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NUCLEAR p26, A SMALL HEAT SHOCK/α-CRYSTALLIN PROTEIN, AND ITS RELATIONSHIP TO STRESS RESISTANCE IN *ARTEMIA FRANCISCANA* EMBRYOS

JULIA K. WILLSIE AND JAMES S. CLEGG*

Section of Molecular and Cellular Biology, and Bodega Marine Laboratory, University of California (Davis), Bodega Bay, CA 94923, USA

*Author for correspondence (e-mail: jsclegg@ucdavis.edu)

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Summary

The role of the small heat shock/α-crystallin protein, p26, in transcription in *Artemia franciscana* embryos was examined using isolated nuclei, containing either control or elevated levels of p26, in transcription run-on assays. Heat shock or anoxia *in vivo* and acid pH *in vitro* were used to transfer p26 into nuclei. The results suggest that parameters other than, or in addition to, p26 are responsible for the reduced transcription rates observed and that decreases in pHi are involved. *In vivo* experiments indicate that RNA synthesis and, to a lesser extent, protein synthesis are downregulated in intact embryos recovering from heat shock and that the precursor pool is not limiting. Confocal microscopy confirmed that p26 moves into nuclei in response to heat shock and anoxia *in vivo*, and to low pH *in vitro*, and indicated that the nuclear distribution of p26 is similar under all three conditions. We present evidence that unstressed (control) embryos containing p26 in all their nuclei will not hatch, even under permissive conditions, and propose that they are unable to terminate diapause.

Potential nuclear targets of p26 chaperone activity are discussed.

Key words: molecular chaperone, small heat-shock protein, nuclear translocation, transcription, heat shock, anoxia, p26, *Artemia franciscana*.

Introduction

Research on the stress (heat-shock) response has increased greatly in recent years, most of it aimed at understanding the structure and function of stress proteins, many of which are molecular chaperones. Feder and Hofmann (Feder and Hofmann, 1999) point out in their review that the literature on these subjects has become so large that even a ‘review of reviews’ is difficult. Their review and following books provide only an entry into this massive literature (Nover, 1991; Morimoto et al., 1994; Feige et al., 1996; Csermely, 1998). Most research has focused on model organisms such as *Drosophila* spp. and *Saccharomyces* spp. and on various cultured cell lines, but there has been increasing interest in evolutionary and ecological aspects of the stress response and its associated proteins (Feder and Hofmann, 1999). Although of intrinsic interest, this approach also provides cellular and molecular insights into the heat-shock response through the study of organisms whose biological settings and capabilities provide unique opportunities for study. The present paper falls in this category.

Artemia franciscana, which belong to a group of primitive crustaceans, produce and release into their hypersaline environment encysted gastrula embryos that are in diapause, a state of developmental and metabolic arrest (Clegg and Conte, 1980; Lavens and Sorgeloos, 1987). In this species, from the South San Francisco Bay, dehydration can terminate diapause and activate the embryos to resume metabolism and development. When rehydrated, activated embryos require only an appropriate temperature and molecular oxygen to resume development, and eventually hatch as larvae. The ability of these embryos to tolerate environmental stresses is extraordinary. They tolerate desiccation to the extent that few water molecules of any physical state can be detected in them (see Clegg and Drost-Hansen, 1990). Repeated cycles of hydration and desiccation offer no threat to their viability (Morris, 1971). They are resistant to damage from ultraviolet and ionizing radiation (for a review, see Clegg and Conte, 1980) and, when dry, survive the conditions of outer space (Gaubin et al., 1983). These embryos are arguably the extremophiles of the animal kingdom, and our interest lies in the molecular and cellular basis of their tolerance.

Diapause-destined embryos synthesize massive amounts of a small heat-shock/α-crystallin protein (Jackson and Clegg, 1996; Liang and MacRae, 1999), which is composed of 26 kDa subunits, hence the name p26. In its native form, p26 forms oligomers of approximately 500 kDa and makes up 10–15 % of the total non-yolk protein (Clegg et al., 1994; Clegg et al., 1995). This protein is embryo-specific, degraded in larvae produced from encysted embryos and has not been detected or induced in any other life history stage (Clegg et al., 1999; Liang and MacRae, 1999). During diapause, nucleic acid

synthesis ceases (Slegers, 1991) and p26 is found in nuclei and cytoplasm in approximately equal amounts (Jackson and Clegg, 1996). With the termination of diapause (activation), most p26 leaves the nuclei, but it re-enters if embryos are subjected to anoxia (Clegg et al., 1994) or temperature stress (Clegg et al., 1999). Translocation to nuclei can also be carried out *in vitro* simply by lowering the pH of homogenates (Clegg et al., 1995), allowing the study of nuclear p26 in the absence of anoxia or heat shock.

The cessation of nucleic acid synthesis during diapause led us to examine the possibility that p26 is involved in specifically inhibiting replication and/or transcription when present in nuclei. As the function of nuclear p26 has not been studied previously, and the specific functions of small heat-shock/α-crystallin proteins are still not well understood (MacRae, 2000), we examined the effect of this protein on transcription in nuclei isolated from control and previously stressed embryos. We found that transcription is depressed in stressed embryos, but evidence from studies in which p26 was translocated *in vitro* by acidic pH suggests that p26 is probably involved in the protection of nuclear components rather than operating as a specific regulator of transcription. Previous work indicated, somewhat unexpectedly, that control embryos also contain p26 (Liang et al., 1997a; Clegg et al., 1999; Liang and MacRae, 1999). We show here that nuclear p26 is found only in those embryos that will not give rise to larvae, and suggest that these embryos may be 'locked' in diapause. Using confocal microscopy, we also demonstrate the presence of p26 in nuclei isolated from anoxic and heat-shocked embryos and from homogenates incubated at low pH, and we discuss possible targets of p26 chaperone activity.

Materials and methods

Sources of Artemia franciscana *embryos and their decapsulation*

Artemia franciscana (Kellog) from salterns in the San Francisco Bay were purchased from San Francisco Bay Brand, Hayward, California, USA, as dried (activated) embryos, and stored at approximately −10 °C under 100 % N_2. Embryos were placed at room temperature (22 °C) for 5 days before use and had a final hatching percentage of 87 %. Dried embryos were hydrated in sea water overnight at 2 °C to suppress metabolism, and the outer shell was removed (decapsulation) prior to heat shock, anoxia or homogenization. Briefly, 5 g of hydrated embryos were washed well with ice-cold distilled deionized (dd) water, blotted, and placed in 200 ml of antiformin (7 % NaOH, 3 % Na_2CO_3, 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 then decanted. These embryos were washed twice with 400 ml of ice-cold 0.5 mol l^{-1} NaCl, followed by 5 min of gentle stirring in 300 ml of 1 % sodium thiosulfate, rinsing with ddH_2O, then 5 min of gentle stirring in 300 ml of 0.1 mol l^{-1} 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 mol l^{-1} NaCl overnight before use. Hatching was monitored after each decapsulation to ensure that hatching levels were not affected.

Hatching assays and immunoblotting

Embryos were placed into 20-well plastic depression plates, each well containing 10–20 embryos in 400 µl of 0.2 µm-filtered sea water. 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. Groups of at least 200 embryos were used to determine hatching percentages.

Embryos were homogenized in buffer K (150 mmol l^{-1} sorbitol, 70 mmol l^{-1} potassium gluconate, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} NaH_2PO_4 and 50 mmol l^{-1} Hepes buffered to pH 7.2), which is suitable for cell fractionation in this system (Clegg et al., 1994). Homogenates (100 mg wet mass ml^{-1}) were centrifuged at 1630 ***g*** for 5 min at 2 °C to obtain supernatant and pellet fractions. Pellets (nuclei, yolk platelets and shell fragments; Clegg et al., 1994) were washed once with 200 times their volume of buffer K and restored to initial volumes. Sample buffer at a concentration of 2× (Laemmli, 1970) was added to samples, the mixture was heated to 100 °C for 5 min, and centrifuged (1630 ***g***, 5 min) to remove shell fragments. For nuclear preparations, 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, and treated as described above. Supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in 12 % polyacrylamide gels, and proteins were detected by staining with Coomassie Blue-G. Proteins separated by SDS–PAGE were also transferred to nitrocellulose sheets and prepared for immunodetection using a polyclonal anti-p26 (Clegg et al., 1994) as the primary antibody and horseradish-peroxidase-conjugated anti-rabbit IgG as the secondary antibody. Bound antibody was identified using a chemiluminescence reaction detected on X-ray film (SuperSignal West Pico, Pierce, Rockford, IL, USA).

Nuclear translocation of p26

Three treatments were used to transfer p26 into nuclei of activated embryos: heat shock and 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 sea water in a Lauda RM40 waterbath (accurate to a 0.05 °C), at a rate of 0.7 °C min^{-1}, from 22 to 42, 46 or 50 °C, then assayed at once. In some studies, embryos heated to 50 °C were also allowed to recover at 25 °C for 2 or 4 h before assay. 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 (approximately 6.5) 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, pH 6.5 or 8.0, and incubated at 25 °C for 15 min. Homogenates were then used for nuclear isolation, and these nuclei used either for transcription assays or for confocal microscopy and p26 immunolocalization, to be described below.

Nuclear preparations

Nuclei were isolated essentially according to the methodology of van Breukelen et al. (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 on ice at 100 mg ml^{-1} in homogenizing medium (10 mmol l^{-1} Tris-HCl buffer, pH 7.5, 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaCl and 0.1 % Nonidet P-40) and filtered through a single layer of Durawipe (Johnson & 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 of 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 of 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 of buffer K and a sample was 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 2000 ***g***) and resuspended at a concentration of 4×10^8 ml^{-1} in buffer K at the appropriate pH and used immediately for transcription assays or confocal microscopy.

Nuclear run-on transcription assays

Once nuclei have been separated from cytoplasmic transcription factors, no new transcription complexes can form and only elongation of previously initiated transcripts can occur (‘run-ons’). Run-on assays were adapted from van Breukelen et al. (van Breukelen et al., 2000). Assay reaction mixtures contained 100 µl of a reaction buffer (100 mmol l^{-1} Tris, 100 mmol l^{-1} maleate, 150 mmol l^{-1} KCl, 40 mmol l^{-1} $(NH_4)_2SO_4$, 4.8 mmol l^{-1} magnesium acetate and 4.0 mmol l^{-1} $MnCl_2$, adjusted to pH 7.6), 15 µl of an rNTP solution (3.3 mmol l^{-1} ATP, 6.7 mmol l^{-1} GTP, 6.7 mmol l^{-1} CTP), 0.5 µl of 20 mmol l^{-1} uridine triphosphate (UTP), 3 µl of 1 mmol l^{-1} dithiothreitol in 0.01 mol l^{-1} sodium acetate, pH 5.2, 0.5 µl of RNAsin (10 units total, Promega, Madison, WI, USA), 2 µl of 37 MBq ml^{-1} [^{3}H]UTP (1.67 TBq $mmol^{-1}$; Amersham, Little Chalfont, UK) and 8.5 µl of ddH_2O. All solutions were made up with diethyl pyrocarbonate (DEPC)-treated water to inhibit RNase. Reaction mixtures were equilibrated for 10 min in an Eppendorf Thermomixer-R at 30 °C and 300 revs min^{-1}. Three samples of nuclei were spun down and restored to their original volumes of buffer K at pH 6.5, 7.5 and 8.0. Transcription was initiated with the addition of 80 µl of a 4×10^8 ml^{-1} nuclear suspension and continued for 15 min. Transcription rates increased linearly during the first 15 min, then leveled off, establishing 15 min as the end point.

Assays were terminated by pipetting 30 µl of the reaction mixture onto triplicate glass fiber filters, which were placed immediately in ice-cold 10 % trichloroacetic acid (TCA) with 1 % pyrophosphate for two 30 min washes, two 10 min washes in 5 % TCA with 0.5 % pyrophosphate and three 10 min washes in 95 % ethanol to remove TCA and unincorporated label. To avoid mechanical damage filters were washed in a perforated chamber within a larger beaker that contained the stir bar (van Breukelen et al., 2000). Blank filters were processed with sample filters for each experiment to measure background radioactivity, which was subtracted from all data. Filters were dried and placed in scintillation vials with 5 ml of CytoScint ES (ICN, Costa Mesa, CA, USA) and analyzed in a Beckman LS 5000 TD liquid scintillation counter. Results in disints min^{-1} were converted to picomoles of [^{3}H]UTP incorporated per 10^8 nuclei for statistical analysis.

Incorporation of [^{14}C]$NaHCO_3$

Artemia franciscana embryos are impermeable to nonvolatile solutes (Clegg and Conte, 1980), but [^{14}C]CO_2 (from [^{14}C]bicarbonate) does penetrate and is incorporated into a variety of amino acids, other organic acids and, under aerobic conditions, pyrimidine nucleotides (Clegg, 1976). In this way, protein and RNA synthesis can be estimated in activated embryos under permissive conditions (Clegg, 1977). Control embryos pre-hydrated at 2 °C but otherwise not incubated, and those recovering from a heat shock at 22–50 °C, were incubated aerobically for 2 or 4 h at 25 °C in 0.4 mol l^{-1} NaCl with 0.05 mol l^{-1} PO_4 at pH 7.2 (25 mg wet embryos ml^{-1}) in corked, swirling flasks, each containing 100 µl [^{14}C]$NaHCO_3$ stock (1.67 GBq $mmol^{-1}$, Amersham) for a final concentration of 925 KBq ml^{-1}. After incubation, embryos were thoroughly washed with ice-cold water and homogenized at 0 °C in 5 % TCA (100 mg wet mass ml^{-1}) and centrifuged (1630 ***g***, 5 min). Samples of the soluble fraction, referred to as precursors, were set aside for scintillation counting and thin-layer chromatography. Pellets were washed twice with 7 ml of cold 5 % TCA, then resuspended in 2.0 ml of 5 % TCA, heated for 1 h at 90 °C, cooled on ice and centrifuged (1630 ***g***, 5 min). Samples of supernatants (crude nucleic acids) were removed for liquid scintillation counting, and pellets were washed with 7 ml of 5 % TCA and centrifuged as above. Pellets (protein fractions) were dissolved in 500 µl of formic acid, and samples were taken for scintillation counting in 4 ml of Cytoscint ES (ICN, Costa Mesa, CA, USA).

Thin-layer chromatography

Samples of cold-TCA-soluble fractions were dried at the base of 100 µm thick cellulose sheets (Eastman Kodak, Rochester, NY, USA) that were developed with solvent composed of 7 vols of isopropanol, 2 vols of water and 1 vol of

88 % formic acid (ascending). After thorough drying, the thin layers were exposed to X-ray film for autoradiography.

Confocal microscopy

Three separate isolations of nuclei were prepared per embryo treatment. Isolated nuclei were placed on poly-L-lysine-coated slides (three slides per isolate), and unhatched cysts (12–15 per population) were gently squashed with coverslips. Samples were 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, nuclei were permeabilized in 0.5 % Triton X-100 in PBS for two 15 min, washed three times for 5 min in PBS, blocked in 0.1 % Tween-20 in PBS twice for 15 min, washed three times for 5 min in PBS, incubated in anti-p26 (polyclonal, goat, 1:500 in PBS) for 50 min, washed three times for 5 min in PBS, blocked in Normal Goat Serum (1:50 in PBS) for 30 min, incubated in Alexa Fluor 488 goat anti-rabbit (1:800 in PBS, Molecular Probes, Inc. Eugene, OR, USA) for 50 min, and washed three times for 5 min in PBS. Slides were either stored at –20 °C or incubated in BO-PRO-3 (1:2500 in PBS, Molecular Probes, Inc. Eugene, OR, USA) for 10 min and rinsed with ddH_2O. Anti-fade medium (90 % glycerol, 10 % PBS, 0.2 % *n*-propyl galate) was added, coverslips placed on top, and the slide sealed and viewed on a BioRad MRC 600 scanning confocal microscope equipped with a krypton/argon laser. Between 10 and 15 fields of view were examined per slide (20×) of isolated nuclei, with the number of nuclei per field of view ranging from 20 to 300. Images were collected at 60× and processed in Adobe Photoshop 5.0.

Statistical analyses

The Sigma Stat 2.0 program was used for all analyses including analysis of variance (ANOVA) and Student's *t*-test.

Results

Effects of heat shock on p26 localization and transcription

Fig. 1A shows that heat-shocking *Artemia franciscana* embryos by increasing the temperature from 22 °C to 50 °C moved approximately 50 % of total p26 from low-speed supernatants into pellets (nuclei, yolk platelets and shell fragments). Control embryos (22 °C) had little p26 associated with pellets, and a heat shock of 42 °C did not affect this distribution (arrow, Fig. 1A). Nuclear translocation of p26 began between 42 and 46 °C, above which translocation increased; at 50 °C, p26 was distributed almost equally between the supernatant and pellet. We know from numerous previous studies using western immunoblotting that the protein band indicated by the arrow is indeed p26 and that all the p26 in the pellet is contained within nuclei (Clegg et al., 1994; Clegg et al., 1995; Clegg et al., 2000; Jackson and Clegg, 1996; Liang et al., 1997a; Liang et al., 1997b).

Fig. 1B shows the relative amounts of nuclear p26 present as a function of temperature. After heating embryos to 50 °C, almost 60 % of the total p26 was present in nuclei, based on

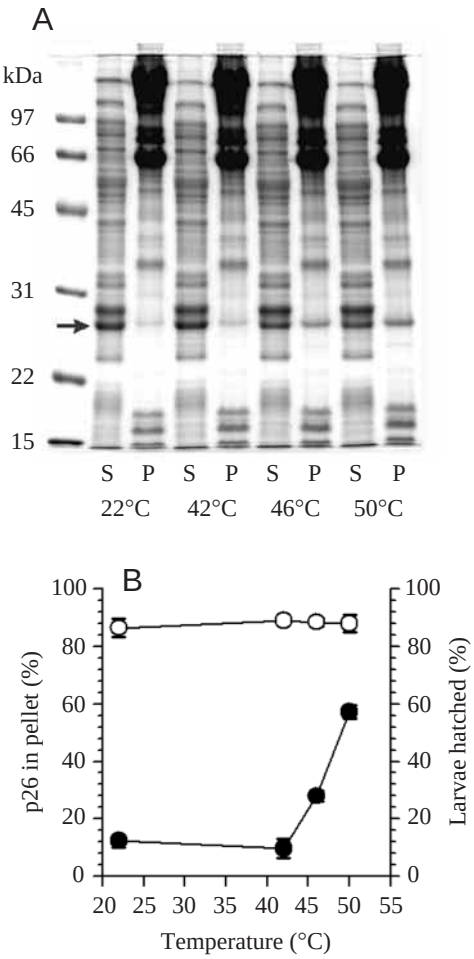

Fig. 1. Effects of heat shock on subcellular localization of p26 and final hatching levels. (A) Embryos were subjected to increasing temperatures (from 22 °C to 50 °C at 0.7 °C min^{-1}) and sampled at 22, 42, 46 and 50 °C. Supernatant (S) and pellet (P) fractions were prepared for SDS–PAGE as described in Materials and methods. The arrow indicates p26 location on this Coomassie-Blue-stained gel. The positions of marker proteins (kDa) are shown. (B) Embryos from the experiment shown in A were also used in hatching assays or analyzed by western immunoblotting. The percentage of total p26 present in the pellet (filled circles) was determined by densitometry of western blots. Open circles indicate the percentage of larvae that hatched. Data points represent the means of three replicate experiments ± S.D.; error bars that are not visible are contained within the symbols.

western immunoblotting and densitometry (Fig. 1B, filled symbols), confirming the data shown in Fig. 1A. There was no significant difference between the final hatching percentages of the 22 °C controls and the embryos subjected to the 42, 46 or 50 °C treatments (P=0.925, ANOVA) (Fig. 1B, open symbols).

We needed to know whether p26 present in nuclei from heat-shocked embryos was retained during isolation, and that it would remain there under assay conditions (15 min at 30 °C in assay buffer at pH 6.5, 7.5 or 8.0). There was concern that p26 might leave nuclei at the two higher pH values but not at pH 6.5, which moves p26 into nuclei *in vitro* (Clegg et al.,

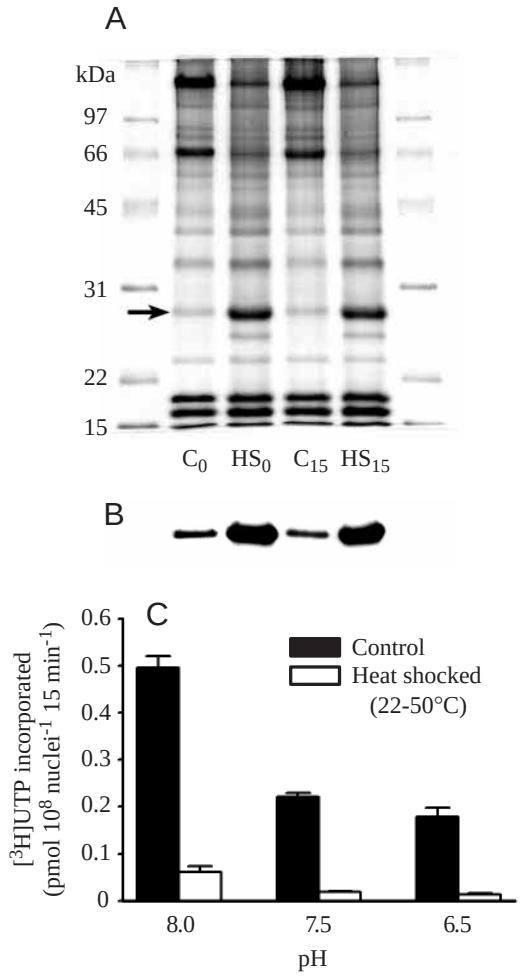

Fig. 2. Effect of heat shock on nuclear localization of p26 and transcription. (A) Coomassie-Blue-stained gel of proteins from nuclei isolated from control (C) and heat-shocked (HS) embryos (22–50 °C) used in 15 min mock run-on assays at pH 8.0. Samples of nuclei were prepared for SDS–PAGE at both initiation (C_0, HS_0) and termination (C_{15}, HS_{15}) of the mock assay. Loading was equalized at 1×10^6 nuclei per lane, and the arrow indicates p26. (B) Western immunoblotting of the samples in A treated with antibody to detect p26. (C) Transcription rates of nuclei from control and heat-shocked embryos. Run-on assays were carried out as described in Materials and methods, at the pH indicated. Error bars are +1 S.D. based on three separate experiments.

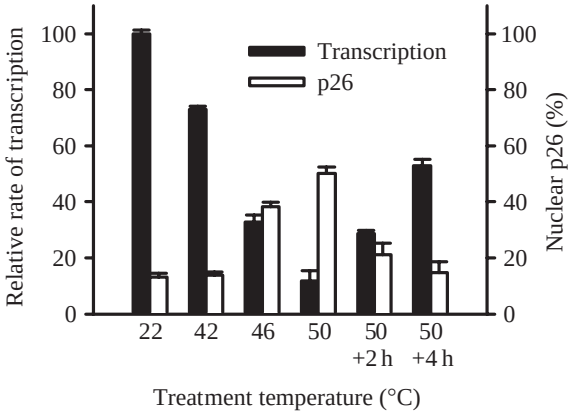

Fig. 3. Effect of heat shock and recovery on rates of transcription and subcellular localization of p26. Nuclear run-on assays measured transcription rates in nuclei isolated from embryos subjected to heat shock. Conditions included 22 °C (control), 22–42 °C, 22–46 °C, 22–50 °C and also 22–50 °C followed by 2 or 4 h of recovery at 25 °C. Nuclei were then isolated and assayed as described in Materials and methods. Transcription data were normalized to control nuclei, and all assays were performed at pH 8.0. Levels of p26 were obtained by immunoblotting and densitometry, and represent nuclear p26 as a percentage of the total. Error bars are +1 S.D., based on three separate experiments.

1995). To address this concern, nuclei from control and heat-shocked embryos were incubated at pH 8.0 in a mock run-on assay (UTP was used in place of [^{3}H]UTP) and sampled at the beginning and end of the incubation. The results from SDS–PAGE (Fig. 2A) show that nuclei from heat-shocked embryos contained large amounts of p26 compared with controls (C_0, HS_0) and that nuclear p26 was not lost during incubation (HS_{15}). Fig. 2B shows the western immunoblot used to identify p26 in the samples shown in Fig. 2A. This confirms that the large band identified by Coomassie staining (arrow in Fig. 2A) was p26 and that no obvious loss of p26 from these nuclei occurred under assay conditions at pH 8.0.

Work by van Breukelen et al. (van Breukelen et al., 2000) showed that the rates of transcription in isolated nuclei were linear for the first 15 min of incubation. Using their methodology, we observed the same results for nuclei from all four treatments used in this study (control, heat shock, anoxia and acid pH; data not shown), confirming 15 min as the incubation end-point.

Nuclei isolated from control and heat-shocked embryos were also used in transcription run-on assays at different pH values (Fig. 2C). Decreasing the assay pH from 8.0 to 7.5 or 6.5 decreased incorporation significantly in control nuclei ($P<0.001$, ANOVA). There was no significant difference between transcription rates in control nuclei at pH 7.5 and 6.5. Nuclei from heat-shocked embryos had significantly reduced transcription rates compared with controls, a 93 % decrease on average, regardless of assay pH (pH 8.0, $P<0.002$; pH 7.5, 6.5, $P<0.001$, Student's *t*-test). No significant differences between transcription rates at pH 7.5 and 6.5 in nuclei from heat-shocked embryos were observed.

Heat shock, nuclear p26 and transcription

To explore further the relationship between these three parameters, embryos were subjected to increasing degrees of heat shock before nuclei were isolated and assayed at pH 8.0 or prepared for western blotting (Fig. 3). Transcription rates in nuclei from control embryos were set to 100 %, and transcription rates in nuclei from heat-shocked embryos expressed as a percentage of controls (filled columns). The open columns in Fig. 3 represent nuclear p26 as a percentage of the total in the sample, as determined by densitometry of western immunoblots. As the amount of p26 present in nuclei

increased in response to the 46 °C and 50 °C heat shocks, a parallel decrease in transcription took place. When embryos were allowed to recover for 2 and 4 h after a 50 °C heat shock, p26 exited the nuclei and transcription rates also began to recover (Fig. 3). Importantly, these data show that mild heat shock (22–42 °C) decreased transcription rates without measurable translocation of p26 to the nuclei. Moreover, after 4 h of recovery from a 22–50 °C heat shock, transcription levels were still only approximately 55 % of normal while p26 had already returned to control levels.

In vivo *incorporation of [^{14}C]carbon dioxide*

The effect of heat shock on nuclear transcription *in vitro* led us to ask whether *in vivo* metabolism would be similarly affected after heat shock. The embryonic cuticle is impermeable to non-volatile compounds, but the incorporation of [^{14}C]CO_2 from [^{14}C]$NaHCO_3$ into several amino acids and pyrimidine nucleotides provides a way of examining metabolism *in vivo* in this system (Clegg, 1976; Clegg, 1977). Control and heat-shocked embryos were incubated in [^{14}C]$NaHCO_3$ at 25 °C for 2 or 4 h, then homogenized and separated into cold-TCA-soluble (precursor amino acids and pyrimidine nucleotides), hot-TCA-soluble (crude nucleic acids) and TCA-insoluble (protein) fractions (Fig. 4). Control embryos showed higher rates of incorporation into protein and nucleic acids than heat-shocked embryos (inset, Fig. 4), but those recovering from heat shock had higher incorporation into precursor amino acids and pyrimidine nucleotides. Nucleic acids in heat-shocked embryos were labeled at a rate almost four times lower than in controls, while the rate of incorporation into heat-shocked embryo protein approached that in controls between 2 and 4 h of recovery (Fig. 4).

To determine whether the decrease in ^{14}C incorporation into nucleic acids in embryos recovering from heat shock was due to a limiting labeled precursor pool, samples of the cold-TCA-soluble fraction (precursors) were analyzed by thin-layer chromatography and autoradiography. The autoradiogram in Fig. 5 shows precursors from control and previously heat-shocked embryos after 2 and 4 h of incubation in [^{14}C]$NaHCO_3$. The distribution of radioactivity was similar in extracts of control and heat-shocked embryos and, overall, heat-shocked embryos contained larger amounts of labeled precursor at both time points.

Transcription is reduced in nuclei from previously anoxic embryos

Anoxia also results in the nuclear translocation of p26 in these embryos (Clegg et al., 1994), but at a much slower rate and possibly less stressful way than with the heat shock used in this study. Transcription rates in nuclei isolated from previously anoxic embryos were therefore examined. Nuclei from control and 2-week-anoxic embryos were also assayed at pH 8.0, 7.5 and 6.5 to evaluate the effects of pH (Fig. 6). The highest transcription rates were, as expected, in control nuclei

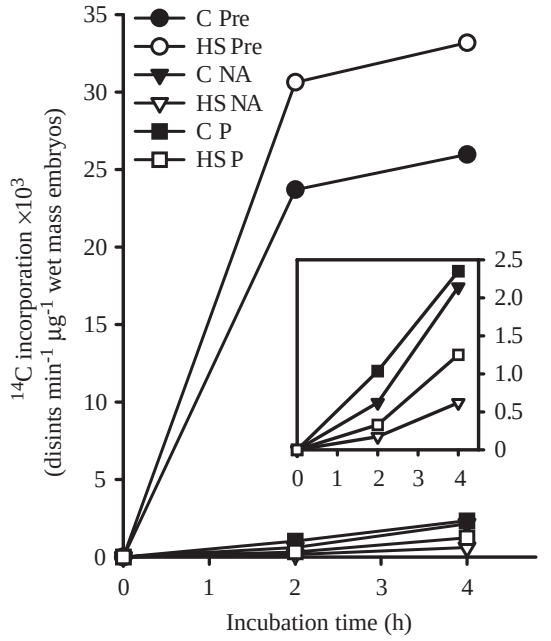

Fig. 4. [^{14}C]CO_2 incorporation in control embryos and in embryos recovering from heat shock. Control (C) and heat-shocked (HS) (22–50 °C) embryos were incubated at 25 °C for 2 or 4 h in [^{14}C]$NaHCO_3$. The protein (P), nucleic acid (NA) and precursor pool (Pre) fractions were obtained as described in Materials and methods, and ^{14}C incorporation was determined by scintillation counting. The inset shows the results for protein and nucleic acid radioactivity on an expanded scale.

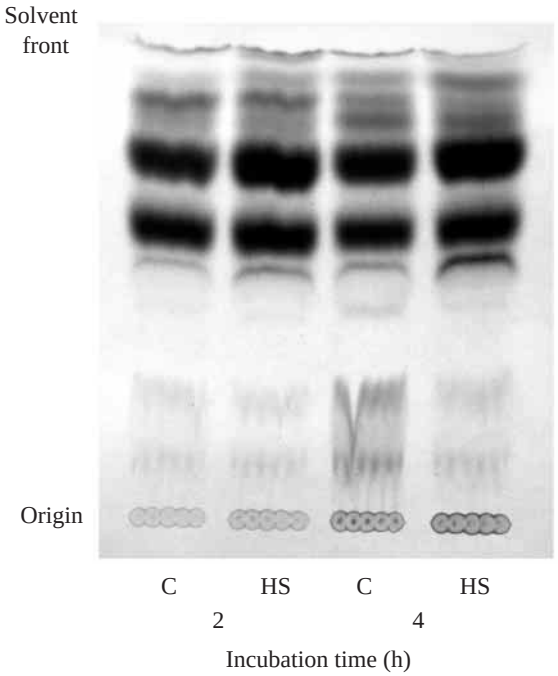

Fig. 5. Autoradiogram of a thin-layer chromatogram of precursor fractions from control and heat-shocked embryos. Cold-TCA-soluble (precursor) fractions were obtained from control (C) and heat-shocked (HS, 22–50 °C) embryos after incubation at 25 °C in the presence of [^{14}C]$NaHCO_3$ for 2 or 4 h.

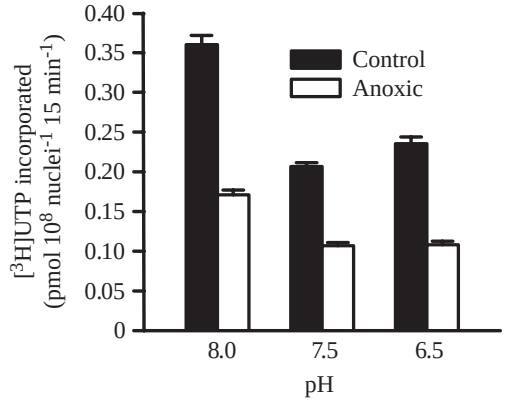

Fig. 6. Transcription rates of nuclei isolated from control and previously anoxic embryos. Embryos were subjected to 2 weeks of anoxia as described in Materials and methods. Nuclei isolated from these embryos were used in nuclear run-on assays at the pH values shown. Error bars represents 1 S.D. for three separate experiments.

at pH 8.0. Control nuclei responded to pH in a manner similar to those in Fig. 2, with decreased transcription rates at pH 7.5 and 6.5 (P<0.001, ANOVA). Nuclei from anoxic embryos showed decreased transcription relative to controls at all pH values (P<0.001, Student's t-test), and they also exhibited decreased transcription in response to lowering the assay pH from 8.0 to 7.5 or 6.5 (P<0.001, ANOVA, Student–Newman–Keuls test). As with heat shock (Fig. 2), there was no significant difference between transcription rates at pH 7.5 and 6.5 in nuclei from control or anoxic embryos (Fig. 6).

Transcription is reduced in nuclei containing p26 translocated by acid pH

Acid pH was used to transfer p26 into nuclei to evaluate the effect of nuclear p26 on transcription without the added stress of heat shock or anoxia. Hydrated embryos were homogenized in buffer K at either pH 8.0 or pH 6.5 and incubated at 25 °C for 10 min. Nuclei were isolated from these homogenates, then analyzed by SDS–PAGE and western blotting or used in run-on assays. The results from a mock run-on assay demonstrate that nuclei isolated from a homogenate incubated at pH 6.5 (lane L_0, Fig. 7A) contained much more p26 than those from one incubated at pH 8.0 (lane C_0) and that was still the case at the end of the mock assay (lanes C_{15}, L_{15}). Western blots probed with an antibody confirmed that p26 was not lost during the assay incubation (Fig. 7B). Nuclear run-on assays showed that nuclei containing p26 loaded by incubation at pH 6.5 have significantly decreased transcription rates at pH 8.0 and 6.5 (Student's t-test, pH 8.0, P<0.001; pH 6.5, P<0.006; Fig. 7C). There was no obvious difference in transcription between assays performed at pH 8.0 and 7.5, in either control or p26-loaded nuclei. This pattern differs from the experiments involving heat shock and anoxia, in which the transcription rates at assay pH 7.5 and 6.5 were similar (Fig. 2, Fig. 6). However, the effect of pH was still significant, whether or not nuclei contained p26. Transcription rates assayed at pH 8.0

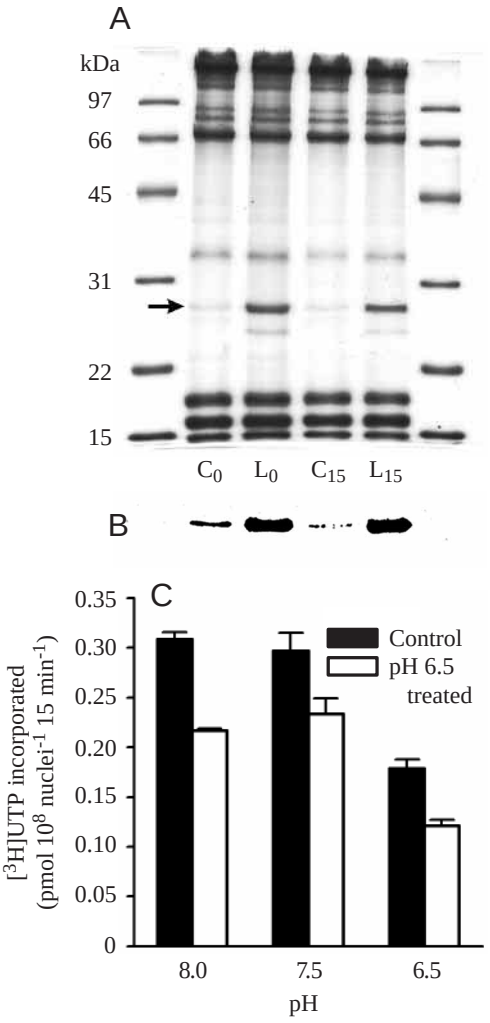

Fig. 7. Effects of pH on subcellular localization of p26 and transcription rates. Homogenates of control embryos were incubated in buffer K at pH 6.5 to move p26 into nuclei, or at pH 8.0 to keep p26 extra-nuclear. Nuclei were then isolated from both homogenates for further study. (A) Nuclei isolated from embryos homogenized at either pH 8.0 (C, control) or pH 6.5 (L, p26-loaded) were used in a 15 min mock run-on assay. Samples of nuclei were prepared for SDS–PAGE at both initiation (C_0, L_0) and termination (C_{15}, L_{15}) of the assay. Equal amounts (1×10^6 nuclei) were loaded per lane, and the arrow indicates p26. (B) Detection of p26 in the samples from part A by western immunoblotting. (C) Transcription rates of nuclei isolated from homogenates incubated at pH 8.0 and pH 6.5. Nuclear run-on assays were carried out as described in Materials and methods, at the indicated pH. Error bars are +1 S.D. based on three separate experiments.

were higher for both control and p26-loaded nuclei than rates assayed at pH 6.5 (ANOVA, P<0.001).

Confocal microscopy

Fig. 8 shows representative nuclei from control (Fig. 8A), heat-shocked (Fig. 8B) and anoxic (Fig. 8C) embryos, and Fig. 8D shows nuclei isolated from embryo homogenates incubated at pH 6.5. The left half of each panel indicates the presence of

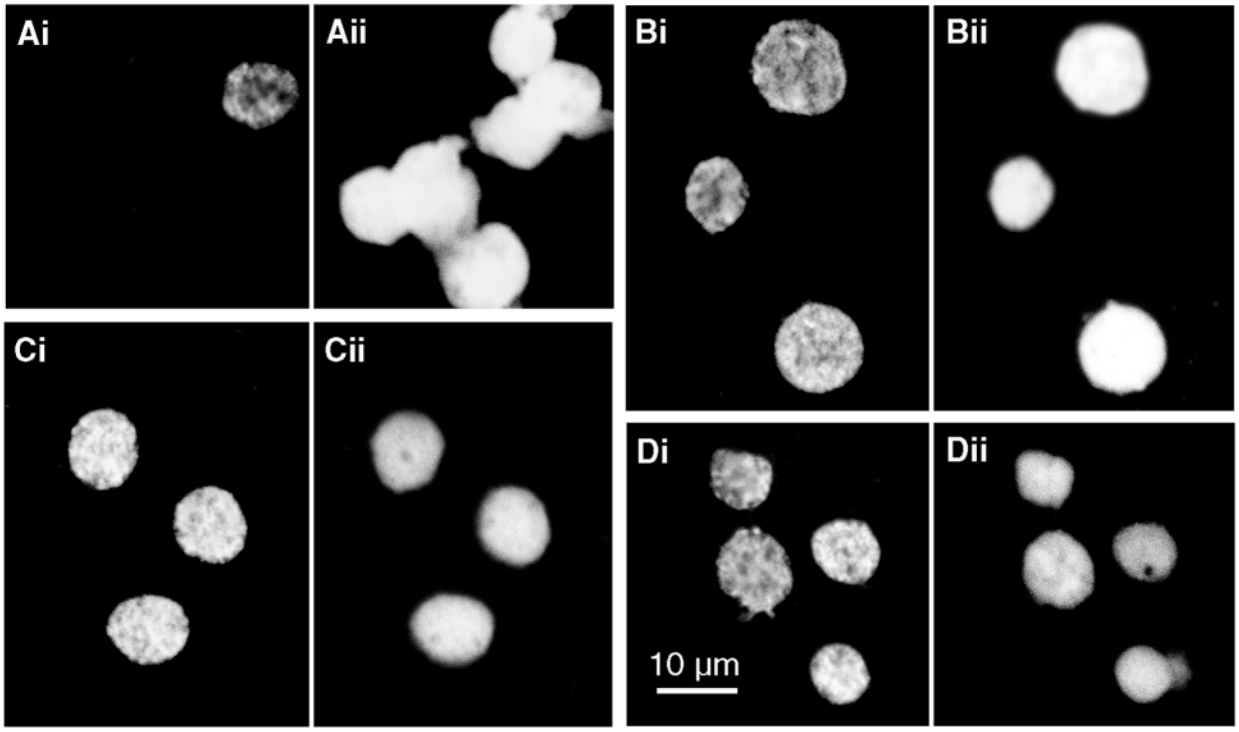

Fig. 8. Confocal microscopy confirms that p26 translocates into nuclei in response to heat shock, anoxia and low pH. Nuclei were double stained using Alexa-Fluor 488 (Ai–Di), and DNA was detected using using Bopro-3 (Aii–Dii). Nuclei were isolated from control embryos (A), embryos heat-shocked from 22 to 50 °C (B), embryos after 2 weeks of anoxia (C) and embryos homogenized and incubated at pH 6.5 (D). The scale bar in D applies to all parts of the figure.

p26 (Fig. 8Ai–Di) while the right side represents DNA (Fig. 8Aii–Dii). All nuclei from embryos treated with heat shock or anoxia, and those from homogenates incubated at acid pH contained p26 distributed unevenly throughout (Fig. 8Bi–Di), with slight variations in signal intensity (for numbers counted see Materials and methods). This description of p26 distribution also applies to only one of the six control nuclei shown in Fig. 8Ai, ruling out the possibility that all nuclei in control embryos contain small amounts of p26 and prompting us to determine the exact percentage of nuclei that did contain p26. Table 1 shows data from two embryo populations with different hatching percentages. The percentage of isolated nuclei containing p26 in both populations was very close to the percentage of embryos that did not hatch, suggesting that nuclear p26 is restricted to those embryos and is absent from viable controls. To examine this possibility further, embryos from both populations were hatched in sea water for 5 days, after which unhatched embryos were collected and prepared for confocal microscopy (see Materials and methods). All 27 unhatched embryos examined contained p26 in all their nuclei (Table 1).

Discussion

Nuclear translocation of p26 in encysted *A. franciscana* embryos during diapause and in response to stress invites the question of whether nuclear p26 acts as a general chaperone or plays a more specific role. One possible function of p26, explored in this study, was to inhibit transcription in diapause and in anoxic embryos when they undergo reversible metabolic arrest (Clegg and Conte, 1980; Slegers, 1991). Many types of eukaryotic transcription repressors have been described (Hanna-Rose and Hansen, 1996; Carlson, 1997) and, given the chaperone characteristics of p26, this protein could act as a direct repressor by interfering with the formation or activity of pre-initiation transcription complexes. Such a role is plausible because p26 only enters nuclei *in vivo* under physiologically stressful conditions or during developmentally programmed diapause, when inhibition of transcription occurs. While transcription was decreased when p26 was translocated into nuclei *via* heat shock or anoxia *in vivo*, or acid pH *in vitro*, this decrease did not suggest that p26 was acting as a specific regulator of transcription. We discuss these results in the following sections, and consider what they indicate for p26 in the role of a nuclear chaperone.

Heat shock

When working with disrupted cells or isolated organelles, one acknowledges that the working material is, in effect, an artifact of isolation, and that certainly applies to nuclei (Paine et al., 1992). Accepting this, we also note that the heat-shock treatment used did not kill embryos (Fig. 1B), indicating that nuclear integrity was not damaged irreversibly prior to isolation and also that isolated nuclei are widely used in run-

on assays. It has been shown that acid pH can cause p26 to exit nuclei in homogenates (Clegg et al., 1995), so we determined that the pH and duration of the nuclear run-on assay did not result in loss of p26 from these isolated nuclei (Fig. 2A,B). Correlation between the movement of p26 into nuclei by heat shock (Fig. 1A, Fig. 2A) and a severe decrease in transcription independent of assay pH (Fig. 2C) allowed for the possibility of a causal connection between nuclear p26 and decreased transcription. The decrease was not surprising because the inhibiting effects of heat shock on overall transcription rates are well-known, and many cells downregulate transcription of non-heat-shock protein (hsp) genes in response to heat shock (Lindquist, 1986; Vazquez et al., 1993; Morimoto et al., 1994; Ghoshal and Jacob, 1996). However, closer examination of p26 translocation and the decrease in transcription associated with heat shock revealed that these were probably separate events (Fig. 3). Transcription was reduced by 25 % after raising embryos to only 42 °C, a temperature at which p26 does not undergo translocation (Fig. 1). In addition, p26 had returned to control levels in nuclei 4 h after heat shock, but transcription was still reduced by 50 % at that time.

We cannot exclude some involvement of nuclear p26 in the depression of transcription, notably after the maximum amount has entered the nuclei. However, we believe that general damage resulting from high temperature alone is a more likely explanation. Examination of transcript patterns before and after heat shock would be worthwhile to determine whether the presence of nuclear p26 coincides with the silencing of specific subsets of genes. Overall, these data suggest strongly the involvement of parameters other than, or in addition to, p26 in the inhibition of transcription by heat shock.

Nuclear run-on assays provide only *in vitro* data, so incorporation of ^{14}C from [^{14}C]bicarbonate was used to examine metabolism *in vivo* in control embryos and in those recovering from heat shock (Fig. 4). The exit of p26 from nuclei *in vivo* is complete in just over 4 h post-heat shock (J. K. Willsie, unpublished data), allowing a window for observation of metabolism in embryos recovering from heat shock and containing varying amounts of p26 in nuclei. The marked decrease in incorporation of radioactive precursors into nucleic acids (RNA) of heat-shocked embryos might involve the thermal sensitivity of the nuclear matrix, where transcription centers are organized and anchored (Berezney et al., 1995; Jolly and Morimoto, 1999). The decreases in incorporation into protein and nucleic acids after heat shock (Fig. 4) were not due to inadequate precursor pools (Fig. 5), which is the same conclusion as that reached in similar experiments using previously anoxic embryos (Clegg and Jackson, 1998).

Table 1. *Relationships between hatching level and nuclei containing p26*

Embryo population	Hatching (%)	Nuclei containing p26 (%)	Nuclei containing p26 in unhatched embryos (%)
1	86.5±3.1	13.8±0.7	100
2	64.4±1.7	33.9±5.1	100

Hatching was determined as described for population 1 in Materials and methods and population 2 was a gift from Kordon/Novalek (Hayward, California).

Nuclei were viewed using a BioRad MRC 600 laser scanning confocal microscope. Two separate isolations of nuclei were prepared from each population and placed on poly-L-lysine coated slides. Unhatched embryos (>5 days) were collected and gently squashed with coverslips. Slides were processed as described in Materials and methods.

Three slides from each preparation of isolated nuclei were examined and 200–300 nuclei counted per slide (3–5 fields of view). 27 unhatched embryos were examined individually: 12 from population 1 and 15 from population 2, with approximately 1,000–2,500 nuclei observed per embryo. Values are means ± S.D.

Anoxia

Nuclei isolated from long-term anoxic embryos contain large amounts of p26 (Clegg et al., 1994), allowing for the possibility that p26 plays a role in the metabolic changes associated with anoxia. The effects of anoxia and metabolic rate reduction have been well studied in this system (Stocco et al., 1972; Hofmann and Hand, 1992; Clegg, 1997; Hand, 1998; van Breukelen et al., 2000), and we investigated how p26 might be involved. We show in Fig. 6 that transcription in nuclei isolated from anoxic embryos was significantly decreased compared with controls, supporting the results of van Breukelen et al. (van Breukelen et al., 2000). They found decreases in transcription of 79 % in nuclei from embryos made anoxic for only 4 h and 88 % in nuclei from embryos made anoxic for 24 h, somewhat larger differences than those we observed (Fig. 6C). The embryos used by van Breukelen et al. (van Breukelen et al., 2000) were from the Great Salt Lake, UT, USA, and nuclei were isolated after incubation under normoxic conditions at 22–23 °C for 4 h, which allowed some pre-emergence development to occur. The result of this incubation is a significant change in the biochemistry of these embryos compared with those hydrated but not allowed to develop (Clegg and Conte, 1980; Slegers, 1991). We used San Francisco Bay *A. franciscana* embryos immediately after hydration overnight at 2 °C and subjected them to 2 weeks of anoxia prior to nuclear isolation. Taken together, the different experimental conditions may explain the observed differences in transcription levels. van Breukelen et al. (van Breukelen et al., 2000) also proposed that p26 was involved in the transcriptional arrest they observed, but this seems doubtful because they isolated nuclei after only 4 or 24 h of anoxia, and even after 24 h only approximately 15 % of total p26 would have entered nuclei (Clegg et al., 1994). Complete nuclear translocation (50 % of total p26) takes 3–4 days under anoxia (Clegg et al., 1994). The results of van Breukelen et al. (van Breukelen et al., 2000) are important, however, because they suggest that transcription decreased in anoxic embryos well before the full complement of p26 had entered the nuclei,

making it clear that, as in the case of heat shock, p26 alone cannot account for the observed reduction in transcription.

pH

Intracellular pH (pHi) is a common thread between heat shock, anoxia, diapause and nuclear translocation of p26. The pHi of embryos under anoxia is approximately 6.5 (see Kwast et al., 1995) and, although the pHi of heat-shocked embryos has not been measured, it is highly likely that it is also acidic since heat shock reduces pHi in many, and possibly all, other systems (Kurkdjian and Guern, 1989; Welch, 1990; Neuhaus-Steinmetz et al., 1996). Embryos within the first 24 h of release from diapause have a pHi 7.9 or greater, and it was determined that the metabolism of these embryos is quite vigorous initially and does not slow to undetectable levels for 3–4 days after release from females (Clegg et al., 1996; Clegg et al., 1999). The argument was made that once the embryos have fully entered metabolic diapause, their pHi will also be acidic (Clegg et al., 1996). On the basis of pHi measurements *in vivo*, and the ability of acid pH to move p26 into nuclei *in vitro* (Clegg et al., 1995; Fig. 7A,B, Fig. 8D), ΔpH emerges as a likely proximate cause of p26 translocation. We therefore asked whether pH played a similar role in the observed decreases in transcription that accompany heat shock, anoxia and diapause. van Breukelen et al. (van Breukelen et al., 2000) demonstrated a linear relationship between pH and RNA synthesis in nuclei isolated from *A. franciscana* embryos. They showed a 65 % decrease in the rate of transcription as the pH of the assay mixture decreased from 7.9 to 6.3. Our results are very similar, in that a 1.5 unit decrease in pH resulted in a decrease in transcription of 40–60 % using control nuclei. In contrast, when nuclei from heat-shocked (Fig. 2C) and anoxic embryos (Fig. 6) were assayed under similar conditions (the same pH values as controls), a much larger decrease in transcription occurred. Thus, pH does not account for the effects of stress on transcription as measured by run-on assays *in vitro*. These results corroborate work by van Breukelen et al. (van Breukelen et al., 2000), who used aerobic acidosis to lower pHi artificially in intact embryos and concluded that only 55 % of the transcriptional arrest observed in *A. franciscana* embryos under anoxia could be attributed to acid pHi. Thus, acid pHi is clearly involved in the inhibition of transcription but, as was indicated above for nuclear p26, pHi alone cannot account for the differences observed between nuclei from stressed and control embryos. It may be that the severity of the stress itself (temperature of heat shock, duration of anoxia) determines the extent to which transcription is inhibited.

Nuclear p26

The first direct evidence that p26 enters the interior of nuclei was an immuno/transmission electron microscopy study on control and anoxic embryos (Clegg et al., 1995). Liang et al. (Liang et al., 1997a) then used confocal and fluorescence microscopy, and published an image of a nucleus from a control embryo in which p26 was present in discrete compartments throughout the nucleus. The seemingly large amount of p26 they observed was unexpected because only approximately 10–12 % of total p26 was normally associated with control nuclei (Clegg et al., 1994; Clegg et al., 1995; Clegg et al., 1999). Previous interpretations of gel and immunoblot data from controls are inconclusive since they could be explained either by the presence of a large amount of p26 in some nuclei or by a small amount in all nuclei. This question of the presence of nuclear p26 in control embryos needed further examination, and the results from our confocal studies appear to have resolved the matter (Fig. 8A). We found in preliminary studies that 80–90 % of the nuclei from control embryos did not contain detectable p26, indicating that Liang et al. (Liang et al., 1997a) might have observed some of the few control nuclei that did. The remaining images confirm what the western immunoblotting of isolated nuclei indicated; namely, that in nuclei isolated from heat-shocked and anoxic embryos, and from homogenates incubated at pH 6.5, p26 was indeed intra-nuclear (Fig. 8Bi–Di). This confirmation is valuable in that it thoroughly substantiates and ties together all previous work examining p26 translocation in response to anoxia, heat shock and acid pH.

The similarity between the percentage of an embryo population that does not hatch and the percentage of nuclei containing p26 isolated from that same population strongly suggests a link between these two parameters (Table 1). This link is further supported by the observation that all unhatched embryos examined from both populations contained p26 in all their nuclei (Table 1). These data give credence to the hypothesis that unhatched embryos are unable to activate and may somehow be locked in diapause (Clegg et al., 1996). Mechanisms that lead to the termination of diapause in *A. fransciscana* are not well understood and variation occurs at both the inter- and intra-population levels (Lavens and Sorgeloos, 1987; Van Stappen et al., 1998). A single round of desiccation is often enough to terminate diapause in San Francisco Bay embryos but, importantly, multiple rounds of desiccation, cold temperatures and/or chemical treatments such as H_2O_2 and NH_4Cl often increase the hatching percentages of embryo populations (Lavens and Sorgeloos, 1987; Clegg and Jackson, 1998; Van Stappen et al., 1998). There is no way of knowing the biological status of the unhatched embryos in this study, but the evidence that hatching can be increased by up to 75 % depending on the strains and treatments used (Van Stappen et al., 1998) suggests that at least some embryos that do not hatch are not literally dead, but simply remain in diapause. The possibility that the exit of p26 from nuclei is part of the mechanism that terminates diapause in these embryos merits further study.

Is p26 a nuclear chaperone?

We have shown that p26 alone cannot account for the decreases in transcription in isolated nuclei containing p26, so roles other than those of a transcription regulator are indicated. We propose that the most plausible role for nuclear p26 is to act as a chaperone, protecting nuclear macromolecules from denaturation and aggregation, and possibly even assisting in

repair. Heat-shock proteins (Hsps) from several families translocate into nuclei in response to stress and associate with nuclear components (Velazquez et al., 1980; Arrigo et al., 1988; Nover, 1991; Parsell and Lindquist, 1993; Jakob and Buchner, 1994; Nover and Scharf, 1997; van de Klundert and de Jong 1999; MacRae, 2000). Both Hsp70 and Hsp90 also undergo nuclear translocation in response to stress in many systems (Morimoto et al., 1994; Csermely, 1998; Csermely et al., 1998; Karlin and Brocchieri, 1998), but we know that neither of these protein families enters the nuclei of anoxic or heat-shocked *A. franciscana* embryos (Clegg et al., 1999). It appears that nuclear p26 in *A. franciscana* plays the same role as those heat-shock protein families do in other systems, translocating into nuclei in response to stress.

Several heat-shock proteins have been reported to form nuclear granules of various sizes in response to stress, including the small heat-shock proteins human hsp27 and murine hsp25 (Arrigo et al., 1988; Welch, 1990; Rogalla et al., 1999). Indirect evidence suggests that p26 is also involved with the formation of nuclear granules in anoxic embryos (Clegg et al., 2000), and these observations also indicated that the targets of nuclear p26 are widespread in this organelle. Fig. 8 in the present study also suggests that nuclear p26 associates with a wide distribution of sites, one of which could be the nuclear matrix (Berezney et al., 1995). Although such a structure is controversial, the consistent presence of this non-chromatin scaffolding network after three different chromatin removal techniques indicates that the nuclear matrix is probably an important and real structural component of the nucleus (Wan et al., 1999). Proteins in the nuclear matrix are reported to be among the most thermally labile of all proteins in cells (Roti Roti et al., 1998), making them obvious chaperone targets (Gerner et al., 1999). Thus, it seems probable that at least some of the translocated p26 is chaperoning nuclear matrix proteins. Associations between small heat-shock proteins and RNA have also been reported (Nover and Hightower, 1991), and Biggiogera et al. (Biggiogera et al., 1996) showed that hsp27 associates with nuclear structures involved in RNA synthesis and processing in response to heat shock, while Nover et al. (Nover et al., 1989) reported associations between hsp20 and non-translated mRNAs after heat shock. Thus, we propose that p26 could be acting as a nuclear chaperone, stabilizing nucleoproteins and/or the nuclear protein scaffolding upon which transcriptional machinery is bound and organized, even if p26 is not interacting specifically with proteins involved in transcription. Determination of nuclear targets for p26 is a current goal.

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