggest that lamins supply mechanical support within the nucleus that is crucial to DNA replication. Morphological observations also suggest that a filamentous lamin network acts as a scaffold for DNA replication factories [Hozák et al., 1993, 1994]. Given these observations, we may suppose that lamins must be protected from stress-induced damage.

Cytoskeletal IF proteins have been regularly reported to interact with HSPs: cytoskeletal elements and α-crystallins in lens cells [Fitz-Gerald and Graham, 1991; Nicholl and Quinlan, 1994], β-Actin and TCP-1 in reticulocytes [Marco et al., 1994], and IF proteins and HSP70 in glandular epithelial cells [Liao et al., 1995] and murine L cells [Pelham et al., 1984]. More recently, it was shown that HSP70 and a TCP-1 like protein are associated with reassembling lamin B2 filaments in the NM fraction of human leukocytes [Gerner et al., 1999]. These reports suggest that there are functional interactions between molecular chaperones and IF proteins and provide a useful model to explore the role of p26 in the protection and/or stabilization of NMPs in *A. franciscana* embryos. The association of HSPs and the NM is not a new topic [Pouchelet et al., 1983; Roti Roti and Turkel, 1994]. Proteins in the NM are reported to be among the most thermally labile of all proteins in the cell [Roti Roti et al., 1998] making them obvious chaperone targets [Pouchelet et al., 1983; Gerner et al., 1999; Lepock et al., 2001]. This is consistent with our finding that p26 associates with NM fractions of heat-shocked and anoxic embryos and homogenates incubated at low pH, and that multiple isoforms of p26, HSP70, and nuclear lamin are present in reassembled NMP complexes. Additionally, confocal microscopy showed that p26 and nuclear lamin have similar distributions within nuclei and nuclear matrices isolated from heat-shocked embryos. Next we discuss these results and the role they indicate for nuclear p26.

Nuclear fractionation showed lamin was limited to two bands near 70 kDa in intact nuclei and NM fractions under all conditions (Fig. 1E–H), similar to nuclear lamins described by other groups [McConnell et al., 1987; Dessev and Goldman, 1988; Lin and Fisher, 1990; Fisher, 1998]. In control embryos, it appears that only those still in diapause contain nuclear p26 [Willsie and Clegg, 2001] and only small amounts of p26 were detected in nuclei from this population (Fig. 1). Relative to controls, NM fractions from all other treatments contained very large amounts of p26. Heat shock is known to increase the amount of protein associated with the NM [reviewed in Roti Roti et al., 1997; VanderWaal et al., 1999], and this increase includes NM-associated HSPs [Roti Roti and Turkel, 1994; VanderWaal et al., 1996; Lepock et al., 2001]. Our data for p26 agree with those results (Table I). The effect of anoxia on

NM-associated proteins is less well studied and was included here because anoxia/hypoxia is an environmental stress that *A. franciscana* embryos encounter in nature [Persoone et al., 1980]. Our results show that anoxia causes a response similar to heat shock in these embryos, translocating p26 to the NM (Fig. 1). This stress response has also been reported in *Drosophila* where anoxia and heat shock both result in nuclear translocation of HSP70 [Velazquez and Lindquist, 1984]. The use of acid pH to translocate p26 into nuclei of *A. franciscana* embryos in vitro resulted in a somewhat different distribution of nuclear p26 (Fig. 1H). The in vitro approach, used to eliminate stresses associated with heat shock and anoxia, showed S and ND nuclear fractions contained increased amounts of p26 relative to nuclei from heat-shocked and anoxic embryos and that p26 in the NM fraction shows a corresponding decrease (Table I). pH_i is thought to act as a translocation "switch" for nuclear p26 [Clegg et al., 1995; Willsie and Clegg, 2001], and while these results support that, suggesting it may be involved in targeting p26 specifically to the NM, the low pH protocol also solubilizes 20% of the p26 that otherwise associates with the NM in response to heat shock and anoxia.

Gerner et al. [1999] were the first, to our knowledge, to provide evidence that there was an association between HSPs and lamins in the NM. Our results add to those observations by showing the association of both p26 and HSP70 with lamin (Fig. 2). All insoluble aggregates were removed by centrifugation prior to reassembly and, since we know that HSP90 is cytoplasmic in *A. franciscana* embryos [Clegg et al., 1999, 2000], we believe our results relate specifically to reassembled NMPs (Fig. 2B). It is not uncommon for HSP90 to undergo nuclear translocation [reviewed by Csermely et al., 1998; Yahara, 1998], but that does not appear to be the case in these embryos. One interesting aspect is that the experiments using human leukocytes did not involve stress of any sort and found HSP70, but not HSP27, associated with NMPs [Gerner et al., 1999]. Our results show that when p26 was present in nuclei (due to heat shock) it and HSP70 were both associated with the reassembled NM fraction (Figs. 2, 3), suggesting small HSPs may interact with the NM specifically as a stress response, while HSP70-NM interacts both constitutively and in response to stress.

HSP70 was not detected after 1-D SDS–PAGE in soluble or insoluble NM fractions from control embryos, but one of the isoforms present in the cytoplasm of these embryos did appear in heat shock NM fractions (Fig. 2). Most of this nuclear HSP70 isoform was present in the reassembled fraction with only a trace in the soluble fraction. These results contradict previous work indicating that nuclear HSP70 levels did not change under the heat shock conditions used in that study [Clegg et al., 1999]. The critical difference between these two studies was the use of a crude nuclear pellet verses an isolated NM preparation that is cleaner and enriched for a subset of nuclear proteins which includes HSP70.

The association of p26 and HSP70 with lamin examined by 2-D gel electrophoresis (Fig. 3) shows that translocation is not isoform-specific for p26, at least in response to heat shock. Nuclear translocation of HSP70 is specific to the lower MW isoforms, but all six detectable isoelectric variants are present in nuclei after heat shock. Two-dimensional protocols require larger amounts of protein be loaded than 1-D SDS–PAGE, explaining why p26 was detected in control NM fractions using 2-D, but not 1-D, gel electrophoresis. There is a p26 isoform unique to NM preparations (control and heat shock) that is even more basic than isoform a, and so we have called it a'. This isoform is not present in purified cytoplasmic p26 and was not detectable in preparations of intact nuclei [Viner et al. unpublished observations]. All of the HSP70 present in nuclear matrices was of the same molecular mass and its distribution supports what was seen in Figure 2: a small amount of HSP70 associated with the unassembled heat shock fraction with the majority in the reassembled/insoluble fraction (Fig. 2A). The pI range of lamin isoforms in these embryos is 5.3–5.8, similar to those published for lamins in *Drosophila* oocytes [5.5–6.8, Maus et al., 1995] and for lamin in several human cell types (5.3–5.4, Gerner et al., 1998].

The similar labeling patterns for p26 and lamin provided by confocal microscopy further support their proposed interaction (Fig. 4). The lamin staining pattern for control nuclei agrees with other published images [Hozák et al., 1995; Spann et al., 1997; Moir et al., 2000a] as does the p26 pattern for nuclei isolated from heat-shocked embryos [Willsie and Clegg, 2001]. Also, nuclear matrices showed no reduction in

signal intensity for either p26 or lamin after the stringent extraction procedures, with p26 and lamin signals again overlapping in the NM preparation. This suggests that the association of p26 with the NM is strong, supporting the idea that p26 chaperones lamin as a core NM filament.

We propose that the majority of p26 translocated into nuclei in response to heat shock, anoxia, low pH and diapause associates with the NM, and more specifically, lamin filaments within the NM. The thermal lability of NMPs makes them logical targets for p26 and other chaperones after heat shock and other stresses. Additionally, we propose that the association of HSP70 with reassembled lamin filament complexes and p26 indicates that multiple isoforms of p26 and HSP70 may work together in protecting and or chaperoning NMPs in these embryos.

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REFERENCES

Arrigo AP, Suhan JP, Welch WJ. 1988. Dynamic changes in the structure and intracellular locale of the mammalian low molecular weight heat shock protein. Mol Cell Biol 8:5059–5071.

Berezney R, Coffey DS. 1974. Identification of a nuclear protein matrix. Biochem Biophys Res Commun 60:1410–1417.

Berezney R, Coffey DS. 1975. Nuclear protein matrix: Association with newly synthesized DNA. Science 189: 291–293.

Berezney R, Jeon KW. 1995. Structural and functional organization of the nuclear matrix. Vol 162B Int Rev Cytol. San Diego: Academic Press, Inc.

Browne RA, Sorgeloos P, Trotman CNA. 1991. *Artemia* Biology. Boca Raton: CRC Press.

Clegg JS. 1997. Embryos of *Artemia franciscana* survive four years of continuous anoxia: The case for complete metabolic rate depression. J Exp Biol 200:467–475.

Clegg JS, Conte FP. 1980. A review of the cellular and developmental biology of *Artemia*. In: Persoone G, Sorgeloos P, Roels O, Jaspers E, editors. The Brine Shrimp *Artemia*, Vol 7. Wetteren, Belgium: Universa Press, p 11–54.

Clegg JS, Drost-Hansen W. 1990. On the biochemistry and cell physiology of water. In: Hochachka PW, Mommsen TP, editors. Biochemistry and molecular biology of fishes, Vol 1: Amsterdam: Elsevier Science Publishers, p 1–23.

Clegg JS, Jackson SA. 1992. Aerobic heat shock activates trehalose synthesis in embryos of *Artemia franciscana*. FEBS Lett 303:45–47.

Clegg JS, Jackson SA, Warner AH. 1994. Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*. Exp Cell Res 212:77–83.

Clegg JS, Jackson SA, Liang P, MacRae TH. 1995. Nuclear-cytoplasmic translocations of protein p26 during aerobic-anoxic transitions in *Artemia franciscana*. Exp Cell Res 219:1–7.

Clegg JS, Drinkwater LE, Sorgeloos P. 1996. The metabolic status of diapause embryos of *Artemia franciscana*. Physiol Zool 69:49–66.

Clegg JS, Willsie JK, Jackson SA. 1999. Adaptive significance of a small heat shock/alpha-crystallin protein in encysted embryos of the brine shrimp, *Artemia franciscana*. Amer Zool 39:836–847.

Clegg JS, Jackson SA, Popov VI. 2000. Long-term anoxia in encysted embryos of *Artemia franciscana*: Viability, ultrastructure, and stress proteins. Cell Tissue Res 301: 433–446.

Cook PR. 1988. The nucleoskeleton: Artefact, passive framework or active site? J Cell Sci 90:1–6.

Crowe JH, Hoekstra SA, Crowe LM. 1992. Anhydrobiosis. Ann Rev Physiol 54:579–599.

Crowe JH, Crowe LM, Petrelski S, Hoekstra FA, Araujo PD, Panek AD. 1997. Anhydrobiosis: Cellular adaptation to extreme dehydration. In: Dantzler WH, editor. Handbook of physiology, section 13, Vol II. New York: Oxford University Press, p 1445–1477.

Csermely P, Schnader T, Sti C, Prohaszka Z, Nardai G. 1998. The 90-kDa molecular chaperone family: Structure, function and clinical applications—A comprehensive review. Pharmacol Ther 79:129–168.

de Jong WW, Caspers GJ, Leunissen JAM. 1998. Genealogy of the alpha-crystallin/small heat-shock protein super family. Int J Biol Macromol 22:151–162.

Decleir W, Moens L, Slegers H, Sorgeloos P, Jaspers E. 1987. *Artemia* research and its applications, Vol 2 Wetteren, Belgium: Universa Press.

Dessev G, Goldman R. 1988. Meiotic breakdown of nuclear envelope in oocytes of *Spisula* solidissima involves phosphorylation and release of nuclear lamin. Dev Biol 130:543–550.

Djabali K, de Néchaud B, Landon F, Portier MM. 1997. αB-crystallin interacts with intermediate filaments in response to stress. J Cell Sci 110:2759–2769.

Drinkwater L, Clegg JS. 1991. Experimental biology of cyst diapause. In: Browne RAP, Sorgeloos P, Trotman CAN, editors. *Artemia* biology. Boca Raton: CRC Press, p 93–118.

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

Ehrnsperger M, Lilie H, Gaestel M, Buchner J. 1999. The dynamics of Hsp25 quaternary structure. Structure and function of different oligomeric species. J Biol Chem 274:14867–14874.

Fey EG, Wan KM, Penman S. 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. J Cell Biol 98:1973–1984.

Fey EG, Krochmalnic G, Penman S. 1986. The nonchromatin substructures of the nucleus: The ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. J Cell Biol 102:1654–1665.

Fisher PA. 1998. Preparation of karyoskeletal protein-enriched fractions from *Drosophila melanogaster* cells and tissues. Methods Cell Biol 53:23–46.

Fisher PA, Lin L, McConnel M, Greenleaf A, Lee M, Smith DE. 1989. Heat shock-induced appearance of RNA polymerase II in karyoskeletal protein-enriched ('nuclear matrix') fractions correlates with transcriptional shutdown in *Drosophila melanogaster*. J Biol Chem 264:3464–3469.

FitzGerald PG, Graham D. 1991. Ultrastructural localization of αA-crystallin to the bovine lens fiber cell cytoskeleton. Curr Eye Res 10:417–436.

Ganea E, Harding JJ. 2000. α-Crystallin assists in the renaturation of glyceraldehyde-3-phosphate dehydrogenase. Biochem J 345:467–472.

Gerner C, Holzmann K, Meissner M, Grimm R, Sauermann G. 1998. Similarity between nuclear matrix proteins of various cells revealed by an improved isolation method. J Cell Biochem 71:363–374.

Gerner C, Holzmann K, Meissner M, Gotzmann J, Grimm R, Sauermann G. 1999. Reassembling proteins and chaperones in human nuclear matrix protein fractions. J Cell Biochem 74:145–151.

Goldberg M, Jenkins H, Allen T, Whitfield WG, Hutchison CJ. 1995. *Xenopus* lamin B3 has a direct role in the assembly of a replication competent nucleus: Evidence from cell-free egg extracts. J Cell Sci 108:3451–3461.

Goldman AE, Moir RD, Montag LM, Stewart M, Goldman RD. 1992. Pathway of incorporation of microinjected lamin A into the nuclear envelope. J Cell Biol 119:725–735.

Head MW, Hurwitz L, Kegel K, Goldman J. 2000. αB-crystallin regulates intermediate filament organization in situ. Neuroreport 11:361–365.

Hendzel MJ, Boisvert FM, Bazett-Jones DP. 1999. Direct visualization of a protein nuclear architecture. Mol Biol Cell 10:2051–2062.

Horwitz J. 1992. Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad USA 89:10449–10453.

Horwitz J, Huang QL, Ding L, Bova MP. 1998. Lens α-crystallin: Chaperone-like activities. Methods Enzymol 290:365–408.

Hozák P, Hassan AB, Jackson DA, Cook PR. 1993. Visualization of replication factories attached to nucleoskeleton. Cell 73:361–373.

Hozák P, Jackson DA, Cook PR. 1994. Replication factories and nuclear bodies: The ultrastructural characterization of replication sites during the cell cycle. J Cell Sci 107:2191–2202.

Hozák P, Sasseville AMJ, Raymond Y, Cook PR. 1995. Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. J Cell Sci 108: 635–644.

Jack RS, Eggert H. 1992. The elusive nuclear matrix. Eur J Biol 209:503–509.

Jackson SA, Clegg JS. 1996. Ontogeny of low molecular weight stress protein p26 during early development of the brine shrimp, *Artemia franciscana*. Dev Growth Differ 38:153–160.

Jackson DA, Yuan J, Cook PR. 1988. A gentle method for preparing cyto- and nucleo-skeletons and associated chromatin. J Cell Sci 90:365–378.

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

Kampinga HH, Jorritsma JBM, Konings AWT. 1985. Heat-induced alterations in DNA polymerase activity of HeLa cells and of isolated nuclei. Relation to survival. Intl J Radiat Biol 47:29–40.

Kampinga HH, Muller E, Brunsting JF, Heine L, Konings AWT, Issels RD. 1993. Association of HSP72 with the nuclear (TX-100-insoluble) fraction upon heating tolerant and non-tolerant HeLa S3 cells. Int J Radiat Biol 9:89–98.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee GJ, Vierling E. 2000. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. Plant Physiol 122:189–197.

Lepock JR, Frey HE, Heynen ML, Senisterra GA, Warters RL. 2001. The nuclear matrix is a thermolabile cellular structure. Cell Stress Chap 6:136–147.

Liang P, MacRae TH. 1999. The synthesis of a small heat shock/α-crystallin protein in *Artemia* and its relationship to stress tolerance during development. Dev Biol 207:445–456.

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

Liao J, Lowthert LA, Ghori N, Omary MB. 1995. The 70-kDa heat shock proteins associate with glandular intermediate filaments in an ATP-dependent manner. J Biol Chem 270:915–922.

Lin L, Fisher PA. 1990. Immunoaffinity purification and functional characterization of interphase and meiotic *Drosophila* nuclear lamin isoforms. J Biol Chem 265:12596–12601.

Lindner RA, Kapur A, Mariani M, Titmuss SJ, Carver JA. 1998. Structural alterations of α- crystallin during its chaperone action. Eur J Biochem 258:170–183.

MacRae TH. 2000. Structure and function of small heat shock/α-crystallin proteins: Established concepts and emerging ideas. Cell Mol Life Sci 57:899–913.

MacRae TH, Bagshaw JC, Warner AH. 1989. Biochemistry and cell biology of *Artemia*. Boca Raton: CRC Press.

Marco S, Carrascosa JL, Valpuesta JM. 1994. Reversible interaction of β-actin along the channel of the TCP-1 cytoplasmic chaperonin. Biophys J 67:364–368.

Martelli AM, Cocco L, Riederer BM, Neri LM. 1996. The nuclear matrix: a critical appraisal. Histol Histopathol 11:1035–1048.

Maus N, Stuurman N, Fisher PA. 1995. Disassembly of the *Drosophila* nuclear lamina in a homologous cell-free system. J Cell Sci 108:2027–2035.

McConnell M, Whalen AM, Smithe DE, Fisher PA. 1987. Heat shock-induced changes in the structural stability of proteinaceous karyoskeletal elements in vitro and morphological effects in situ. J Cell Biol 105:1087–1098.

Meier J, Campbell KH, Ford CC, Stick R, Hutchison CJ. 1991. The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of *Xenopus* eggs. J Cell Sci 98:271–279.

Moir RD, Montag-Lowy M, Goldman RD. 1994. Dynamic properties of nuclear lamins: Lamin B is associated with sites of DNA replication. J Cell Biol 125:1201–1212.

Moir RD, Spann TP, Goldman RD. 1995. The dynamic properties and possible functions of nuclear lamins. In: Berezney R, Jeon KW, editors. Structural and functional organization of the nuclear matrix. Int Rev Cytol 162B. San Diego: Academic Press, Inc., p 141–182.

Moir RD, Spann TP, Herrmann H, Goldman RD. 2000a. Disruption of nuclear lamin organization blocks the elongation phase of DNA replication. J Cell Biol 149: 1179–1191.

Moir RD, Spann TP, Lopez-Soler RI, Yoon M, Goldman AE, Khuon S, Goldman RD. 2000b. Dynamics of the nuclear lamins during the cell cycle-relationship between structure and function. J Struct Biol 129:324–334.

Morris JE. 1971. Hydration, its reversibility, and the beginning of development in the brine shrimp, *Artemia salina*. Comp Biochem Physiol 39A:843–857.

Muchowski PJ, Valdez MM, Clark JI. 1999. αB-crystallin selectively targets intermediate filament proteins during thermal stress. Invest Ophthalmol Vis Sci 40:951–958.

Nakayasu H, Berezney R. 1991. Nuclear matrins: Identification of the major nuclear matrix proteins. Proc Natl Acad Sci USA 88:10312–10316.

Newport JW, Wilson KL, Dunphy WG. 1990. A lamin-independent pathway for nuclear envelope assembly. J Cell Biol 111:2247–2259.

Nicholl ID, Quinlan RA. 1994. Chaperone activity of α-crystallins modulates intermediate filament assembly. EMBO J 13:945–953.

Nickerson JA, Krockmalmic G, Wan KM, Penman S. 1997. The nuclear matrix revealed by eluting chromatin from a cross-linked nucleus. Proc Natl Acad Sci USA 94:4446–4450.

Pederson T. 1998. Thinking about a nuclear matrix. J Mol Biol 277:147–159.

Pederson T. 2000. Half a century of "The nuclear matrix". Mol Biol Cell 11:799–805.

Pelham H, Lewis M, Lindquist S. 1984. Expression of a *Drosophila* heat shock protein in mammalian cells: Transient association with nucleoli after heat shock. Philos Trans R Soc Lond B Biol Sci 307:301–307.

Perng MD, Cairns L, van den P, Prescott A, Hutcheson AM, Quinlan RA. 1999. Intermediate filament interactions can be altered by hsp27 and αB-crystallin. J Cell Sci 112:2099–2112.

Persoone G, Sorgeloos P, Roels O, Jaspers E. 1980. The Brine Shrimp, *Artemia*, Vol 2 Wetteren, Belgium: Universa Press.

Pouchelet M, St-Pierre E, Bibor-Hardy V, Simard R. 1983. Localization of the 70,000 dalton heat-induced protein in the nuclear matrix of BHK cells. Exp Cell Res 149:451–459.

Rao V, Horwitz J, Zigler JS. 1993. Alpha-crystallin, a molecular chaperone, forms a stable complex with carbonic anhydrase upon heat denaturation. Biochem Biophys Res Commun 190:786–793.

Roti Roti JL, Turkel N. 1994. Heat-induced changes in nuclear associated proteins in normal and thermotolerant HeLa cells. Radiat Res 139:73–81.

Roti Roti JL, Wright WD, VanderWaal RP. 1997. The nuclear matrix: A target for heat shock effects and a determinant for stress response. Crit Rev Eukaryot Gene Expr 7:343–360.

Roti Roti JL, Kampinga HH, Malyapa RS, Wright WD, VanderWaal RP, Xu M. 1998. Nuclear matrix as a target for hyperthermic killing of cancer cells. Cell Stress Chap 3:245–255.

Spann TP, Moir RD, Goldman AE, Stick R, Goldman RD. 1997. Disruption of nuclear lamin organization alters the distribution of replication factors and inhibits DNA synthesis. J Cell Biol 136:1201–1212.

Stewart M. 1993. Intermediate filament structure and assembly. Curr Opin Cell Biol 5:3–11.

Stuurman N, Heins S, Aebi U. 1998. Nuclear lamins: Their structure, assembly, and interactions. J Struct Biol 122: 42–66.

van Breukelen F, Maier R, Hand SC. 2000. Depression of nuclear transcription and extension of mRNA half-life under anoxia in *Artemia franciscana* embroys. J Exp Biol 203:1123–1130.

van de Klundert FAJ, de Jong WW. 1999. The small heat shock proteins hsp20 and alphaB-crystallin in cultured cardiac myocytes: Differences in localization and solubilization after heat stress. Eur J Cell Biol 78:567–572.

van de Klundert FA, Gijsen ML, van den PR, Snoeckx LH, de Jong WW. 1998. Alpha B-crystallin and hsp25 in neonatal cardiac cells–differences in cellular localization under stress conditions. Eur J Cell Biol 75:38–45.

VanderWaal R, Thampy G, Wright WD, Roti Roti JL. 1996. Heat-induced modifications in the association of specific proteins with the nuclear matrix. Radiat Res 145:746–753.

VanderWaal RP, Wright WD, Roti Roti JL. 1999. The effects of heat-shock on nuclear matrix-associated DNA-replication complexes. Crit Rev Eukaryot Gene Expr 9:363–371.

Vanhoudt J, Aerts T, Abgar S, Clauwaert J. 1998. Quaternary structure of bovine α-crystallin: Influence of temperature. Int J Biol Macromol 22:229–237.

Velazquez JM, Lindquist S. 1984. Hsp70: Nuclear concentration during environmental stress and cytoplasmic storage during recovery. Cell 36:655–662.

Viner RI, Clegg JS. 2001. Influence of trehalose on the molecular chaperone activity of p26, a small heat shock/α-crystallin protein. Cell Stress Chap 6:126–135.

Wan KM, Nickerson JA, Krockmalnic G, Penman S. 1999. The nuclear matrix prepared by amine modification. Proc Natl Acad Sci USA 96:933–938.

Wang K, Spector A. 2000. α-crystallin prevents irreversible protein denaturation and acts cooperatively with other heat-shock proteins to renature the stabilized partially denatured protein in an ATP-dependent manner. Eur J Biochem 267:4705–4712.

Warner AH, MacRae TH, Bagshaw JC. 1989. Cell and molecular biology of *Artemia* development. NY: Plenum Press.

Willsie JK, Clegg JS. 2001. Nuclear p26, a small heat shock/α-crystallin protein, and its relationship to stress

resistance in *Artemia franciscana* embryos. J Exp Biol 204:2339–2350.

Xu M, Wright W, Higashikubo R, Roti Roti JL. 1998. Intracellular distribution of hsp70 during long duration moderate hypothermia. Int J Hypertherm 14: 211–225.

Yahara I. 1998. Structure and function of the 90-kD stress protein Hsp90. In: Fink AL, Goto Y, editors. Molecular chaperones in the life cycle of proteins. New York: Marcel Dekker, p 183–192.

Zbarskii IB, Debov SS. 1948. On the proteins of the cell nucleus. Dokl Akad Nauk SSSR 63:795–798.