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Developmental regulation of the heat shock response by nuclear transport factor karyopherin-α3

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SUMMARY

During early stages of *Drosophila* development the heat-shock response cannot be induced. It is reasoned that the adverse effects on cell cycle and cell growth brought about by Hsp70 induction must outweigh the beneficial aspects of Hsp70 induction in the early embryo. Although the *Drosophila* heat shock transcription factor (dHSF) is abundant in the early embryo it does not enter the nucleus in response to heat shock. In older embryos and in cultured cells the factor is localized within the nucleus in an apparent trimeric structure that binds DNA with high affinity. The domain responsible for nuclear localization upon stress resides between residues 390 and 420 of the dHSF. Using that domain as bait in a yeast two-hybrid system we now report the identification and cloning of a *Drosophila* nuclear transport protein karyopherin-α3 (dKap-α3). Biochemical methods demonstrate that the dKap-α3 protein binds specifically to the dHSF's nuclear localization sequence (NLS). Furthermore, the dKap-α3 protein does not associate with NLSs that contain point mutations, which are not transported in vivo. Nuclear docking studies also demonstrate specific nuclear targeting of the NLS substrate by dKap-α3. Consistant with previous studies demonstrating that early *Drosophila* embryos are refractory to heat shock as a result of dHSF nuclear exclusion, we demonstrate that the early embryo is deficient in dKap-α3 protein through cycle 12. From cycle 13 onward the transport factor is present and the dHSF is localized within the nucleus thus allowing the embryo to respond to heat shock.

Key words: *Drosophila melanogaster*, dKap-α3, Heat shock factor, Nuclear transport

INTRODUCTION

Temporal and spatial regulation of specific transcription factor activity is a key process in embryonic development. Activation of specific transcription factor activity in response to extracellular stimuli is also an important process both during and post embryonic development. Some of the regulatory mechanisms include restricted transcription factor expression by promoter regulatory elements during development (Arnone and Davidson, 1997), activation of a dormant pre-existing molecule into an active form by protein modification (Karin et al., 1997; Schindler and Darnell, 1995), and restricted nuclear entry (Kaffman and O'Shea, 1999). In mammals the well-characterized NF-κB transcription factor is released from an inhibitory molecule IκB bound to the NLS of NF-κB by specific phosphorylation and degradation of the inhibitor (Woronicz et al., 1997; Zandi et al., 1998; Zandi et al., 1997a). The released NF-κB molecule can now enter the nucleus and activate specific gene transcription. Conditional nuclear import is also used by the NF-AT transcription factors. NF-ATs are cytoplasmic in unstimulated T cells but upon engagement of the T cell receptor and CD28 coreceptor they rapidly translocate into the nucleus and activate the transcription of an array of cytokine genes (Zhu et al., 1998). NF-AT nuclear import is induced by the calcium-dependent phosphatase calcineurin (Jain et al., 1992). In *Drosophila* regulated nuclear entry of the NF-κB/Rel-related protein Dorsal plays a key role in the establishment of the dorsal-ventral axis during early embryogenesis (Morisato and Anderson, 1995; Steward, 1987). Dorsal is retained in the cytosol by Cactus, which shares significant homology with IκB family members (Geisler et al., 1992). Cactus is phosphorylated in response to a signaling cascade that leads to its degradation allowing Dorsal to enter the nucleus (Kidd, 1992; Rushlow et al., 1989).

Nuclear restriction also plays a role in the regulation of the heat shock response during early development in *Drosophila* (Wang and Lindquist, 1998). Although many heat shock proteins (HSPs) of *Drosophila* are maternally supplied, including Hsp83 and the small HSPs, Hsp70 is not (Arrigo and Tanguay, 1991; Zimmerman and Cohill, 1991). In fact Hsp70 is not inducible by heat shock in the early *Drosophila* embryo, nor is any other HSP gene (Graziosi et al., 1980). Indeed Hsp70 is not inducible in embryos of a wide variety of organisms in addition to flies including mice, frogs and sea urchins (Heikkila et al., 1985; Morange et al., 1984; Roccheri et al., 1982). Despite the fact that in the *Drosophila* embryo the heat shock transcription factor (dHSF; which is responsible for the heat-induced transcription activation of HSP genes) is maternally

supplied and abundant, the embryo remains refractory to heat shock until cycle 13. Wang and Lindquist have shown that the dHSF does not enter the nucleus until cycle 13 at which point Hsp70 induction can occur (Wang and Lindquist, 1998). Developmental regulation of the nuclear localization of dHSF, therefore, plays a key role in the establishment of the heat shock response in the early embryo.

The nuclear localization sequence of the dHSF has been identified and characterized (Zandi et al., 1997b). To identify protein(s) that bind to the NLS and may be involved in the nuclear transport of dHSF, a yeast two-hybrid screen was conducted using the NLS as 'bait'. Several positive *Drosophila* cDNAs were identified of which one belongs to the karyopherin family of nuclear transport proteins, and has been designated *Drosophila* karyopherin-α3 (dKap-α3) (Gorlich et al., 1994; Kohler et al., 1997). Biochemical analysis of dKap-α3 demonstrates specific and functional interactions of the nuclear transporter with dHSF in vitro. Examination of the temporal and spatial expression pattern of dKap-α3 revealed that it is not expressed until cycle 13 of embryogenesis. These observations strongly support the notion that dKap-α3 is the nuclear transporter of the dHSF in vivo, and that developmental regulation of dKap-α3 synthesis determines the time at which the heat shock response can be activated in the early embryo.

MATERIALS AND METHODS

Two-hybrid screening and protein expression

A 129 bp dHSF DNA fragment that encodes the bait protein containing NLS was amplified by PCR and cloned into the pAS2 GAL4-DB (DNA binding domain) vector using *Bam*HI and *Nde*I restriction sites. The fusion plasmid DNA was then amplified and purified from *E. coli*. A *Drosophila* embryonic cDNA library was carried in pAC2 GLA4-AD (activation domain) vector (Clonetech). Both plasmids were co-transformed into yeast y190 cells and plated on SD/-His/-Trp/-Leu to select for His^+ transformants. All positives were then tested for expression of the second reporter gene by colony lift β-galactosidase assays. Finally, individual positive clones were co-transformed with a pAS2 vector that carries the NLS-deleted bait to eliminate the false positives. Three positive pAC2 plasmids were isolated from yeast and sequenced to assemble the full-length coding region of dKap-α3. PCR primers were then designed according to the c-DNA sequence, and a full-length dKap-α3 DNA was cloned by PRC using a λgt11 cDNA library.

dKap-α3 was expressed in *E. coli* cells by subcloning the full-length cDNA into a GST fusion protein vector pGEX-2t. The GST-tagged dKap-α3 was then bound to a GST-affinity resin (Stratagene) and eluted with 10 mM reduced glutathione in 50 mM Tris (pH 8). The GST tag was cleaved with thrombin for 60 minutes at a ratio of 3 units protease per mg of recombinant protein in 50 mM Tris (pH 8), 150 mM NaCl and 2.5 mM $CaCl_2$.

Colony lift β-galactosidase filter assay

The primary His^+ transformants were grown in 5 ml selective SD medium at 30°C to OD 0.5. Cells were then re-streaked onto a 150 mm SD/-His/-Trp/-Leu agar plate and incubated for 72 hours at 30°C. A sterile Whatman no. 5 filter was placed over the surface of the agar plate. As soon as the filter was completely wet it was carefully lifted off the plate and quick frozen in liquid nitrogen for 1 minute. A second sterile Whatman no. 5 filter was pre-soaked in 3.5 ml of Z buffer/X-gal solution. The frozen filter was thawed at room temperature and carefully placed on the second filter, colony side up. Both filters were incubated at room temperature for up to 8 hours until the appearance of blue colonies.

In vitro cross-linking assay

A 191 aa 6HIS-tagged polypeptide from dHSF (mini-probe) containing the NLS was over-expressed in DE3 cells and purified by Ni-NTA affinity column (Qiagen). In addition, a similar 154 aa polypeptide with the NLS deleted (mini-ΔNLS probe) was also over-expressed in DE3 as were all polypeptides with point mutant NLS (mini-mNLS probes). All of the protein probes were labeled with [γ-^{32}P]ATP by MAPK phosphorylation in vitro.

dKap-α3 (1 μg/μl) was incubated in D buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 1 mM EDTA and 0.2% Triton X-100) with 2 μl of γ-^{32}P-mini probes (1 ng/μl, approx. 80000 cpm/μl) for 20 minutes at 25°C in a 20 μl reaction volume. Cross-linking was subsequently carried out by addition of 2 μl of 20 mM DSS and incubation for 15 minutes at 25°C. The reaction was quenched by the addition of 2 μl of 200 mM lysine for 10 minutes. Protein-protein adducts were analyzed by 6% SDS-PAGE and autoradiography.

Cloning, expression and purification of *Drosophila* karyopherin (importin) β

Four PCR primers were designed according to the partial genomic sequence published in GenBank (accession number g92598391):

5′GCGCGCGAATTCCATATAGAGAGGAAAAGAG3′
5′GCGCGCCTCGAGCATAGTGCTTGGACAC3′
5′GCGCGCCTCGAGGTGCTCTGCAGTTCCTG3′
5′GCGCGCTCTAGACTACTGTGCGATGGACCTGGGT3′

Two amplified fragments corresponding to –26 to 962 and 963 to 2655 of the cDNA sequence of karyopherin β were obtained using the above primers and a *Drosophila* embryonic cDNA library (λt11) as PCR template. The two fragments were ligated into pBluescript (Stratagene) and sequenced. The full-length karyopherin β was fused to a GST tag using vector pGEX-2t (Smith and Johnson, 1988), expressed in *E. coli*, and purified with GST affinity resin (Stratagene). The GST tag was removed with thrombin.

In vitro nuclear docking assays

In vitro nuclear docking assays were performed according to the methods developed by (Stochaj and Silver, 1992). To study the binding of *Drosophila* heat shock factor NLS, Schneider cells were allowed to attach to polylysine-coated slides for 20 minutes on ice. Then the cells were permeabilized with 45 μg/ml digitonin in Buffer A (20 mM Hepes (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 'complete protease inhibitors' from Boehringer for 20 minutes on ice. NLS-EGFP fusion proteins, dKap-α3 and dKap-β were pre-incubated on ice for 20 minutes, then were incubated with permeabilized cells at room temperature for 20 minutes. Cells were washed with Buffer A and fixed with Histochoice Tissue Fixative MB (Amresco). Slides were mounted in Buffer A/90% glycerol containing 1 mg/ml o-phenylenediamine.

Immunofluorescence staining

Mouse monoclonal antibodies were raised against recombinant dKap-α3 using standard immunization procedures. Three monoclonal lines were characterized: 5E3, 5F6, 6G7 and all three reacted with an epitope present in the N-terminal 100 amino acids of dKap-α3. This domain is unique among the dKap-α family members and therefore should eliminate any cross reactivity. Western analysis with these antibodies reveal only a single strongly reactive protein species of the correct molecular mass.

18×18 mm no. 1 cover slips were coated in 1 mg/ml poly-L-lysine for 15 minutes and air dried. 0.5 ml of *Drosophila* SL2 cells with a density of 4×10^5 cells/ml were placed onto each cover slide and incubated for 15 minutes at room temperature. Cells were then heat

shocked at 37°C for various times, washed with PBST, and fixed with Histochioce tissue fixative MB (Amresco) for 15 minutes on ice. After several washes to remove the fixative, the fixed cells were incubated with 1:1000 dilution of monoclonal anti-dKap-α3 antibody (5E3) in PBS buffer containing 0.5% bovine serum albumin (BSA) for 2 hours at room temperature. Cells were then washed with PBST 4 times for 10 minutes each to remove unbound first antibody. Fluorescein-coupled goat anti-mouse IgG (Pierce) secondary antibody was then added at 1:100 dilution in 0.5% BSA in PBS and incubated at 4°C overnight. Cell nuclei were visualized by co-staining with DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride) for 10 minutes. Finally, the cover slips were washed with PBST 4 times and mounted onto microscope slides in 90% glycerol/PBS containing 2.5% DABCO (1,4-diazabicyclo [2,2,2] octane; Sigma). The fluorescent images were viewed and photographed using a Zeiss Axioplan microscope with UV irradiation and appropriate filters.

Fixed *Drosophila* embryos were rehydrated in a methanol/PBST series: 15 minutes each in 75%, 50%, 25% and 30 minutes in PBST. Immunofluorescence staining was then carried out as described previously (Patel, 1994). All antibodies were pre-incubated with 0- to 12-hour embryos overnight at 4°C. Anti-dKap-α3 antibodies were diluted 1:500 and anti-dHSF antibodies were diluted 1:250. Fluorescent dye-labeled secondary antibodies were diluted 1:100. All primary antibody incubations were at 4°C overnight and secondary antibody incubations were at room temperature for 90 minutes. Images were taken using confocal microscopy (Zeiss, LSM310) or Axioplan microscopy. The embryonic stages were determined by co-staining with DAPI (4′, 6-Diamindine-2′-phenylinedole dihydrochloride).

Developmental western blots

0- to 2-hour, 0- to 4-hour and 0- to 6-hour embryos were collected and washed with 0.03% Triton X-100/0.9% NaCl. Non-shocked or heat shocked (37°C for 15 minutes) embryos were rinsed twice with homogenization buffer (50 mm Tris (pH 7.5), 140 mM NaCl, 5 mM $MgCl_2$, 0.05% NP-40, 1 mM PMSF, 1 μg/ml pepstain A, 1-2 μg/ml aprotinin, 1 μg/ml leupeptin), and then homogenized in 2 volumes of homogenization buffer. Extracts were then centrifuged to remove cell debris and the supernatants were mixed with SDS-PAGE gel loading buffer and electrophoresed in 8% SDS-PAGE. The separated proteins were then transferred to nitrocellulose and blocked with 5% non-fat dry milk overnight. The blot was then probed with anti-dDKap-α3 monoclonal antibody 5E3 and developed with anti-mouse alkaline phosphatase conjugated antibodies.

Embryo preparation

Drosophila embryos were collected in the population cages with different time span. No-shock embryos were processed immediately, while heat-shock samples were incubated in a 37°C water bath for 15 minutes. All embryos were washed with NaCl-Triton (0.9%NaCl, 0.03%Triton X-100), dechorionated in 50% bleach for 3 minutes and fixed in *n*-heptane/formaldehyde/PBS (5:1:5) for 30 minutes. After removing vitelline membranes in *n*-heptane/methanol (1:1) by vigorous shaking, embryos were washed three times with methanol and stored in ethanol at –20°C. For western blotting, embryonic extracts were made without the fixation step and stored at –70°C.

RESULTS

The *Drosophila* HSF contains a 33-residue bi-partite NLS that is required for nuclear localization of the HSF during heat stress (Zandi et al., 1997b). Deletion of the NLS prevents nuclear localization yet allows the spontaneous oligomerization of the HSF in the cytosol, thus generating an active DNA binding form of the factor. These observations suggest that cytosolic factor(s) may interact, at least in part, with the NLS to prevent oligomerization as well as nuclear localization. The cytosolic factor(s) might function as a direct repressor of nuclear entry, similar to Iκ-B. Alternatively, a novel nuclear localization process may be involved in which the monomer HSF is associated with a specific nuclear localization transport molecule.

To identify components of this regulatory system, a 43 amino acid segment of the *Drosophila* HSF which includes the NLS (Fig. 1A) was used as bait in a yeast two-hybrid system screen (Bartel et al., 1993; Chien et al., 1991; Fields and Song, 1989). This screen allowed a search of a *Drosophila* cDNA embryonic library for proteins capable of specifically binding to the NLS. The screen revealed one primary class of NLS-binding proteins with sequence similarity to nuclear transport proteins.

Cloning *Drosophila* karyopherin-α3

Three strong positive clones were isolated and sequenced from the yeast two-hybrid screen of 5×10^6 cDNAs from a *Drosophila* embryonic library (see Materials and Methods). All three clones contained overlapping amino acid sequences (Fig. 1B). One of the three contained an apparent full-length cDNA sequence encoding a 514-residue protein. A gene bank sequence search identified a matching sequence with a previously unpublished *Drosophila* karyopherin-α3 protein (dKap-α3). Based on sequence homology, dKap-α3 is a member of the nuclear import α-proteins (importins) (Gorlich et al., 1994; Kohler et al., 1997). Also based on homology, the dKap-α3 contains an internal NLS and *Drosophila* karyopherin-β (dKap-β) binding domain in the N terminus as other previously characterized karyopherins (Moroianu et al., 1996), and eight ‘arm-repeats’ (armadillo repeats) in the carboxy portion of the protein (Fig. 1C) (Conti et al., 1998). At the amino acid level dKap-α3 shows 45% identity to *Drosophila* Kap-α1, 44% identity to *Drosophila* Kap-α2 and 67% identity to human Kap-α3.

Karyopherin-α3 binds specifically to the *Drosophila* HSF NLS

In vitro protein cross-linking was used to determine the capability and specificity of dKap-α3 binding to the *Drosophila* HSF NLS. A 191 amino acid protein segment derived from the dHSF containing a centrally located NLS was cloned into pET11a and expressed in *E. coli* (Fig. 1A). The purified protein was labeled in vitro with MAP kinase and [γ-^{32}P]ATP at fortuitous MAP kinase sites present within the protein segment; this probe was termed the ‘mini-NLS’. An otherwise identical protein segment containing a deletion of the NLS was similarly labeled with MAP kinase and termed ‘mini-ΔNLS’ (Fig. 1A). Mini-NLS was incubated with recombinant dKap-α3, molecularly cross-linked using disuccinimidyl suberate (DSS) and the product of this reaction analyzed by SDS-PAGE (Fig. 2 lane 1). A complex of approximately 90 kDa was identified by autoradiography of the gel. This complex is only observed when dKap-α3 was present in the reactions, and immunoprecipitation with anti-Kap-α3 antibodies demonstrates that Kap-α3 is present in the complex (data not shown). Similar analysis with the mini-ΔNLS revealed no complex formation (Fig. 2 lane 9). These results demonstrate that the NLS is required for dKap-α3 binding to the mini probes.

To determine the specificity of dKap-α3 interaction with the NLS, a series of point mutants within the NLS that are known to affect NLS function in vivo (Zandi et al., 1997b; also summarized in the upper portion of Fig. 2) were tested for their in vitro binding properties. Biochemical cross-linking studies with labeled mutant mini-NLS probes revealed a close correlation between the transport properties of these mutants and their ability to bind to the mini probes. As shown in Fig. 2, those mutants that are not transported in vivo do not bind dKap-α3 in vitro (K405M, lane 3; L404P, lane 4; N408S/R411L, lane 5 and K400E/R401L, lane 6). Two mutants, that are transported in vivo, do bind dKap-α3 in vitro although one more weakly than wild type (Q399l/K400R/L404R, lane 2; E406P, lane 8). A constitutively localized mutant Q403L binds efficently to dKap-α3 (lane 7). Overall these results support the view that the cloned dKap-α3 protein can specifically bind to the dHSF NLS in vitro.

Karyopherin-β enhances dKap-α3 binding to the NLS of *Drosophila* HSF

Active nuclear transport complexes in vivo include an α/β-karyopherin heterodimer with the α subunit bound to the NLS of the cargo (Enenkel et al., 1995; Rexach and Blobel, 1995). It has been shown that in vitro binding of recombinant yeast Kap-α (kap 60) to NLS domains is cooperatively enhanced by Kap-β (kap 95) (Rexach and Blobel, 1995). The cross-linking experiments described above show that in the absence of dKap-β the NLS and dKap-α3 bind specifically to each other. To determine what quantitative role dKap-β may have in the dNLS-dKap-α3 interaction we cloned the *Drosophila* Kap-β cDNA using primers derived from the known genomic sequence (see Materials and Methods). dKap-β was expressed in *E. coli* and purified using the GST-tag system and the GST-tag removed prior to use (Smith and Johnson, 1988). α and β proteins were combined and incubated with ^{32}P-labeled mini probes cross-linked with DSS and examined by SDS-PAGE. The addition of dKap-β to the reactions modestly stimulated dKap-α3 binding to the NLS (Fig. 3A; compare lanes 1, 2 and 3). Interestingly, the molecular mass of the complex was not altered by the presence of dKap-β, suggesting that either the dKap-β association is transient in vitro or the interaction is such that it cannot be cross-linked with DSS.

The karyopherin-β binding domain of dKap-α3 is required for NLS binding in vitro

A comparison of the human and mouse dKap-α3 protein

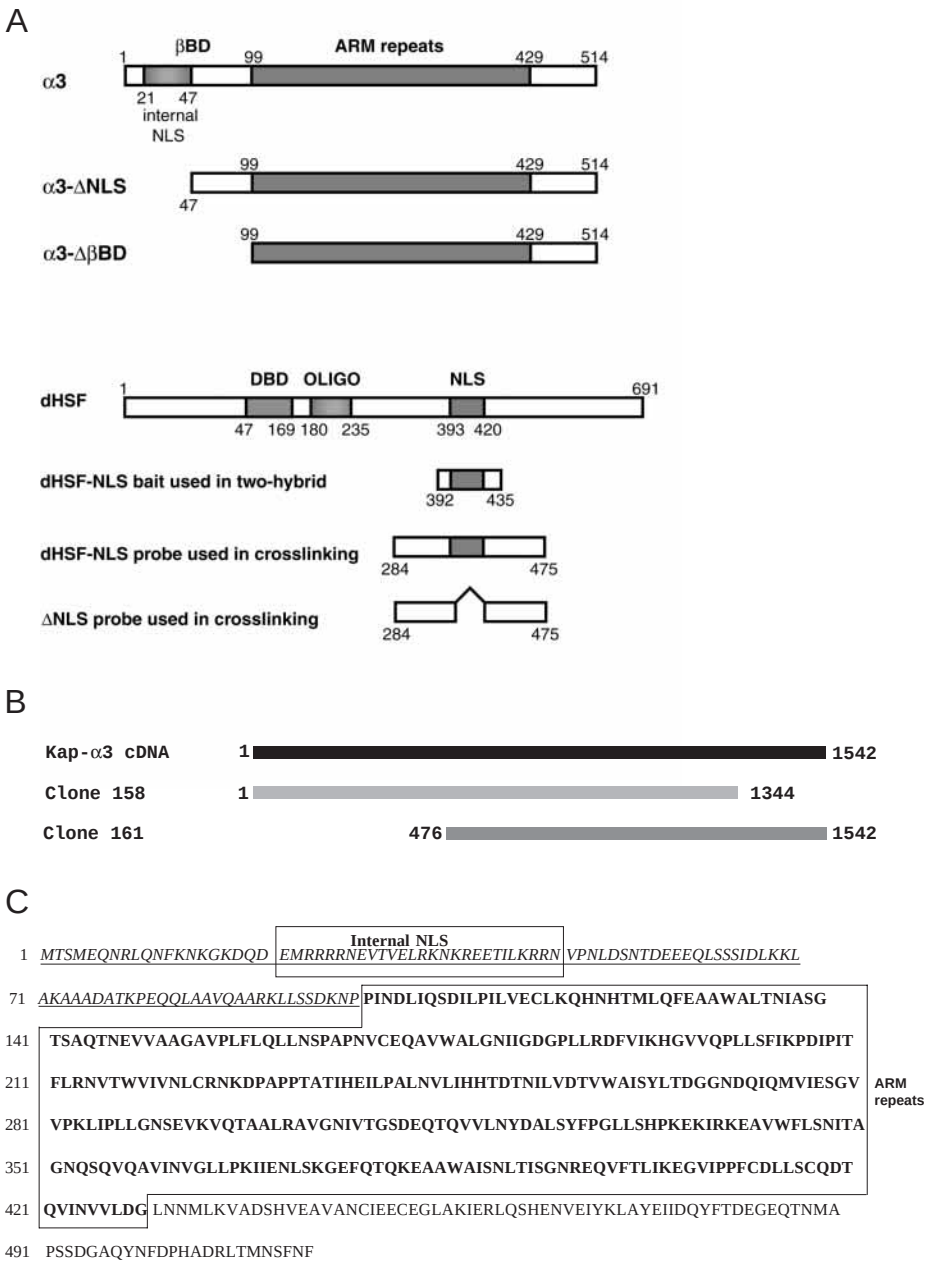

Fig. 1. (A) Schematic diagram of the domain organization of karyopherin-α3 and *Drosophila* HSF. Amino acid endpoints for each region, as well as their proposed functions, are indicated. Two Kap-α3 deletion mutants used in the crosslinking experiments, α3-ΔNLS and α3-ΔβBD, as well as three dHSF-NLS probes used in two hybrid screening or crosslinking experiments, are also shown. DBD, DNA-binding domain of dHSF; OLIGO, oligomerization domain of dHSF. (B) Two-hybrid cloning and amino acid sequence of dKap-α3. Schematic diagram of the dKap-α3 cDNAs isolated by two-hybrid screening. Two of the three distinctive positive clones, #158 and #161, were found to cover the entire coding region of dKap-α3. (C) Amino acid sequence of *Drosophila* karyopherin-α3. Amino acid sequence of full-length karyopherin-α3 is shown. The functional domains of dKap-α3 are illustrated as follows: boxed sequences include the internal NLS of dKap-α3 and ARM repeats, respectively; dKap-α3 binding domain is underlined and in italics.

Lane	1	2	3	4	5	6	7	8	9
NLS probe	wtNLS	Q399L K400R L404R	K405M	L404P	N408S R411L	K400E R401L	Q403L	E406P	ΔNLS
Localization (25°C)	Cyt	Cyt	Cyt	Cyt	Cyt	Cyt	Nuclear	Nuclear	Cyt
Localization (37°C)	Nuclear	Nuclear	Cyt	Cyt	Cyt	Cyt	Nuclear	Nuclear	Cyt

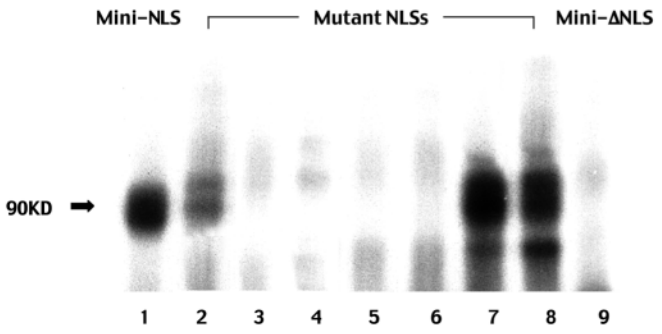

Fig. 2. Cross-linking of dKap-α3 to mutant forms of dHSF-NLS polypeptides. The upper table summarizes the in vivo subcellular localization of the wild-type and mutant NLSs determined by immunostaining (Zandi et al., 1997b). Recombinant wild-type dKap-α3 protein (1 μg) was cross linked to the following P^{32}-labeled forms of mini-NLS: mini-NLS (lane 1); Q399L, K400R, L404R mini-NLS (lane 2); K405M mini-NLS (lane 3); L404P mini-NLS (lane 4); N408S, R411L mini-NLS (lane 5); K400E, R401L mini-NLS (lane 6); Q403L mini-NLS (lane 7); E406P mini-NLS (lane 8) and mini-ΔNLS (lane 9). All probes were labeled with [γ^{32}P]ATP and MAPK. 2 ng of probe (approximately 150,000 cpm) was used in each reaction. The NLS mutation Q399L/K400R/L404R (lane 2), and two constitutive nuclear entry mutations (Q403L, lane 7 and E406P, lane 8) formed complexes with dKap-α3 of approximately the same size (approx. 90 kDa) as the wild-type mini-NLS probe (lane 1). The remaining mutant forms, which are not transported in vivo (lanes 3-6), did not form specific complexes in vitro. The lower molecular mass complexes seen are dimers of probe and are mini-NLS probe specific.

sequences to the *Drosophila* α3 sequence reveals several regions of homology. One homologous segment corresponds to the internal NLS of the human and mouse proteins, and is located between residues 22 and 41 of *Drosophila* dKap-α3 (Moroianu et al., 1996). A second homologous region present in dKap-α3 is the karyopherin-β binding domain (Moroianu et al., 1996). This domain is located in the first 115 amino acids of the N terminus of karyopherin-α3.

To investigate the role of these domains in dKap-α3 binding to the *Drosophila* HSF NLS, N-terminal deletions of dKap-α3 were examined. One deletion removed the internal NLS (deleting residues 1-47 of the N terminus) and the other had the entire N-terminal 99 amino acid β-binding domain deleted. These proteins were cross-linked to ^{32}P-labeled mini-NLS and mini-ΔNLS probes and the results are shown in Fig. 3B, lanes 3-6. *Drosophila* Kap-α3, deleted of its internal NLS, bound the mini-NLS probe very well but not the mini-ΔNLS probe (lanes 3 and 4, respectively). Deletion of the entire β-binding domain, however, eliminates mini-NLS probe binding (lane 5). This observation suggests that the β-binding domain of *Drosophila* Kap-α3 is necessary for dHSF-NLS binding. This is unexpected because previous biochemical and structural studies with yeast and human proteins have demonstrated that karyopherin-α3 binds to its target NLS-peptide cargo within the arm repeats. Indeed, an N-terminal β-binding domain-deleted form of the yeast α3 protein was sufficient for crystallization with an SV-40 NLS peptide (Conti et al., 1998). It is possible that the use of a significantly larger cargo in our experiments requires a more significant portion of dKap-α3 for docking.

Karyopherin-α3 is required for nuclear docking in vitro

Nuclear docking experiments were performed to determine if dKap-α3 and dKap-β can target the dHSF-NLS to the nuclear pore complex. These experiments employed Schneider (SL2) cells, which were depleted of nuclear transport factors along with other cytosolic proteins by digitonin permeabilization

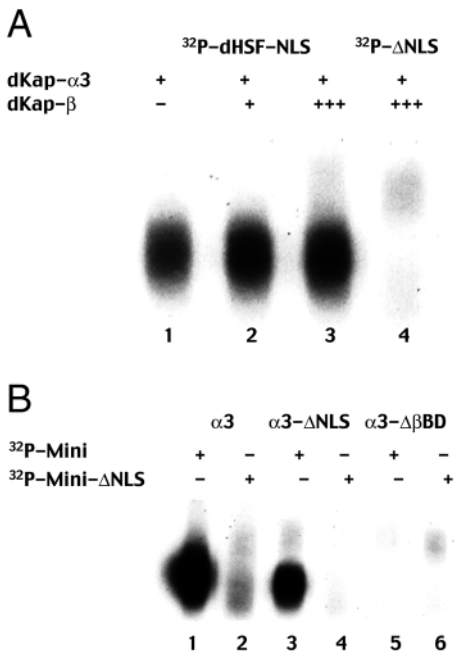

Fig. 3. (A) The effect of dKap-β on dKap-α3 binding to *Drosophila* HSF's NLS. 200 ng of recombinant dKap-α3 protein was cross linked to either mini-NLS or mini-ΔNLS probe. Increasing amounts of recombinant dKap-β was added to the indicated reactions with mini-NLS probe (lanes 1-3). Lane 1, no dKap-β; lane 2, 200 ng dKap-β; lane 3, 1 μg dKap-β. No enhancement of dKap-α3 binding to the mini-ΔNLS reaction was observed even with the addition of 1 μg of dKap-β (lane 4). (B) Domains of dKap-α3 required for *Drosophila* HSF NLS binding. Recombinant wild-type or mutant dKap-α3 protein (1 μg) was cross linked to either mini-NLS or mini-ΔNLS probes, which were labeled with [γ^{32}P]ATP using MAP kinase. 2 ng of probe (150,000 cpm) was used in each reaction. Both wild-type dKap-α3 and dKap-α3-ΔNLS cross-linked to mini-NLS probe very efficiently (lanes 1 and 3). Deletion of the dKap-β binding domain, dKap-α3-ΔβBD, eliminated binding to the mini-NLS probe (lane 5). The mini-ΔNLS probe did not bind to any of the dKap-α3 proteins, and no complex is observed (lanes 2,4 and 6).

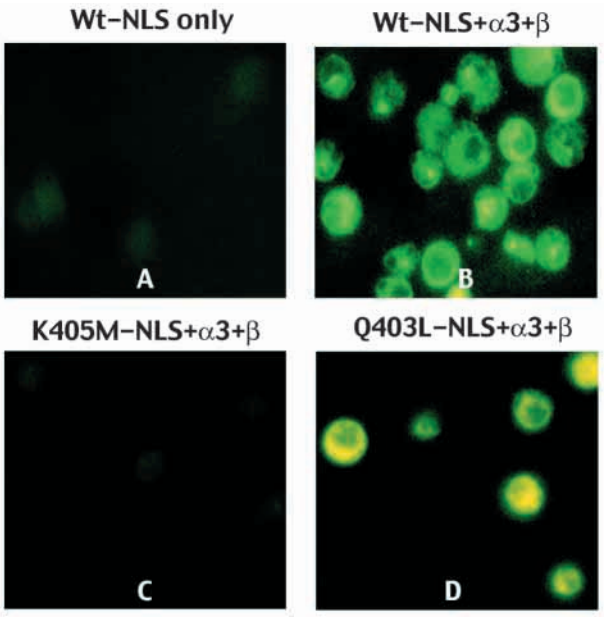

Fig. 4. Karyopherin-α3 is required for nuclear docking in vitro. Digitonin-permeabilized SL2 cells were incubated with NLS-EGFP fusion proteins, recombinant dKap-α3 and dKap-β. The nuclear docking of EGFP fusion proteins was examined by fluorescent microscopy. (A) Wild-type NLS-EGFP fusion protein only. (B) Wild-type NLS-EGFP fusion protein, kap-α3 and kap-β. (C) K405M mutant NLS-EGFP fusion protein, dKap-α3 and dKap-β. (D) Q403L mutant NLS-EGFP fusion protein, dKap-α3 and Kap-β.

(Smith and Johnson, 1988). Nuclear transport factors are added to the cells as purified recombinant proteins along with NLS-EGFP fusion proteins to serve as the cargo protein. As shown in Fig. 4, wild-type NLS fused to EGFP was effectively docked on the nuclear periphery in the presence of both α and β proteins (Fig. 4B). The NLS-EGFP protein alone was not able to dock on the nuclear membrane (Fig. 4A), indicating that dKap-α3 and dKap-β are required for docking. Consistent with the biochemical crosslinking experiments, and in vivo localization studies, the K405M NLS mutant did not show nuclear docking in the presence of dKap-α3 and dKap-β (Fig. 4C). The constitutive nuclear-localized mutant, Q403L NLS, was able to dock on the nuclear pore complexes in the presence of dKap-α3 and β, as expected (Fig. 4D).

Drosophila karyopherin-α3 is excluded from the nucleus after heat shock

Biochemical and immunocytochemical studies of HeLa and *Drosophila* SL2 cells have shown that vertebrate HSF1 and dHSF are distributed in a diffuse pattern over the cytoplasm and nucleus, under normal growth conditions. Upon heat shock, HSF is localized into the nucleus, rapidly and forms large nuclear granules (Cotto et al., 1997; Zandi et al., 1997b). Because dKap-α3 appears to be the nuclear import factor involved in this process in *Drosophila*, we examined the cellular distribution of dKap-α3 protein in response to heat shock. In normally growing SL2 cells the immunofluorescent staining pattern of dKap-α3 is uniformly distributed throughout the cell. After five minutes of heat shock the majority of dKap-α3 is localized in the cytoplasm (Fig. 5).

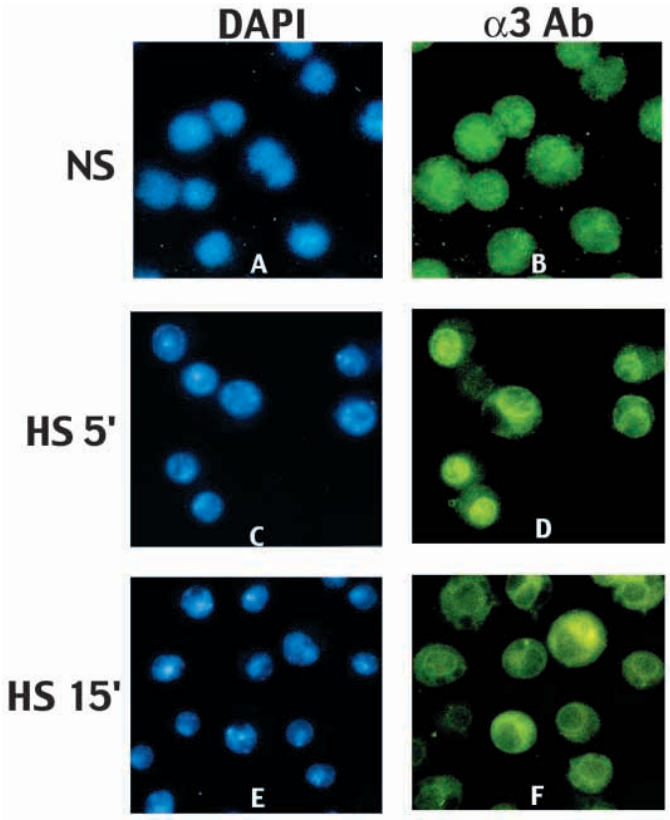

Fig. 5. Effect of heat shock on the sub-cellular distribution of dKap-α3 in SL2 cells. Schneider cells stained with DAPI are shown on the left side and immunofluorescent staining with anti-dKap-α3 monoclonal antibodies at 1:1000 dilution is shown on the right side. NS, Non shocked cells; HS, cells heat shocked at 37°C.

However, after 15 minutes of heat shock, dKap-α3 is localized exclusively in the cytoplasm around the nuclear periphery. This peripheral nuclear staining increases strikingly with increasing duration of heat shock.

Developmental regulation of karyopherin-α3 protein synthesis correlates with nuclear entry of dHSF

The developmental profile of dKap-α3 expression was examined by western blotting. Extracts were prepared from early *Drosophila* embryos at 0-2, 0-4 and 0-6 hours after egg deposition. The results of the western analysis are shown in Fig. 6 using anti-dKap-α3 monoclonal antibodies (the epitopes for these antibodies are present in the α-3 specific N terminus). It is evident from the blot that dKap-α3 is expressed at very low levels if at all at 0-2 hours (Fig. 6; lanes 1 and 2). At 0-4 and 0-6 hours, dKap-α3 is abundantly expressed (Fig. 6; lanes 3, 4 and 5). The total amount of protein present in each lane was normalized by Coomassie staining; and demonstrated that equivalent amounts of total protein were loaded for each developmental stage (Fig. 6B).

In situ immunostaining of *Drosophila* embryos with an anti-dHSF monoclonal antibody was used to assess the temporal and spatial expression of both dHSF and dKap-α3 proteins (Fig. 7). At cycle 11, dHSF is clearly seen in the cytosol of the embryo and excluded from the nucleus (Fig. 7E). At cycle 13 dHSF is localized within the nucleus in the absence of heat

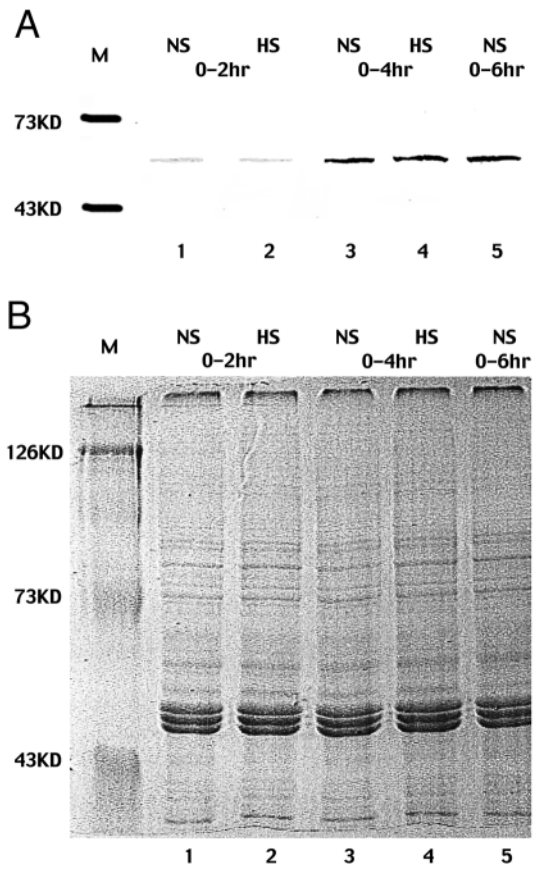

Fig. 6. Developmental western analysis of dKpa-α3 expression in *Drosophila* embryos. 0- to 2-hour, 0- to 4-hour and 0- to 6-hour (after egg laying) embryos were collected and homogenized. Whole embryo extracts obtained from homogenization were then analyzed by SDS-PAGE, probed with anti-α3 antibody for western blotting analysis (A) or stained with Coomassie Blue (B). M, molecular mass standards; NS, non-shock embryos; HS, heat-shocked embryos

shock (Fig. 7F). Similarly staged embryos were also stained with anti-dKap-α3 antibodies. Non-shocked embryos demonstrated essentially no staining in early cycles (cycle 11, Fig. 7I), but significant staining is seen in cycle 13 embryos, predominantly in the cytosol (Fig. 7J).

Analysis of the distribution of dHSF in heat-shocked embryos shows that dHSF is present only in the cytosol of heat-shocked cycle 12 embryos (Fig. 7G). Despite the fact that these embryos have been heat shocked, the transcription factor cannot enter the nucleus. Heat-shocked cycle 13 embryos, however, demonstrate a striking puntate/granular pattern to the nuclear staining (Fig. 7H). A similar pattern of staining is seen in heat-shocked *Drosophila* SL2 cells (Zandi et al., 1997b). Note the absence of detectable dKap-α3 protein in the heat-shocked cycle 12 embryo (Fig. 7K), correlating well with the absence of dHSF nuclear entry. At cycle 13 there is significant dKap-α3 protein observed in the cytosol, and the dHSF can now enter the nucleus.

Similar analyses of the mRNA distribution of dHSF and dKap-α3 are in agreement with the protein distribution patterns both temporally and spatially. The absence of appreciable dKap-α3 mRNA in the early embryo is evident in Fig. 7M (panel 1), where a cycle 10-11 embryo is compared with a cycle 13-14 embryo that shows considerable mRNA accumulation. It is interesting to note, however, the presence of a small amount of dKap-α3 mRNA in the posterior of the cycle 10-11 embryo where the pole cells will arise (indicated by the arrow; Fig. 7M, panel 1). These observations demonstrate that dKap-α3 expression during the first 12 cycles of embryogenesis is restricted to the very posterior of the embryo. Examination of the posterior region of cycle 12 embryos with anti-dHSF antibodies shows clear localization of dHSF protein within the nuclei of the poll cells (Fig. 7M, panel 2). These data further strengthen the correlation between the presence of dKap-α3 and the nuclear localization of dHSF.

DISCUSSION

Early embryos of animals as diverse as flies, frogs, sea urchins and mice are unable to induce Hsp70 transcription (Heikkila et al., 1985; Morange et al., 1984; Roccheri et al., 1982). The presence of large quantities of Hsp70, or the modification of the transcription apparatus that heat shock might cause, could be deleterious to the early embryo. It has been shown that elevated levels of Hsp70 can impede cell growth and division at normal growth temperatures (Feder et al., 1992; Krebs and Feder, 1997). This may also be the case at stress temperatures in the early embryo. The interruption of the normal developmental transcription program by the activation of heat shock protein synthesis may simply be too damaging to the early embryo and provide little advantage to the organism. Wang and Lindquist previously demonstrated that nuclear exclusion of the dHSF in the early embryo is correlated with the absence of Hsp70 induction (Wang and Lindquist, 1998). In this report we show that the mechanism that restricts the nuclear entry of the dHSF is the absence of a key nuclear transporter, *Drosophila* karyopherin-α3.

Biochemical analysis of dKap-α3

dKap-α3 is likely to be the bona fide nuclear transporter for dHSF for a number of reasons. First, the two-hybrid system used to screen for NLS binding proteins selected dKap-α3 as the primary interacting protein. Second, dKap-α3 was the only gene isolated from this family; indeed, neither dKap-α1 nor dKap-α2 were identified in this screen although both cDNAs are present in the embryonic library that was used (unpublished observations; Kussel and Frasch, 1995; Torok et al., 1995). Third, point mutations in the NLS of dHSF, which block nuclear entry in vivo, also prevent dKap-α3 binding in vitro. Finally, dKap-β promotes binding of the NLS to dKap-α3 in vitro and together the α3 and β proteins allow NLS-EGFP fusion proteins to dock to nuclei in digitonin-treated cells. Successful docking to the nuclei is also sensitive to mutations in the NLS that prevent nuclear entry in vivo.

Localization of dKap-α3

In normally growing SL-2 cells, dKap-α3 protein is uniformly localized throughout the cells. After heat shock, the transporter relocates to the nuclear membrane and within 15 minutes is entirely excluded from the nucleus. The significance of this localization may be to prevent interactions of dHSF with the transporter while it is involved in the activation of transcription. Alternatively, the dKap-α3 may become associated with the

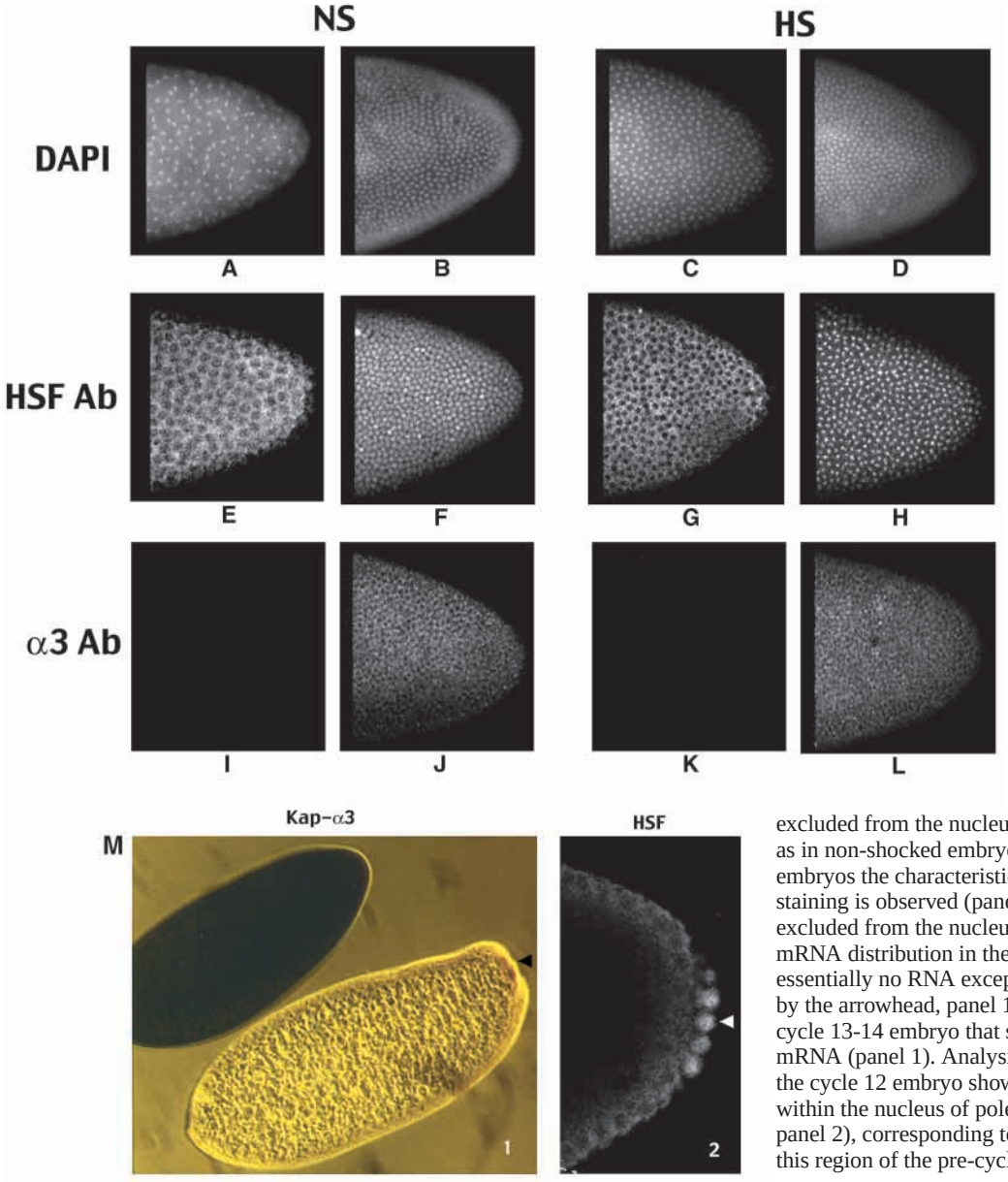

Fig. 7. Developmental timing of dKap-α3 expression correlates with nuclear entry of dHSF. (A-L) Cycle-11 to cycle-13 embryos were stained with either DAPI, anti-dHSF monoclonal antibodies (HSF panels) or anti-dKap-α3 monoclonal antibodies (Kap-α3 panels). NS, non-shocked *Drosophila* embryos; HS, heat-shocked embryos. (A,E,I) Cycle 11 embryos; (C,G,K) cycle 12 embryos; (B,F,J,D,H,L) cycle 13 embryos. Clearly present but excluded from the nucleus is dHSF in both non-shocked cycle 11 embryos (E) and heat-shocked cycle 12 embryos (G). Similarly staged embryos display essentially no dKap-α3 staining (I and K). At cycle 13 in non-shocked embryos the dHSF is constitutively nuclear and dKap-α3 is expressed yet largely excluded from the nucleus (J). In heat-shocked cycle 12 embryos the dHSF remains excluded from the nucleus 9 (G) and no dKap-α3 is observed as in non-shocked embryos (K). At cycle 13 in heat-shocked embryos the characteristic puntate pattern of dHSF nuclear staining is observed (panel H) and dKap-α3 remains excluded from the nucleus (L). (M) Analysis of the Kap-α3 mRNA distribution in the cycle 9-10 embryo demonstrates essentially no RNA except in the posterior region (indicated by the arrowhead, panel 1). Adjacent to this embryo is a cycle 13-14 embryo that shows significant levels of Kap-α3 mRNA (panel 1). Analysis of dHSF protein distribution in the cycle 12 embryo shows that dHSF is clearly present within the nucleus of pole cells at this early stage (arrowhead, panel 2), corresponding to the presence of Kap-α3 mRNA in this region of the pre-cycle 13 embryo (panel 1).

nuclear membrane and not be able to undergo the normal nuclear transporter recycling events during heat stress.

The spatial and temporal aspects of dKap-α3 expression in the early embryo demonstrate that the dKap-α3 protein is not expressed until 13 cycle, at which point significant RNA and protein accumulation is observed. Remarkably, this correlates precisely with that of dHSF nuclear entry and Hsp70 heat inducibility. The absence of dKap-α3 expression is coincident with the refractory period of Hsp70 induction and the nuclear exclusion of dHSF. Taken together with the biochemical analysis, these data present a compelling case that dKap-α3 is the nuclear transporter of the dHSF in vivo. Further genetic analysis will be necessary to demonstrate that mutations in dKap-α3 alter dHSF subcellular localization and function.

The role of dKap-α3

Western blotting and immunoprecipitation of dKap-α3 in cultured *Drosophila* Kc cells have shown that dKap-α3 is present in significantly greater quantities than dHSF (data not shown). It is therefore reasonable to suppose that dKap-α3 transports a number of other proteins into the nucleus. Indeed, a recent report using similar methods to identify proteins interacting with the *Drosophila* germ cell-less protein identified the same dKap-α3 described in this report (Dockendorff et al., 1999). Interestingly, the Gcl protein also contains a bi-partite NLS of approximately 30 amino acids. Comparison of the two NLSs revealed essentially no sequence similarity other than the presence of basic residues. Computer projections of the putative structure of the dHSF NLS suggests that it is α-helical (Zandi et al., 1997b), whereas the Gcl NLS contains two proline residues that would interrupt an α-helical structure.

Previous northern analysis and whole-mount in situ hybridization results have suggested that dKap-α3 is ubiquitously expressed throughout early development (Dockendorff et al., 1999). These observations do not agree

with our analysis of protein and RNA expression. Although we do not observe any appreciable accumulation of either dKap-α3 protein or mRNA until cycle 13, there is a small amount mRNA in the posterior of cycle 10-11 embryos as described in the Results section. This RNA may provide dKap-α3 for the developing pole cells and hence transport Gcl; it is clear that in the cycle 12 pole cells dHSF is nuclear. Thus, early expression of dKap-α3 in the posterior of the embryo may facilitate nuclear entry of critical proteins like Gcl into the developing pole cells.

Domains of dKap-α3

Remarkably, deletion of the dKap-α3 β-binding domain eliminates binding of the dHSF NLS to dKap-α3 in vitro. Previous structural studies have shown that for a fragment of the yeast dKap-α3 protein, which lacks the β-domain, two binding sites exist for an SV-40 NLS peptide within the arm-repeat domain (Conti et al., 1998). A recent structural study of mouse importin α using full-length protein shows that the N-terminal β-binding domain is capable of interacting intramolecularly with the arm repeats to form a self-inhibitory structure (Kobe, 1999). In this case no exogenous NLS was present in the crystals. It is likely that the significant size difference between the dHSF mini-NLS cargo used in this report and the SV-40 peptide may explain why other domains of the α-3 protein are needed for binding.

Early embryonic transcription and nuclear transport

Early development in *Drosophila* is characterized by series of rapid zygotic nuclear divisions without appreciable transcription until cycles 8 and 9 (Erickson and Cline, 1993). It has been demonstrated that components of the basal transcription machinery are transported into the nuclei at different division cycles. The RNA polymerase IIC subunit is found within the nucleus at cycle 7 whereas TFIID's TATA-binding protein (TBP) is localized within the nucleus between cycles 8 and 9 (Wang and Lindquist, 1998). The timing of dHSF entry into the nucleus is independent of these two general factors and this is probably due to the requirement of other nuclear transport molecules for the nuclear localization of these basal factors.

Developmental regulation of the heat shock response by a nuclear transporter represents a novel form of transcription regulation for a specific group of genes. It is possible that the absence of transcription during early embryonic stages may, in general, be due to the absence of specific nuclear transporters, at least for those transcription factors that are maternally provided. Indeed, this mechanism could represent a general explanation for the lack of transcription of early acting genes in embryonic nuclei. It will be very interesting to determine whether the nuclear entry of specific transcription factors as well as members of the basal transcription machinery correlates with the presence of specific nuclear transporters.

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