

Developmentally regulated nuclear transport of transcription factors in *Drosophila* embryos enable the heat shock response

Zhaohui Wang¹ and Susan Lindquist^{2,*}

¹Committee on Developmental Biology, The University of Chicago, Chicago, IL 60637, USA

²Howard Hughes Medical Institute, 5841 S. Maryland Avenue, MC1028, Room AMB N339, The University of Chicago, Chicago, IL 60637, USA

*Author for correspondence (e-mail: s-lindquist@uchicago.edu)

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SUMMARY

Hsp70 is a broadly conserved thermotolerance factor, but inhibits growth at normal temperatures and cannot be induced in early embryos. We report that in *Drosophila* embryos the temporal and spatial patterns of Hsp70 inducibility were unexpectedly complex, with striking differences between the soma and the germline. In both, regulation occurred at the level of transcription. During the refractory period for Hsp70 induction, HSF (heat-shock transcription factor) exhibited specific DNA-binding activity characteristic of activation in extracts of heated embryos. Remarkably, however, HSF was restricted to the cytoplasm in intact embryos even after heat shock. HSF moved from the cytoplasm to the nucleus in the absence of heat precisely when the capacity to induce Hsp70 was acquired (cycle 12 of the germline, cycle 13 in the soma). During oogenesis, Hsp70 inducibility was lost in nurse cells around stage 10, in a posterior-to-anterior gradient and HSF redistributed from nucleus to cytoplasm in the same spatiotemporal pattern. In a highly inbred derivative of the Samarkind strain, HSF moved into embryonic nuclei

earlier than in our standard wild-type strain. Correspondingly, Hsp70 was inducible earlier, confirming that nuclear transport of HSF controls the inducibility of Hsp70 in early embryos. We also report for the first time the nuclear import patterns of two general transcription factors, RNA polymerase subunit IIc and TATA binding protein (TBP). Both enter nuclei in a highly synchronous manner, independently of each other and of HSF. The import of TBP coincides with the first reported appearance of transcripts in the embryo. We suggest that the potentiation of general and heat shock-specific transcription in *Drosophila* embryos is controlled by the developmentally programmed relocalization of general and heat shock-specific transcription factors. Restricted nuclear entry of HSF represents a newly described mechanism for regulating the heat-shock response.

Key words: HSF, Hsp70, *Drosophila melanogaster*, Embryo, Transcription factors, Nuclear import

INTRODUCTION

Proteins that are vital in one cellular context can be detrimental in another. Tightly controlled regulatory systems have evolved to ensure that such proteins become active at the right time and place but are otherwise silent. The heat-shock proteins (HSP) are an example of highly conserved proteins whose expression is tightly regulated. A particularly salient example is the Hsp70 protein of *Drosophila*.

Drosophila cells constitutively express several Hsp70 relatives (Hsc70s) at high levels under normal conditions (Craig et al., 1983). In contrast, Hsp70, the heat-inducible form, is undetectable at normal temperatures but rapidly becomes one of the most abundant proteins in the cell upon a shift to high temperatures (Velazquez et al., 1983). This induction is the most rapid and massive known for any metazoan protein. Indeed, in *Drosophila*, Hsp70 dominates the entire protein synthetic profile in most cell types during heat shock (Parsell and Lindquist, 1994).

This remarkable induction is achieved via a wide range of regulatory strategies including: (1) the presence of multiple *Hsp70* genes in the genome (Ish-Horowicz et al., 1979); (2) the maintenance of an open chromatin configuration on these genes even at normal temperatures (Wu, 1980), with RNA polymerase engaged but arrested at the transcription start site (Rougvie and Lis, 1988); (3) the activation of a pre-existing transcription factor (HSF) within one minute of temperature shift (Lis and Wu, 1993); (4) the lack of introns, which allows messages to escape the disruption of splicing that occurs during heat shock and to rapidly exit the nucleus (Yost and Lindquist, 1986); (5) the stabilization of the normally unstable hsp70 mRNA at heat-shock temperatures (Petersen and Lindquist, 1988); and (6) the preferential translation of this mRNA at elevated temperatures (McGarry and Lindquist, 1985).

These elaborate regulatory mechanisms act not only to ensure rapid induction of Hsp70 upon heat shock, but also to maintain Hsp70 expression at an undetectable level at normal temperatures. Indeed, it is just as important to restrict Hsp70

expression at normal temperatures as it is to induce it with heat shock. If Hsp70 is expressed from a heterologous promoter in cultured cells, it greatly increases survival when cells are heat shocked, but stops cell growth and division at normal temperatures (Feder et al., 1992; Solomon et al., 1991). Moreover, transgenic flies with extra copies of the *Hsp70* genes acquire thermotolerance more rapidly with severe heat shocks (Welte et al., 1993; Feder et al., 1996) but, with milder heat shocks, have slower rates of development, and reduced survival to adulthood (Krebs and Feder, 1997).

Preblastoderm embryos, characterized by rapid rates of nuclear division, have adopted an even more extreme regulatory strategy. Other major HSPs of *Drosophila*, Hsp83 and the small HSPs, are maternally supplied and present constitutively in the embryo (Arrigo and Tanguay, 1991; Zimmerman and Cahill, 1991; and Z. Wang, unpublished observation). In contrast, Hsp70 is not maternally supplied, not constitutively expressed, and not inducible with heat shock (Graziosi et al., 1980). This restriction is maintained even after the activation of zygotic transcription and makes early embryos extremely sensitive to heat killing (Welte et al., 1993). Apparently, in early embryos the high rates of nuclear division cause the detrimental effects of Hsp70 on cell division to outweigh its beneficial effects on survival, resulting in the evolution of mechanisms to restrict its expression absolutely.

The embryonic restriction of Hsp70 expression is widespread. Although virtually all organisms, and most cell types in multicellular organisms, express Hsp70 in response to heat, organisms as diverse as fruit flies, sea urchins (Roccheri et al., 1982), frogs (Heikkila et al., 1985) and mice (Morange et al., 1984) restrict Hsp70 inducibility in early embryos. The regulatory mechanisms that control this restriction are unknown. Because the mechanisms that regulate Hsp70 in other *Drosophila* cell types are so well characterized, and many tools are available for its analysis, we sought to understand the mechanism that restricts its expression in the early embryos of this organism. We report that an unprecedented developmental regulation of HSF nuclear import is a key element in determining when and where Hsp70 can be expressed. Our results also have implications concerning the regulation of general transcription in early embryos.

MATERIALS AND METHODS

Fly cultures

Flies were raised on standard cornmeal-agar media at 25°C. The wild-type strain was Oregon-R, maintained in a population cage. A highly inbred wild-type line, Sam1236, was also used. It derived from the Samarkind strain and was obtained from T. Mackay. *Res1 (st kni^{14F} pp Res1/TM3Sb, hb-Z)* was kindly provided by D. Ruden. Embryos were collected from a population cage after two or three pre-collections to reduce the number of retained eggs. Each collection was divided into several samples and aged at 25°C. Control samples were processed immediately. Heat-treated samples were incubated for 15-30 minutes in a 35.5°C water bath to ensure even heating as described by Welte et al. (1995).

Whole mount in situ hybridization

Hsp70 mRNAs in whole embryos were detected by in situ hybridization as described by Tautz and Pfeifle (1989) with minor modifications. RNA probes (240-250 nucleotides) derived from the

entire hsp70-3'UTR or its complementary sequence were synthesized with T3 or T7 polymerase in the presence of digoxigenin-11-UTP (Boehringer Mannheim). Fixed embryos were treated with proteinase K (50 mg/ml) for 8 minutes. Prehybridization and hybridization (probe concentration 100 ng/ml) were performed at 50°C, washing at 53°C. Digoxigenin was detected with alkaline phosphatase-conjugated antibodies at 1:2000 dilution (Boehringer Mannheim). Images were acquired by conventional microscopy.

Immunostaining

Embryos were fixed and stained as described by Patel (1994) with modifications. Monoclonal antibody 7FB specifically recognizes the heat-inducible member of *Drosophila* Hsp70 family (Velazquez et al., 1983). Anti-*Drosophila* HSF (Westwood et al., 1991), TBP and RNA polymerase IIc (Pol-IIc) antisera were generously provided by C. Wu, J. Kadonaga, and A. Greenleaf, respectively. Mouse anti-chick β -tubulin antibody was from Amersham. Primary antibodies were diluted 1:1000 or 1:2000 (except for Pol-IIc, 1:100) and incubations were at 4°C overnight. Fluorescent dye-conjugated secondary antibodies (Jackson IRL) were diluted 1:300-400, and incubations were at room temperatures for 2 hours. Images were acquired by confocal microscopy (Zeiss, LSM410). The embryonic stage was determined by co-staining with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) and counting nuclei within a 2200 μm^2 area on the embryo surface as described by Foe and Alberts (1983).

Electrophoretic mobility shift assay

Whole embryo extracts were prepared by freezing dechorionated embryos in liquid N₂, and grinding the frozen pellet in 350 mM NaCl, 10 mM HEPES pH 7.9, 0.1 mM EGTA, 5% glycerol, 0.4 mM PMSF, and 0.5 mM DTT. Lysates were spun for 3-5 minutes at 14,000 rpm in a microcentrifuge. Pellets and supernatants were resuspended by repetitive pipetting through plastic tips. After a second centrifugation for 30 minutes at 100,000 g, pellets were discarded and supernatants were stored at -80°C. Each microliter of extract contained proteins from two embryos (3-5 $\mu\text{g}/\mu\text{l}$). Probes were made from complementary synthetic oligonucleotides annealed and end-labeled by [γ -³²P]ATP. Wild-type (consensus) and mutant sequences of the HSE probes were:

wild-type	5'-tcgactagaagcttctagaagcttag-3'
mutant	5'-tcgactagaagcttaaagaagcttag-3'

Each 25 μl reaction contained 8 μl extract (approx. 40 μg), 1-2 μl of probe (3×10^4 cts/minute), 5 μl of 5 \times binding buffer (750 mM NaCl, 50 mM Tris pH 7.5, 2.5 mM EDTA, and 30% Ficoll), and 80 ng/ μl poly(dI-dC). After a 20-minute incubation at room temperature, the sample was quantified on a 4% polyacrylamide gel (Sarge et al., 1993). Radioactivity in bands was quantified by a PhosphorImagerTM (Molecular Dynamics) and ImageQuant software.

Analysis of proteins

Embryos were ground individually in 10 μl of sample buffer (2% SDS, 10% glycerol, 5% β -ME, 60 mM Tris, and 0.025% bromophenol blue) in culture tubes (6 \times 50 mm; Kimble) and immediately boiled. Pestles were made from 200 μl -micropets (Clay Adams, N.J.) by burning either end of the glass pipette to seal the opening. Proteins were separated on 6% or 7.5% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Blots were reacted sequentially with Hsp70 (1:5000 7Fb) and HSF (1:10,000) antibodies. Horseradish peroxidase-conjugated secondary antibodies were detected with ECL (Amersham) or SuperSignalTM (Pierce) reagents.

For 2-D electrophoresis and detection of HSF, whole embryos were manually disrupted in buffer containing 8 M urea, 2% Triton X-100, 1:50 pharmalyte pH 3-10, and 0.3% DTT. Proteins were electrophoretically separated with the Immobiline DryStrip Kit (Pharmacia), using a pH 4-7 DryStrip for the first dimension and 8-18% gradient gel for the second dimension. Proteins from 65-80

embryos were loaded per gel strip and samples were focused for at least 55,000 v-hour. Proteins were transferred to Immobilon-P membranes (Millipore), and sequentially incubated with HSF antisera and monoclonal antibody 7.10, which recognizes both heat-inducible and the constitutive members of Hsp70 family (Kurtz et al., 1986).

Heat treatment and fixation of ovaries

Flies in stock vials were submerged in 37°C water bath (80-120 minutes), and anesthetized on ice. Ovaries were dissected in PBS (non-stressed ovaries) or in 0.1% Triton X-100 in PBS (stressed ovaries). Dissection without Triton X-100 produced a stronger HSF signal, but detection of Hsp70 required detergent. Although Triton X-100 reduced the intensity of HSF signals, it did not alter HSF distribution. Ovaries were fixed in 6 volumes of heptane, and 1 volume of 6% formaldehyde in 100 mM KH₂PO₄/K₂HPO₄ pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM MgCl₂, with vigorous shaking for 20 minutes. Ovarioles were separated by hand in PBS/0.1% Triton X-100 after fixation.

RESULTS

Hsp70 inducibility in early embryos correlates with transcript accumulation

In the *Drosophila* embryo, some genes are transcribed as early as nuclear cycles 8 and 9 (Erickson and Cline, 1993), but a general increase in transcriptional activity occurs upon cellularization, at cycle 14 (Pritchard and Schubiger, 1996). Previous analyses with bulk preparations of embryos suggested that Hsp70 first becomes heat inducible at this stage (Graziosi et al., 1980). To define Hsp70 inducibility more precisely we examined individual embryos, by immunostaining with an Hsp70-specific antibody (Fig. 1). This revealed that Hsp70 first became inducible at cycle 12 in a subset of pole cells (germline cells). (A detailed analysis of Hsp70 expression and thermotolerance in the germline will be reported elsewhere; L. Yue, G. Lin, J. Feder, Z. Wang, and S. Lindquist, manuscript in preparation.) In the soma, induction was first observed in cycle 13.

To investigate the basis for this complex expression pattern, we compared the inducibility of Hsp70 protein and RNA on a cell-by-cell basis in embryos by immunofluorescent staining and in situ hybridization. The stage of each embryo was determined by counting nuclei co-stained with the fluorescent DNA-binding dye DAPI. Hsp70 messages were detected with a probe complementary to the 3'UTR specific for the heat-inducible form of Hsp70 (Dellavalle et al., 1994). No Hsp70 protein (Fig. 1A, HSP70) or RNA signals (Fig. 1B, upper panel) were detected without heat treatment, and no RNA signals in heat-shocked embryos were detected with a probe from the sense strand of the Hsp70 3'UTR

(Fig. 1B, lower panel), demonstrating the specificity of the probes.

In response to heat, the induction of Hsp70 mRNA exhibited a stage-dependent and cell-type-specific pattern identical to that of the protein (Fig. 1C and D). Both protein and mRNA signals were first observed in the pole cells of approximately one third of cycle 12 embryos (Fig. 1C and D, cycle 12). In those embryos that were positive for Hsp70 protein and RNA, only a subset of the pole cells showed induction. The same pattern was observed in ectopic pole cells (Ephrussi and Lehmann, 1992) induced at the anterior end in flies carrying an *oskar-bicoid 3'UTR* fusion gene (not shown). The induction of protein and RNA in pole cells was weaker in cycle 13, and was lost during cellularization (Fig. 1C and D, cycle 13 and 14 inserts). In contrast, Hsp70 protein and transcripts were not detectable in the soma until cycle 13; both were detected in about 2/3 of cycle 13 embryos and were abundant in all embryos at cycle 14 (Fig. 1C and D). Within an individual

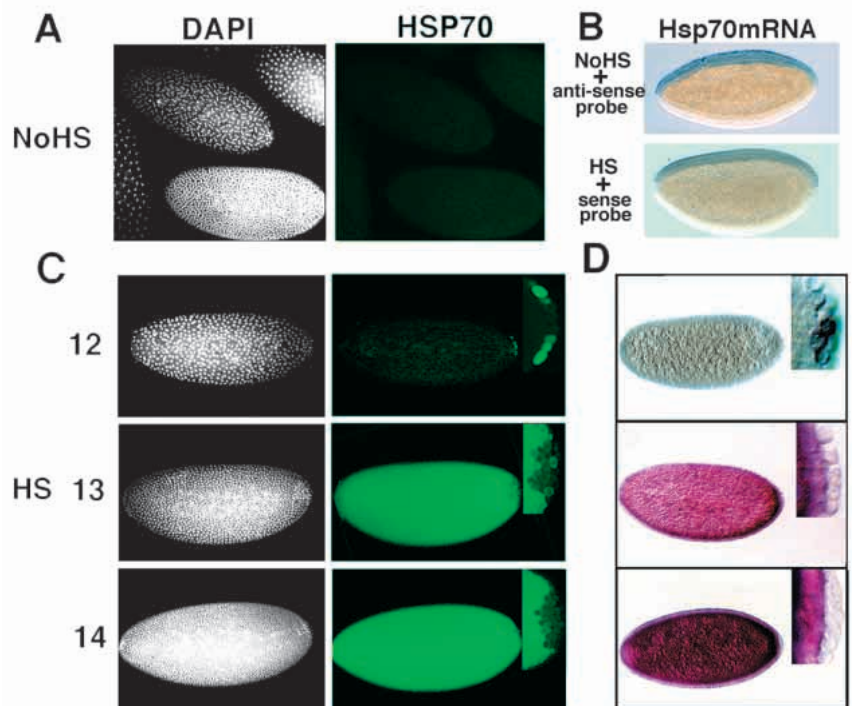


Fig. 1. The cell-type and stage-specific induction of Hsp70 RNA corresponds closely with that of Hsp70 protein. (A) Embryos raised at 25°C were fixed and stained with DAPI to visualize DNA (left), and with the antibody 7FB, specific for Hsp70. The signal of Hsp70 was detected by a secondary antibody conjugated to FITC. Embryos exhibited no Hsp70 expression at any stage without heat stress. (B) Top, embryos raised at 25°C were fixed and hybridized with a probe complementary to Hsp70 mRNA 3'UTR. Bottom, embryos treated at 35.5°C for 30 minutes were hybridized with a probe containing 3'UTR sequence of Hsp70 mRNA. Cellularized embryos are shown, but embryos of other ages also showed no hybridization signal in these control experiments. (C) Embryos heat shocked at 35.5°C for 30 minutes were stained as described in A. Embryos shown are representatives of nuclear cycle 12, 13, and 14. Hsp70 can first be induced in a subset of pole cells in cycle 12 embryos and in soma of cycle 13 embryos. At cycle 14, no Hsp70 is detectable in any pole cells but high levels are observed in soma of every embryo. Inserts provide a higher magnification view of the posterior end, with pole cells. (D) Embryos heat-treated as in C were hybridized in situ with a probe complementary to the 3'UTR of Hsp70 mRNA. The pattern of Hsp70 RNA accumulation is remarkably similar to that of Hsp70 protein expression.

embryo, when Hsp70 mRNA and protein were induced, the entire soma stained with a similar intensity. Thus, in the soma the induction of Hsp70 is a sudden, and an all-or-none phenomenon.

The close temporal (early vs. late) and spatial (pole cell vs. soma) correlation between the appearance of Hsp70 protein and mRNA demonstrates that the complex pattern of developmental restriction in Hsp70 expression is mediated by restricting the accumulation of RNA. The short induction period employed (15 to 30 minute heat treatment immediately prior to the fixation of samples) suggests that regulation occurs at the level of transcription.

HSF is competent to bind DNA upon heat stress even when Hsp70 cannot be induced

The transcriptional activation of *Hsp70* in *Drosophila* is mediated by heat-shock factor (HSF) which trimerizes in response to heat and then binds to heat-shock elements (HSE) upstream of the gene (Wu, 1995). We asked if the failure to induce Hsp70 was due to an inhibition of HSF's DNA-binding ability, using the electrophoretic mobility-shift assay. Three developmental windows, defined by minutes after egg laying (AEL), were examined: (a) 60 to 80 minutes AEL, the preblastoderm stage (cycles 8-10) when Hsp70 is not inducible, even in pole cells; (b) 90 to 110 minutes AEL, the syncytial blastoderm stage (cycles 12 and 13) when Hsp70 is becoming inducible; and (c) 150 to 170 minutes AEL (cycle 14) when Hsp70 is highly inducible in all embryos. The nuclear cycle was directly determined for each collection, by DAPI staining, to ensure that staging was correct.

Extracts from unheated embryos had no detectable HSE-binding activity at any of these stages (Fig. 2, 'C' lanes). Surprisingly, extracts from heat-treated embryos produced an HSE mobility shift at each stage (Fig. 2, 'H' lanes). The specificity of this shift was confirmed in three ways. First, unlabeled wild-type HSEs competed with radiolabeled HSEs for binding. This competition was dose-dependent, with most

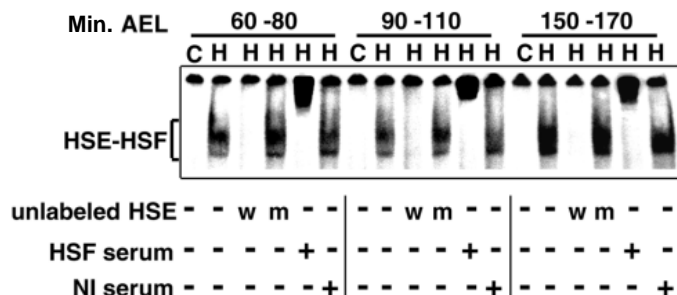


Fig. 2. Even at stages when Hsp70 is not inducible, HSF in extracts of heated embryos binds heat-shock elements with high specificity. Extracts were prepared from embryos of different ages maintained at 25°C (C, control) or heat shocked at 37°C for 15 minutes (H), and incubated with the same quantity of ³²P-labeled HSE-containing DNA oligomers. The lanes marked with 'w' or 'm' indicate reactions that contained an excess of unlabeled wild-type or mutant HSE, respectively. The '+' signs indicate the addition of rabbit anti-HSF serum or non-immune (NI) serum. The position of the HSF-HSE complex is marked by the bracket on the left. Free probe, which ran at the bottom of the gel, is omitted in this figure. Min. AEL, minutes after egg laying.

of the signal eliminated at 3 pg/μl (Fig. 2, 'w' and data not shown). Second, mutant HSEs, carrying a 2-nucleotide substitution in the HSE consensus sequence, did not compete even at 30 pg/μl (Fig. 2, 'm'). Third, HSF antiserum retarded the migration of HSE complexes at a 1:100,000 dilution (Fig. 2, HSF serum). Non-immune serum, at a 1:20,000 dilution, did not (Fig. 2, NI serum).

Older embryos consistently yielded a stronger gel-shift signal than younger embryos (about two-fold higher by phosphor-imaging). However, immunoblot analysis of the extracts demonstrated that older embryos also had a higher concentration of HSF than younger ones (Fig. 3A), presumably due to the continuous translation of maternally supplied HSF mRNA during this period. Since younger embryos have fewer nuclei to provide potential targets for HSF, on a per-nucleus basis the HSE-binding activity of younger embryos is roughly comparable to that of older ones. Therefore, the inherent capacity of HSF to trimerize and bind HSEs in response to heat is not compromised at the early stages when Hsp70 cannot be induced.

Hyper-phosphorylation of HSF is apparently unnecessary for Hsp70 induction

Heat-induced hyper-phosphorylation of HSF is correlated with transcriptional activation in yeast and human cells (Larson et al., 1988; Winegarden et al., 1996). The immunoblot analysis used to examine HSF levels in extracts for gel-shift assays also revealed a heat-induced reduction in HSF mobility (Fig. 3A) that correlated with the change in Hsp70 inducibility. Samples exhibiting a strong mobility change with heat-shock were

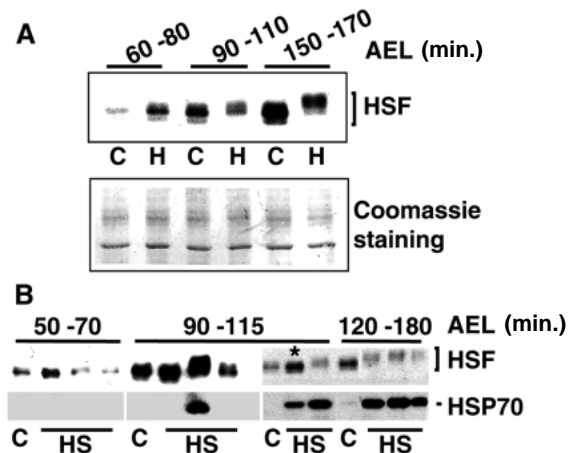


Fig. 3. Hyper-phosphorylation of HSF is apparently not required for Hsp70 induction. (A) Extracts used in gel shift assays of Fig. 2 were analyzed by western blotting. The blot was stained with Coomassie blue to demonstrate equal loading and even migration. HSF was detected by reacting with HSF antiserum followed by peroxidase-conjugated secondary antibody. HSF concentrations increased with the age of embryos. A shift in the mobility of HSF with heat shock was more pronounced in the older embryos, which expressed Hsp70. (B) Hsp70 induction and HSF mobility shifts were analyzed in lysates of individual embryos. Three representative western blots are shown. Hsp70 and HSF were probed on the same blot. The change in HSF mobility reflects hyper-phosphorylation of HSF. * marks the sample exhibiting Hsp70 induction without HSF hyper-phosphorylation. AEL (min.), minutes after egg laying.

Table 1. Summary of data obtained from individual embryos: heat-induced hyper-phosphorylation and Hsp70 induction

	Embryo age (min. AEL)				
	0-70 <i>n</i> =21	90-115 <i>n</i> =27	120-180 <i>n</i> =19		
HS-induced HSF mobility shift	-	-	-	+	+
Hsp70 induction	-	-	+	+	+
Number	21	12	5	10	19

n is the number of heat-shocked embryos analyzed for each developmental stage; '+' or '-' indicates presence or absence of HSF hyper-phosphorylation or Hsp70 induction.

treated with calf intestinal phosphatase. The mobility of samples from heat-treated embryos was greatly reduced and this reduction was blocked by the phosphatase inhibitor sodium pyrophosphate (data not shown). These results confirmed that the heat-induced mobility shift in HSF was due to hyper-phosphorylation.

To investigate more rigorously the possibility that the failure to hyper-phosphorylate HSF is responsible for restricting Hsp70 expression in early embryos, we analyzed the proteins of individual embryos. Little change in HSF mobility and no induction of Hsp70 were observed in any heat-treated embryos between 0 and 70 minutes AEL (Fig. 3B and data not shown). In all cellular blastoderm embryos (120-180 minutes AEL), HSF was hyper-phosphorylated following heat stress and Hsp70 was induced. During the transition period (cycles 12 and 13, 90 to 115 minutes AEL), most embryos showing induction of Hsp70 also showed a change in HSF mobility. However, in five of the 27 embryos analyzed, Hsp70 was induced with no obvious change in HSF mobility (Fig. 3B, lane *, and Table 1). It is unlikely that the absence of hyper-phosphorylation in these samples is due to artifactual activation of a phosphatase during lysis. Embryos were lysed directly into SDS sample buffer and immediately boiled. Moreover, Hsp70 induction in the absence of hyper-phosphorylation was observed only in cycle 13 embryos. Hyper-phosphorylation seemed to be associated with a higher level of Hsp70 expression.

However, hyper-phosphorylation of HSF is not required for the Hsp70 induction by heat. Thus, the absence of HSF hyper-phosphorylation in early embryos cannot be responsible for the failure to induce Hsp70.

A heat-independent change in HSF phosphorylation was not detected

HSF possesses multiple target sites for phosphorylation (Clos et al., 1990). It is quite possible that individual phosphorylations, not distinguished on one-dimensional gels, are required to activate HSF. Two-dimensional gels provide high resolution of phosphoprotein isoforms, but single embryos don't provide sufficient material for this technique. Therefore, we could not apply it to the analysis of heterogeneous heat-treated embryos. Instead we asked if a subtle, heat-independent change in phosphorylation might occur in embryos maintained at 25°C, which could lead to the dramatic potentiation of HSF's transcriptional activity at cycle

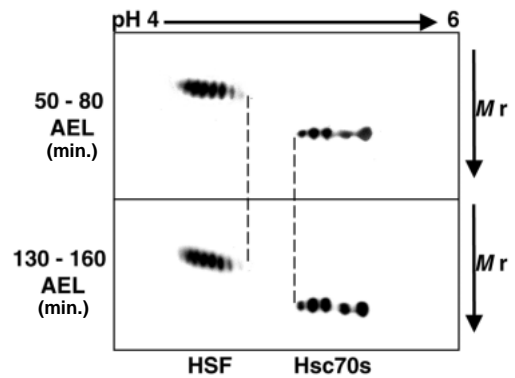


Fig. 4. The two-dimensional electrophoretic patterns of HSF are identical whether or not Hsp70 is inducible. A 30-minute collection of embryos was divided in half and aged at 25°C. Lysates of 80 preblastoderm (50'-80') and 65 cellular-blastoderm (130'-160') embryos were used to equalize the quantity of HSF loaded. pH dimension is horizontal, mass dimension vertical (M_r). HSF and the constitutively expressed relatives of Hsp70 (Hsc70s) were detected by immunoblotting. Hsc70 proteins serve as reference points, as their migration does not change during embryogenesis. AEL (min.), minutes after egg laying.

13. At least 7 differentially charged species of HSF were detected in non-stressed embryos (Fig. 4). (The protein profile of the constitutive relatives of Hsp70, which does not change during embryonic development, served as a reference point for HSF migration; Fig. 4 and K. Palter, personal communication). The distribution of HSF isoforms in unheated embryos was identical in all samples, including stages when Hsp70 was not inducible in any embryos (50-80 minutes AEL, cycle 6-10) and stages when it was highly inducible in all embryos (130 to 160 minutes AEL, cycle 14).

HSF moves from cytoplasm to nucleus in a cell-type and stage specific manner in the absence of stress

In all organisms and cell types studied to date, including *Drosophila* tissue culture cells, HSF is either constitutively nuclear (Morimoto et al., 1992; Wu, 1995; and our own unpublished observations), or concentrated in nuclei in response to heat (Zandi et al., 1997). Unexpectedly, we found that in early *Drosophila* embryos, HSF was not constitutively nuclear, nor could it enter nuclei in response to heat.

We examined the localization of HSF in interphase nuclei to avoid confusion caused by the fast nuclear divisions and frequent breakdown of the nuclear envelope in preblastoderm embryos. As determined by co-staining with β -tubulin antibodies, all embryos shown in Fig. 5 were in interphase: no tubulin staining was detected in the nucleus and no spindle structure was present. In the absence of heat shock, HSF exhibited a striking change in localization that correlated with the change in Hsp70 inducibility in a cell-type and stage-specific manner. Initially, HSF was cytoplasmic in both pole cells and soma. HSF first appeared in the nucleus of pole cells at cycle 12 (Fig. 5, arrowhead), the same stage at which Hsp70 first becomes inducible in pole cells. In the soma, HSF was excluded from nuclei until cycle 13, the transition stage for Hsp70 induction. At this stage, some embryos displayed cytoplasmic localization of HSF (Fig. 5, cycle 13a), and

others displayed nuclear localization (cycle 13b). In both cases, the pattern of HSF staining was uniform. That is, when one somatic nucleus displayed HSF staining, all other somatic nuclei in that embryo did as well (Fig. 5 and data not shown). Every cycle 14 embryo exhibited nuclear staining of HSF in all cells. Further, with the single exception of developing egg chambers described below, in all of the many different cells and tissue-types we have examined in older embryos, larvae, and adults (data not shown), HSF staining was always nuclear.

Nuclear targeting of HSF is a prerequisite to Hsp70 induction

We observed an absolute correlation between HSF nuclear localization in the absence of heat and Hsp70 inducibility with heat before and after cycle 13. However, at cycle 13, a discrepancy appeared. Two-thirds of cycle 13 embryos showed Hsp70 induction with heat shock, but HSF was nuclear in just one-quarter of unheated embryos (Fig. 5 and data not shown). This discrepancy was explained when cycle 13 embryos were stained for HSF after heat shock. Nuclear HSF staining increased to nearly two-thirds (Fig. 6; 125/193). This might be because the nuclear localization of HSF is heat-inducible only in some embryos and only at this stage. More likely it is because the embryos continued to develop during the 15 minute heat treatment, progressing from early cycle 13 to late cycle 13. Indeed, in separate experiments, we monitored the developmental progression of embryos during the mild heat treatments employed in these experiments (35.5°C). We found that prior to cycle 14 heat treatment did not stop progression from one cycle to the next, whereas it did block progression at cycle 14 (Wang, 1998). This is notable because heat shock blocks cell cycle progression in all cell types and in all organisms examined (Francis and Barlow, 1988; Roti Roti et al., 1992; Maldonado-Codina et al., 1993; Nunes and Siede, 1996; Nitta et al., 1997). The failure of heat shock to block cell cycle progression prior to cycle 14 is reminiscent of the absence of checkpoint controls for DNA damage in embryos prior to cycle 14, but we have not yet investigated whether heat-inducible checkpoints operate by the same mechanism.

In any case, only a few heat shocked embryos showed nuclear HSF without Hsp70 induction (11 out of 193). These were presumably fixed at a time when HSF had moved to the nucleus but before the Hsp70 genes could be transcribed and the Hsp70 message transported and

translated. A small fraction of embryos (12/193) were fixed during mitosis when HSF concentrations were equivalent in the nucleus and the cytoplasm and only about half of these embryos exhibited Hsp70 induction (Fig. 6; 'cyto&nuc'). Clearly, in all of the cycle 13 embryos where HSF was still concentrated in the cytoplasm, Hsp70 was not induced (Fig. 6, cytoplasmic). These observations demonstrate that the developmentally programmed nuclear entry of HSF is an immediate prerequisite to the transcriptional activation of *Hsp70* genes and suggest it may be the primary mechanism regulating Hsp70 inducibility.

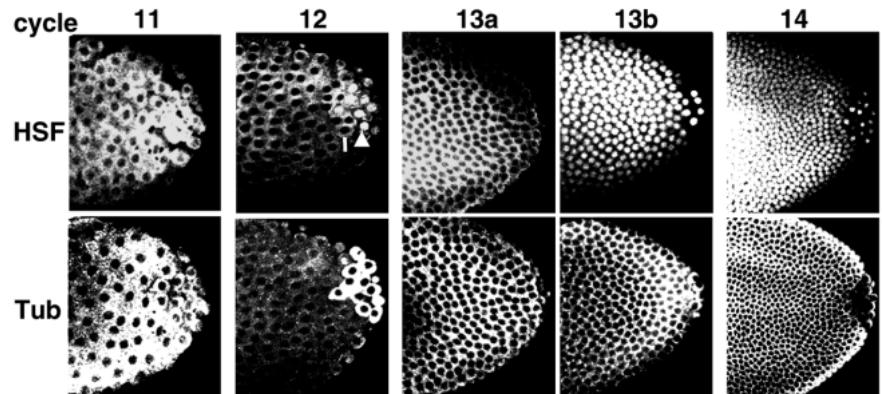


Fig. 5. Developmentally programmed relocalization of HSF from the cytoplasm to the nucleus in the absence of heat stress. Embryos maintained at 25°C were fixed and stained for HSF and tubulin (Tub). HSF and tubulin were visualized with secondary antibodies conjugated to Texas-red or FITC, respectively. Nuclear division cycle was determined by DAPI staining (not shown). All embryos exhibited cytoplasmic HSF staining in some prior to cycle 13 and all embryos exhibited nuclear staining after cycle 13. During cycle 13, some embryos exhibited cytoplasmic staining (13a), while others nuclear staining (13b). Only the posterior portion of the embryo is shown, but in each embryo, all soma showed the same staining pattern. The intensity of tubulin staining in pole cells varies in individual embryos for unknown reasons.

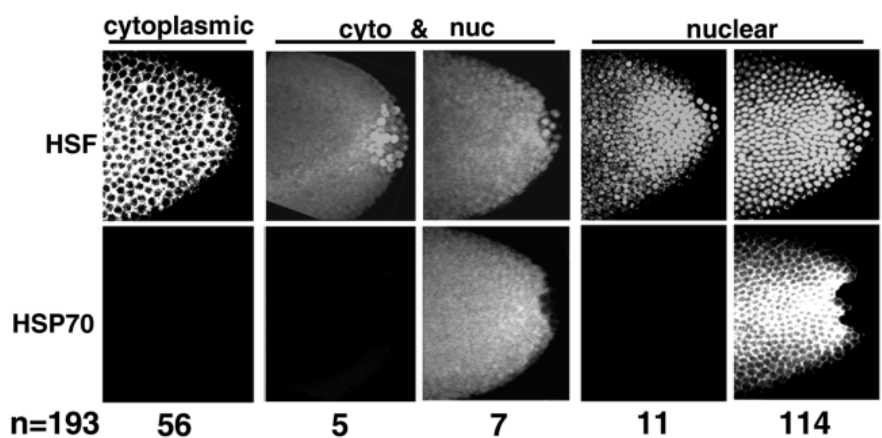


Fig. 6. Hsp70 inducibility correlates with HSF nuclear localization in cycle 13 embryos. Embryos were heat-treated for 15 minutes at 35.5°C and stained with DAPI (not shown), HSF antiserum, and Hsp70 monoclonal antibody. Hsp70 and HSF were visualized using secondary antibodies conjugated to FITC or Texas-red, respectively. 193 embryos of cycle 13 were scored and categorized according to their HSF patterns (numbers below the columns). The majority of embryos exhibited nuclear localization of HSF (nuclear) and most of these showed induction of Hsp70; a small fraction exhibited uniform HSF staining (cyto & nuc) with or without Hsp70 induction; no embryo with cytoplasmic HSF localization was able to express Hsp70 (cytoplasmic).

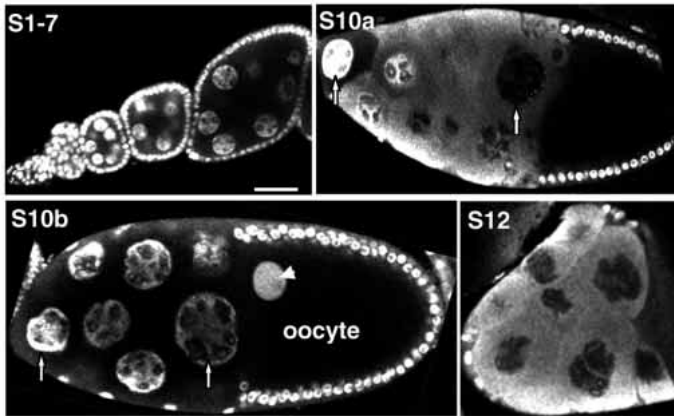


Fig. 7. HSF moves from nucleus to cytoplasm in nurse cells during oogenesis. Ovaries from females maintained at 25°C were fixed and incubated with HSF antiserum, then with Texas-red-conjugated secondary antibody. Egg chambers of stages 1-7 (S1-7), two stage 10 egg chambers (S10a and S10b), and nurse cells of stage 12 (S12) are shown. Arrowhead indicates oocyte nucleus. Nurse cells with polyploid nuclei are anterior to the oocyte. Small follicle cells outline the egg chamber. Arrows point to nurse cell nuclei. Scale bar, 10 μm. Left, anterior.

HSF relocates from nucleus to cytoplasm during oogenesis

In early embryos, HSF is maternally supplied. Previous *in situ* hybridization studies demonstrated that germline-derived cells in ovaries express *Hsp70* mRNA in response to heat stress, but become recalcitrant to heat induction at about stage 10 of oogenesis (Zimmerman et al., 1983). To determine when the cytoplasmic localization of HSF is established, we examined HSF distribution during oogenesis. *Drosophila* oocytes develop within an egg chamber, surrounded by soma-derived follicle cells. In the egg chamber, 1 of the 16 germline-derived cystocytes develops into an oocyte, the remaining cystocytes become polyploid and act as 'nurse' cells, supplying the oocyte with most components required for early development.

In flies maintained at 25°C, HSF relocated from the nucleus to the cytoplasm of nurse cells around stage 10 (Fig. 7). In general, HSF was nuclear before stage 10 and cytoplasmic after stage 11, but the precise timing of relocation varied. In Fig. 7, one of the stage 10 egg chambers is beginning to show HSF relocation (S10a) whereas another, which appears slightly more mature, still shows nuclear localization (S10b). Notably, when relocation occurred, it exhibited a sharp spatial gradient, with the nurse cells more proximal to the oocyte showing cytoplasmic localization first, while distal cells still displayed nuclear concentration. Oocyte nuclei seemed to follow the same pattern as nurse cell nuclei, but because the vitelline membrane forms at stage 10, they could not be scored in all egg chambers.

Next, we examined the relationship between HSF localization and *Hsp70* inducibility. From stage 1 through 9, *Hsp70* was induced in all germline cells (Fig. 8 and data not shown). Starting at stage 10, *Hsp70* inducibility gradually decreased, and eventually disappeared, as HSF redistributed from the nucleus to the cytoplasm. This change in *Hsp70* inducibility also exhibited a spatial gradient, with the nurse cells closer to the oocyte becoming refractory to *Hsp70*

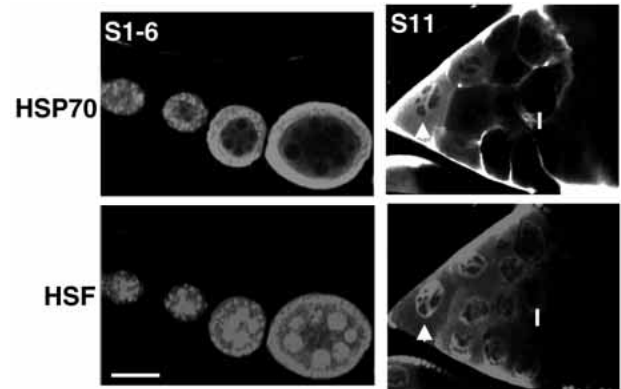


Fig. 8. HSF redistribution correlates with the change in *Hsp70* inducibility of nurse cells. Ovaries from females subjected to heat treatment at 37°C for 80-120 minutes were fixed and stained with antibodies against *Hsp70* (7FB) and HSF (rabbit serum) as described in Fig. 6. Prior to stage 10, *Hsp70* is induced in both somatic and germline cells (S1-6). In the nurse cells of heat-treated flies *Hsp70* staining was weaker than in other tissues, but much stronger than in the nurse cells of non-heated flies, which was virtually undetectable. In a stage 11 egg chamber (S11, only nurse cells are shown), *Hsp70* is induced only in the more anterior cells where HSF is nuclear (arrowhead). Vertical bars indicate the nurse cell in which HSF is mostly cytoplasmic and *Hsp70* is not induced. Scale bar, 10 μm.

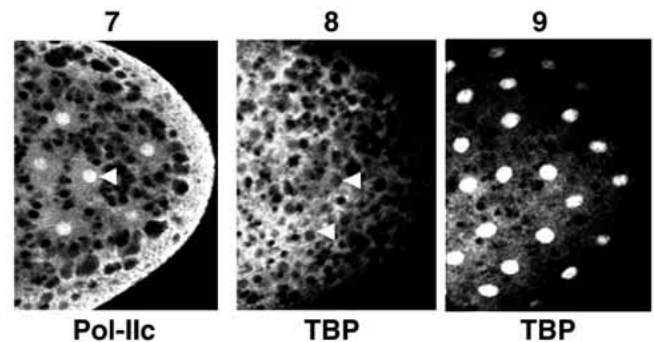


Fig. 9. A general transcription factor also exhibits developmentally programmed relocalization, but the timing is distinct from that of HSF. Embryos maintained at 25°C were fixed and stained with antiserum against RNA polymerase IIc (Pol-IIc) or TBP. Secondary antibodies were fluorescent-dye conjugates. Numbers refer to nuclear cycles. Arrowheads indicate nuclei. Both nuclei and lipid droplets exclude TBP at cycle 8.

induction first. Note that *Hsp70* became refractory to induction at a stage when most, but not all HSF had redistributed to the cytoplasm (Fig. 9; S11 and vertical bars). A modification of HSF, a change in its associations, or a general change in nuclear architecture may inactivate it before cytoplasmic transport is complete.

HSF does not become nuclear when cycle 12 is prolonged

In early embryogenesis nuclear divisions are very rapid, occurring once every 10 minutes in cycles 1-11. The 12th cycle is slightly longer, approx. 12 minutes, and the 13th cycle is longer still, approx. 20 minutes (Foe and Alberts, 1983). To

determine if it is simply the lengthening of the cell cycle that allows HSF to concentrate in the nucleus during cycle 13, we examined HSF localization in the presence of a dominant mutation, *Res1*, which prolongs the duration of cycle 12 to approx. 18 minutes (Ruden and Jackle, 1995). This mutation was originally isolated because the longer cell cycle allows precocious expression of the *knirps-related* gene, and thereby rescues *knirps* mutants. At normal temperatures HSF did not enter nuclei in *Res1* embryos prior to cycle 13. Next, we scored 49 heat-treated cycle 12 embryos. None showed nuclear HSF localization or Hsp70 induction (data not shown).

Genetic background affects HSF relocalization

During the course of our studies we observed strain-dependent changes in the developmental timing of HSF nuclear relocalization. Oregon-R is a commonly used wild-type strain. In the many thousands of preblastoderm embryos scored from this strain in the past three years, as well as in the hundreds of embryos scored from the *Res1* line, we never observed HSF in somatic nuclei prior to cycle 13. Inbred lines, however, can expose trait variation due to homozygosity. Indeed, in a highly inbred wild-type derivative of the Samarkind strain (SamI236), many cycle 12 embryos, and even in a few cycle 10 and 11 embryos, exhibited precocious nuclear localization of HSF. This afforded an opportunity to test the importance of HSF nuclear entry in regulating the timing of Hsp70 inducibility. We scored 22 heat-shocked cycle 12 embryos from the SamI236 stock. Among these, 12 showed HSF nuclear localization and 6 exhibited Hsp70 induction (data not shown). That earlier nuclear entry of HSF was associated with earlier activation of Hsp70 provides a compelling argument that it is the developmental restriction of HSF nuclear import that restricts Hsp70 inducibility in the early embryo.

Developmentally programmed HSF relocalization is distinct from that of other transcription factors

We asked if the cycle 13 relocalization of HSF in Oregon-R was part of a global change in transcription factor localization occurring just prior to the large scale transcriptional activation of the zygotic genome at cycle 14. RNA polymerase subunit IIC was nuclear in about half of cycle 7 embryos (Fig. 9), and nuclear in all cycle 8 embryos. TATA-binding protein (TBP) however, underwent a different pattern of re-localization. Prior to cycle 8, TBP was cytoplasmic (data not shown). In cycle 8, it was cytoplasmic in most embryos (Fig. 9) and nuclear in a few (data not shown). At cycle 9, many, but not all embryos exhibited nuclear localization (Fig 9). Notably within an individual embryo, all nuclei exhibited nuclear concentration of RNA polymerase subunit IIC or TBP at the same time. Thus, the nuclear targeting of these transcription factors is also developmentally regulated in an all-or-nothing manner but the timing is distinct from that of HSF.

DISCUSSION

The forms and features of embryogenesis differ greatly among eukaryotes. Yet embryos from organisms as diverse as sea urchins, fruit flies, frogs, and mice fail to express Hsp70 in response to heat shock (Roccheri et al., 1982; Morange et al., 1984; Heikkilä et al., 1985; Arrigo and Tanguay, 1991). This

restriction occurs in embryos that are transcriptionally active and is all the more striking because the induction of HSPs is a universal protective mechanism that operates in nearly all cell types. The failure to induce Hsp70 leaves embryos extremely vulnerable to environmental stresses. Experiments in *Drosophila* have provided a likely explanation for this puzzling phenomenon. Hsp70 protects the organism from the toxic effects of heat, but it impedes growth and division at normal temperatures (Feder et al., 1992; Krebs and Feder, 1997). Apparently, early embryos forgo the potential benefits of Hsp70 in stress tolerance to avoid its potential interference with the rapid and highly stereotyped divisions that characterize this stage of development. Here, we report the mechanism that restricts Hsp70 expression in *Drosophila* embryos: a cell-type and stage-specific restriction on the nuclear entry of HSF. This is the first report of developmentally regulated HSF transport, and the first case in which a mechanism for the embryonic restriction of Hsp70 synthesis has been elucidated.

The link between Hsp70 inducibility and HSF nuclear import

The cytoplasmic localization of HSF is initially established during oogenesis. HSF moves from the nucleus to the cytoplasm in a specific spatiotemporal pattern, beginning in stage 10 egg chambers with the nurse cells most proximal to the oocyte. This mirrors the spatiotemporal change in Hsp70 inducibility during oogenesis. The ability to express Hsp70 in response to heat is first lost at stage 10, in the nurse cells most proximal to the oocyte. It is not clear whether the relocalization of HSF in egg chambers involves HSF-specific regulatory factors or is simply a consequence of the global shut-down of nurse cell transcription which occurs at about this stage. It is clear, however, that once the cytoplasmic localization of HSF is established, re-entry into nuclei is regulated in a highly specific manner, which depends upon cell type and developmental stage, rather than on environmental stress

In embryos of the wild-type Oregon-R strain, HSF first enters the nuclei of pole cells at cycle 12, the stage and cell-type in which Hsp70 is first inducible by heat stress. HSF enters the somatic nuclei of some embryos at cycle 13, and Hsp70 is then inducible in the soma of these embryos. Confirming the importance of HSF relocalization in potentiating the heat shock response, we found that in a different genetic background (SamI236) HSF enters nuclei at an earlier stage and, correspondingly, Hsp70 is inducible at an earlier stage in this background. Nuclear localization of HSF is not sufficient for Hsp70 induction. Heat-shock is still required in the soma, and even heat-shock only activates a subset of pole cells. Our data indicate that the developmental program that restricts HSF from the nucleus is the primary constraint on the embryo's capacity to respond to heat shock.

HSF relocalization: specificity of the program

The relocalization of HSF is not simply a result of the lengthening of the cell cycle during later nuclear divisions. A mutation (*Res1*) that prolongs cycle 12 to nearly the length of cycle 13 did not allow precocious nuclear concentration of HSF. This conclusion was confirmed by the observation that HSF enters the nuclei of SamI236 embryos at an earlier stage, when the cell cycle is still short. There must, then, be genetic factors that regulate relocalization, and these differ in the

SamI236 and Oregon-R strains. Because the nuclear entry of HSF is temporally distinct from that of RNA polymerase IIc or TATA-binding protein, we conclude that HSF relocalization during embryo development is under the control of a highly specific program.

Relationship to other studies

Our observation of cytoplasmic HSF was unexpected. In virtually all organisms and cell types studied to date, HSF is constitutively nuclear (Morimoto et al., 1992; Wu, 1995). One exception is a specific HSF variant in mammalian cells, HSF2, which is partially cytoplasmic in the absence of stress and concentrates in nuclei upon heat-shock (Sistonen et al., 1994; Shelton and Kingston, 1993). Another exception might be HSF in certain *Drosophila* S2 cell lines, where constitutive cytoplasmic localization and heat-induced nuclear entry has been reported (Zandi et al., 1997). However, we and others have found that HSF is constitutively nuclear in *Drosophila* S2 cells (Westwood et al., 1991; our unpublished data). It may be that cell culture conditions or genetic differences in cell lines with unstable karyotypes can influence HSF localization. If so, tissue culture cells with cytoplasmic HSF might provide a more biochemically tractable system for isolating regulatory factors than *Drosophila* embryos. We have attempted by several methods to isolate factors that might be complexed with HSF and restrict its transport to nuclei prior to cycle 12. These efforts were thwarted by the low concentration of HSF and the low ratio of cytoplasm to yolk in embryos at that stage. It might also be possible to identify such factors genetically, exploring differences between the Oregon-R and Samarkind lines.

In any case, within the context of *Drosophila* development, we see little evidence for regulation of the heat-shock response by heat-inducible relocalization of HSF. In all of the many cell types we have examined in late embryos, larvae, and adults, HSF is constitutively nuclear, with the exception of early embryos and germline-derived cells of late oogenesis. Furthermore, prior to the developmentally programmed entry of HSF into nuclei, HSF cannot be targeted to nuclei even with a heat shock. Thus, relocalization is developmentally programmed and independent of heat.

Our experiments also provide information on the relationship between HSF hyper-phosphorylation and transcriptional activation. Previous investigations with yeast and human cells have yielded ambiguous results on the importance of heat-induced hyper-phosphorylation of HSF (Wu, 1995). Indeed, with lysates of pooled embryos, we observed an apparent correspondence between HSF hyper-phosphorylation and Hsp70 induction. But in analyzing individual embryos, this correspondence was broken: some embryos showed Hsp70 induction without HSF hyper-phosphorylation. *Drosophila* embryos furnish a particular advantage for examining this issue because each embryo contains an exquisitely synchronized population of somatic nuclei uniform in their response to heat. Of course, we do not exclude the possible importance of individual phosphorylations in HSF activation. Moreover, with respect to hyper-phosphorylation, our data suggest it enhances the level of Hsp70 expression. However, the unique features of *Drosophila* embryos demonstrate that hyper-phosphorylation is not an obligate precondition for the activation of Hsp70 in response to heat.

Gene activation in the preblastoderm embryo

We also found that two general transcription factors exhibit highly coordinated nuclear localization in *Drosophila* embryos. RNA polymerase IIc localized to nuclei in cycle 7 embryos and in all cycle 8 embryos. Within a given cycle 7 embryo, when one nucleus showed nuclear localization, all did. TBP concentrated in the nuclei of some embryos at cycle 8, in many at cycle 9. Again, all nuclei of an individual embryo showed nuclear localization of TBP at the same time.

The timing of HSF entry into nuclei is regulated independently of these general transcription factors. Nuclear entry occurs at different times in the germline and the soma, and in the soma some embryos execute translocation in cycle 13, others in cycle 14. However, within a given embryo, all germ cells exhibit nuclear entry at the same time and, even more strikingly, the entire soma effects relocation at the same time, whether it be at cycle 13 or at cycle 14. The differences in the timing of transcription factor relocalization between embryos combined with the extraordinary synchrony of relocalization within an embryo indicate that nuclear import is regulated by signals that can propagate across the entire embryo very rapidly.

Once the soma acquires the capacity to respond to heat shock, every somatic cell in the embryo expresses Hsp70 with a similar intensity. This pattern contrasts with the activation of other embryonic transcripts such as *ftz*. *Ftz* is regulated by a maternally supplied transcriptional repressor and is activated when the nuclear/cytoplasmic ratio reaches a critical threshold. Stochastic differences in the concentrations of the repressor cause *ftz* to be activated in different embryos at different stages and each nucleus in an embryo acquires this potential independently of the others (Pritchard and Schubiger, 1996).

Thus, diverse mechanisms regulate the translocation of transcription factors in early *Drosophila* embryos. Some operate in a nucleus-autonomous manner; others show some variation in timing between embryos but are synchronous for every nucleus within an embryo. HSF exhibits distinct patterns in the germline and soma, but within these cell types, relocalization is synchronous.

Our results indicate that it is the regulation of HSF relocalization that determines when embryos acquire the potential to induce Hsp70 in response to stress. In this respect, the timing of TBP nuclear entry is of interest to the broader question of general transcriptional activation. Zygotic transcripts are first detectable in *Drosophila* nuclei at nuclear cycles 8 and 9 (Erickson and Cline, 1993). We suggest that the transcriptional activity of the embryo might be initially restricted by the cytoplasmic retention of TBP, and perhaps other general transcription factors, and that transcriptional activation is potentiated by highly synchronized import of these factors to the nucleus.

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